Evaluation of the role of PGIPs in plant defense responses

by

John van Wyk Becker

Dissertation presented for the Degree of Doctor of Philosophy at Stellenbosch University.

February 2007

Promoter:
Prof MA Vivier

Co-promoter:
Prof KJ Denby

DECLARATION

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

John Becker   13 February 2007

Name of candidate   **Date**

SUMMARY

Plants have developed sophisticated means of combating plant diseases. The events that prepare the plant for, and follow plant-pathogenic interactions, are extremely complex and have been the topic of intensive investigation in recent years. These interactions involve a plethora of genes and proteins, and intricate regulation thereof; from the host and pathogen alike. Studying the contribution of single genes and their encoded proteins to the molecular dialogue between plant and pathogen has been a focus of plant molecular biologists.

To this end, a gene encoding a polygalacturonase-inhibiting protein (PGIP) was recently cloned from *Vitis vinifera*. These proteins have the ability to inhibit fungal endopolygalacturonases (ePGs), enzymes which have been shown to be required for the full virulence of several fungi on their respective plant hosts. The activity of PGIP in inhibiting fungal macerating enzymes is particularly attractive for the improvement of disease tolerance of crop species. The VvPGIP-encoding gene was subsequently transferred to *Nicotiana tabacum* for high-level expression of VvPGIP. These transgenic plants were found to be less susceptible to infection by *Botrytis cinerea* in an initial detached leaf assay. Also, it was shown that ePG inhibition by protein extracts from these lines correlated to the observed decrease in susceptibility to *B. cinerea*. This study expands on previous findings by corroborating the antifungal nature of the introduced PGIP by whole-plant, time-course infection assays. Six transgenic tobacco lines and an untransformed wild-type (WT) were infected and the lesions measured daily from day three to seven, and again at day 15. The transgenic lines exhibited smaller lesions sizes from three to seven days post-inoculation, although these differences only became statistically significant following seven days of incubation. At this point, four of the six lines exhibited significantly smaller lesions than the WT, with reductions in disease susceptibility ranging between 46 and 69% as compared to the WT. Two of the lines exhibited disease susceptibility comparable to the WT. In these resistant plant lines, a correlation could be drawn between *Vvpgip1* expression, PGIP activity and ePG inhibition. These lines were therefore considered to be PGIP-specific resistant lines, and provided ideal resources to further study the possible *in planta* roles of PGIP in plant defense.

The current hypothesis regarding the role(s) of PGIP in plant defense is two-fold. Firstly, PGIPs have the ability to specifically and effectively inhibit fungal ePGs. This direct inhibition results in reduced fungal pathogenicity. Alternatively, unhindered action of these enzymes results in maceration of plant tissue and ultimately, tissue necrosis. Subsequently, it could be shown that, *in vitro*, the inhibition of ePGs prolongs the existence of oligogalacturonides, molecules with the ability to activate plant defense responses. Thus, PGIPs limit tissue damage by inhibition of ePG; this inhibition results in activation of plant defense responses aimed at limiting pathogen ingress.

Several publications reported reduced susceptibility to *Botrytis* in transgenic plant lines overexpressing PGIP-encoding genes. However, none of these publications could expand on the current hypotheses regarding the possible *in planta* roles of PGIP in plant defense. In this study we used transgenic tobacco lines overexpressing *Vvpgip1* as resources to study the *in planta* roles for PGIP. Transcriptomic and hormonal analyses were performed on these lines and a WT line, both before and following inoculation with *Botrytis cinerea.*

Transcriptomic analysis was performed on uninfected as well as infected tobacco leaf material utilizing a *Solanum tuberosum* microarray. From the analysis with healthy, uninfected plant material, it became clear that genes involved in cell wall metabolism were differentially expressed between the transgenic lines and the WT. Under these conditions, it could be shown and confirmed that the gene encoding tobacco xyloglucan endotransglycosylase (XET/XTH) was downregulated in the transgenic lines. Additionally, genes involved in the lignin biosynthetic pathway were affected in the individual transgenic lines. Biochemical evidence corroborated the indication of increased lignin deposition in their cell walls. Additionally, phytohormone profiling revealed an increased indole-acetic acid content in the transgenic lines. These results show that constitutive levels of PGIP may affect cell wall metabolism in the *Vvpgip1*-transgenic lines which may have a positive impact on the observed reduced susceptibilities of these plants. An additional role for PGIP in the contribution to plant defenses is therefore proposed. PGIP may directly influence defense responses in the plant leading to the strengthening of cell walls. This might occur by virtue of its structural features or its integration in the cell wall. These reinforced cell walls are thus “primed” before pathogen ingress and contribute to the decrease in disease susceptibility observed in lines accumulating high levels of PGIP.

Transcriptional and hormonal analyses, at the localized response, were performed on *Botrytis*-infected leaf tissue of the transgenic lines and a WT line. Several *Botrytis* responsive genes were found to be upregulated in both the WT and the transgenic lines. Although limited differential expression was observed between the two genotypes, the analyses identified a gene which was upregulated two-fold in the transgenic lines, as compared to WT. This was confirmed by quantitative Real-Time PCR. This gene is involved in the lipoxygenase pathway, specifically the 9-LOX branch, leading to the synthesis of the divinyl ether oxylipins colneleic and colnelenic acid, which show inhibitory effects on *Botrytis* spore germination. Phytohormone profiling revealed that the transgenic lines accumulated more of the defense-related hormone pool of jasmonates. These are formed via the 13-LOX pathway and have been shown to be important for the restriction of *Botrytis* growth at the site of infection. Collectively, the results from the infection analyses indicate that in these transgenic lines, both branches of the lipoxygenase pathway are differentially induced at the level of the localized response to *Botrytis* infection. Similarly, an

increased induction of the synthesis of the defense-related hormone salicylic acid could be observed, although this hormone did not accumulate to significantly higher levels. These results are the first report of differential induction of a defense-related pathway in *pgip*-overexpressing lines and substantiate the proposal that following ePG inhibition by PGIP, signaling which activates plant defense responses, takes place.

Taken together, these results significantly contribute to our understanding of the *in planta* role of PGIP in plant defense responses.

OPSOMMING

Plante het deur evolusie gesofistikeerde meganismes teen die aanslag van plantsiektes ontwikkel. Die gebeure wat die plant voorberei, asook dié wat op plant-patogeen interaksies volg, is uiters kompleks en vorm die kern van verskeie navorsingstemas die afgelope paar jaar. Etlike plant- én patogeengene en proteïene is by hierdie interaksies betrokke en aan komplekse reguleringsprosesse onderworpe. Die bestudering van die bydrae van enkelgene en hul gekodeerde proteïene tot die molekulêre interaksie tussen 'n plant en patogeen is 'n sterk fokus van plant-molekulêre bioloë.

Met hierdie doel as fokus, is 'n geen wat vir 'n poligalakturonase-inhiberende proteïen (PGIP) kodeer, van *Vitis vinifera* gekloneer. Hierdie proteïene beskik oor die vermoë om fungiese endopoligalakturonases (ePG's), ensieme wat benodig word vir die virulensie van verskeie fungi op hul gasheerplante, te inhibeer. Die inhibisie van ePG's deur PGIP en die gepaardgaande verminderde weefseldegradasie is 'n baie belowende strategie vir die verbetering van verboude gewasse se patogeentoleransie. Die VvPGIP-enkoderende geen is gevolglik na *Nicotiana tabacum* oorgedra vir hoëvlak-uitdrukking van VvPGIP. Daar is gevind dat hierdie transgeniese plante minder vatbaar vir *Botrytis cinerea*-infeksies was in 'n inisiële antifungiese toets wat gebruik gemaak het van blaarweefsel wat van die moederplant verwyder is. Daar is ook 'n korrelasie gevind tussen *B. cinerea*-siekteweerstand en ePG-inhibisie deur proteïenekstrakte van die transgeniese populasie. Die huidige studie bou voort op en bevestig vorige bevindinge betreffende die antfungiese aard van die heteroloë PGIP in die heelplant en oor tyd. Ses transgeniese tabaklyne en 'n ongetransformeerde wilde-tipe (WT) is geïnfekteer en die lesies is vanaf dag drie tot sewe, en weer op dag 15, gemeet. Die transgeniese lyne het in die tydperk van drie tot sewe dae ná-inokulasie kleiner lesies as die WT getoon, alhoewel hierdie verskille slegs statisties beduidend geword het na sewe dae van inkubasie. Op daardie tydstip het vier van die ses lyne aansienlik kleiner lesies as die WT getoon, en verlagings in siektevatbaarheid het, in vergelyking met die WT, van 46% tot 69% gewissel. Twee van die lyne het siektevatbaarheid getoon wat vergelykbaar was met dié van die WT. In die siekteweerstandbiedende plantlyne was daar 'n verband tussen *Vvpgip1*-ekspressie, PGIP-aktiwiteit en ePG-inhibisie. Hierdie plantlyne is dus as PGIP-spesifieke siekteweerstandslyne beskou en dien dus as ideale eksperimentele bronne vir die ontleding van die moontlike *in planta*-funksies van PGIP in plantsiekteweerstandbiedendheid.

Die huidige hipotese betreffende die funksie(s) van PGIP in plantsiekteweerstand is tweeledig. Eerstens het PGIP die vermoë om fungus-ePG's spesifiek en doeltreffend te inhibeer. Hierdie direkte inhibisie veroorsaak 'n vermindering in patogenisiteit van die fungus op die gasheer. Indien ePG's egter hulle ensimatiese aksie onverstoord voortsit, sal weefseldegradasie en uiteindelik

weefselnekrose die gevolg wees. Daar kon ook bewys word dat die *in vitro*-inhibisie van ePG's deur PGIP die leeftyd van oligogalakturoniede, molekules wat die vermoë het om die plantweerstandsrespons aan te skakel, kan verleng. PGIP het dus nie net die vermoë om ePG's, en dus weefseldegradasie, te inhibeer nie; maar hierdie inhibisie lei ook daartoe dat plantweerstandsresponse aangeskakel word met die oog op die vermindering van patogeenindringing.

Verskeie publikasies het reeds gerapporteer oor verminderde *Botrytis*-vatbaarheid in PGIP transgeniese plantlyne. Geeneen van hierdie publikasies kon egter uitbrei op die huidige hipotese aangaande die moontlike *in planta*-funksie van PGIP in plantsiekteweerstand nie. In hierdie studie is transgeniese tabaklyne wat PGIP ooruitgedruk gebruik om hierdie moontlike *in planta*-funksies vir PGIP uit te klaar. Transkriptoom- en hormonale analises is op hierdie plantlyne en ‘n WT voor en ná inokulasie met die nekrotroof *Botrytis cinerea* uitgevoer,.

Transkriptoomanalises is uitgevoer op ongeïnfekteerde, sowel as geïnfekteerde tabakblaarmateriaal deur gebruik te maak van ‘n *Solanum tuberosum*-mikroraster. Die analises met gesonde, ongeïnfekteerde plantmateriaal het daarop gewys dat gene betrokke by selwandmetabolisme tussen die transgeniese lyne en die WT verskillend uitgedruk was. Dit kon bewys word dat, sonder infeksiedruk, die geen wat xiloglukaan-endotransglikosilase (XET) kodeer, in die transgeniese lyne afgereguleer was. Gene wat betrokke is in die lignien-biosintetiese pad was ook in die individuele transgeniese lyne beïnvloed. Biochemiese toetse het ook die aanduiding van verhoogde ligniendeposisie in die transgeniese lyne se selwande bevestig. Addisionele fitohormoonprofiele het getoon dat hierdie lyne ook beskik oor verhoogde vlakke van indoolasynsuur (IAA). Hierdie resultate wys daarop dat konstitutiewe vlakke van PGIP selwandmetabolisme in die *Vvpgip1*-transgeniese lyne moontlik kan beïnvloed, wat plantsiekteweerstand in dié lyne positief kan beïnvloed. Dit wil dus voorkom asof PGIP 'n bykomende funksie in plantsiekteweerstand het. Plantweerstandsreponse kan direk deur PGIP beïnvloed word, wat tot die versterking van plantselwande kan lei; dit kan geskied by wyse van die strukturele eienskappe van die proteïen of die integrasie daarvan in die selwand. Hierdie selwande is dus “voorberei” alvorens patogeenindringing plaasvind en kon bydra tot die verminderde siektevatbaarheid wat waargeneem is in lyne wat hoë vlakke van PGIP akkumuleer.

Transkriptoom- en hormonale analises is ook uitgevoer op *Botrytis*-geïnfekteerde blaarmateriaal van beide die transgeniese lyne en ‘n WT. Verskeie *Botrytis*-responsgene is in beide die transgeniese lyne en die WT opgereguleer. Differensïele geenekspressie tussen die twee genotipes was taamlik beperk, maar in die analises kon ‘n geen geïdentifiseer word wat tweevoudig in die transgeniese lyne opgereguleer was in vergelyking met die WT. Hierdie resultaat is ook bevestig met behulp van die “Real-Time” Polimerasekettingreaksie (PKR). Hierdie geen is betrokke in die lipoksigenase (LOX) -pad (spesifiek die 9-LOX-

arm), wat tot die sintese van die diviniel-eter oksilipiene “colneleic-” en “colnelenic”-suur lei. Daar is al bewys dat hierdie twee verbindings *Botrytis*-spoorontkieming kan inhibeer. Fitohormoonprofiele van die geïnfekteerde plante het gewys dat die transgeniese lyne verhoogde vlakke van die poel van jasmonate wat plantsiekteweerstands-hormone is, ná inokulasie akkumuleer. Hierdie hormone word in die 13-LOX-arm van die lipoksigenase pad gevorm en is belangrik vir die beperking van *Botrytis* by die infeksiesetel. Die resultate van die analises wat op *Botrytis*-infeksie volg, dui daarop dat beide arms van die lipoksigenasepad in die transgeniese lyne verskillend by die lokale respons geïnduseer word. ‘n Verhoogde induksie van ‘n ander plantsiekteweerstandshormoon, salisielsuur, kon ook opgemerk word, alhoewel die totaal geakkumuleerde vlakke nie beduidend hoër was as dié van die WT nie. Hierdie resultate is die eerste wat onderskeidende induksie van ‘n siekteweerstandspad in enige van die *pgip*-ooruitgedrukte plantlyne rapporteer. Daarmee ondersteun dit ook die hipotese dat, seintransduksie wat plantweerstandsresponse aanskakel, ná inhibisie van ePG deur PGIP plaasvind.

Die resultate wat met hierdie studie verkry is, dra dus beduidend by tot die huidige kennis van die *in planta*-funksie van PGIP in plantsiekteweerstandsresponse.

This dissertation is dedicated to Nelmarie.
Hierdie proefskrif is opgedra aan Nelmarie.

BIOGRAPHICAL SKETCH

John van Wyk Becker was born in Cape Town on the 23rd of January 1975 and matriculated from Roodepoort High School in 1993. John enrolled at Rand Afrikaans University in 1995 and obtained a BSc degree, with major subjects Botany and Biochemistry, in 1997. He subsequently enrolled at Stellenbosch University for the degrees HonsBSc (Wine Biotechnology) and MSc (Wine Biotechnology), the latter being awarded *cum laude*.

ACKNOWLEDGEMENTS

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

Nelmarie Becker, for endless support, love and encouragement;

My parents, for all their support and understanding;

Prof MA Vivier, for acting as supervisor and helping to shape my career as researcher;

Prof KJ Denby, for acting as co-supervisor and for advice and critical discussions;

Lab colleagues, for providing a pleasant working atmosphere, countless fruitful discussions and a life away from the lab;

The National Research Foundation, the **Harry Crossley Foundation**, **The Institute for Wine Biotechnology** and **Winetech** for financial support.

PREFACE

This dissertation is presented as a compilation of six chapters. Each chapter is introduced separately and is written according to the style of the journal Molecular Plant-Microbe Interactions to which Chapters 4 and 5 shall be submitted for publication. Chapter 3 was published in Transgenic Research.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
PGIP, pathogens and plant protection

Chapter 3 **Research Results**
The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases

Chapter 4 **Research Results**
Constitutive levels of PGIP influence cell wall metabolism in transgenic tobacco

Chapter 5 **Research Results**
Transgenic tobacco plants with a PGIP-specific resistance phenotype show increased induction of jasmonate synthesis and *des1* expression in the local response following *Botrytis cinerea* infection

Chapter 6 **General Discussion and Conclusions**

I hereby declare that I was a co-contributor to the multi-author manuscript presented in Chapter 3. My contribution involved the analysis of transgenic tobacco in the whole-plant, time-course infection assay. Dr DA Joubert and Dr AR Slaughter contributed the majority of the experimental data. I was the primary contributor with respect to the experimental data presented on the multi-author manuscripts presented in Chapters 4 and 5.
Mr AGJ Tredoux was involved in the adoption and adaptation of the method involving the profiling of phytohormones.
My supervisors Prof MA Vivier and Prof KJ Denby were involved in the conceptual development of the study, and continuous critical evaluation of the research and of the resulting manuscript.

CONTENTS

CHAPTER 1. GENERAL INTRODUCTION AND PROJECT AIMS 1

1.1 Introduction
1.2 Polygalacturonase-Inhibiting Proteins and their Roles in Plant Defense 1
1.3 Specific Project Aims 3
1.4 References 4

CHAPTER 2. LITERATURE REVIEW: PGIP, PATHOGENS AND PLANT PROTECTION 6

2.1 Introduction 6
2.2 The Role of Endopolygalacturonases (ePGs) in Fungal Pathogenicity 6
2.3 ePG Inhibitors (PGIP) 10
2.3.1 Genomic Organization 10
2.3.2 *pgip* Genes are Expressed in Response to Stress 14
2.3.3 PGIP Inhibition Spectra Reveals Their Role in Defense 15
2.4 Structures of PGIP, ePG and Their Interaction 17
2.5 Overexpression of *pgip* Reduces Susceptibility to *Botrytis* 19
2.6 Vitis PGIP: What do we know? 21
2.7 Summary 22
2.8 References 23

CHAPTER 3. RESEARCH RESULTS: THE GRAPEVINE POLYGALACTURONASE-INHIBITING PROTEIN (VVPGIP1) REDUCES *BOTRYTIS CINEREA* SUSCEPTIBILITY IN TRANSGENIC TOBACCO AND DIFFERENTIALLY INHIBITS FUNGAL POLYGALACTURONASES 28

3.1 Abstract 28
3.2 Introduction 29
3.3 Materials and Methods 30
3.3.1 Plant growth and light conditions 30
3.3.2 Vector constructs and plant transformation 30
3.3.3 PCR and Southern blot analyses of transgenic lines 30
3.3.4 Northern blot analysis of *Vvpgip1* transcript levels 31
3.3.5 Detection of VvPGIP1 activity in transgenic tobacco 31
3.3.6 Fungal endopolygalacturonases 31
3.3.7 Plant infections and disease assessment 32
3.3.8 Isolation and purification of VvPGIP1 from transgenic tobacco plants 32

3.3.9 Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) 33
3.3.10 Quantitative endopolygalacturonase inhibition assays with purified VvPGIP1 33
3.4 Results 33
3.4.1 Construction of a plant expression cassette and subsequent tobacco transformations 33
3.4.2 Crude protein extracts from transgenic tobacco over-expressing *Vvpgip1* can inhibit crude polygalacturonase (PG) preparations from *Botrytis cinerea* 34
3.4.3 Transgenic tobacco plants over-expressing *Vvpgip1* are less susceptible to *B. cinerea* infection 34
3.4.4 VvPGIP1 purified from transgenic tobacco differentially inhibits endo-polygalacturonases from *Aspergillus niger* (AnPGs) and *B. cinerea* (BcPGs) 37
3.5 Discussion 38
3.6 Acknowledgements 41
3.7 References 42

CHAPTER 4. RESEARCH RESULTS: CONSTITUTIVE LEVELS OF PGIP INFLUENCE CELL WALL METABOLISM IN TRANSGENIC TOBACCO 44

4.1 Abstract 44
4.2 Introduction 45
4.3 Materials and Methods 46
4.3.1 Plant Material and Growth Conditions 46
4.3.2 Microarray Analyses 47
4.3.2.1 RNA Extraction 47
4.3.2.2 cDNA Synthesis and Hybridization 47
4.3.3 Quantitative Real-Time PCR 49
4.3.4 Determination of Lignin Content 49
4.3.4.1 Histochemical Assays for Determining Lignin Content 49
4.3.4.2 Lignin Quantification 50
4.3.5 Simultaneous Analysis of Phytohormones Using GC/MS 50
4.3.5.1 Chemicals 50
4.3.5.2 Sample Preparation for Vapor Phase Extraction 50
4.3.5.3 Vapor Phase Extraction 51
4.3.5.4 Instrumentation 51
4.4 Results 52
4.4.1 Microarray Analyses 52
4.4.2 Quantitative Real-Time PCR 54
4.4.3 Determination of Lignin Content 55
4.4.4 Simultaneous Analysis of Phytohormones Using GC/MS 57

4.5 Discussion 58
4.6 References 62
4.7 Acknowledgements 65
ADDENDUM A 66

CHAPTER 5. RESEARCH RESULTS: TRANSGENIC TOBACCO PLANTS WITH A PGIP-SPECIFIC RESISTANT PHENOTYPE SHOW INCREASED INDUCTION OF JASMONATE SYNTHESIS AND *DES1* EXPRESSION IN THE LOCAL RESPONSE FOLLOWING *BOTRYTIS CINEREA* INFECTION 68

5.1 Abstract 68
5.2 Introduction 69
5.3 Materials and Methods 70
5.3.1 Plant Material and Growth Conditions 70
5.3.2 Fungal Inoculation on Tobacco Plants 70
5.3.3 Microarray Analyses 71
5.3.3.1 RNA Extraction 71
5.3.3.2 cDNA Synthesis and Hybridization 71
5.3.3.3 Microarray Data Analysis 72
5.3.4 Quantitative Real-Time PCR 72
5.3.5 Simultaneous Analysis of Phytohormones Using GC/MS 73
5.3.5.1 Chemicals 73
5.3.5.2 Sample Preparation for Vapor Phase Extraction 73
5.3.5.3 Vapor Phase Extraction 74
5.3.5.4 Instrumentation 74
5.4 Results 75
5.4.1 Microarray Analyses 75
5.4.2 Quantitative Real-Time PCR 79
5.4.3 Simultaneous Analysis of Phytohormones Using GC/MS 80
5.5 Discussion 81
5.6 References 85
5.6 Acknowledgements 88

CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS 89

6.1 General Discussion and Conclusions 89
6.2 References 94

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 Introduction

Living organisms are continually exposed to a barrage of environmental stresses. Both biotic and abiotic stress can be averted by organisms with mobile ability. Conversely, most plants feature developed root systems which anchor them in a static position. Through evolution, plants have developed mechanisms which allowed them to adapt to these environmental stresses.

Undoubtedly, the risk posed to agriculture by biotic stress has major implications for crop production and the eventual quality thereof. Biotic stress includes pathogenesis by a variety of organisms- bacteria, viruses and fungi. Of these, fungi arguably constitute the greatest potential loss to the pre- and post-harvest crop. For the infection and ultimate colonization of host plant tissue, these phytopathogenic fungi have developed sophisticated tools. When aiming to infect the plant, fungi are confronted by the plant cell wall, a physical barrier that has to be breached for successful colonization. Enzymes with the ability to degrade the pectic component of plant cell walls greatly aid fungi in their infective ability on plant tissue. Foremost among these are the endopolygalacturonases (ePGs), shown to be important for the virulence of numerous fungi on their respective hosts. Strategies involving the inhibition of the action of these enzymes would no doubt prove favorable for the management of not only phytopathogenic fungi, but also other organisms utilizing ePGs for their infective or feeding ability on plants; including bacteria, fungi, nematodes and insects (Di Matteo et al., 2006 and references therein).

Proteins with the ability to inhibit these enzymes are found in numerous plant species (De Lorenzo et al., 2001; De Lorenzo et al., 2002). These proteins are termed polygalacturonase-inhibiting proteins (PGIPs) and specifically and effectively inhibit ePGs from fungal, but not plant origin.

1.2 Polygalacturonase-Inhibiting Proteins and Their Roles in Plant Defense

The contribution of PGIP to plant defense has been studied for well over 20 years. Several aspects of these proteins, their encoding genes and their regulation imply that they play important roles in plant defense (De Lorenzo et al., 2001; De Lorenzo et al., 2002). These proteins specifically and effectively inhibit the action of fungal ePGs *in vitro* (De Lorenzo et al., 2001); although clear *in planta* evidence is yet to be shown for most ePG-PGIP interactions. ePGs have been shown to be important for the pathogenicity of several fungi on their respective hosts (Shieh et al., 1997; ten Have et al., 1998; Isshiki et al., 2001; Oeser et al., 2002; Li et al., 2004; Kars et al., 2005). These enzymes, among the first to be secreted upon fungal attack (Jones et al., 1972), cleave the linkages of galacturonic acid residues (Esquerré-Tugayé et al.,

2000) in the plant pectic backbone and expose the rest of the plant cell wall to degradation by other wall-modifying enzymes (Annis and Goodwin, 1997). The rate of depolymerization of cell wall fragments *in planta* is hypothesized to be slowed when PGIPs inhibit ePGs, although this has been shown for the protein interaction *in vitro* (Cervone et al., 1989). Should these enzymes continue their action unhindered, large-scale tissue maceration and necrosis of plant tissue would ultimately be the outcome (Kars et al., 2005; Joubert et al., 2007).

Direct evidence for the involvement of PGIPs in plant defense has been obtained by overexpressing their encoding genes in different plant backgrounds. Overexpression of *pgip* genes in numerous backgrounds (Powell et al., 2000; Ferrari et al., 2003; Aguero et al., 2005; Manfredini et al., 2005; Joubert et al., 2006 (Chapter 3 of this dissertation)) has provided the respective plant species with tolerance to infection by an economically important fungus, *Botrytis cinerea*. Furthermore, plant susceptibility to fungal pathogens has been shown to be dependent on the presence of appreciable levels of PGIP before fungal infection (Abu-Goukh et al., 1983; Salvi et al., 1990; Johnston et al., 1993; Powell et al., 2000; Ferrari et al., 2003; Aguero et al., 2005; Manfredini et al., 2005; Joubert et al., 2006; Ferrari et al., 2006).

As previously mentioned, PGIPs directly inhibit the action of fungal ePGs. The eventual outcome of the continued action of these enzymes is tissue maceration and necrosis (Kars et al., 2005; Joubert et al., 2007). Additionally, Cervone et al. (1989) proposed that the shift towards cell wall fragments (oligogalacturonides) with plant defense elicitor activity is favored when PGIP slows the action of ePG on the pectic component of plant cell walls. Thus, the authors proposed that PGIP may, in inhibiting PG, activate plant defense responses. Thus, the current understanding of PGIP in decreasing fungal susceptibility is two-fold; firstly, by inhibiting macerating enzymes; followed by defense signaling that is a result of the inhibition of these enzymes.

Several authors have described overexpression of *pgip* genes associated with decreased susceptibility to a fungal pathogen. These lines provide ideal resources to further elucidate the *in planta* roles of PGIP in plant defense. For instance, the downstream events following infection in PGIP-accumulating lines as compared to their untransformed counterparts have not been elucidated, even though transgenic lines expressing heterologous *pgips* have been available for some time. Transgenic tobacco plants overexpressing *Vitis vinifera pgip1* (*Vvpgip1*) (Joubert et al., 2006) were used in this regard to evaluate the antifungal ability of the heterologously overexpressed PGIP, as well as gaining a better understanding of *how* PGIPs protect plants against fungal pathogens.

1.3 Specific Project Aims

Since the hypothesis of Cervone et al. (1989) was put forward, our understanding of the specific role(s) of PGIP in plant defense responses has not increased substantially. The aim of this study was to expand the current knowledge regarding the *in planta* roles of PGIP in plant defense responses. Plant lines accumulating high levels of VvPGIP and showing reduced susceptibility to *Botrytis* infection were considered ideal experimental resources to this end.

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

1) To confirm the strong antifungal nature of the protein encoded by the gene *Vitis vinifera polygalacturonase-inhibiting protein 1 (Vvpgip1) in planta*, in a transgenic tobacco background where this gene was overexpressed.

> (i) Elucidating disease susceptibility in transgenic tobacco plants overexpressing *Vvpgip1* and challenged with the fungus *Botrytis cinerea,* in a whole-plant, time-course antifungal assay.

2) To evaluate the possible transcriptomic differences and their physiological effect(s) induced by overexpression of *Vvpgip1* (i.e. any possible effects linked to the mere presence of PGIP at high levels).

> (i) Determination of relative gene expression in *Vvpgip1* transgenic lines, as compared to an untransformed WT line, by means of microarray analysis of healthy, uninfected plants;
>
> (ii) The confirmation of genes differentially expressed by Real-Time Quantitative PCR;
>
> (iii) The confirmation of the involvement of any differentially regulated pathways with biochemical methods; and
>
> (iv) The measurement of the phytohormone content under the test conditions of both WT and transgenic plant lines overexpressing *Vvpgip1*.

3) To elucidate the *in planta* contribution of VvPGIP to the reduced fungal susceptibility pertaining to the induced or active plant defenses following *Botrytis cinerea* inoculation, at the localized response.

> (i) Elucidate differential expression between WT and *Vvpgip1* transgenic lines by means of transcriptomic analyses, in tissues at and surrounding the infection sites;
>
> (ii) Confirmation of genes differentially expressed between the WT and transgenic lines at different time points following infection; and
>
> (iii) The determination of the phytohormone profiles of the aforementioned lines following infection at several time points.

1.4 References

Abu-Goukh, A.A., Strand, L.L., and Labavitch, J.M. 1983. Development-related changes in decay susceptibility and polygalacturonase inhibitor content of “Bartlett” pear fruit. Physiol. Plant Pathol. 23: 101-109.

Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce’s disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pathol. 6: 43-51.

Annis, S.L., and Goodwin, P.H. 1997. Recent advances in the molecular genetics of plant cell-wall degrading enzymes produced by plant pathogenic fungi. Eur. J. Plant Pathol. 103: 1-14.

Cervone, F. Hahn, M.G., De Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. 90: 542-548.

De Lorenzo, G., D’Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu. Rev. Phytopath. 39: 313-335.

De Lorenzo, G., and Ferrari, S. 2002. Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. Curr. Opin. Plant Biol. 5: 1-5.

Di Matteo, A., Bonivento, D., Tsernoglou, D., Federici, L., and Cervone, F. 2006. Polygalacturonase-inhibiting protein (PGIP) in plant defence: a structural view. Phytochemistry 67: 528-533.

Esquerré-Tugayé, M-T., Boudart, G., and Dumas, B. 2000. Cell wall degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. Plant Physiol. Biochem. 38: 157-163.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. 2003. Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins (PGIP) are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15: 93-106.

Ferrari, S., Galletti, R., Vairo, D., Cervone, F., and De Lorenzo, G. 2006. Antisense expression of the *Arabidopsis thaliana AtPGIP1* gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. Mol. Plant Microbe Interact. 8: 931-936.

Isshiki, A., Akimitsu, K., Yamamoto, M., and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Mol. Plant Microbe Interact. 14: 749-757.

Johnston, D.J., Ramanathan, V., and Williamson, B. 1993. A protein from immature raspberry fruits which inhibits endopolygalacturonases from *Botrytis cinerea* and other micro-organisms. J. Exp. Bot. 44: 971-976.

Jones, T.M., Anderson, A.J., and Albersheim, P. 1972. Host-pathogen interactions IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. 2: 153-166.

Joubert, D.A., Slaughter, A.R., Kemp, G., Becker, J.V.W., Krooshof, G.H., Bergmann, C., Benen, J., Pretorius, I.S., and Vivier, M.A. 2006. The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. DOI 10.1007/s11248-006-9019-1.

Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and van Kan, J.A.L. 2007. A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *B. cinerea* in *N. benthamiana* leaves without any evidence for *in vitro* interaction. Mol. Plant Microbe Interact. (in print).

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and van Kan, J.A. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris.* Plant J. 43: 213-225.

Li, R., Rimmer, R., Buchwalt, L., Sharpe, A.G., Seguin-Swartz, G., and Hegedus, D.D. 2004. Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of *endo*-and *exo*-polygalacturonases expressed during the saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

Manfredini, C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M., and De Lorenzo, G. 2005. Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol. Mol. Plant Pathol. 67: 108-115.

Oeser, B., Heidrich, P.M., Muller, U., Tudzynski, P., and Tenberge, K.B. 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. Fungal Genetic. Biol. 36: 176-186.

Powell, A.L., van Kan, J.A., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. 13: 942-950.

Salvi, G., Giarrizzo, F., De Lorenzo, G., and Cervone, F. 1990. A polygalacturonase-inhibiting protein in the flowers of *Phaseolus vulgaris* L. J. Plant Physiol. 136: 513-518.

Shieh, M.T., Brown, R.L., Whitehead, M.P., Cary, J.W., Cotty, P.J.,Cleveland, T.E., and Dean, R.A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63: 3548-352.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant Microbe Interact. 11: 1009-1016.

LITERATURE REVIEW

2.1 Introduction

Living organisms find themselves in relationships with other living organisms. These relationships are defined by the outcome of the interaction between them, whether it be beneficial for both or to the detriment of either. This also holds true for plants and the habitats where they occur naturally, or as a result of cultivation. Unable to escape their exposure to a multitude of biota, plants have evolved numerous responses to aid their survival when confronted by these organisms. Foremost amongst the organisms detrimental to plants are the phytopathogenic fungi, responsible for crop losses totaling millions of dollars annually.

Plant pathogenic fungi have at their disposal an array of enzymes facilitating their colonization of host tissue. Pectic enzymes with the ability to depolymerize the pectin fragment of the cell wall are included in this group. A number of these pectic enzymes have been shown to be important for the disease causing ability of several pathogenic fungi; not only being among the first enzymes secreted by invading fungal pathogens, but also exposing the rest of the primary wall to degradation by other classes of wall-hydrolyzing enzymes. The endopolygalacturonases (ePGs), a class of pectic enzymes secreted soon after infection, have been shown to be important for the pathogenicity of several fungal species on their respective plant hosts. Not only do these enzymes macerate host tissue but the resulting wall fragments provide the fungi with nutrients for their growth.

Plants exposed to these fungi and their macerating ePGs have evolved specific and effective inhibitors of the latter. These are termed polygalacturonase-inhibiting proteins (PGIPs) and are present in both mono- and dicotyledonous plants. These proteins and their encoding genes form the focus of this review. A section outlining the importance of ePGs, the ligands of PGIPs, commences this review. Several aspects of PGIP-encoding genes are discussed, including genomic organization and gene regulation, as they relate to plant-pathogen interactions. In the same vein, PGIP inhibition spectrum and structure are examined. Several *pgip*-overexpression strategies, which have been proven to be successful to reduce fungal susceptibility of transgenic plants, are discussed. This review is concluded by a brief summary of our current knowledge of grapevine PGIP and its encoding gene.

2.2 The Role of Endopolygalacturonases (ePGs) in Fungal Pathogenicity

Endopolygalacturonases (ePGs) secreted by phytopathogenic fungi are amongst the first tissue macerating enzymes secreted upon fungal infection (Jones et al., 1972). Not only do these enzymes degrade a key component of the pectin backbone of plant cell walls, they also expose it to the action of other wall-degrading enzymes, including the cellulases and hemicellulases (Annis and Goodwin, 1997). ePGs

hydrolyze the α-1,4 linkages of unesterified galacturonic acid residues of the homogalacturonan domain (Esquerré-Tugayé et al., 2000).

In the case of three phytopathogenic fungi, there is some evidence to suggest that the size of the ePG gene family correlates to its host range (Esquerré-Tugayé et al., 2000). The necrotrophic fungus *Botrytis cinerea*, with a host range of over 200 plant species (Jarvis, 1977) harbors a gene family of six members (ten Have et al., 1998). Moreover, *Sclerotinia sclerotiorum*, a broad host range fungus, contain ePGs encoded for by a multigene family of seven members (Fraissinet-Tachet et al., 1995). In contrast, *Colletotrichum lindemuthianum*, which only successfully colonizes bean plants, contains a family consisting of only two ePG-encoding genes (Centis et al., 1997). Detailed characterization of all fungal ePG members of the mentioned fungi, on a broad range of host plants, will no doubt shed more light on this generalization.

The contribution of ePGs to the ability of phytopathogenic fungi to infect plants has been substantiated for numerous fungi (Shieh et al., 1997; ten Have et al., 1998; Isshiki et al., 2001; Oeser et al., 2002; Li et al., 2004; Kars et al., 2005). Of these, the ePGs of the necrotroph *B. cinerea* has enjoyed the most interest, and will be discussed in further detail. The typical infection process of *B. cinerea* involves several steps and is detailed in an excellent review by Van Kan (2006; and references therein). Briefly, when the pathogen comes to rest on the leaf surface, in the form of conidia (asexual, non-motile spores of the fungus), this surface has to be breached by the pathogen. Appressoria, fungal infection structures that aid in the penetration of the leaf cuticle, are then formed. Presumably cutinases and lipases are excreted from this structure to aid in the penetration of the plant cuticle, but this process is still poorly understood. The part of the appressorium which penetrates the cuticle is referred to as the penetration peg, which subsequently grows into the anticlinal wall of the epidermal plant cell directly below the penetration point. This wall is pectin-rich and requires the action of the pectinolytic enzymes of *B. cinerea*, specifically the endopolygalacturonases, for successful invasion. Consequently, host tissue is killed and the formation of primary lesions ensues. This decomposition of plant tissue serves as nutrient for the growth of the fungus. Nutrients are sensed and signaling cascades activated which ultimately result in increased fungal biomass and the spread of the primary lesions beyond the initial infection site (Van Kan, 2006; and references therein).

B. cinerea ePGs display differences in substrate specificity, degradation rate and pH optimum for activity (Kars et al., 2005). pH optima for the ePGs were found to be 4.2 (BcPG1), 4.9 (BcPG4) and 4.5 (BcPG2 and BcPG6). A pH optimum of between 3.2 and 4.5 was observed for BcPG3, using PGA as substrate. On the same substrate, specific activity differences were found for the ePGs, with BcPG2, BcPG3 and BcPG6 exhibiting higher specific activities than either BcPG1 or BcPG4. Using pectin with different degrees of methylation (DM), the authors could show that for BcPG1, BcPG2 and BcPG4, the preferred substrate was unmethylated PGA. BcPG3 and BcPG6 exhibited the highest activity on pectin with 7 and 22% DM, respectively.

Also, bond hydrolysis rates of oligogalacturonides of defined length differed by up to 100-fold for the different ePGs (Kars et al., 2005). Undoubtedly, these different characteristics aid the fungus in hydrolyzing a wide range of pectic substances and the concerted action of the different ePGs help maintain the wide host range of the necrotroph.

The ePGs BcPG1 and BcPG2 have been studied in more detail and shown to be important for its pathogenicity on particular hosts (ten Have et al., 1998; Kars et al., 2005). A gene replacement mutant of *B. cinerea*, deficient in the *Bcpg1* gene, exhibited significant reductions in lesion growth rate on fruits of apple and tomato, as well as on tomato leaves (ten Have et al., 1998) (Figure 2.1). These mutants were found to retain their primary infection ability as compared to the control strains, but the growth of the lesions beyond the primary infection site was significantly decreased (ten Have et al., 1998).

Figure 2.1. Infective ability of a mutant and wild-type strain of *Botrytis cinerea* on tomato leaf tissue, following inoculation of conidia on the leaflet. (TOP HALF) Mutant Δ39, with the *Bcpg1* gene replaced with a gene encoding hygromycin resistance, exhibits a significant reduction in lesion growth rate at 144 hours post-inoculation (hpi). (BOTTOM HALF) Lesions formed by wild-type strain B05.10 at 144 hpi have spread to cover a much larger leaf area, indicative that BcPG1 is involved in lesion expansion in the infection process of tomato plants. Adopted from ten Have et al. (1998).

Similarly, for *Bcpg2* mutants, replaced by either the hygromycin or nourseothricin antibiotic resistance genes, was reduced pathogenicity of the fungus observed on tomato and broad bean tissue (Kars et al., 2005). Not only was the expansion phase of primary lesions delayed by 24 hours in the leaves infected with these mutants, but the expansion rate of the resulting lesions was reduced by between 50 and 85% when compared to the wild-type strain B05.10. The authors concluded that *Bcpg2* fulfilled a major role in both the initial infection and the subsequent spread of the lesion beyond this site (Kars et al., 2005). Also, an active site mutant of BcPG2, lacking enzyme activity but retaining the correct protein structure, was unable to hydrolyze polygalacturonic acid or cause any symptoms when infiltrated in broad bean or tomato tissue, even at high concentrations, indicating that enzyme activity is essential for BcPG2 to cause tissue necrosis (Kars et al., 2005). In contrast, an inactive BcPG1, harboring an active site mutation, was still able to cause necrosis

and tissue damage to a similar extent as the wild-type when infiltrated into *Nicotiana benthamiana* leaf tissue (Joubert et al., 2007). Additionally, the necrotizing activity of the ePGs BcPG1 and BcPG2 were found to be much higher than any of the other four *Botrytis* PGs on bean and tomato leaf tissue (Kars et al., 2005). The authors proposed that these two enzymes may cleave specific linkages in the pectin backbone which play critical roles in the maintenance of the integrity of the pectic matrix, but such linkages remain, as yet, unidentified. Taken together, the results to date suggest a major role for the ePGs BcPG1 and BcPG2 in the pathogenicity of the fungus *B. cinerea* on specific host plants. Future work will no doubt include the assessment of the role of the remaining four ePGs in the pathogenicity of the broad host range fungus *B. cinerea* on other plant hosts.

In addition to *Botrytis*, the fungi *Claviceps purpurea*, *Alternaria citri* and *Aspergillus flavus* rely on ePGs for their pathogenicity. Mutants of *C. purpurea* were rendered nearly nonpathogenic on rye (*Secale cereale*) when the two polygalacturonase-encoding genes, *cppg1* and *cppg2*, were replaced with a gene encoding phleomycin resistance (Oeser et al., 2002). The ability of *A. citri* to cause Alternaria rot on citrus or tissue maceration of potato tubers was considerably reduced when an ePG was disrupted (Isshiki et al., 2001). Introduction of the gene encoding P2c, an *A. flavus* polygalacturonase, into a strain lacking the gene increased the aggressiveness of the infection of the strain on cotton bolls (Shieh et al., 1997). Conversely, deletion of the same gene in a $P2c^{+}$ strain reduced the pathogenicity of the strain on the same host.

Agro-infiltration of a construct containing the gene encoding *C. lindemuthianum* ePG (clPG) resulted in an active ePG being expressed in tobacco three days post-infiltration (dpi) (Boudart et al., 2003). At four dpi, progressive cell wall degradation initiated, culminating in the spread of tissue degradation extending to the whole intercellular space after seven dpi. Adjacent cells were showing evidence of cell separation as the middle lamella was degraded. None of these symptoms was observed for empty vector infiltrations.

ePGs seem to be critical for fungal infection of plant tissue. In-depth analyses of *B. cinerea* ePGs has significantly added to our understanding of their role in plant pathogenesis. Also, several authors have examined the inhibition of these ePGs by the plant inhibitors, PGIP. For BcPG1, an enzyme crucial for full *Botrytis* pathogenicity, inhibition by various PGIPs is reported. However, for BcPG2, equally important for *Botrytis* pathogenicity, *in vitro* inhibition by PGIP is yet to be shown. Recently, however, Joubert et al. (2007) could show that *in planta*, the action of this enzyme is indeed inhibited by VvPGIP, even though no *in vitro* interaction and inhibition could be observed. The authors proposed that the PGIP interacted with the pectic substrate (as proposed by Spadoni et al., 2006) and not directly with BcPG2. These findings highlighted the need to include *in vivo* methodologies in the study of ePGs and their plant inhibitors.

2.3 ePG Inhibitors (PGIP)

2.3.1 Genomic Organization

Genes encoding PGIP have been isolated from numerous monocotyledonous and dicotyledonous plant species (De Lorenzo et al., 2001; De Lorenzo et al., 2002). In fact, sequences for more than 120 *pgip* genes have been deposited in GenBank (Gomathi and Gnanamanickam, 2004). PGIP-like activity has also been reported in the floral nectary of a tobacco line overproducing nectar (Thornburg et al., 2003). Whether this activity is indeed resultant from a PGIP protein; and whether it is present in plant organs other than the floral nectary, remains to be seen. The aim of this review is not to be an exhaustive account of all cloned *pgip* genes and their encoded proteins, but will instead focus on recent data regarding PGIPs encoded for by multigene families in *Arabidopsis* (Ferrari et al., 2003), French bean (*Phaseolus vulgaris*) (D'Ovidio et al., 2004), *Brassica napus* (Li et al., 2003), soybean (*Glycine max*) (D'Ovidio et al., 2006) and the monocots rice and wheat (Janni et al., 2006). In the next section, the regulation of the expression of these multigene families in response to stress conditions will be discussed.

The four members of the *pgip* gene family and their encoded proteins from *P. vulgaris* has enjoyed the most interest of all cloned *pgip* genes, and the structure of PvPGIP2 is, to date, the only PGIP to have its structure solved (Di Matteo et al., 2003). Leckie et al. (1999), in characterizing the first two members were able to show that a single amino acid change between PvPGIP1 and PvPGIP2 was able to confer a new recognition capability towards ePG of *Fusarium monoliforme*. More recently, the full complement of *Pvpgip* genes in *P. vulgaris* genotypes BAT93 (Figure 2.2) and Pinto were characterized (D'Ovidio et al., 2004). Four intronless *pgip* genes are located on a 50 kb locus, with distances of 17, 15 and 8 kb separating the *Pvpgip* genes 1-4, respectively. From Pinto, small variations in *Pvpgip2* (*Pvpgip2.1* and *Pvpgip2.2*) include four synonymous amino acid substitutions in the coding region and a 2 bp insertion in the untranslated region. It was concluded that these changes were due to the nature of the seed composition of the commercially available Pinto, which includes several bean varieties. In both genotypes the four genes could be divided into two distinct groups based on their sequence similarity. The pair *Pvpgip1/Pvpgip2* could be separated from *Pvpgip3/Pvpgip4* by the presence of two amino acid insertions in the *Pvpgip3/Pvpgip4* gene products. The two encoded proteins, PvPGIP1 and PvPGIP2, also lack the typical first N-linked glycosylation site. PvPGIP3 is additionally separated from PvPGIP4, in both genotypes, by the presence of an additional Cys residue in the C terminal domain and two amino acid deletions in two of the leucine-rich repeats (LRR). Comparison of BAT93 and Pinto genotypes revealed that they differed with a nonsynonymous nucleotide change in both *Pvpgip1* and *Pvpgip3*, while *Pvpgip4* genes were identical. Most strikingly, *Pvpgip2* from BAT93 shows a nine nucleotide deletion as compared to Pinto

Pvpgip2.2. This deletion includes the stretch which harbors the residue shown to be critical for recognition of *F. monoliforme* ePG. Interestingly, differential inhibition of fungal ePGs was observed for all four PGIP proteins, while, in addition, those of the group *Pvpgip3/Pvpgip4* also inhibited ePGs of insect origin.

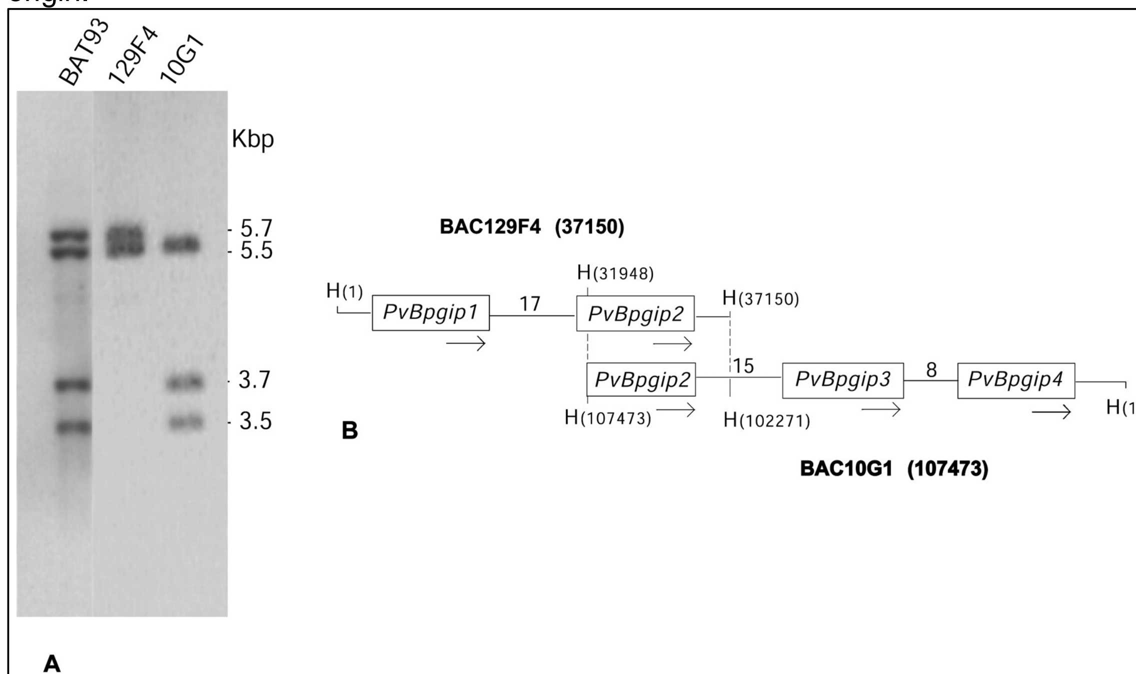


Figure 2.2. Genomic organization of the *Phaseolus vulgaris pgip* gene family in the BAT93 genotype. **(A)** Southern blot analysis of DNA digested with both *Eco*RI and *Hin*dIII from BAT93 and the two plasmids containing *Pvpgip* sequence: BAC clones 129F4 and 10G1. The presence of the 5.5 kb overlapping fragment is observed in both BAC clones following hybridization to a stretch of the *Pvpgip1* gene from genotype Pinto. **(B)** Schematic representation of the organization of the *Pvpgip* gene family as found on the two BAC clones. An overlapping stretch of 5.5 kb includes the *Pvpgip2* gene in both clones. The direction of the coding region, from ATG to stop codon, is indicated by the arrows. *Pvpgip1-4* share the same orientation. *Hin*dIII sites (H) and their positions in the BAC clones are indicated in brackets after H. The distances between *Pvpgip* ORFs are indicated in kb between them (17, 15 and 8 kb between *Pvpgip1-4*, respectively). Adopted from Di Matteo et al., 2004).

The *pgip* family from the closely related *G. max* (soybean) has also been characterized (D'Ovidio et al., 2006). French and soybean plants fall within a subdivision of the Leguminoseae family, the Phaseoleae tribe. Not surprisingly, a small family of at least four *pgip* genes (*Gmpgip1-4*) was found to be present in the soybean genome. As with French bean, these genes could be divided in pairs based on sequence similarity. The two pairs (*Gmpgip1/ Gmpgip2* and *Gmpgip3/ Gmpgip4*) share 77% nucleotide and 63% amino acid similarity. The pair *Gmpgip1/ Gmpgip2* both contains an additional Cys residue; *Gmpgip1* in the first and *Gmpgip2* in the fourth LRR. The distance between the former is about 3 kb and the latter a maximum of 60 kb. However, functional redundancy, as seen in PvPGIPs, was not observed since GmPGIP3 proved to be the only protein with inhibitory capability towards fungal ePGs.

Tandemly repeated *pgip* genes located within 507 bp of each other were cloned from *Arabidopsis thaliana* (Ferrari et al., 2003). The genes *Atpgip1* and

Atpgip2 are interrupted by short introns. Nucleotide homology of 78% and amino acid similarity of 76% is shared between these genes and their encoded proteins.

Four *pgip* genes were found to be present in *B. napus* (Li et al., 2003). The two tandemly repeated *Bnpgip3* and *Bnpgip4* genes share 80% sequence similarity to *Bnpgip1*. Both *Bnpgip1* and *Bnpgip2* contain short introns of 86 and 72 bp, respectively, as observed for the two *Atpgip* genes.

Multigene families encoding PGIPs are also present in monocotyledonous plants. Their relationship and sequence similarities and those of several dicots are shown in Figure 2.3. Small *pgip* gene families have been cloned from the monocots rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Janni et al., 2006). Four rice *pgip* genes (*Ospgip1-4*), distributed over a 30 kb region, are located on the short arm of chromosome 5. Chromosome 7B and 7D were found to be the location of two wheat *pgip* genes (*Tapgip1* and *Tapgip2*), respectively. *Ospgip1-4* contains open reading frames (ORFs) of 927, 1029, 1020 and 1050 bp, respectively. The shorter ORF of *Ospgip1* is reflected in the translated OsPGIP1- containing a shorter signal peptide and exhibiting the absence of the seventh LRR. Thus, OsPGIP1 contains only nine LRR modules compared to the ten of OsPGIP2-4, which is typical of PGIP topology. These three rice PGIPs also contain an extra Cys residue in the C-terminal region, compared to the characteristic eight residues found in dicotyledonous PGIPs and OsPGIP1. The potential glycosylation sites in these proteins vary between four and ten, of which three occur in corresponding positions in all four proteins. The calculated pI values of these proteins (OsPGIP1-4 have pIs of 6.6, 4.6, 5.7 and 7.8, respectively) seem to be lower than the typical values found in the dicotyledonous PGIPs so far isolated (pI between 8 and 9).

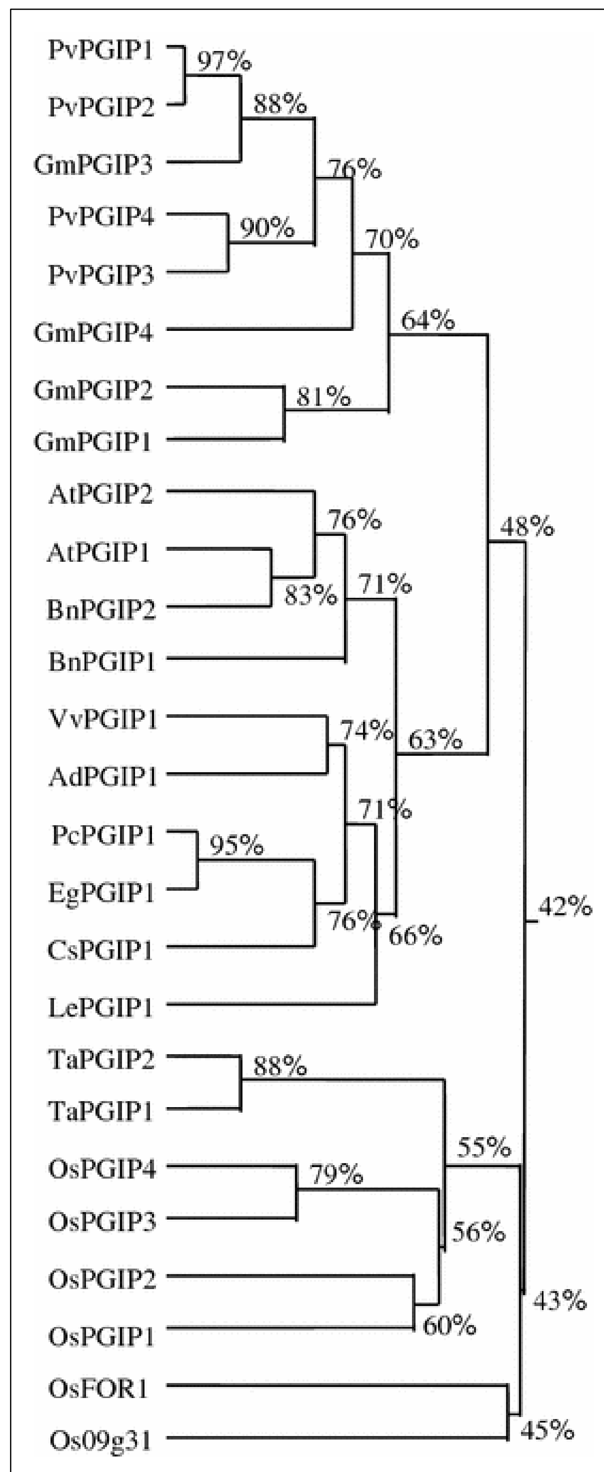


Figure 2.3. Phylogenetic tree depicting the similarity between mature PGIP proteins from both monocotyledonous and dicotyledonous plant species. The species and GenBank accession numbers of dicots are: AdPGIP1 (*Actinidia deliciosa*, Z49063); AtPGIP1 and AtPGIP2 (*Arabidopsis thaliana*, AF229249, AF229250); BnPGIP1 and BnPGIP2 (*Brassica napus*, AF529691, AF529693); CsPGIP1 (*Citrus sinensis*, Y08618); EgPGIP1 (*Eucalyptus grandis*, AF159167); GmPGIP1-4 (*Glycine max*, AJ972660-AJ972663); LePGIP1 (*Lycopersicon esculentum*, L26529); PcPGIP1 (*Pyrus communis*, L09264); PvPGIP1-4 (*Phaseolus vulgaris*, AJ786408-AJ786411); VvPGIP1 (*Vitis vinifera*, AF499451). The monocots are: TaPGIP1 and TaPGIP2 (*Triticum aestivum*, AM180656, AM180657); OsPGIP1-4 (*Oryza sativa*, AM180652-AM180655); OsFOR1 (*Oryza sativa*, AF466357); Os09g31 (*Oryza sativa*, AC108762). Rice (OsPGIPs) and wheat PGIPs (TaPGIPs) form a cluster which separates from the dicotyledonous PGIPs. Within each cluster (monocot or dicot), a similar extent of sequence variability is observed. Adopted from Janni et al. (2006).

The rice *pgips* could also be separated in subgroups based on sequence homology. OsPGIP1/OsPGIP2 and OsPGIP3/OsPGIP4 share 60% and 79% sequence identity. An additional gene encoding a protein with PGIP activity against *Aspergillus niger* ePG, *OsFOR1* (*Oryza sativa floral organ regulator1*), has previously been cloned from rice (Jang et al., 2003), and was the first monocot *pgip* gene to be reported. Apart from its ability to inhibit ePGs, this protein regulates the number of floral organs in rice, as the name implies.

The wheat PGIP, TaPGIP2, is distinguished from TaPGIP1 by short deletions in the N-terminal region and single substitutions spread along the length of the mature protein, resulting in sequence similarity of 88% on amino acid level. Both proteins contain five putative glycosylation sites and exhibit comparable pI values (6.25) to that of the monocot rice.

From these observations, it is apparent that plants have retained small *pgip* gene families through evolution. The advantage of maintaining multiple members becomes apparent when considering their differential responses to various environmental stimuli (section 2.3.2) and inhibition spectra (section 2.3.3). Thus, *pgips* from multigene families are not only differentially regulated, but harbor different inhibitory capabilities.

These small gene families encode proteins exhibiting the characteristic PGIP topology, as described by Di Matteo et al. (2003), following crystallographic analysis (described in section 2.4). This includes a short signal peptide, followed by the N-terminal domain. The 10 module LRR domain is present in all PGIPs described, save OsPGIP1, which harbors nine; followed by the short C-terminus. The LRR consensus sequences of these genes also precisely match that of the plant extracellular LRR (eLRR) resistance genes (Ferrari et al., 2003; Li et al., 2003; D'Ovidio et al., 2004; D'Ovidio et al., 2006; Janni et al., 2006).

2.3.2 *pgip* Genes are Expressed in Response to Stress

Transcript analyses following several biotic and abiotic stress conditions indicate that the regulation of *pgips* in gene families has diversified (Table 2.1). Within the gene families, different members can be seen to be upregulated in response to various stresses. For example, *Pvpgip2*, encodes a protein with a broad inhibition spectrum (section 2.3.3) and is upregulated rapidly by four different stimuli. *Pvpgip1* and *Pvpgip3* were only upregulated following wounding or oligogalacturonide treatment respectively, while *Pvpgip4* did not respond to any of these inductions. While all *Gmpgips* responded to *Sclerotinia* infection, *Gmpgip1, 3* and *4* were also responsive to wounding.

B. napus and *A. thaliana pgip* inductions provide further evidence of differential regulation of the two *pgip* genes in both genotypes. Both *Bnpgip1* and *Bnpgip2* were induced following jasmonate treatment and *Sclerotinia* infection, however, *Bnpgip1* was additionally upregulated by low temperature, herbivory and

wounding. Unfortunately, the *Brassica* proteins were not tested for inhibitory ability against fungal or insect PGs. *Atpgip1* and *Atpgip2* were induced following wounding and *Botrytis* infection. Both these inhibitors are active against crude preparations of *B. cinerea* ePG, and it is therefore interesting to note that in the case of *Atpgip1*, this gene is also induced following oligogalacturonide treatment and low temperature exposure. *Atpgip2*, on the other hand, was inducible by methyl jasmonate treatment.

Table 2.1. Regulation of the expression of several *pgip* genes following stress-related inductions. *Pgip* genes were differentially induced by different biotic or abiotic stress treatments.

Plant species	Transcript	Induced by	Induced within	Treatments tested	Reference
Phaseolus vulgaris	*Pvpgip1*	W	3 h	W, SA, OG, FG	D'Ovidio et al., 2004
	Pvpgip2	W, SA, OG, FG	3 h (all treatments)		
	Pvpgip3	OG	3 h		
	Pvpgip4	n/i	n/i		
Glycine max	*Gmpgip1*	W, Ss	8 h(W), 8 h(Ss)	W, Ss	D'Ovidio et al., 2006
	Gmpgip2	Ss	48 h(Ss)		
	Gmpgip3	W, Ss	8 h(W), 8 h(Ss)		
	Gmpgip4	W, Ss	8 h(W), 16 h(Ss)		
Brassica napus	*Bnpgip1*	JA, W, low °C, Ss herb	1 h(JA), 0.5 h(W), days(low°C), 24 h(Ss), 7 h(FB)	JA, SA, W, low °C, FB, Ss, D	Li et al., 2003
	Bnpgip2	JA, Ss	1 h(JA), 24 h(Ss)		
Arabidopsis thaliana	*Atpgip1*	W, Bc, OG, low °C	8 h(W), 48 h(Bc), 1.5 h(OG), 24 h(low °C)	W, Bc, OG, low °C, SA, MeJA	Ferrari et al., 2003
	Atpgip2	W, Bc, MeJA	8 h(W), 48 h(Bc), 48 h(MeJA)		

Legend: W, wounding; SA, salicylic acid; JA, jasmonic acid; MeJA, methyl jasmonate; OG, oligogalacturonides; FG, fungal glucan; Bc, *Botrytis cinerea* infection; Ss, *Sclerotinia sclerotiorum* infection; low °C, low temperature (4°C); FB, flea beetle; D, dehydration; n/i, no induction under any of the treatments tested.

These data provide some insight into the evolution of the host-pathogen interaction in the case of *Botrytis* ePG and *Arabidopsis* PGIP. Should the pathogen *Botrytis* evolve mechanisms to circumvent the induction of a specific plant defense pathway (e.g. methyl jasmonate) leading to *pgip* expression (for example *Atpgip2*), a gene encoding a PGIP with activity against *Botrytis* ePG (AtPGIP1) would still be induced, although via another signal transduction pathway.

2.3.3 PGIP Inhibition Spectra Reveals Their Role in Defense

The different inhibition spectra of PGIPs encoded for by multigene families provides compelling evidence that these proteins have evolved to inhibit a multitude of polygalacturonases from different sources. Analysis of the French bean PGIP family

reveals that the pairs PvPGIP1/PvPGIP2 and PvPGIP3/PvPGIP4 are specialized for defense against fungi and insects (D'Ovidio et al., 2004; D'Ovidio et al., 2006). PvPGIP2, arguably the most potent inhibitor of fungal ePGs analyzed to date, has the ability to inhibit ePGs from *S. sclerotiorum, F. monoliforme, F. graminearum, Botrytis aclada, B. cinerea, A. niger and C. acutatum* (D'Ovidio et al., 2004; D'Ovidio et al., 2006). Among the four PvPGIPs, it is also the only inhibitor to show activity against ePG from *F. monoliforme* (FmPG). Leckie et al. (1999) were able to show that a single amino acid substitution from PvPGIP1 to a corresponding residue in PvPGIP2 conferred to it the ability to inhibit FmPG. All four PvPGIPs inhibit PGs from *Stenocarpella maydis*, *C. acutatum* and *B. cinerea*, albeit with different efficiencies. Generally less PvPGIP1 or PvPGIP2 protein was necessary for the inhibition of these ePGs (4.5-200 ng), compared to the pair PvPGIP3/PvPGIP4 (65-1400 ng). *A. niger* ePG is inhibited by all, save PvPGIP3. In addition to the fungal inhibition spectra of the PvPGIP3/PvPGIP4 pair, it was found that these two proteins are, remarkably, also able to inhibit PGs from two mirid bugs, *Lygus rugulipennis* and *Adelphocoris lineolatus* (Di Matteo et al., 2004). Therefore, in *P. vulgaris*, the two pairs form two distinct classes- those evolved to effectively inhibit fungal ePGs, but not insect PG (PvPGIP1/PvPGIP2); and the PvPGIP3/PvPGIP4 pair which has evolved to inhibit insect PG, with some weaker activity against fungal ePGs. In the closely related *G. max* (D'Ovidio et al., 2006), fungal ePG inhibitory activity is only observed for the member GmPGIP3, of a small family of four PGIPs. However, testing a larger panel of fungal ePGs may reveal inhibitory activity for these PGIPs. This protein is able to inhibit the same set of fungal ePGs as PvPGIP2, with comparable efficiencies.

Rice and *Arabidopsis* PGIPs also inhibit ePGs from different sources. Interestingly, the rice PGIP, OsPGIP1, which lacks the entire seventh LRR repeat, retains the ability to interact with and inhibit fungal ePGs (Janni et al., 2006). ePGs from *S. sclerotiorum*, *F. graminearum*, *A. niger* and *B. cinerea* are among those inhibited by the rice protein. *A. thaliana* PGIPs inhibit ePGs from *Colletotrichum gloeosporoides*, *S. maydis* and *B. cinerea*. Although *B. cinerea* ePGs are equally efficiently inhibited by AtPGIP1 and AtPGIP2, those from *C. gloeosporoides* and *S. maydis* are more efficiently inhibited by AtPGIP1 (Ferrari et al., 2003).

Data regarding the inhibition of ePGs by PGIP has largely been substantiated by *in vitro* studies. A need for complementing the *in vitro* inhibition studies by *in vivo* means has arisen and will greatly add to the growing body of *in vitro* knowledge. Data emerging from *in planta* studies, including the recent work of Joubert et al. (2007), have shown that a lack of *in vitro* inhibition is not necessarily a reflection of what transpires in the *in planta* context, whether this inhibition is direct or by other means. Also, the individual ePGs inhibited by different PGIP proteins remain relatively uncharacterized. ePG inhibition studies, more often than not, rely on crude preparations of these enzymes, therefore the inhibition spectra reflected is that of all ePGs expressed in the growing fungal culture, by whichever means cultivated.

2.4 Structures of PGIP, ePG and Their Interaction

The structure of *P. vulgaris* PGIP2 (Figure 2.4) was solved at 1.7 Å resolution (Di Matteo et al., 2003). This is, to date, the only LRR protein of plant origin to have its structure solved. The overall protein structure is shown in Figure 2.4a. Amino acid residues 53-289 represent the central LRR domain, consisting of 10 tandem repeats of 24 residues each. This central domain forms a right-handed superhelix. Within this domain, the residues form two parallel β-sheets (B1 and B2), with B1 occupying the inner concave side of the protein, while the additional sheet B2 characterizes the fold of the protein.

Sheet B1, at its centre, forms a negative pocket of charged residues. These consist of three aspartic residues, conserved in all PGIP proteins (De Lorenzo et al., 2001), and a serine and two threonine residues. Bishop (2005) noted that seven of nine sites, identified as positively selected by phylogenetic codon-substitution models, were scattered around this negatively charged pocket. Leckie et al. (1999) have shown that within this region, a single amino acid substitution could confer a new ePG recognition capability to a PGIP unable to previously interact with it. Nine 3_{10}-helices are situated opposite and nearly parallel to sheet B1, representing the secondary structural elements of the convex side of PGIP2 (Di Matteo et al., 2003).

The β-sheet B2 found in PGIP2 is typically absent in LRR proteins. The B1 sheets and helices in these proteins are connected by loops or β-turns. Figure 2.4b shows the homology between several plant extracellular LRRs (eLRRs) and PGIP2. These plant-derived eLRR proteins are therefore most likely to contain the additional β-sheet as observed in PGIP2. Di Matteo et al. (2003) argued that this additional sheet may form a second ligand interaction surface.

The protein termini as well as conserved residues in the LRR serve to ensure the formation of the hydrophobic core of the protein. At the N-terminus, the α-helix and a small β-sheet interact with the B1 sheet. Two disulfide bridges at both the N- and C-termini, as well as the structure of the C-terminus (the last two 3_{10}-helices, one strand of the B2 sheet and loop) help to shape the hydrophobic core. Conserved residues of the LRR motif are involved in stacking interactions that also help to form the hydrophobic solenoid.

Residues in both PvPGIP1 and PvPGIP2 have been shown to be critical for the binding of these inhibitors to plant cell wall pectin (Spadoni et al., 2006). A pectin binding motif is located between the β-sheets B1 and B2, in close proximity to the negative cleft that is proposed to be involved in ligand binding (Di Matteo et al., 2003). This cleft shows a binding affinity for blockwise demethylated stretches of pectin. The pectin binding motif consists of three arginine residues and one lysine residue (R183, R206, K230 and R252), creating a cluster of positive charges evenly exposed to the solvent. Single mutations in the motif caused altered binding ability of the plant inhibitor to polygalacturonic acid, while double, triple and quadruple mutations completely abolished this binding capability. These mutations, however,

did not affect the binding of PGIP to its ligand. Also, the authors could show that upon formation of the PGIP-ePG complex, PGIP was displaced from its binding with pectin. This displacement was consistent with the specificity of the interaction between the inhibitor and ePG. Under *in vitro* conditions, PGIP bound most effectively to the uronic-acid rich cell wall fraction, at a pH of 5.0 and in the presence of Ca^{2+}. At higher pH values (pH 7.0) no binding of PGIP to polygalacturonic acid was observed. Since pathogen attack is associated with a rise in apoplastic pH values (Bolwell et al., 2002), it is plausible that PGIP may be released from pectin under these conditions to participate in the inhibition of the fungal macerating activity of fungal ePGs.

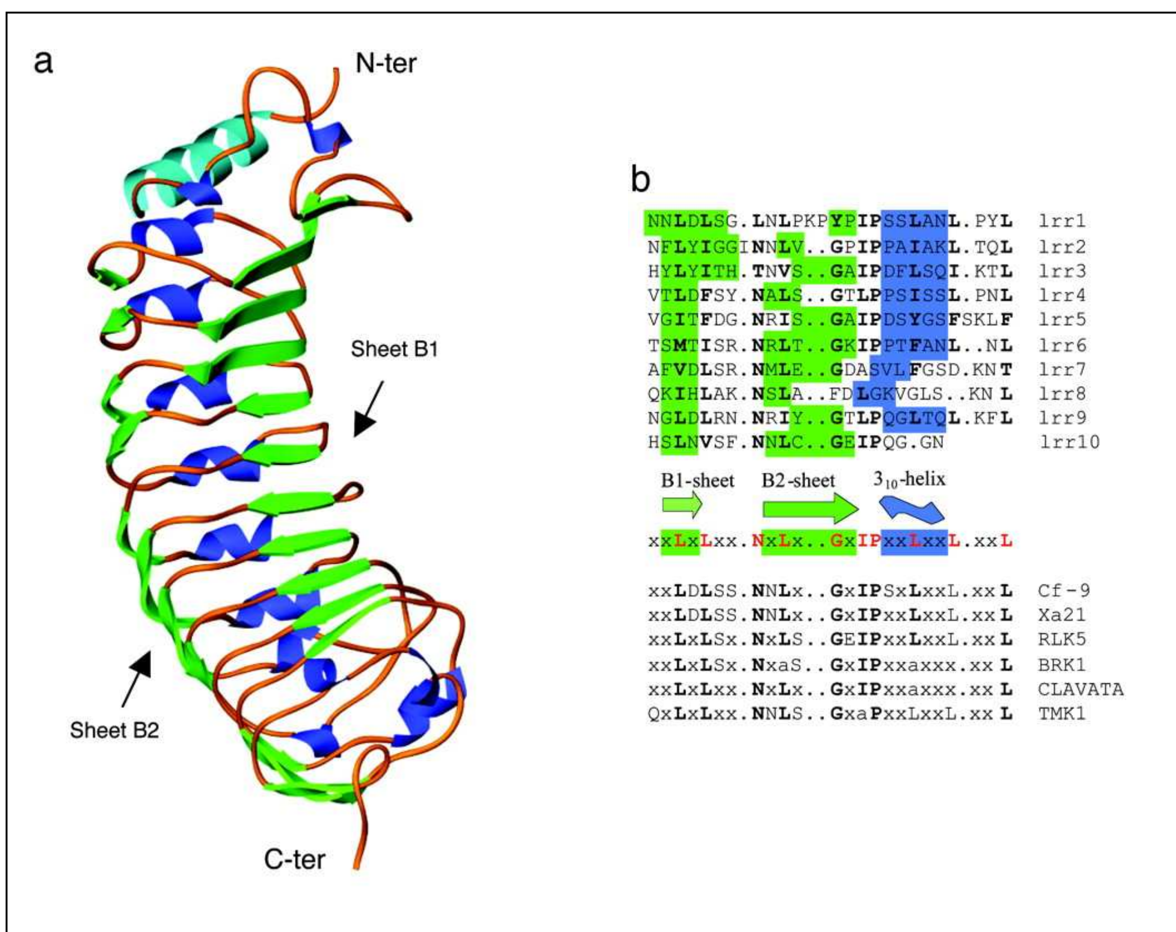


Figure 2.4. (a) The structure of *Phaseolus vulgaris* PGIP2. The two parallel β-sheets (B1 and B2) are colored in green, the N-terminal α-helix in light blue, the 3_{10}-helices in the central LRR dark blue. **(b)** The secondary structure of PvPGIP2 residues 53-289, forming the LRR motif. Amino acid residues which help to form this structure in the protein are indicated: green for the two β-sheets and blue for the 3_{10}-helices. Plant proteins harboring the eLRR consensus sequence and showing homology to PvPGIP2 are shown. Cf-9, Jones et al. (1994); Xa21, Song et al. (1995); RLK5, Walker (1993); BRK1, Djakovic et al., 2006; CLAVATA, Clark et al. (1997) and TMK1, Chang et al. (1992). Adopted from Di Matteo et al., 2003.

The crystal structures of two fungal ePGs have been solved, and helps shed some light on its interaction with PvPGIP2. *F. monoliforme* ePG (*Fm*PG) (Federici et al., 2001) and *A. niger* ePGII (AnPGII) (van Santen et al., 1999) were solved at

1.73 Å and 1.68 Å, respectively. FmPG and AnPGII are remarkably similar and almost completely superimposable (Federici et al., 2001). Identical features between the two enzymes include the β-helix turn, containing an equal number of turns; β-strands of equal length and position; and the number of disulphide bridges, also occurring in the same positions. Conserved sites in the two ePGs include His-188, Lys-269 and Arg-267, and are important for the formation of contacts in the PG-PGIP complex. The latter two residues are putatively involved in substrate binding, and no specific role has been attributed to His-188. The authors also mutated this residue to a proline, together with insertion of a Trp after Ser-270, which mimics the consensus found in plant ePGs. While the modification of His-188 decreased the affinity for the formation of the ePG-PGIP complex, insertion of the Trp eliminated any remaining affinity. These findings help elucidate why plant ePGs escape inhibition by PGIP.

His-188 is located in the active cleft of the enzymes, at the opposite side of the active cleft where Lys-269 and Arg-267 reside (Federici et al., 2001). This poses an interesting mechanism by which PGIP may inhibit ePG: when the inhibitor is bound to ePG, the enzyme is prevented from binding to the substrate as the residues deemed necessary (Lys-269 and Arg-267), are in complex with PGIP. The multiple contacts with the three residues will also simultaneously cause the ePG active site, located between His-188 and Lys-269, to be covered (Federici et al., 2001). This is consistent with the competitive mode of inhibition observed for *Fm*PG and PvPGIP2. PGIPs may also exhibit a non-competitive (Lafitte et al., 1984; Johnston et al., 1993; Stotz et al., 2000) or a mixed-type inhibition (Yao et al., 1995).

These findings contribute significantly to the understanding of the interaction between ePG and PGIP and the formation of the complex between them. However, an aspect that remains unresolved is the events following the formation of this complex. Cervone et al. (1989) found that, *in vitro,* upon formation of the complex and subsequent inhibition of ePG, the lifespan of elicitor-active oligogalacturonides generated from polygalacturonic acid were extended. In turn, these oligogalacturonides may be able to induce defense-related pathways in plants. However, data supporting or refuting this hypothesis are yet to emerge. Chapter 5 of this dissertation addresses this issue in further detail.

2.5 Overexpression of *pgip* Reduces Susceptibility to *Botrytis*

The role of PGIPs in plant defense has been substantiated in transgenic plants overexpressing different *pgip* genes and showing reduced susceptibility towards infection by *B. cinerea*. These plant species include tomato, tobacco, *Arabidopsis* and grapevine (Powell et al., 2000; Ferrari et al., 2003; Aguero et al., 2005; Manfredini et al., 2005; Joubert et al., 2006 (Chapter 3, this work)). In the case of grapevine (Aguero et al., 2005), an increased tolerance to *Xylella fastidiosa*, the causal agent of Pierce's disease, was also observed.

Transgenic tobacco plants overexpressing the genes encoding *P. vulgaris* PGIP2 (*Pvpgip2*) (Manfredini et al., 2005) and *Vitis vinifera* PGIP1 (*Vvpgip1*) (Joubert et al., 2006, Chapter 3 of this dissertation) exhibited reduced susceptibility towards *Botrytis* infections. In both cases, transgenic tobacco plants exhibited significantly smaller lesions in detached leaf assays three days post-inoculation, employing very high fungal inocula. Tobacco plants overexpressing *Vvpgip1* were also shown to be less susceptible in whole-plant infection assays. The infections of wild-type and transgenic lines were followed for a period of fifteen days. Lesions on the untransformed plants expanded at a more rapid rate as compared to *Vvpgip1*-overexpressing lines, and this difference became apparent and statistically separable in the period between seven and 15 dpi, although transgenic lines showed an overall reduction in lesion size over the first seven days. Heterologously expressed PGIP was purified from these transgenic lines and shown to strongly inhibit *Botrytis* PG1 (BcPG1). This macerating enzyme is required for the full virulence of this fungus on several hosts (ten Have et al., 1998). These results suggest that the reduction of fungal disease development could, at least in part, be attributed to the inhibition of ePG by *Vitis* PGIP (Joubert et al., 2006, Chapter 3, this work).

Arabidopsis plants overexpressing native *pgip1* or *pgip2* (Ferrari et al., 2003), and antisense expression of the gene encoding AtPGIP1 (Ferrari et al., 2006) have confirmed the significance of appreciable levels of PGIP in the host at the time of fungal infection. Following *Botrytis* inoculation, plants overexpressing either *Atpgip1* or *Atpgip2* revealed smaller lesion diameters that were statistically separable from untransformed and non-expressing transgenic lines. *Arabidopsis* lines unable to accumulate PGIP (Ferrari et al., 2006) following infection not only exhibited a greater percentage of wet, spreading lesions, but these lesions were also significantly larger than wild-type lines that had the ability to accumulate these proteins. Transgenic *Arabidopsis* overexpressing *Pvpgip2* were also afforded a degree of protection from infection by this pathogen (Manfredini et al., 2005).

The economically important fruit crops, tomato and grapevine, also exhibited reduced susceptibility to *Botrytis* infections when overexpressing the pear *pgip* gene (Powell et al., 2000; Aguero et al., 2005). Homozygous transgenic tomato lines showed a reduction of fruit colonization by *Botrytis* of up to 15%, while reductions in lesion size of up to 24% for these lines were observed when tomato leaf material was inoculated. Similarly, for transgenic *V. vinifera* cvs. Thompson Seedless and Chardonnay reduced lesion expansion on infected leaves was observed in all transgenic lines tested, and in a high percentage of these (67%), the difference was statistically significant. Unfortunately, the fruits of these transgenic lines were not tested for *Botrytis* susceptibility. Some of the transgenic vines also exhibited an increased tolerance to the causal agent of Pierce's disease, *Xylella fastidiosa*. Although the bacterium was present in stem segments of the transgenic lines, it was at a considerably lower titer compared to that of the untransformed control plants (Aguero et al., 2005).

Although the aforementioned authors could show the efficacy of PGIP as antifungal strategy, none could shed light on the events following *Botrytis* infection in the transgenic lines. As the hypothesis of Cervone et al. (1989) suggests, differential induction of plant defense responses are expected in plants overexpressing *pgip* genes following fungal inoculation. This work (Chapter 5) is the first to address the differential induction of defense responses in lines with appreciable levels of PGIP.

The enhanced tolerance to two devastating grapevine pathogens (causing grey rot and Pierce's disease) using single gene transformation holds enormous promise for the grapevine industry. Additionally, in tomato overexpression of a *pgip* gene resulted in soft fruit exhibiting less severe disease symptoms when infected with *B. cinerea*. The efficacy of the overexpression of these genes in reducing pathogenicity has compellingly been shown for at least one economically important fungus. Concerning the specificity of these gene products, it is conceivable that soon this overexpression strategy may be utilized for controlling more plant diseases relying on, amongst others, ePGs for their pathogenicity (Di Matteo et al., 2006 and references therein).

2.6 Vitis PGIP: What do we know?

The gene encoding *Vitis vinifera* polygalacturonase-inhibiting protein 1 (VvPGIP1) was cloned from a 7.2 kb subgenomic fragment from a library constructed from the *Vitis* cultivar Pinotage. The intronless gene (Genbank accession AF499451) comprises an open reading frame of 1002 bp which encodes a deduced polypeptide of 333 amino acids. Indications are that *Vvpgip1*, curiously, does not belong to a multigene family. Expression analyses of the gene showed that it was strictly expressed in grape berries, at the onset of ripening. Expression levels increased following the veráison stage of berry ripening, after which it declined to seemingly undetectable levels. Promoter analysis revealed the presence of TATA and CAAT boxes, which were shown to be important for *Vvpgip1* expression. Additionally, the start of transcription was found to be 17 bp upstream of the putative ATG (De Ascensao-Slaughter, A., Joubert, DA., Becker, JVW., Pretorius, IS., Bellstedt, DU., Caprari, C., De Lorenzo, G. and Vivier, MA., unpublished results).

VvPGIP1 was subsequently purified from veráison berries and found to give rise to three polypeptides, ranging in mass from 37 to 41 kDa, presumably as a result of differential glycosylation of the protein. The protein was found to strongly inhibit crude ePG preparations from *B. cinerea* in a non-competitive manner. The protein also inhibited ePGs from *A. alternata*, *C. gloeosporoides*, *Monolinia laxa, Mycor* spp., *Penicillium expansum* and *Rhizopus stolonifer*, but to a lesser extent (De Ascensao-Slaughter, A., Joubert, DA., Becker, JVW., Pretorius, IS., Bellstedt, DU., Caprari, C., De Lorenzo, G. and Vivier, MA., unpublished results).

The gene was subsequently expressed to high levels in transgenic tobacco plants. The generation, analysis and subsequent resistance phenotypes conferred by

this gene are described in Joubert et al. (2006, Chapter 3 of this dissertation). The heterologous protein was also purified and shown to inhibit BcPG1 (Joubert et al., 2006), an ePG from *Botrytis* required for the full virulence of the fungus (ten Have et al., 1998).

Joubert et al. (2007) examined the effect of the transient expression of the gene encoding BcPG2 (*Bcpg2*), alone, and in combination with expression of *Vvpgip1*, in *Nicotiana benthamiana.* BcPG2, like BcPG1, is required for the full virulence of *Botrytis*. Infiltration of *Bcpg2* resulted in severe wilting and necrosis of the infiltrated tissue, but co-infiltration with *Vvpgip1* substantially reduced these symptoms. Surprisingly, no *in vitro* interaction between these gene products could be observed. Previously, it was found that PGIP may bind to pectin (Spadoni et al., 2006). These observations might suggest that *in planta*, a context is provided for the inhibition of BcPG2, although not *via* direct inhibition of BcPG2 by VvPGIP, but rather by shielding the pectic components vulnerable to digestion by BcPG2.

The work presented in this dissertation aims to add to this body of knowledge to eventually elucidate the full role of VvPGIP in plant defense.

2.7 Summary

Several aspects regarding polygalacturonase-inhibiting proteins (PGIPs) infer or confirm their role in defense against phytopathogenic fungi. These proteins are capable of inhibiting an extensive range of ePGs, from not only fungal, but also of insect origin. The inhibition potential of these proteins may extend to a much wider range of organisms, to date most of those tested have been shown to be inhibited to some degree. In reality, certain bacteria and nematodes also rely on ePGs for their pathogenicity, and it is conceivable that soon their activity will be screened for inhibition by PGIP. Small *pgip* gene families seem to have been maintained through evolution, providing plants with PGIPs with distinct inhibitory capabilities, which may also be regulated by separate signal transduction pathways. Furthermore, the structure of PGIP allows it to be targeted to the cell wall, where it interacts with ePGs.

Numerous genetic backgrounds in which *pgip* genes were overexpressed have been afforded a degree of protection from fungal virulence, more specifically the necrotrophic fungus *B. cinerea*. These include not only the model plants *Nicotiana tabacum* and *A. thaliana*, but the economically important fruit crops tomato and grapevine. In the case of the latter, an increased tolerance to a bacterial pathogen of grape was also observed. However, none of these authors were able to describe how the decrease in fungal susceptibility was afforded to these transgenic plants. Cervone et al. (1989) were able to show, *in vitro*, that PGIPs could favor the accumulation of elicitor-active molecules capable of initiating certain pathways of the plant defense response. Whether this will prove to be the case *in planta*, remains an open question.

eLRR proteins showing homology to PGIP have been shown to have roles other than the inhibition of ePG (Worrall et al., 1998; Becraft, 2002; Guyon et al.,

2004). Whether PGIP proteins exhibit functions other than the inhibition of ePG, which may ultimately aid in the reduction of fungal susceptibility, has not been investigated in any of the backgrounds where *pgip* genes were overexpressed.

The work described in this dissertation (Research Results) aims to shed some light on the mechanisms by which PGIP orchestrated the observed reduction in fungal susceptibility. Chapter 4 describes the possible physiological roles of *V. vinifera* PGIP, other than the inhibition of ePG, which may contribute to the observed decreased susceptibility; while chapter 5 aims to elucidate the pathways affected in transgenic lines overexpressing the VvPGIP encoding gene, following *B. cinerea* infection, specifically at the local infection sites.

2.8 References

Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pathol. 6: 43-51.

Annis, S.L., and Goodwin, P.H. 1997. Recent advances in the molecular genetics of plant cell-wall degrading enzymes produced by plant pathogenic fungi. Eur. J. Plant Pathol. 103: 1-14.

Becraft, P.W. 2002. Receptor kinase signaling in plant development. Annu. Rev. Cell Dev. Biol. 18: 163-192.

Bishop, J.G. 2005. Directed mutagenesis confirms the functional importance of positively selected site in polygalacturonase-inhibitor protein. Mol. Biol. Evol. 22: 1531-1534.

Bolwell, G.P., Bindschedler, L.V., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C., and Minibayeva, F. 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J. Exp. Bot. 53: 1367-1376.

Boudart, G., Charpentier, M., Lafitte, C., Martinez, Y., Jauneau, A., Gaulin, E., Esquerré-Tugayé, M-T., and Dumas, B. 2003. Elicitor activity of a fungal polygalacturonase in tobacco requires a functional catalytic site and cell wall localization. Plant Physiol. 131: 93-101.

Centis, S., Guillas, L., Séjalon, N., Esquerré-Tugayé, M-T., and Dumas, B. 1997. Endopolygalacturonase genes from *Colletotrichum lindemuthianum*: cloning of *clpg2* and comparison of its expression to that of *clpg1* during saprophytic and parasitic growth of the fungus. Mol. Plant Microbe Interact. 10: 769-775.

Cervone, F. Hahn, M.G., De Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. 90: 542-548.

Chang, C., Schaller, G.E., Patterson, S.E., Kwok, S.F., Meyerowitz, E.M., and Bleecker, A.B. 1992. The TMK1 gene from *Arabidopsis* codes for a protein with structural and biochemical characteristics of a receptor protein kinase. Plant Cell 4: 1263-1271.

Clark, S.E., Williams, R.W., and Meyerowitz, E.M. 1997. The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89: 575–585.

De Lorenzo, G., D'Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu. Rev. Phytopath. 39: 313-335.

De Lorenzo, G., and Ferrari, S. 2002. Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. Curr. Opin. Plant Biol. 5: 1-5.

Di Matteo, A., Federici, L., Mattei, B., Salvi, G., Johnson, K.A., Savino, C., De Lorenzo, G., Tsernoglou, D., and Cervone, F. 2003. The crystal structure of polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein involved in plant defense. Proc. Natl. Acad. Sci. USA 17: 10124-10128.

Di Matteo, A., Bonivento, D., Tsernoglou, D., Federici, L., and Cervone, F. 2006. Polygalacturonase-inhibiting protein (PGIP) in plant defence: a structural view. Phytochemistry 67: 528-533.

Djakovic, S., Dyachok, J., Burke, M., Frank, M.J., and Smith, L.G. 2006. BRICK1/HSPC300 functions with SCAR and the ARP2/3 complex to regulate epidermal cell shape in *Arabidopsis.* Development 133: 1091-1100.

D'Ovidio, R., Raiola, A., Capodicasa, C., Devoto, A., Pontiggia, D., Roberti, S., Galletti, R., Conti, E., O'Sullivan, D., and De Lorenzo, G. 2004. Characterization of the complex locus of bean encoding polygalacturonase-inhibiting proteins reveals subfunctionalization for defense against fungi and insects. Plant Physiol. 135: 2424-2435.

D'Ovidio, R., Roberti, S., Di Giovanni, M., Capodicasa, C., Melaragni, M., Sella, L., Tosi, P., and Favaron, F. 2006. The characterization of the soybean polygalacturonase-inhibiting proteins (*Pgip*) gene family reveals that a single member is responsible for the activity detected in soybean tissues. Planta 224: 633-645.

Esquerré-Tugayé, M-T., Boudart, G., and Dumas, B. 2000. Cell wall degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. Plant Physiol. Biochem. 38: 157-163.

Federici, L., Caprari, C., Mattei, B., Savino, C., Di Matteo, A., De Lorenzo, G., Cervone, F., and Tsernoglou, D. 2001. Structural requirements of *endo*polygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein). Proc. Natl. Acad. Sci. USA 98: 13425-13430.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. 2003. Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins (PGIP) are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15: 93-106.

Ferrari, S., Galletti, R., Vairo, D., Cervone, F., and De Lorenzo, G. 2006. Antisense expression of the *Arabidopsis thaliana AtPGIP1* gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. Mol. Plant Microbe Interact. 8: 931-936.

Fraissinet-Tachet, L., Reymond-Cotton, P., and Fevre, M. 1995. Characterization of a multigene family encoding an endopolygalacturonase in *Sclerotinia sclerotiorum*. Curr. Genet. 29: 96-99.

Gomathi, V., and Gnanamanickam, S.S. 2004. Polygalacturonase-inhibiting proteins in plant defence. Curr. Sci. 87: 1211-1217.

Guyon, V., Tang, W.H., Monti, M.M., Raiola, A., De Lorenzo, G., McCormick, S., and Taylor, L.P. 2004. Antisense phenotypes reveal a role for SHY, a pollen-specific leucine-rich repeat protein, in pollen tube growth. Plant J. 39: 643-654.

Isshiki, A., Akimitsu, K., Yamamoto, M., and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Mol. Plant Microbe Interact. 14: 749-757.

Jang, S., Lee, B., Kim, C., Kim, S-J., Yim, J., Han, J-J., Lee, S., Kim, S-R., and An, G. 2003. The *OsFOR1* gene encodes a polygalacturonase-inhibiting protein (PGIP) that regulates floral organ number in rice. Plant Mol. Biol. 53: 357-369.

Janni, M., Di Giovanni, M., Roberti, S., Capodicasa, C., and D'Ovidio, R. 2006. Characterization of expressed *Pgip* genes in rice and wheat reveals similar extent of sequence variation to dicot PGIPs and identifies an active PGIP lacking an entire LRR repeat. Theor. Appl. Genet. DOI 10.1007/s00122-006-0378-z.

Jarvis, W.R. 1977. *Botryotinia* and *Botrytis* species; taxonomy, physiology and pathogenicity. Monograph No. 15, Canadian Department of Agriculture, Ottawa.

Johnston, D.J., Ramanathan, V., and Williamson, B. 1993. A protein from immature raspberry fruits which inhibits polygalacturonases from *Botrytis cinerea* and other micro-organisms. J. Exp. Bot. 44: 971-976.

Jones D.A., Thomas C.M., Hammond-Kosack K.E., Balint-Kurti, P.J., and Jones, J.D.G. 1994. Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789–793.

Jones, T.M., Anderson, A.J., and Albersheim, P. 1972. Host-pathogen interactions IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. 2: 153-166.

Joubert, D.A., Slaughter, A.R., Kemp, G., Becker, J.V.W., Krooshof, G.H., Bergmann, C., Benen, J., Pretorius, I.S., and Vivier, M.A. 2006. The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. DOI 10.1007/s11248-006-9019-1.

Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and van Kan, J.A.L. 2007. A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *B. cinerea* in *N. benthamiana* leaves without any evidence for *in vitro* interaction. Mol. Plant Microbe Interact. (in print).

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and van Kan, J.A. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 43: 213-225.

Lafitte, C., Barthe, J.P., Montillet, J.L., and Touzé, A. 1984. Glycoprotein inhibitors of *Colletotrichum lindemuthianum* endopolygalacturonase in near isogenic lines of *Phaseolus vulgaris* resistant and susceptible to anthracnose. Physiol. Plant Pathol. 25: 39-53.

Leckie, F., Mattei, B., Capodicasa, C., Hemmings, A., Nuss, L., Aracri, B., De Lorenzo, G., and Cervone, F. 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. EMBO J. 18: 2352-2363.

Li, R., Rimmer, R., Yu, M., Sharpe, A.G., Séguin-Swartz, G., Lydiate, D., and Hegedus, D.D. 2003. Two *Brassica napus* polygalacturonase inhibiting protein genes are expressed at different levels in response to biotic and abiotic stresses. Planta 217: 299-308.

Li, R., Rimmer, R., Buchwalt, L., Sharpe, A.G., Seguin-Swartz, G. and Hegedus, D.D. 2004. Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of *endo*-and *exo*-polygalacturonases expressed during the saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

Manfredini, C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M., and De Lorenzo, G. 2005. Polygalactuonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol. Mol. Plant Pathol. 67: 108-115.

Oeser, B., Heidrich, P.M., Muller, U., Tudzynski, P., and Tenberge, K.B. 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. Fungal Genet. Biol. 36: 176-186.

Powell, A.L., van Kan, J.A., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. 13: 942-950.

Shieh, M.T., Brown, R.L., Whitehead, M.P., Cary, J.W., Cotty, P.J., Cleveland, T.E., and Dean, R.A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63: 3548-3552.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C., and Ronald, P. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science 270: 1804–1806.

Spadoni, S., Zabotina, O., Di Matteo, A., Mikkelsen, J.D., Cervone, F., De Lorenzo, G., Mattei, B., and Bellincampi, D. 2006. Polygalacturonase-inhibiting protein interacts with pectin through a binding site formed by four clustered residues of arginine and lysine. Plant Physiol. 141: 557-564.

Stotz, H.U., Bishop, J.G., Bergmann, C.W., Koch, M., Albersheim, P., Darvill, A.G., and Labavitch, J.M. 2000. Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. Physiol. Mol. Plant Pathol. 56: 117-130.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant Microbe Interact. 11: 1009-1016.

Thornburg, R.W., Carter, C., Powell, A., Mittler, R., Rizhsky, L. and Horner, H.T. 2003. A major function of the tobacco floral nectary is defense against microbial attack. Plant Syst. Evol. 238: 211-218.

van Santen, Y., Benen, J.A., Schroter, K.H., Kalk, K.H., Armand, S., Visser, J., and Dijkstra, B.W. 1999. 1.68-Å crystal structure of endopolygalacturonase II from *Aspergillus niger* and identification of active site residues by site-directed mutagenesis. J. Biol. Chem. 274: 30474-30480.

Van Kan, J.A.L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11: 247-253.

Walker, J.C. 1993. Receptor-like protein kinase genes of *Arabidopsis thaliana*. Plant J. 3: 451–456.

Worrall, D., Elias, L., Ashford, D., Smallwood, M., Sidebottom, C., Lillford, P., Telford, J., Holt., C., and Bowles, D. 1998. A carrot leucine-rich repeat protein that inhibits ice recrystallization. Science 282: 115-117.

Yao, C., Conway, W.S., and Sams, C.E. 1995. Purification and characterization of a polygalacturonase-inhibiting protein from apple fruit. Phytopathology 85: 1373-1377.

Transgenic Res
DOI 10.1007/s11248-006-9019-1

ORIGINAL PAPER

The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases

Dirk A. Joubert · Ana R. Slaughter · Gabré Kemp · John V. W. Becker · Geja H. Krooshof · Carl Bergmann · Jacques Benen · Isak S. Pretorius · Melané A. Vivier

Received: 20 December 2005 / Accepted: 6 June 2006
© Springer Science+Business Media B.V. 2006

Abstract Polygalacturonase-inhibiting proteins (PGIPs) selectively inhibit polygalacturonases (PGs) secreted by invading plant pathogenic fungi. PGIPs display differential inhibition towards PGs from different fungi, also towards different isoforms of PGs originating from a specific pathogen. Recently, a PGIP-encoding gene from *Vitis vinifera* (*Vvpgip1*) was isolated and characterised. PGIP purified from grapevine was shown to inhibit crude polygalacturonase extracts from *Botrytis cinerea*, but this inhibitory activity has not yet been linked conclusively to the activity of the *Vvpgip1* gene product. Here we use a transgenic over-expression approach to show that the PGIP encoded by the *Vvpgip1* gene is active against PGs of *B. cinerea* and that over-expression of this gene in transgenic tobacco confers a reduced susceptibility to infection by this pathogen. A calculated reduction in disease susceptibility of 47–69% was observed for a homogeneous group of transgenic lines that was statistically clearly separated from untransformed control plants following infection with *Botrytis* over a 15-day-period. VvPGIP1 was subsequently purified from transgenic tobacco and used to study the specific inhibition profile of individual PGs from *Botrytis* and *Aspergillus*. The heterologously expressed and purified VvPGIP1 selectively inhibited PGs from both *A. niger* and *B. cinerea*, including BcPG1, a PG from *B. cinerea* that has previously been shown to be essential for virulence and symptom development. Altogether our data confirm the antifungal nature of the VvPGIP1, and the in vitro inhibition data suggest at least in part, that the VvPGIP1 contributed to the observed reduction in disease symptoms by inhibiting the macerating action of

Dirk A. Joubert and Ana R. Slaughter contributed equally to this work.

D. A. Joubert · J. V. W. Becker · M. A. Vivier (✉)
Department of Viticulture & Oenology, Institute for Wine Biotechnology, Stellenbosch University, Stellenbosch 7600, South Africa
e-mail: mav@sun.ac.za

A. R. Slaughter
Department of Biochemistry, Institute of Botany, University of Neuchatel, Rue Emile-Argand 11, CH-2007 Neuchatel, Switzerland

G. Kemp
Department of Plant Sciences, University of the Free State, Bloemfontein 9300, South Africa

C. Bergmann
Complex Carbohydrate Research Centre, University of Georgia, Athens, Georgia 30602-4712, USA

G. H. Krooshof · J. Benen
Laboratory of Microbiology, Wageningen University, Wageningen 6703 HA, The Netherlands

I. S. Pretorius
The Australian Wine Research Institute, Waite Road, Urrbrae, Adelaide, SA 5064, Australia

certain *Botrytis* PGs *in planta*. The ability to correlate inhibition profiles to individual PGs provides a more comprehensive analysis of PGIPs as antifungal genes with biotechnological potential, and adds to our understanding of the importance of PGIP:PG interactions during disease and symptom development in plants.

Keywords *Vitis vinifera* · Polygalacturonase-inhibiting protein · Polygalacturonases · *Botrytis cinerea* · *Aspergillus niger*

Introduction

Polygalacturonase-inhibiting proteins (PGIPs) have been isolated from many mono- and dicotyledonous plants and in recent years, a large amount of data has emerged regarding the regulation, structure and function of PGIPs (for recent reviews, see De Lorenzo et al. 2001; De Lorenzo and Ferrari 2002; Gomathi and Gnanamanickam 2004). PGIPs are cell wall proteins with a role(s) in plant defence, most notably their interaction with and inhibition of fungal polygalacturonases (PGs). PGs are among the first enzymes secreted by a number of fungal and bacterial pathogens when breaching plant cell walls (Herlache et al. 1997; De Lorenzo et al. 2001; Idnurm and Howlett 2001). Fungal pathogens such as *Botrytis cinerea*, *Aspergillus flavus* and *Altenaria citri* are all dependent on PG genes to maintain full virulence on their respective hosts (Shieh et al. 1997; ten Have et al. 1998; Kars et al. 2005). PGs and their action on plant cell walls have been comprehensively studied and the contribution of this interaction to the molecular dialogue between host and pathogen is well established (Esquerré-Tugayé et al. 1999). PGs cleave the α-1,4 linkages of non-esterified galacturonic acid residues within the homogalacturonan domain (Esquerré-Tugayé et al. 1999), but the specific activity, pH optimum and substrate preference of PGs can vary considerably (De Lorenzo and Ferrari 2002; Kars et al. 2005).

PGIPs may specifically inhibit PGs by either interacting with residues within the active cleft of the enzyme, thereby inhibiting binding of the PG to its substrate while simultaneously blocking the active site (De Lorenzo and Ferrari 2002; Federici et al. 2001), or by binding to the opposite site of the PG molecule, preventing the conformational changes necessary for ligand binding (King et al. 2002). These observations are consistent with the hypothesis that the action of PGIPs during fungal attack is 2-fold; the physical interaction between PGIPs and PGs slows down the infection rate of the fungus and facilitates the prolonged existence of mid-sized oligogalacturonides (degree of polymerisation 11–20) which in turn can elicit a general defence response from the plant (De Lorenzo et al. 1994; Hahlbrock et al. 1995; Reymond et al. 1995).

The importance of PGIPs in defence against fungal pathogens has been further demonstrated by the over-expression of various PGIP-encoding genes in native as well as heterologous hosts. These include the over-expression of pear *pgip* in tomato (Powell et al. 2000), the over-expression of bean *pgip* in tobacco (De Lorenzo and Ferrari 2002) and the over-expression of *Arabidopsis pgip* genes in *Arabidopsis* (Ferrari et al. 2003). In all cases, a reduction in disease symptoms was reported when transgenic plants were infected with *B. cinerea*. This reduction of susceptibility towards *B. cinerea* can presumably be attributed to the inhibition of a PG(s) that is an important pathogenicity factor(s) for the fungus. None of the previous studies explored the nature of the observed resistance further and no data exist regarding the inhibition of individual PGs from these transgenic experiments. It was recently shown that *B. cinerea* has at least six PGs that are differentially regulated during the infection process and contribute differentially to virulence and symptom development (Wubben et al. 1999; ten Have et al. 2001; Kars et al. 2005). The study of PGIPs and individual PGs from fungal pathogens not only provides valuable insight into the nature and dynamics of these interactions, but helps also to identify promising candidate genes for biotechnological approaches to improve disease resistance.

A grapevine PGIP encoding gene, *Vvpgip1*, has previously been isolated in our laboratory and PGIP purification from grapevine berries yielded a protein with strong inhibition activity against a crude extract of PGs from *B. cinerea*

(De Ascensao 2001). Here we over-express the *Vvpgip1* gene in tobacco, confirm PGIP activity in the heterologous host and show that *B. cinerea* symptom development is reduced in transgenic lines. VvPGIP1 was subsequently purified from transgenic tobacco and used to evaluate its interaction with and inhibition of individual PGs from *Aspergillus niger* and *B. cinerea*.

Materials and methods

Plant growth and light conditions

In vitro tobacco (*Nicotiana tabacum* L. Havana petit SR1) plantlets were cultured on MS medium (Murashige and Skoog 1962) and incubated at 26°C with a 16 h light 8 h dark photoperiod regime. Tobacco leaf discs transformed with the grapevine polygalacturonase-inhibiting protein encoding gene (*Vvpgip1*) were allowed to form shoots on MS media supplemented with 0.5 μg/ml 6-benzyl-aminopurine (BAP) and 100 μg/ml kanamycin. Rooting was induced on MS media supplemented with 0.1 μg/ml naphthalenacetic acid (NAA) and 100 μg/ml kanamycin. To establish greenhouse plants, seeds were germinated in peat moss (Jiffy International AS, Kristiansand, Norway) and plants were maintained in a greenhouse under natural light at 26°C and 65% humidity.

Vector constructs and plant transformation

Oligonucleotide primers 5′-<u>CTGCAG</u>ATGGAGACTTCAAAACTTTT-3′ (*Pst*I site underlined) and 5′-<u>GGATCC</u>ACTTGCAGCTCTGGAGTGGAG-3′ (*Bam*HI site underlined) were used to amplify the 1002 bp ORF of the *Vvpgip1* gene (Genbank accession: AF499451). The fragment was cloned into the pGEM-T-Easy vector (Promega Corporation, Madison, USA) and confirmed by sequencing. The *Vvpgip1* insert was subcloned into the *Pst*I and *Bam*HI sites of pBluescriptSK(+) (Stratagene, La Jolla, USA) for mobilisation into a plant expression vector. The fragment was subsequently excised from pBluescriptSK(+) with *Eco*RV and *Sac*I and cloned into the *Sma*I and *Sac*I sites of the binary vector pBI121 (Jefferson et al. 1987), replacing the β-glucuronidase gene to yield pBI(Vvpgip1) and placing the gene under control of the 35S CaMV promoter and nopaline synthase (NOS) terminator. The construct was mobilised into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation. Tobacco was transformed with pBI(Vvpgip1) using the leaf disc method of Gallios and Marinho (1995) and plantlets were regenerated under kanamycin selection (100 μg/ml). Several putative primary transgenic lines were recovered and acclimatised for growth in a greenhouse (T0 lines). The T0 lines were allowed to self-pollinate and the offspring (T1 lines) were germinated and grown as described.

PCR and Southern blot analyses of transgenic lines

Transgene integration in primary transformants was confirmed by PCR with the *Vvpgip1* gene specific primers (results not shown). All PCR analyses were conducted in a Whatman Biometra Trio-thermoblock automated temperature cycler (Göttingen, Germany). Typical PCRs consisted of 1× PCR buffer, 0.25 μM primer, 0.2 μM dNTPs, 100 ng genomic DNA as template and 0.5 U of *Taq* DNA polymerase in a 50 μl reaction volume. PCR cycle conditions typically included an initial denaturation step at 95°C for 3 min and 30 amplification cycles consisting of denaturation at 95°C for 30 s, annealing at 58°C for 40 s and elongation at 72°C for 40 s. Reactions also typically included a final elongation step of 5 min at 72°C.

Genomic DNA was extracted from 0.1 g of tobacco leaves obtained from greenhouse acclimatised T0 and T1 generation transformants according to the method of McGarvey and Kaper (1991). For Southern blot analysis, genomic DNA from the transgenic tobacco lines (10 μg) was digested with *Eco*RV and separated on a 0.8% (w/v) agarose gel. *Eco*RV cuts on the 3′ end (just 5′ of the *Vvpgip1* gene) of the 35S CaMV promoter, but not in the *Vvpgip1* gene, providing an estimate of transgene insertion events. Gels were transferred to positively charged Hybond-N nylon membranes according to Sambrook et al. (1989). Pre-hybridisation (2 h) and hybridisation (16–20 h) reactions were carried out at 42°C in DIG

Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany). The *Vvpgip1* gene was used as a probe and digoxigenin-labelled according to the DIG System User's Guide (Roche Diagnostics GmbH, Mannheim, Germany). After hybridisation, membranes were washed sequentially at room temperature (two washes with double strength SSC [1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0] and 0.1% SDS [w/v] for 5 min each) and 68°C (two washes with half strength SSC and 0.1% SDS [w/v] for 15 min each). Chemiluminescent detection of nucleic acids was done using CSPD as substrate (Roche Diagnostics GmbH, Mannheim, Germany).

Northern blot analysis of *Vvpgip1* transcript levels

Total RNA was extracted from the leaves of greenhouse acclimatised T0 and T1 generation transformants using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's specifications. Total RNA (10 μg per lane) was size fractionated by electrophoresis on a 1.2% (w/v) formaldehyde agarose gel and blotted to Hybond-N nylon membranes using standard techniques as described by Sambrook et al. (1989). The membranes were pre-hybridised at 50°C for 4 h in DIG Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were then hybridised in the same solution with the addition of the digoxigenin-labelled *Vvpgip1* probe. After hybridisation, membranes were washed twice in double strength SSC and 0.1% SDS (w/v) at room temperature for 15 min and twice in half strength SSC and 0.1% SDS (w/v) at 68°C for 15 min. Chemiluminescent detection proceeded as previously described for Southern blot detection.

Detection of VvPGIP1 activity in transgenic tobacco

Crude protein extracts of independent transgenic tobacco lines (T0 generation) and untransformed control tobacco plants were analysed for the presence of PGIP activity by an agarose diffusion assay (Taylor and Secor 1988). This assay uses polygalacturonic acid (PGA) as substrate for polygalacturonases (PGs) and inhibition of PG activity by active PGIP results in a size-reduction of clearing zones. Finely crushed leaf tissue (0.4 g) was homogenised in extraction buffer consisting of 0.1 M sodium acetate buffer (pH 6.0), 10 mM β-mercaptoethanol and 1% (w/v) PVP-40. The homogenate was centrifuged at 10,000*g* for 15 min, followed by two more extractions in extraction buffer without PVP-40, each followed by centrifugation. The remaining insoluble tissue was resuspended in 2 volumes of 50 mM sodium acetate buffer (pH 5.2) also containing 1 M NaCl and stirred for 1 h at 4°C. The insoluble debris was removed by centrifugation at 10,000*g* for 20 min. The proteins precipitating at 80% (w/v) saturated ammonium sulphate were collected, resuspended in 20 mM sodium acetate (pH 5.2) and dialysed extensively at 4°C against 20 mM sodium acetate (pH 5.2). The protein concentration was determined according to Bradford (1976), using a Bio-Rad (Hercules, USA) protein assay kit and bovine serum albumin (BSA) as standard. The dialysed samples were assayed for PG inhibition by the agarose diffusion assay using crude PGs from *Botrytis cinerea* (see next section). All agarose diffusion assays were done in triplicate and allowed to proceed for 16 h at 30°C. A boiled sample of crude extract was also loaded on the plates to confirm the proteinaceous origin of the reduction in clearing zones. Zones were visualised by staining of the plates with 0.05% Ruthenium red. Equal amounts of total protein were used in the assay. The diameter of the clearing zones as a result of PG hydrolysis on the pectic substrate was compared with those formed when plant cell extracts containing inhibitor was added, and expressed as a percentage reduction.

Fungal endopolygalacturonases

Crude PG preparations from *B. cinerea* were prepared by culturing the fungal spores in citrate phosphate buffer (pH 6.0), supplemented with 1% (w/v) citrus pectin, 2 mM $MgSO_4 \cdot 7H_2O$, 0.6 μM $MnSO_4 \cdot H_2O$, 25 mM KNO_3, 30 μM $ZnSO_4 \cdot 7H_2O$, 0.9 μM $CuSO_4$ and 65 μM $FeSO_4$ for 10 days at 22°C. The cultures were filtered through Whatman No. 1 paper and precipitated

overnight with 80% $(NH_4)_2SO_4$ at 4°C. Proteins were recovered by centrifugation at 10,000*g* for 20 min at 4°C and resuspended in 40 mM sodium acetate (pH 5.0). Purified PGs from *Aspergillus niger* (AnPGII, AnPGA and AnPGB) and *B. cinerea* (BcPG1, BcPG3, BcPG4 and BcPG6), were obtained as previously reported (Pařenicová et al. 1998; Kars et al. 2005).

Plant infections and disease assessment

Pathogenic cultures of *B. cinerea* were isolated from a South African vineyard and were maintained on sterile halved apricots (Naturlite, Tiger Food Brands Limited, South Africa), in a dark growth chamber at 23°C until sporulation occurred. Spore inocula were harvested using sterile distilled water following incubation on apricot halves for 12 days. Mycelial debris was removed from the spores by filtration. Spore viability was evaluated by plating an aliquot of the spore suspension on 0.8% (w/v) water agar (Difco, Detroit, MI) and calculating the percentage of spores that had germinated following incubation for 24 h at 23°C.

Susceptibility to *B. cinerea* of the T0 population was evaluated using a detached leaf assay according to Carstens et al. (2003). Leaves of 7- to 8-week-old greenhouse acclimatised plants (including an untransformed control) were detached with a scalpel blade and placed in a sealed tissue culture container, with the petiole embedded in 0.8% (w/v) water agar. Three leaves per plant line were inoculated on the adaxial side, without wounding the surface, with two aliquots of 5×10^3 spores per spot. The containers were sealed to maintain 100% humidity and placed in a growth room at 23°C. Disease symptoms were scored 3 days after inoculation by measuring the diameter of the lesions, this data was used to calculate the percentage decrease in disease susceptibility in the transgenic lines compared to the untransformed control.

The detached leaf assay was followed by a whole plant infection on 7- to 8-week-old T1 progeny of six of the independent transgenic lines over a period of 15 days. Lines 1, 5, 24, 37, 45 and 47 were randomly selected for this assay and used together with untransformed control plants. For the whole plant infections, 5 μl of a spore suspension (1×10^3 in a 50% grape juice medium) was spotted on the adaxial surface of leaves without detaching or wounding the leaves. Three leaves per plant line were inoculated with four spots per leaf. Mature, fully expanded leaves from positions 3, 4 and 5 were selected for inoculation and care was taken to ensure that leaf position of inoculated leaves were consistent between plant lines. Plants were placed in a humidity chamber at 100% relative humidity and incubated for a period of 15 days following inoculation at 23°C. The success of the infections was monitored and disease susceptibility was determined by measuring the diameters of the lesions daily from day three to seven and again at day 15. The decrease in disease susceptibility was calculated by comparing the average lesion diameter at day 15 of all lines to that of the untransformed control. Two independent whole plant infections were carried out on the mentioned lines. Significant differences and homogeneous groups were calculated by performing a one-way Analysis of Variance (ANOVA) using the STATISTICA 6 (StatSoft Inc., Tulsa, OK, USA) software package at 95% confidence intervals.

Isolation and purification of VvPGIP1 from transgenic tobacco plants

Purified PGIP was isolated from transgenic tobacco plants (line 37) using a modified approach from Favaron et al. (1994). Healthy leaves from 6- to 8-week-old T1-generation tobacco plants (450 g) were homogenised in a blender with 1 l of acetone. The pulp was filtered through a nylon cloth, squeezed, dried and blended with 300 ml of acetone (repeated three times), and allowed to dry out completely (overnight at room temperature). The dried pulp was soaked in 1 l of extraction buffer (20 mM sodium acetate, pH 5.5, 1 M NaCl) and stirred for 24 h at room temperature. The material was centrifuged at 9000*g* for 30 min. The supernatant was recovered, filtered through Whatmann GF/A glass filter paper and dialysed against 20 mM sodium acetate (pH 5.5) over a period of 3 days in standard cellulose dialysis tubing (12,000–14,000 molecular weight

cut-off) with at least six buffer changes. The dialysed sample was centrifuged for 30 min at 10,000*g* and the supernatant recovered.

The protein content was separated by cation exchange chromatography using a 5 ml Econo-Pac high S cartridge (Biorad, Hercules, USA) at a flow rate of 1 ml/min. The column was equilibrated with buffer A (20 mM sodium acetate, pH 5.0) and the bound fraction was pulse-eluted with buffer B (20 mM sodium acetate, 300 mM NaCl, pH 5.0). The eluted fraction was diluted 1:2 in double strength ConcavalinA (ConA) buffer (200 mM sodium acetate, pH 6.0, 2 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$) (Favaron et al. 1994) and diluted again 1:2 in ConA buffer. The sample was loaded onto a ConA Sepharose 4B column (Amersham Biosciences, NJ, USA) the flow-through collected and eluted with ConA elution buffer (250 mM α-methyl-D-manno-pyranoside in ConA buffer) (Favaron et al. 1994). The flow-through was loaded on the column again and eluted as described. This procedure was repeated one more time to ensure maximum recovery of VvPGIP1.

The eluted sample was concentrated to 20 ml and diluted 1:10 in 20 mM sodium acetate (pH 5.0). This procedure was repeated three times to remove all traces of the elution buffer. Before proceeding to chromatography, the final concentrate was diluted 1:10 in 20 mM sodium acetate (pH 5.0). The diluted sample was loaded onto a Hi-Trap S cation exchange column (Amersham Biosciences, NJ, USA) at a flow rate of 1 ml/min. The column was again equilibrated with buffer A and eluted with buffer B, using a gradient of 1% buffer B per minute. Protein content in the fractions were quantified according to Bradford (1976) using a Bio-Rad protein assay kit and BSA as a standard. Eighty 0.5 ml fractions were analysed for PGIP activity against PGB from *Aspergillus niger* (AnPGB).

Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)

Fractions eluted from the Hi-Trap S cation exchange column that contained PGIP activity were analysed on a Hewlett Packard G2025A MALDI-TOF mass spectrometer (Palo Alto, CA, USA). The matrix used was a saturated sinapinic acid (3,5 dimethoxy-4-hydroxy-*trans*-cinnamic acid) solution in 50% acetonitrile. Equal volumes of sample and matrix (approximately 1 μl) were loaded onto the probe and were dried under vacuum. The laser energy ranged from 10 to 14 μJ.

Quantitative endopolygalacturonase inhibition assays with purified VvPGIP1

Endopolygalacturonase inhibition by the purified grapevine PGIP was followed by measuring the hydrolytic activity of the PGs on a pectic substrate in the absence and presence of the inhibitor. Approximately 100 ng purified VvPGIP1 was used to test inhibition against BcPGs 1, 3, 4 and 6 as well as AnPGs II, A, and B at pH 3.75, 4.0, 4.2, 4.5, 4.75, 5.0, 5.5 and 6.0. Approximately 50 ng PG was used in each assay. Reactions were incubated for 1 h at room temperature with the respective PGs in 0.025% (w/v) polygalacturonic acid (Sigma, St Louis, USA) as substrate and buffered in 50 mM sodium acetate. Following incubation, the inhibition was quantified by measuring the decrease in the release of reducing sugars spectrophotometrically at 410 nm, using the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985). Controls lacking PGs were included to compensate for possible reducing sugars and native PGs present in the chromatographic fractions. To compensate for the possible influence of the chromatography buffer gradient on the enzyme activity, equivalent amounts of salt were added to all assays. All assays were done in triplicate.

Results

Construction of a plant expression cassette and subsequent tobacco transformations

The complete coding region of the grapevine polygalacturonase inhibiting protein encoding

gene (*Vvpgip1*) was inserted between the CaMV 35S promoter and nopaline synthase terminator in the plant expression vector pBI121. pBI(Vvpgip1) was mobilised into *Agrobacterium tumefaciens* and subsequently used for tobacco transformation. Independent transgenic tobacco lines were identified with PCR and Southern blot analyses. PCR analysis performed on 46 independent primary transgenic tobacco lines yielded a fragment corresponding to the size (1002 bp) of the *Vvpgip1* gene in all of the lines tested (results not shown). Untransformed lines were used as negative controls and did not result in any amplification. Southern blot analysis (Fig. 1) confirmed integration of the *Vvpgip1* ORF in most lines tested. The integration number of the transgenes varied per transformed line (T0 generation) and was estimated from the Southern results to range from one to approximately eight or more insertion events. Expression of the *Vvpgip1* transgene in the transformed tobacco lines was analysed by Northern blot analysis using the *Vvpgip1* gene as probe. Hybridisation analysis of total RNA from leaf tissue demonstrated that *Vvpgip1* transcripts of the correct size were expressed in 19 transgenic lines. No transcripts could be detected in untransformed plants (Fig. 2). The transgenic population was phenotypically analysed and compared to untransformed controls and did not show any abnormalities with regards to growth, size or reproduction.

Crude protein extracts from transgenic tobacco over-expressing *Vvpgip1* can inhibit crude polygalacturonase (PG) preparations from *Botrytis cinerea*

To assess the activity of VvPGIP1 in transgenic tobacco plants over-expressing the *Vvpgip1* gene, crude protein extracts prepared from greenhouse acclimatised T0 transgenic tobacco leaves (all 19 Northern positive lines) were used to assess inhibition of crude PG preparations from *B. cinerea* in an agarose diffusion assay described by Taylor and Secor (1988). The results confirmed PGIP activity in the crude extracts from all the transgenic lines, showing clear reductions in the clearing zones when the plant extracts were incubated with the PGs on the pectic substrate. The levels of PG inhibition ranged from 25 to 80% (Table 1).

Transgenic tobacco plants over-expressing *Vvpgip1* are less susceptible to *B. cinerea* infection

Initial experiments to determine the effect of VvPGIP1 over-expression in transgenic tobacco on disease susceptibility was conducted using greenhouse acclimatised T0 Northern positive transgenic lines in a detached leaf assay. Successful infections occurred on all leaves and proceeded aggressively in the untransformed controls. Lesion sizes were significantly reduced

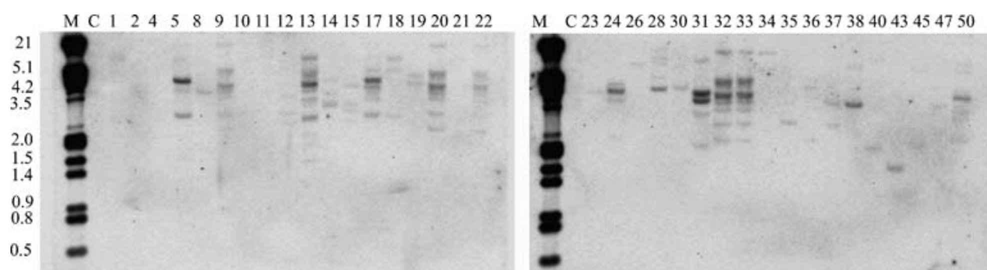


Fig. 1 Southern blot analyses of T0 generation tobacco plants transformed with the grapevine PGIP1 encoding gene. Genomic DNA from tobacco plants were digested with *Eco*RV and hybridised with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of the VvPGIP1 encoding gene. Digoxigenin-labelled lambda DNA was used to visualise the molecular marker (lambda DNA digested with *Eco*RI and *Hin*dIII). The numbers identify each independent transgenic plant tested. Untransformed tobacco genomic DNA digested with *Eco*RV is shown in lane C. The marker lane (M) contains *Eco*RI and *Hin*dIII digested lambda DNA. Sizes of the standard DNA fragments are indicated in kb

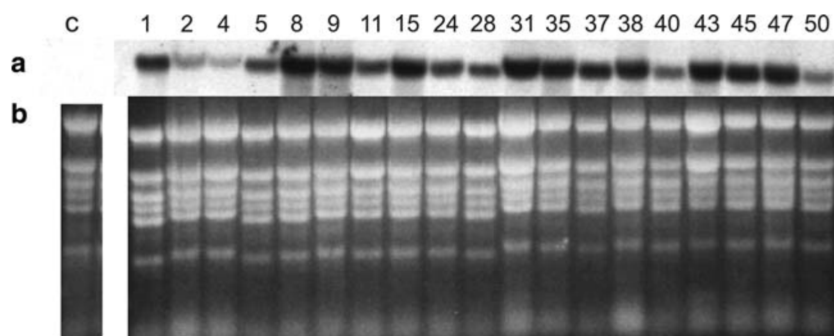


Fig. 2 Northern blot analysis of the expression of the grapevine polygalacturonase-inhibiting protein encoding gene (*Vvpgip1*) in T0 generation transgenic tobacco lines. Total RNA was extracted from leaf tissue and probed with a digoxigenin-labelled 1002 bp fragment corresponding to the coding region of the grapevine *pgip1* gene as shown in **a**. Ethidium bromide staining of the formaldehyde agarose gel is shown in **b**. Numbers identify each transgenic plant line that showed mRNA *pgip1* expression. Total RNA extracted from an untransformed tobacco plant is shown in lane C

Table 1 Analysis of transgenic tobacco over-expressing a grapevine PGIP: Inhibition of polygalacturonases (PGs) from *Botrytis cinerea* by protein extracts[a] from the transgenic plants and assessment of lesion development on detached leaves of the transformed plants when infected by *B. cinerea*

PG control and pant lines[b]	PGIP activity assay		Detached leaf assay	
	Clearing zone (mm) in agarose plate assay	PG inhibition[c] (%)	Lesions (mm) on detached leaves infected with *B. cinerea*	Decrease in lesion size of detached leaves infected with *B. cinerea* (%)
PG control	21 ± 2.0	N/A	N/A	N/A
Untransformed	20.5 ± 1.8	2.4	16.17 ± 1.5	0
Boiled extract	20.5 ± 0.5	2.4	N/A	N/A
1	7.7 ± 0.6	63.3	8.17 ± 0.6	49.5
2	15 ± 1.0	28.6	10.50 ± 1.2	35.1
4	14.8 ± 1.6	29.5	10.00 ± 1.2	38.1
5	7 ± 1.0	66.7	7.83 ± 0.9	51.5
8	8.2 ± 1.0	61.0	8.83 ± 1.2	45.4
9	6.5 ± 0.5	69.0	7.33 ± 1.1	54.6
11	8.3 ± 1.2	60.5	9.33 ± 1.0	42.3
15	7.3 ± 1.2	65.2	8.33 ± 0.9	48.5
24	11.3 ± 0.6	46.2	10.50 ± 1.2	35.1
28	10.3 ± 0.6	51.0	9.67 ± 0.9	40.2
31	4.2 ± 0.3	80.0	6.33 ± 1.5	60.8
35	5 ± 0.5	76.2	7.00 ± 1.9	56.7
37	5.7 ± 0.6	72.9	7.17 ± 1.5	55.7
38	4.7 ± 0.6	77.6	6.33 ± 1.4	60.8
40	11 ± 0.0	47.6	10.17 ± 0.9	37.1
43	9.5 ± 0.5	54.8	9.00 ± 0.9	44.3
45	8.5 ± 0.9	59.5	9.50 ± 1.0	41.2
47	8.2 ± 1.3	61.0	9.00 ± 0.8	44.3
50	15.8 ± 1.3	24.8	11.33 ± 1.1	29.9

[a]Crude extracts were prepared from leaves

[b]Numbers identify each independent transgenic line tested

[c]PG inhibition was determined by the agarose diffusion assay (Taylor and Secor, 1988), 100 ng of extract was used for each sample

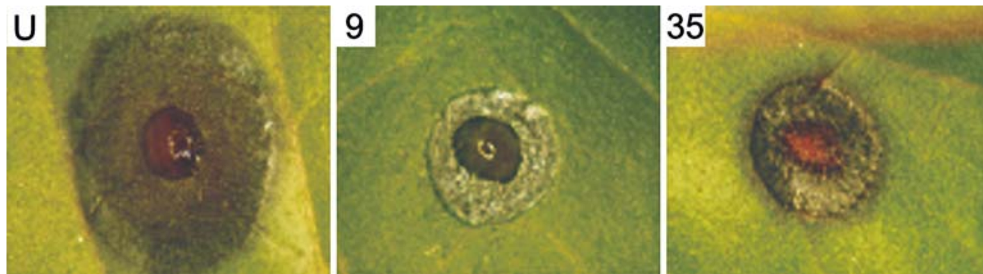


Fig. 3 *Botrytis cinerea* lesion development on detached leaves of untransformed and transgenic T0 generation *Nicotiana tabacum* L. Havana petit SR1 plants. Shown is the lesion development on tobacco plants lines 9 and 35 over-expressing the grapevine polygalacturonase inhibiting protein (VvPGIP1) relative to the untransformed control plants (U). Lesions were photographed 3 days after inoculation using similar magnification conditions

in the transgenic population at 3 days post-infection (dpi) and up to 61% reduction in lesion size was obtained (Table 1 and Fig. 3).

To assess whether transgenic tobacco whole plants expressing VvPGIP1 showed reduced susceptibility to *B. cinerea* infection, untransformed as well as six *Vvpgip1* expressing T1 progeny lines were infected with *B. cinerea* spores. The infection was followed for 15 days. The high spore-load as well as the incubation conditions favoured disease development and 75–92% of inoculum sites developed into primary lesions within three dpi. Lesion size was scored daily for the period 3–7 dpi and again after 15 dpi (see Table 2 for the lesion size at 15 dpi). Comparing lesion development over the 15 days, lesion development as well as lesion size remained comparable in all transgenic lines for up to 7 dpi, yet consistently showed reduced lesion sizes compared to the untransformed control (Fig. 4). During the analysis period, lesions on the untransformed plants remained wet and started to expand very rapidly, while lesions on transgenic plants expanded at significantly reduced rates and became dry and necrotic.

Statistically significant differences in lesion development and size between the various transgenic lines as well as between transgenic lines and untransformed control only occurred in the period between 7 and 15 dpi (Fig. 4). Statistical analysis grouped the transgenic lines with high confidence into two distinct homogeneous groups based on lesion sizes (at 15 dpi) (Table 2). Lines 5, 24, 37 and 45 were placed in one group, while lines 1 and 47 were placed with the untransformed plants in a separate group. Group two

Table 2 Analysis of lesion development on transgenic tobacco lines over-expressing a grapevine PGIP following whole plant infections with *Botrytis cinerea*

Plant line	Percentage of successful infections per plant line[a]	Average lesion diameter (mm) [a]	Percentage decrease in disease susceptibility (compared to WT) [a]	Homogenous groups[a]
Wild-type	75%	40.94 ± 3.50	0	1
VvPGIP1	75%	37.66 ± 4.24	8.0	1
VvPGIP 5	83%	12.58 ± 1.36	69.2	2
VvPGIP 24	83%	15.43 ± 0.94	62.3	2
VvPGIP 37	83%	12.60 ± 1.06	69.2	2
VvPGIP45	92%	21.84 ± 3.10	46.7	2
VvPGIP 47	92%	36.77 ± 3.24	10.2	1

The decrease in disease susceptibility was calculated by comparing the average lesion diameter at day 15 of all lines to that of the untransformed control

[a]Two independent whole plant infections were carried out on the mentioned lines. Significant differences and homogeneous groups were calculated by performing a one-way Analysis of Variance (ANOVA) using the STATISTICA 6 (StatSoft Inc, Tulsa, OK, USA) software package at 95% confidence intervals

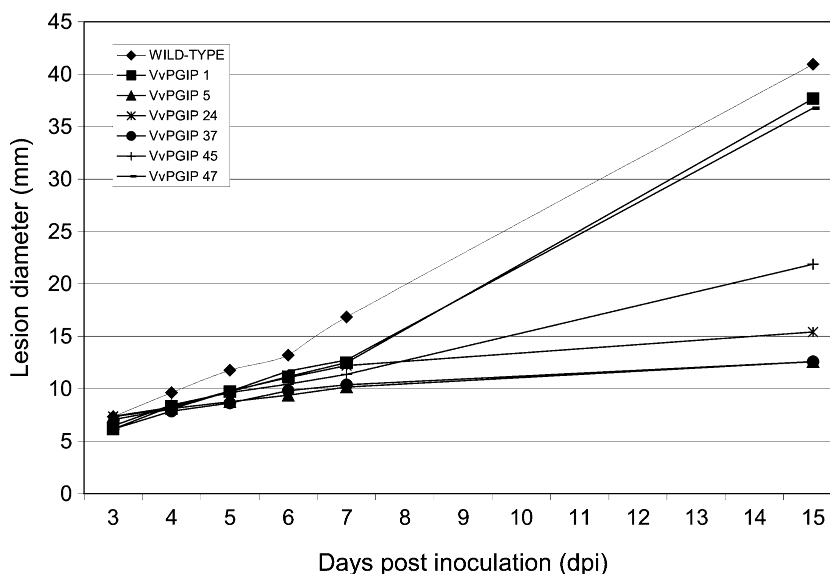


Fig. 4 Disease assessment of *Botrytis cinerea* infections on untransformed and VvPGIP1 transgenic tobacco lines at the whole-plant level. *B. cinerea* spores were inoculated on the adaxial surface of the leaves and the infection progression followed for a period of 15 days. Lesions were measured daily from day 3 to 7 and again at day 15. The mean lesion diameter of developing lesions at the indicated days post-inoculation of untransformed wild-type and transgenic lines are shown. One-way ANOVA indicated significant differences between mean lesion diameters of plant lines at 15 dpi. Lesion diameters and respective standard error at 15 dpi of all lines are indicated in Table 2

plants showed reductions in disease susceptibility ranging from 47 to 69% (Table 2) and lesions on these plants did not exhibit the same rapid expansion phenotype as those on wild-type plants (Fig. 4). Plants from group one showed high susceptibility to *B. cinerea* infection.

VvPGIP1 purified from transgenic tobacco differentially inhibits endo-polygalacturonases from *Aspergillus niger* (AnPGs) and *B. cinerea* (BcPGs)

To evaluate the inhibition of individual PGs from *A. niger* and *B. cinerea* by VvPGIP1, PGIP was purified from transgenic tobacco line 37. Eighty 0.5 ml fractions were collected from the final cation exchange chromatography step and tested for PGIP inhibitory activity against AnPGB (Fig. 5). Fractions containing PGIP inhibitory activity were further analysed with MALDI-TOF MS to assess the purity and integrity of the samples. All PGIP-containing fractions showed three peaks corresponding to 37, 40, and 42 kDa. A contaminating peak of about 24 kDa was observed in the fractions with the highest PGIP activity, but inhibition assays with fractions not containing this peak showed that it did not influence PGIP activity in any way (results not shown). The MALDI-TOF profile of fraction 45 (highest PGIP inhibitory activity) is shown in Fig. 6.

The activity of seven fungal PGs; AnPGA, AnPGB, AnPGII, BcPG1, BcPG3, BcPG4 and BcPG6 were assayed over a wide pH range in the presence or absence of the grapevine inhibitor. The enzymatic activity of the PGs tested displayed variable pH optimums on a homogalacturonan substrate using reducing sugar analysis (Fig. 7). The AnPGs A and B were most active at pH 4.2 and pH 5.5, respectively (Fig. 7a and b), whereas the AnPGII displayed optimum activity around pH 4.75 (Fig. 7c). BcPG1, BcPG3 and BcPG4 required a more acidic pH that ranged between 3.75 and 4.0 (Fig. 7d–f) for optimal activity while BcPG6 was most active in a less acidic environment with an optimum pH of 5.5 (Fig. 7g).

The addition of PGIP caused a significant reduction in activity of AnPGA and AnPGB, as well as BcPG1 and BcPG6, whereas the activity of BcPG3 was virtually unchanged by VvPGIP1

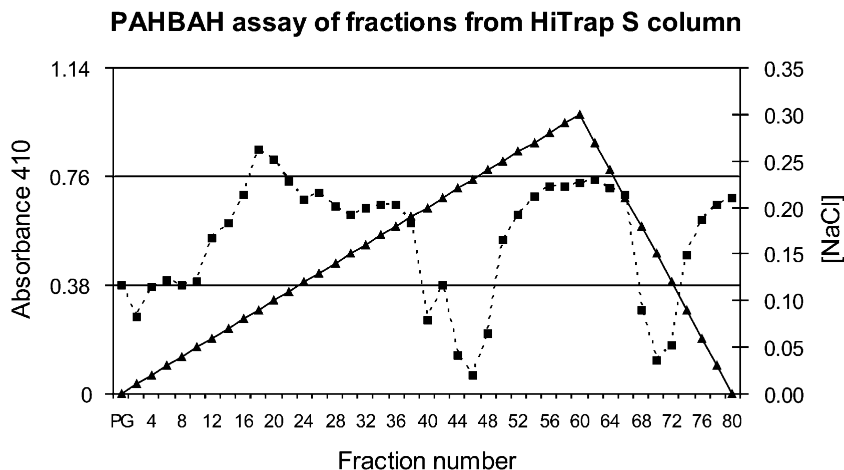


Fig. 5 Analysis of grapevine polygalacturonase-inhibiting protein (VvPGIP1) activity in 80 fractions collected from the final cation exchange chromatography step during the purification of VvPGIP1 from transgenic tobacco. Fractions were eluted with a NaCl gradient (solid line, ▲) and assayed against *Aspergillus niger* PGB (AnPGB). Inhibition was quantified by measuring the decrease in the release of reducing sugars spectrophotometrically at 410 nm, using the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985). The relative activity of AnPGB (as indicated by the A_{410} values) alone (PG) or in combination with the different fractions (numbers 2–80) is indicated on the graph (dotted line, ■). Every second fraction was assayed for PGIP activity

(Fig. 7). BcPG4 was only inhibited in the lower pH range (3.75–4.75), whereas AnPGII was not inhibited by VvPGIP1. The inhibition assays were repeated with fraction 69 (Fig. 4) which was less homogeneous, but did not contain the 24 kDa contaminating peak that fraction 45 contained. The inhibition data for this fraction did not differ significantly from fraction 45 (results not shown).

Discussion

In recent years, several studies have reported that polygalacturonase-inhibiting proteins (PGIPs) are effective in reducing the disease susceptibility of plants towards pathogens (De Lorenzo et al. 2001 and references therein). Apart from these transgenic over-expression studies, biochemical

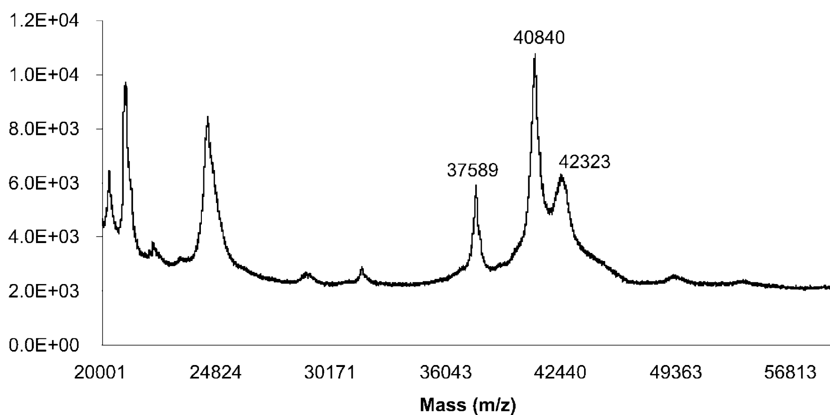
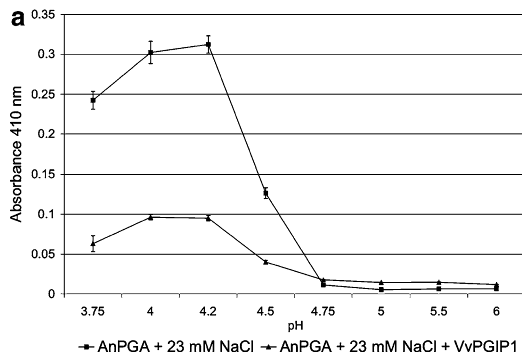
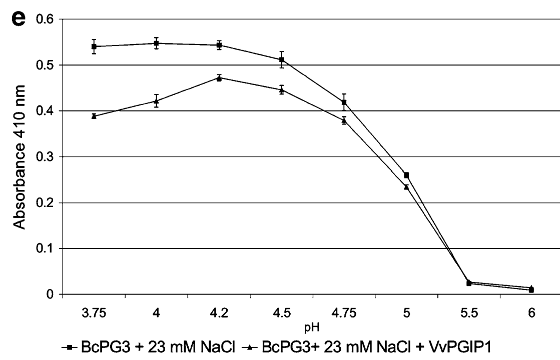
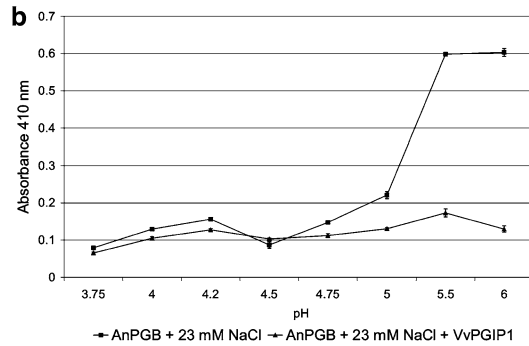


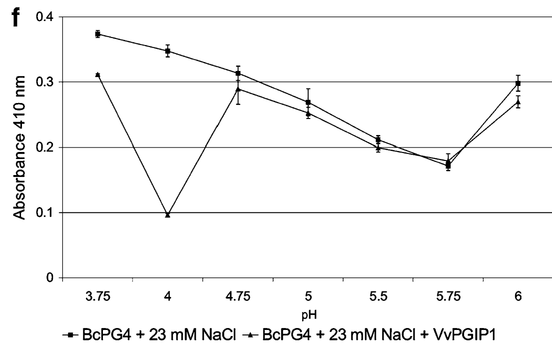
Fig. 6 Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) analysis of the fraction containing the highest PGIP activity eluted from the final cation exchange chromatography step during the purification of VvPGIP1 from leaves of transgenic tobacco. Samples were analysed on a Hewlett Packard G2025A MALDI-TOF mass spectrometer to assess the integrity and purity of PGIP containing fractions. The matrix used was a saturated sinapinic acid (3,5 dimethoxy-4-hydroxy-*trans*-cinnamic acid) solution in 50% acetonitrile. Equal volumes of sample and matrix (approximately 1 µl) were loaded onto the probe and dried under vacuum. The laser energy ranged from 10 to 14 μJ. VvPGIP1-containing fractions showed three peaks corresponding to 37, 40 and 42 kDa

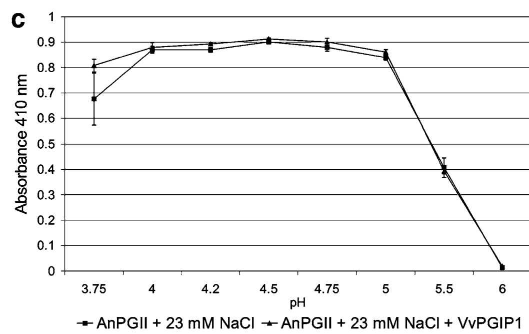
Springer

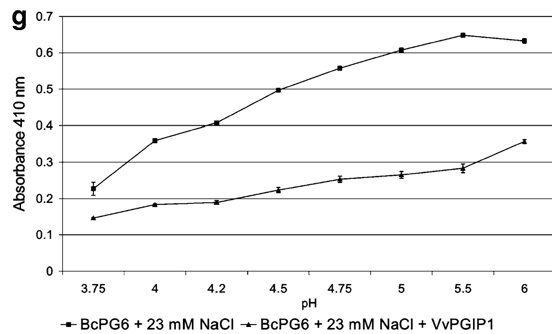


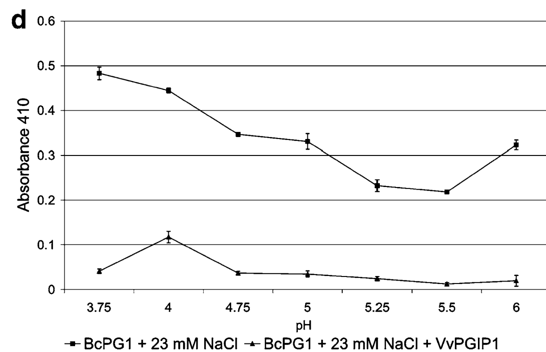












analysis and protein–protein interaction data confirmed the direct inhibition of fungal polygalacturonases (PGs) by PGIPs. Moreover, *pgip* genes have been shown to be transcriptionally upregulated by pathogen infection and the presence of defence signaling molecules. Although a role for PGIPs in plant defence is well-established, more work is needed to explore their role(s) *in*

◀ **Fig. 7** The inhibition profile of VvPGIP1 purified from transgenic tobacco was determined against polygalacturonases (PGs) from *Aspergillus niger* and *Botrytis cinerea*. Inhibition was quantified by measuring the release of reducing sugars spectrophotometrically at 410 nm, using the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York et al. 1985) by fungal polygalacturonases (PGs) alone (■) or in combination with VvPGIP1 (▲). Assays were done at room temperature using the PAHBAH reagent across a wide pH range with 0.025% polygalacturonic acid as substrate. For the assays with PGs alone, NaCl was added to the same concentration as that of the fraction containing VvPGIP1 used in the combined assays. All assays were done in triplicate. (**a**–**c**) Reducing sugar (PAHBAH) assays of *Aspergillus niger* PGs A, B and II, respectively. (**d**–**g**) Reducing sugar (PAHBAH) assays of *Botrytis cinerea* PGs 1, 3, 4 and 6

planta in the progression and signaling of the defence response in plants following infection.

Powell et al. (2000) were the first to report that the heterologous expression of pear PGIP in tomato successfully conferred resistance against *B. cinerea* by targeting and inhibiting the tissue-macerating functions of the fungus. The availability of PG encoding genes, purified PGs, and data regarding the contribution of individual PGs to virulence and symptom development now facilitate studies to evaluate PGIPs more accurately and comprehensively.

A PGIP encoding gene has been isolated from grapevine and it was shown that grapevine has strong PGIP activity against a crude extract of PGs from *B. cinerea* (De Ascensao 2001). PGIPs in grapevine are strictly regulated, and expression has been found to be tissue specific, developmentally regulated and are inducible by *B. cinerea* infection amongst other factors (DA Joubert, G de Lorenzo, IS Pretorius and MA Vivier, unpublished data). In this study, the *pgip1* gene from *Vitis vinifera* cv Pinotage was over-expressed in tobacco plants, resulting in the accumulation of *Vvpgip1* transcripts in 19 individual transgenic tobacco lines. These lines were used to show using an in vitro plate assay, that the over-expression of this gene resulted in active PGIP in the plant extracts of the transgenic lines, causing inhibition of crude *B. cinerea* PGs (Table 1). A boiled plant extract sample did not show any PGIP activity, confirming that the PG inhibitions observed was not an artefact of the extraction procedure.

Having established that the grapevine PGIP gene is indeed active in the heterologous host, its ability to decrease disease susceptibility was first assessed with a detached leaf assay to screen all primary transformants accumulating VvPGIP1. At 3 dpi a clear reduction in lesion sizes was observed in the transgenic population compared to the untransformed control (Table 1 and Fig. 3). A very high spore load and high humidity conditions ensured very effective infection rates. From these detached leaf assays, reductions in disease susceptibility ranging from 30 to 61% were observed. These promising results were corroborated by whole plant infections conducted over 15 days on six of the transgenic lines, as well as on untransformed control plants. The whole plant infection assay was carried out on T1 progeny lines randomly chosen and yielded statistically significant separation of four of the six lines from the untransformed control. Successful *Botrytis* infections occurred with high percentages, with primary lesions developing at the inoculation sites within 3 dpi. The lines over-expressing VvPGIP1 showed a reduction in lesion size, spread and subsequent expansion of fungal mass over the 15-day evaluation period, with the most significant statistical differences being observed at 15 dpi. Calculated reductions in disease susceptibility, expressed relative to the untransformed control approached 70% in the least susceptible lines in this analysis. The detached leaf assay and the whole plant infections confirmed that the VvPGIP1 can influence plant defence positively, presumably through the observed inhibitory activity towards *B. cinerea* PGs. Further investigation is ongoing to clarify the susceptible phenotype of transgenic lines 1 and 47.

The *Vvpgip1* gene was isolated from *V. vinifera* cv Pinotage and as with most of the cultivars within *V. vinifera*, it has rather poor resistance to most fungal pathogens. Our results of the grapevine PGIP in transgenic tobacco indicate it to be an effective antifungal gene, but in the native genetic background it clearly does not act with the same efficiency. During grape berry maturation an accumulation of numerous developmentally regulated defence-associated proteins occur up to *véraison*, and the green berries are quite resistant to fungal pathogens. Some of the most prominent

defence-proteins in the pre-*véraison* stage include acidic chitinases, thaumatin-like proteins and lipid-transfer proteins (Salzman et al. 1998; Davies and Robinson 2000); grapevine *pgip* is also strongly expressed at *véraison* (DA Joubert, G de Lorenzo, IS Pretorius and MA Vivier, unpublished data). During the post-*véraison* stages (comprising ripening-related events such as accumulation of sugars and pigments as well as softening of cell walls) most of these genes, including *pgip*, are down regulated and the berries also become highly susceptible to fungal infections. It has been speculated that the berries remain resistant to fungal attack until the seeds are fully formed (*véraison*-stage) and that from an evolutionary perspective there is no need (for the plant) after that to protect the berries. Grapevine susceptibility to *B. cinerea* should therefore not primarily be used to gauge the efficiency of the grapevine PGIP as a possible antifungal agent.

During fungal infection, it is thought that spreading lesion development and the expansion of fungal biomass is dependent on the expression and secretion of PGs (ten Have et al. 2002; Kars et al. 2005). *Botrytis* has been shown to express at least six PGs (encoded by *Bcpg1-6*) during the infection process (ten Have et al. 2001). BcPG1 and 2 have been shown to be important pathogenicity factors (ten Have et al. 1998; Kars et al. 2005).

Tobacco transgenic lines were also used to purify VvPGIP1; this provided a mechanism to link a specific PG inhibition profile to the *Vvpgip1* encoded PGIP. PGIP purified from grape berries showed inhibition against PGs from several fungal pathogens (De Ascensao 2001), but the inhibition profile of individual grapevine PGIPs has not been determined yet. Leaf material from line 37 was used in a purification protocol that yielded several active fractions; these were confirmed to contain polypeptides of the expected sizes corresponding to previous data regarding the glycoforms of the PGIP purified from grapevine berries (De Ascensao 2001). The heterologously produced and purified VvPGIP1 were also used to evaluate the interaction and inhibition of this inhibitor towards individual PGs from *B. cinerea* and *Aspergillus niger*. These PGs have earlier been shown to be homogeneous (Kemp et al. 2004; Krooshof et al. 2004; Kars et al. 2005). Differential inhibition was observed for PGs from both *A. niger* and *B. cinerea*. VvPGIP strongly inhibited BcPG1 and 6 under all conditions tested, whereas inhibition towards BcPG3 was less pronounced. The inhibition of BcPG4 was very pH dependent, as was the inhibition for AnPGA and B. Unlike the results of Kemp et al. (2004) for a PGIP from *Phaseolus vulgaris* (PvPGIP2), PG activation could not be detected.

These inhibition assays confirmed the ability of VvPGIP1 to inhibit fungal PGs. The observed decrease in *Botrytis* symptom development in tobacco lines over-expressing VvPGIP1, could therefore be due to the inhibition of the fungal PGs, specifically the strong inhibition of BcPG1. BcPG1 has been shown to be essential for pathogen virulence and causes tissue maceration, chlorosis and necrosis when applied in purified form to tobacco plants (Kars et al. 2005). The ability of PGIP to inhibit fungal PGs in vitro suggests that the *in planta* role of PGIPs includes protection of the plant cell walls by inhibiting pathogen PG action.

In conclusion, the over-expression of a grapevine PGIP encoding gene in tobacco and the subsequent purification of the inhibitor confirmed that this gene encodes a PGIP with strong activity against *Botrytis* PGs. Moreover, the VvPGIP1 differentially inhibits individual PGs from *B. cinerea*, as well as *A. niger* and this data provide one of the first reports of detailed inhibition studies of a PGIP and the individual PGs produced by a pathogen. Transgenic tobacco plants expressing VvPGIP1 displayed reduced symptom development when inoculated with *Botrytis*, suggesting that the in vitro PG inhibition by this PGIP also occurred *in planta*, at least in part contributing to the observed reduced disease symptoms. This study provides a strong base to study the currently available and other potential grapevine PGIPs further, to identify inhibitors with specialised PG inhibition profiles and to also study and understand the *in planta* roles of PGIPs in disease responses and signalling.

Acknowledgements This work was supported by grants from the South African Wine Industry (Winetech), the National research foundation of South Africa (NRF), the US Department of Energy (DOE) (DE-FG02-96ER20221) and the DOE-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG02-93ER20097).

References

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254

Carstens M, Vivier MA, Pretorius IS (2003) The *Saccharomyces cerevisiae* chitinase, encoded by the *CTS1-2* gene, confers antifungal activity against *Botrytis cinerea* to transgenic tobacco. Transgenic Res 12:497–508

Davies C, Robinson SP (2000) Differential screening indicates a dramatic change in mRNA profiles during grape berry ripening. Cloning and characterization of cDNAs encoding putative cell wall and stress response proteins. Plant Physiol 122:803–812

De Ascensao A (2001) Isolation and characterization of a polygalacturonase-inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L. Ph.D. Thesis, Stellenbosch University, Stellenbosch, RSA

De Lorenzo G, Ferrari S (2002) Polygalacturonase-inhibiting proteins in defence against phytopathogenic fungi. Curr Opin Plant Biol 5:295–299

De Lorenzo G, Cervone F, Bellincampi D, Caprari C, Clark AJ, Desiderio A, Devoto A, Forrest R, Leckie F, Nuss L (1994) Polygalacturonase, PGIP and oligogalacturonides in cell–cell communication. Biochem Soc Trans 22:394–397

De Lorenzo G, D'Ovidio R, Cervone F (2001) The role of polygalacturonase-inhibiting proteins (PGIPs) in defence against pathogenic fungi. Annu Rev Phytopathol 39:313–335

Esquerré-Tugayé MT, Boudart G, Dumas B (1999) Cell wall-degrading enzymes, inhibitory proteins and oligosaccharides participate in the molecular dialogue between plants and pathogens. Plant Physiol Biochem 38:157–163

Favaron F, D'Ovidio RD, Porceddu E, Alghisi P (1994) Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. Planta 195:80–87

Federici L, Caprari C, Mattei B, Savino C, Di Matteo A, De Lorenzo G, Cervone F, Tsernoglou D (2001) Structural requirements of endopolygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein). Proc Natl Acad Sci USA 98:13425

Ferrari S, Vairo D, Ausubel FM, Cervone F, De Lorenzo G (2003) Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15:93–106

Gallois P, Marinho P (1995) Leaf disc transformation using *Agrobacterium tumefaciens*-expression of heterologous genes in tobacco. Methods Mol Biol 49:39–48

Gomathi V, Gnanamanickam SS (2004) Polygalacturonase-inhibiting proteins in plant defence. Curr Sci 87:1211–1217

Hahlbrock K, Scheel D, Logemann E, Nurnberger T, Parniske M, Reinold S, Sacks WR, Schmelzer E (1995) Oligopeptide elicitor-mediated defence gene activation in cultured parsley cells. Proc Natl Acad Sci USA 92:4150–4157

Herlache TC, Hotchkiss AT, Burr TJ, Collmer A (1997) Characterization of the *Agrobacterium vitis pehA* gene and comparison of the encoded polygalacturonase with the homologous enzymes from *Erwinia carotovora* and *Ralstonia solanacearum*. Appl Environ Microbiol 63:338

Hood EA, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218

Idnurm A, Howlett BJ (2001) Pathogenicity genes of phytopathogenic fungi. Mol Plant Pathol 2:241–255

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a selective and versatile gene fusion marker in higher plants. EMBO J 13:3901–3907

Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JAE, Van Kan JAL (2005) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J 43:213–225

Kemp G, Stanton L, Bergmann CW, Clay RP, Albersheim P, Darvill A (2004) Polygalacturonase-inhibiting proteins can function as activators of polygalacturonase. Mol Plant Microbe Interact 17:888–894

King D, Bergmann C, Orlando R, Benen JAE, Kester HCM, Visser J (2002) Use of amide exchange mass spectrometry to study conformational changes within the endopolygalacturonase II-homogalacturonan-polygalacturonase inhibiting protein system. Biochemistry 41:10225–10233

Krooshof GH, Joosten R, Kester HCM, Kars I, Van Kan JAL, Benen JAE (2004) Endopolygalacturonases from *Botrytis cinerea*: biochemical properties and interaction with inhibiting proteins. XIII *Botrytis* symposium. Antalya, Turkey

McGarvey P, Kaper JM (1991) A simple and rapid method for screening transgenic plants. Biotechniques 11:428–432

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Pařenicová L, Benen JAE, Kester HCM, Visser J (1998) PgaE encodes a fourth member of the endoploygalacturonases of *Aspergillus niger*. Eur J Biochem 251:72–80

Powell ALT, Van Kan JAL, ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM (2000) Transgenic expression of pear PGIP in tomato limits fungal colonisation. Mol Plant Microbe Interact 9:942–950

Reymond P, Grunberger S, Paul K, Muller M, Farmer EE (1995) Oligogalacturonide defence signals in plants: large fragments interact with the plasma membrane *in vitro*. Proc Natl Acad Sci USA 92:4145–4149

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

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shieh MT, Brown RL, Whitehead MP, Cary JW, Cotty PJ, Cleveland TE, Dean RA (1997) Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl Environ Microbiol 63:3548–3552

Taylor RJ, Secor GA (1988) An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopathology 78: 1101–1103

ten Have A, Mulder W, Visser J, Van Kan JAL (1998) The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol Plant Microbe Interact 11:1009–1016

ten Have A, Oude Brueil W, Wubben J, Visser J, van Kan JAL (2001) *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. Fungal Genet Biol 33:97–105

ten Have A, Tenberge KB, Benen JAE, Tudzynski P, Visser J, van Kan JAL (2002) The contribution of the cell wall degrading enzymes to pathogenesis of fungal plant pathogens. In: Kempken F (ed) The Mycota XI, agricultural applications. Springer-Verlag, Berlin, pp 341–358

Wubben JP, Mulder W, ten Have A, van Kan JAL, Visser J (1999) Cloning and partial characterisation of endopolygalacturonase genes from *Botrytis cinerea*. Appl Environ Microbiol 65:1596–1602

York WS, Darvill AG, McNiel M, Stevenson TT, Albersheim P (1985) Isolation and characterization of plant cell walls and plant cell wall components. Methods Enzymol 118:3–40

RESEARCH RESULTS

Constitutive levels of PGIP influence cell wall metabolism in transgenic tobacco

John V.W. Becker[1], Andreas G.J. Tredoux[1], Katherine J. Denby[2,3] and Melané A. Vivier[1]

[1]Institute for Wine Biotechnology, Department of Viticulture and Oenology, Faculty of AgriSciences, Stellenbosch University, Stellenbosch, South Africa

[2]Department of Molecular and Cell Biology, University of Cape Town, Private Bag, Rondebosch, Cape Town, South Africa

[3] Warwick HRI and Warwick Systems Biology, University of Warwick, Wellesbourne, Warwick, CV35 9EF, United Kingdom (current address)

4.1 Abstract

Polygalacturonase-inhibiting proteins (PGIPs) in plant cell walls inhibit fungal endopolygalacturonases (ePGs). This inhibition interaction directly limits the effective ingress of the pathogen relying on functional ePGs to breach the plant cell wall and is hypothesized to facilitate the prolonged presence of elicitor active molecules to upregulate the plant's defense response.

Overexpression and silencing studies of PGIP encoding genes provide ample proof that PGIPs confer resistance phenotypes to their respective transgenic plant backgrounds. Recently, PGIP activity, ePG inhibition and decreased *Botrytis* susceptibility could be correlated in tobacco plants overexpressing a grapevine (*Vitis vinifera* L.) PGIP-encoding gene. Building on that study, two transgenic lines with prominent resistance phenotypes were selected to be compared to the untransformed control on the transcriptomic and hormonal level. Here the results obtained when analyzing the plant lines without inducing the resistance phenotype are reported. Microarray analysis showed that 52 genes were differentially expressed between the wild-type and the transgenic lines under these uninducing conditions. Statistical analysis showed that several genes involved in cell wall metabolism were affected by the presence of constitutive copies of PGIP. Transcriptional differences in the *Vvpgip1*-expressing lines, when compared to the wild-type, included downregulation of a gene encoding tobacco xyloglucan endotransglycosylase (XET), and altered expression of genes involved in the lignin biosynthetic pathway. Real-Time quantitative PCR confirmed that tobacco *XET* was downregulated in both the transgenic lines analyzed in the microarray analysis, as well as in the other lines with a similar resistance phenotype. The transcriptomic analysis was followed with an analysis of lignin content in the lines and biochemical data corroborate the increase in lignin content in the transgenic lines. A hormone profile of the plant lines showed that in *Vvpgip1*-overexpressing lines, indole-acetic acid levels (IAA) were elevated. The PGIP-specific resistance phenotypes could be linked to one of the most basic mechanisms plants use to defend themselves, namely strengthening of cell walls. The fact that this analysis was done under uninfecting and uninducing conditions, points to an additional role for PGIP beyond the direct inhibition of ePGs and defense signaling that is currently accepted. It might indicate that the structural features of PGIP and/or its integration in cell wall complexes could possibly

trigger defense responses, perhaps leading to a permanent defense-primed state. PGIP expression is typically upregulated by infection; having constitutive levels of this known defense protein might directly activate general defense responses such as strengthening of cell walls. Linking this "priming" phenomena with the PGIP-specific resistance phenotypes under uninfecting conditions does significantly contribute to understanding *how* PGIPs might improve disease resistance and also provide insight into the *in planta* roles of PGIPs.

4.2 Introduction

Polygalacturonase-inhibiting proteins (PGIPs) are extracellular-leucine rich repeat (eLRR) proteins, found in all plant species thus far examined, with recognition and inhibition capabilities towards fungal endopolygalacturonases (ePGs) (De Lorenzo et al., 2001). ePGs are among the first enzymes to be secreted during fungal infection and are capable of hydrolyzing the homogalacturonan component of plant cell wall pectin. These enzymes have been shown to play a major role in the virulence of several phytopathogenic fungal and bacterial species (Rodriguez-Palenzuela et al., 1991; Shieh et al., 1997; ten Have et al., 1998; Isshiki et al., 2001; Tans-Kersten et al., 2001; Oeser et al., 2002; Li et al., 2004; Kars et al., 2005). Most notably, two ePGs of the necrotrophic fungus *Botrytis cinerea* are required for its full virulence on different plant hosts (ten Have et al., 1998; Kars et al., 2005). Typically, the genes encoding PGIP are inducible by a wide range of environmental stimuli (reviewed in De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002). This is also true of a grapevine PGIP encoding gene, *Vvpgip1*, which is induced by *Botrytis cinerea* infection, wounding, osmotic stress and hormonal cues (Joubert, DA., De Lorenzo, G., Pretorius, IS. and Vivier, MA., unpublished results).

PGIPs differentially inhibit ePGs from not only a diverse range of fungi, but also different ePG isoforms of the same fungus (De Lorenzo et al., 2001). This is well illustrated for VvPGIP1 and ePGs from *Aspergillus niger* and *B. cinerea*, where differential inhibition towards these ePGs was observed (Joubert et al., 2006) in *in vitro* assays. Further *in vitro* evidence suggests that the interaction and resultant inhibition of ePG by PGIP results in the prolonged existence of molecules with the ability to upregulate a plant's natural defense response (Cervone et al., 1989). Thus, PGIPs are believed to protect plants from fungal infection by not only inhibiting fungal macerating enzymes and thereby directly limiting tissue damage, but also by switching on plant defense pathways as a result of this inhibition. Although these *in vitro* experiments have contributed significantly to our understanding of the dialogue between PGIPs and their ligands, ePGs, evidence is yet to emerge to substantiate the *in planta* roles of PGIP and the resultant events following the formation of the inhibition complex. Recent findings have highlighted the need to include *in vivo* experimentation when studying PGIP-ePG interactions. It was shown that the VvPGIP1 reduces the symptoms of the BcPG2 from *B. cinerea* in tobacco leaves, without any evidence for an *in vitro* interaction (Joubert et al., 2007). From this work it

appeared that the *in vivo* environment provided a context for this specific PGIP-ePG pair that could not be duplicated *in vitro*. It was proposed that VvPGIP1 might bind to pectin as was shown for bean PGIPs (Spadoni et al., 2006) and that VvPGIP1 did not directly inhibit BcPG2, but perhaps rather shielded the most exposed and vulnerable positions in the pectin (Joubert et al., 2007), thereby affording indirect protection against the actions of the ePG.

Numerous studies, where high level expression resulted in reduced susceptibility towards *B. cinerea*, have been reported that substantiated the role of PGIP in plant defense. These included overexpression of the pear *pgip* gene in tomato (Powell et al., 2000) and grapevine (Aguero et al., 2005); *Arabidopsis pgip* genes overexpressed in *Arabidopsis* (Ferrari et al., 2003); a bean *pgip* gene in tobacco (Manfredini et al., 2005) and recently the grapevine *pgip* gene in tobacco (Joubert et al., 2006, Chapter 3 of this dissertation). Also, antisense expression of *Arabidopsis AtPGIP1* resulted in enhanced susceptibility towards this pathogen (Ferrari et al., 2006). From these investigations, it is clear that the level of PGIP expression at the time of pathogen inoculation is critical. This is further illustrated by the fact that PGIP levels correlate with increasing resistance in immature raspberry fruit (Johnston et al., 1993) and bean hypocotyls of increasing age (Salvi et al., 1990). Ripening pear fruits show an increased susceptibility towards *Botrytis* and *Dothiorella* which correlates with a decline in PGIP level in the fruit (Abu-Goukh et al., 1983).

Transgenic tobacco plants overexpressing *Vvpgip1* and accumulating high levels and activity of VvPGIP have convincingly been demonstrated to be less susceptible to *B. cinerea* infection in both detached leaf and whole-plant, time-course antifungal assays (Joubert et al., 2006). These lines are considered to be PGIP-specific resistant lines (i.e. the resistance phenotype could be correlated to the overexpression, acitivity levels and the ePG-inhibition profiles of the VvPGIP) and as such provide excellent genetic resources to study the possible *in planta* role(s) of PGIPs in mediating the observed resistance phenotypes. To this end, transcriptomic analysis and hormone profiling was conducted on two PGIP-specific resistant lines in comparison with the wild type. These analyses revealed that under uninducing/uninfecting conditions, the presence of PGIP caused altered cell wall metabolism.

4.3 Materials and Methods

4.3.1 Plant Material and Growth Conditions

Wild-type and transgenic *Nicotiana tabacum* SR1 (Petit Havana) plants were grown in a soil and peat moss (Jiffy Products International AS, Norway) mixture in a growth room at 24°C and 55% relative humidity. Plants were supplemented with a commercially available liquid organic fertilizer every two weeks (Nitrosol®, Fleuron

(Pty) Ltd, South Africa). A light intensity of 120 µmol.m^2.s^{-1} was maintained for the 16 h light photoperiod.

Untransformed WT and transgenic lines VvPGIP 37 and VvPGIP 45 (as described in Joubert et al. (2006; Chapter 3 of this dissertation) were utilized for all analyses. (In quantitative Real-Time PCR, additional transgenic lines were utilized). Expression of the *Vvpgip1* transgene was confirmed by Northern blotting in all transgenic lines (results not shown). These two transgenic lines exhibited a marked decrease in susceptibility towards *Botrytis* infection in detached leaf and whole-plant antifungal assays (Joubert et al., 2006), and were statistically separable from the untransformed wild-type. These lines were considered PGIP-specific resistant phenotypes and were analysed, in comparison with the wild-type without any infection or disease inducing condition.

Leaf material (positions three to five) of healthy six to eight week old tobacco plants were collected, flash frozen in liquid nitrogen and stored at -80℃ for RNA extraction, lignin analyses and phytohormone profiling.

4.3.2 Microarray Analyses

4.3.2.1 RNA Extraction

RNA was extracted using a sodium perchlorate-based method. An extraction buffer consisting of 5 M sodium perchlorate, 0.3 M Tris-HCl (pH 8.3), 8.5% polyvinylpolypyrollidone (PVPP), 2.0% PEG 4000, 1.0% β-mercapto-ethanol and 1.0% SDS was added to plant material finely ground in liquid nitrogen. The tissue was allowed to thaw in this buffer and shaken for 30 min at room temperature. Following centrifugation, the supernatant was passed through a syringe plugged with cotton wool to remove insoluble reagents and plant debris. Several phenol/chloroform extractions were performed before precipitating the RNA with 2.5 M LiCl at -20°C overnight. The pellet was washed with 70% ethanol and the resuspended RNA was purified using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). RNA was quantified using the Nanodrop (Wilmington, DE, USA).

4.3.2.2 cDNA Synthesis and Hybridization

Two cDNA synthesis reactions of 25 µg each were set up for each test or reference RNA sample in a total of 30 µL. Before denaturation at 70ºC for 10 min, 2 µL of oligo d(T) primers (500 µg/mL) were added. Following denaturation, first strand buffer and DTT were added according to the manufacturer's recommendation (InVitrogen, Carlsbad, CA, USA). A 2:3 (aa-dUTP:dTTP) aminoallyl-dNTP (Ambion, Austin, TX, USA) mix was added to a 1X concentration (0.5 mM each of dATP, dCTP and dGTP; 0.2 mM aa-dUTP and 0.3 mM dTTP) before incubation at 46ºC for 2 min, after which 200 U of SuperScript III Reverse Transcriptase (InVitrogen, Carlsbad, CA, USA) was

added. The same amount of enzyme was added following incubation for 4 h at 46ºC, after which cDNA synthesis proceeded overnight.

RNA was hydrolyzed by the addition of 10 µL each of 1 M NaOH and 0.5 M EDTA solutions and incubated at 65ºC for 15 min. To neutralize the pH, 10 µL of a 1 M HCl solution was added following hydrolysis. Unincorporated aminoallyl dUTP and free amines were removed by purification using the RNeasy Mini Kit. The eluted cDNA was quantified using a Nanodrop and similar quantities of test and reference cDNA were dried down to volumes of less than 1 µL in a vacuum drier. Five microliters of a 0.1 M Na_2CO_3 buffer, pH 9.0, was added to the cDNA, mixed well, and incubated at 37ºC for 10 min. Cy3 or Cy5 (4.5 µL) (Amersham Biosciences, Buckinghamshire, UK) ester was added and the coupling allowed to proceed for 1 h in the dark at room temperature. Uncoupled dyes were removed by purification with the RNeasy Mini Kit. Probe labeling with Cy-esters were estimated by measuring the absorbance at 550 nm and 650 nm on a Nanodrop and calculating the fold of incorporation of dye. Similar amounts of labeled probe were hybridized to the microarray slides. Slides were prehybridized with a solution containing 5X SSC (from 20X SSC: 3 M NaCl, 1.5 M sodium citrate, pH 7.0), 0.1% SDS and 1% BSA, prewarmed to 42ºC. The solution was pipetted under lifterslips (Erie Scientific, Portsmouth, NH, USA) and the slides incubated at 42ºC for at least 30 min. Following prehybridization, the slides were washed in five washes of deionized water and finally briefly submerged in ethanol before centrifugation for 5 min at 1000 rpm. The combined Cy-labelled probes (28 µL) were mixed with 30 µL of a 2X hybridization buffer (50% formamide, 5X SSC and 0.2% SDS), to which was added 1 µL each of COT1 DNA (1 µg/µL) and poly(A)-DNA (12 µg/µL), for a total volume of 60 µL. The probes were denatured at 90ºC for 3 min, and subsequently applied to the slide under lifterslips. The microarray slides were enclosed in ArrayIt (Telechem International, Sunnyvale, CA, USA) hybridization chambers and submersed in a heated water bath and incubated for 16 h at 42ºC. The slides were successively washed in low stringency (2X SSC, 0.5% SDS; heated to 55ºC), medium stringency (0.5X SSC) and high stringency (0.05X SSC) wash buffers for 5 min each. Prior to centrifugation (5 min, 1000 rpm) the slides were briefly submerged in ethanol. Scanning was performed with an Axon GenePix 4000A scanner (Molecular Devices, Sunnyvale, CA, USA). Photomultiplier tube (PMT) settings for each channel were optimized during prescans to obtain equal output from the red and green channels and to minimize the amount of saturated spots, before proceeding to full scans. GenePix results (gpr) files were generated using GenePix 5.1 (Molecular Devices, Sunnyvale, CA, USA) software, without normalization. The results files were normalized with DNMAD (Vaquerizas et al., 2004) using print-tip loess and inconsistent replicates were removed. The normalized values were subsequently downloaded and analyzed with the Multiexperiment Viewer (MeV) in the TM4 software suite (Saeed et al., 2003). Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) was performed to obtain genes differentially expressed between

the untransformed control and *Vvpgip1*-transformed lines. The SAM delta value was adjusted to obtain a maximum of 5% falsely discovered genes.

4.3.3 Quantitative Real-Time PCR

RNA for quantitative PCR analyses was extracted from leaf material of untransformed and transgenic lines in a similar fashion to the protocol utilized for extracting RNA for microarray analyses. A microgram of RNA was utilized for each cDNA synthesis reaction, using SuperScript III Reverse Transcriptase according to the manufacturer's specifications. Both oligo d(T) and random primers were added to ensure full length cDNA was obtained. Real-Time PCR analysis was performed using the LightCycler Instrument (Roche Diagnostics GmbH, Mannheim, Germany). cDNA from each sample was amplified using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). Primer sequences for the tobacco genes xyloglucan endotransglycosylase (*XET*, Genbank Acc AB017025.1) (recently re-annotated as *XTH*, Rose et al., 2002; hereafter referred to as *XTH*) and tubulin (*TUB*, Genbank Acc AB052822) were: *XTH* forward 5'-AGTCCAAGTTTGTAACACC-3' and reverse 5'-TCTGTCCTTAGTGCATTCTG-3', giving rise to an amplification product 175 bp in length; *TUB* forward 5'-TCTGGCTGCTCTGGAAA-3' and reverse 5'-GCATACAAGACACCATCAAAT-3', priming a 197 bp fragment of the tobacco tubulin gene. cDNA amplification conditions were as follows: denaturation at 95ºC for 10 min, followed by 45 cycles of denaturation, 95ºC for 10 s; primer annealing at 58ºC for 10 s and primer extension at 72ºC for 8 s, during which a single data acquisition per cycle was performed. A melting curve analysis was performed at the end of the 45 cycles by raising the temperature from 65ºC to 95ºC, increasing the temperature by 0.1ºC per second in continuous acquisition mode. PCR efficiencies for each sample were calculated using LinRegPCR, software described in Ramakers et al. (2003). Fluorescence per cycle of each sample is exported from which the efficiencies of individual samples were calculated. These efficiencies were used to calculate relative expression in a mathematical model described by Pfaffl (2001).

4.3.4 Determination of Lignin Content

4.3.4.1 Histochemical Assays for Determining Lignin Content

The lignin content of the WT and PGIP-specific resistant lines (VvPGIP 37 and VvPGIP 45) were estimated in leaf sections with potassium permanganate staining followed by transmission electron microscopy according to Fromm et al. (2003). Additionally, the lignin content in the stems of the same lines was estimated by staining with a solution of phloroglucinol according to Ruzin (1999).

4.3.4.2 Lignin Quantification

Lignin was quantified in the WT and transgenic lines VvPGIP 37 and VvPGIP 45 using the acetyl bromide method as described by De Ascensao and Dubery (2003), with some modifications. Dried tobacco leaf material (leaves three to five of eight week old plants) was utilized to isolate the alcohol-insoluble residue (AIR), consisting of cell wall material. Leaf tissue was ground in liquid nitrogen and extracted twice with 80% aqueous methanol following homogenization. Following centrifugation at 12 000x*g* for 10 min, the pellets were washed three times with 96% ethanol and twice with a solution of 96% ethanol:hexane (2:1). The resulting AIR pellets were dried overnight at 70ºC. Five to ten mg of the AIR was used to determine the percentage of lignin contained therein. The AIR was washed with 25% acetyl bromide (in acetic acid), after which it was incubated in 1 ml of the same solution at 70ºC for 30 min. The mixture was cooled to room temperature, and 0.9 ml of NaOH and 0.1 ml of hydroxylamine hydrochloride (0.1 M) added. The volume was subsequently made up to 10 ml with acetic acid. The solution was left overnight and the absorbance measured at 280 nm with a procedural blank. The lignin content of the samples was calculated as follows: % lignin content = (absorbance X 100) ÷ (SAC X sample concentration ($g.l^{-1}$)); where SAC is the specific absorption coefficient of lignin, for which the value of 20 $g.l^{-1}.cm^{-1}$ was used.

4.3.5 Simultaneous Analysis of Phytohormones Using GC/MS

4.3.5.1 Chemicals

Salicylic acid, indole-3-acetic acid, methyl jasmonate, *o*-anisic acid, trimethylsilyldiazomethane, hexane and 1-propanol were purchased from Sigma-Aldrich (Steinheim, Germany). Dichloromethane and acetic acid were purchased from BDH (Poole, England). Hydrochloric acid was obtained from Saarchem (Wadeville, SA). Water was deionized using a MilliQ water purification system (MilliQ, Billerica, MA, USA).

4.3.5.2 Sample Preparation for Vapor Phase Extraction

Leaf tissue flash frozen in liquid nitrogen was extracted according to the method of Schmelz et al. (2004), with some modifications. Approximately 100 mg of tissue was ground to a fine powder in eppendorf tubes prior to the addition of extraction solvent (n-propanol/water/HCl) and internal standard (*o*-anisic acid), of which 30 ng per sample was added. Samples were vortexed to ensure homogenization prior to partitioning with dichloromethane. For the conversion of phytohormone acids to their corresponding methyl esters, the organic phase (dichloromethane/propanol) was derivatized in 4 mL glass vials for 30 minutes using 4 µL of a 2 M

trimethylsilyldiazomethane solution in hexane. The activity of derivatization agent was subsequently quenched with 4 µL of a 2 M acetic acid solution, also in hexane.

4.3.5.3 Vapor Phase Extraction

Extraction of the derivatized organic phase proceeded according to Schmelz et al. (2004), with the exception that commercially available Super Q filters were purchased from Analytical Research Systems, Inc. (Gainesville, FL, USA). Briefly, the derivatized sample was evaporated at 70ºC and passed through a Super Q filter under a N_2 flow of 500 mL/min. To ensure complete vaporization of less volatile compounds the vial was subsequently heated to 200ºC for 2 min while passing the vapor through the filter. The analytes were eluted from the Super Q filter with 150 uL CH_2Cl_2 and analyzed by EI-GC/MS as described below.

4.3.5.4 Instrumentation

A Trace Gas Chromatograph (GC) (ThermoFinningan, Milan, Italy) coupled to a Trace Mass Spectrometer (MS) (Thermo MassLab, Manchester, UK) was used for all analyses. GC/MS conditions were amended from that described by Schmelz et al. (2004). Briefly, 2 uL of the dichloromethane eluent was injected in the split/splitless injector of the GC, operated in the splitless mode (purge time 3.5 min., 50 mL/min.) at 280ºC. Compounds were separated on a Factor Four VF5-MS capillary column (Varian, Palo Alto, CA, USA) with dimensions 30 m l. x 0.25 mm i.d. x 0,25 um f.t. Flow of the carrier gas (Helium) through the column was 0.7 mL/min. in the constant flow mode. The oven program used was 40ºC, hold 1 min., ramp 15ºC/min to 250ºC, hold 5 min., ramp 20ºC/min., and hold 2 min. In order to avoid carryover a post-run was performed after each analysis at 280ºC under a head-pressure of 300 kPa. The temperature of the MS interface was kept at 280ºC and the source at 200ºC. The MS-detector was operated in Electron Impact (EI) mode at 70 eV and Selected Ion Monitoring (SIM) mode. The electron multiplier voltage was set at 500 V.

Three carboxylic acid methyl ester analytes were detected and quantified using SIM with retention times and ion mass to charge ratios (m/z) as follows: methyl salicylate (8.39 min., m/z 92, 120, 152); methyl jasmonate (12.38 min., m/z 83) and methyl indole-3-acetate (13.81 min., m/z 130). The internal standard, *o*-anisic acid methyl ester was eluted at 9.70 min. with m/z 92, 120 and 152.

For quantitation the internal standard method was used. Calibration curves were constructed for each analyte over the range from 2-200 $ng.mL^{-1}$. The regression equations and their correlation co-efficients obtained for SA, MeJA and IAA are detailed respectively: $y=0.0370x+0.2511$ ($r^2=0.9924$), $y=0.0087x+0.0303$ ($r^2=0.9954$) and $y=0.0120x+0.0238$ ($r^2=0.9964$). The limit of quantification (LOQ) was established to be 2 pg for all analytes. Statistical analysis was performed in STATISTICA 6 (Statsoft Inc., Tulsa, OK, USA).

4.4 Results

Vvpgip1-overexpressing tobacco lines from Joubert et al. (2006; this work, Chapter 3) were used in this study. These lines are considered to have a PGIP-specific resistance phenotype since a correlation could be drawn between overexpression of the gene, PGIP activity as well as ePG inhibition and the resistance phenotypes obtained. Microarray analysis and hormone profiling was performed on healthy (uninfected) transgenic lines in comparison with the wild type control.

4.4.1 Microarray Analyses

The integrity of RNA extracted from leaf material from uninfected wild-type (WT) and transgenic lines VvPGIP 37 and VvPGIP 45 was visualized on 1.2% formaldehyde gels (results not shown). No degradation of RNA was visible in any of the samples. The purity of the samples was satisfactory (absorbance ratios of 260/230 and 260/280 >2). cDNA synthesis from a total of 50 µg of RNA yielded more than a microgram of cDNA, and equal amounts of reference and test cDNA were indirectly coupled to the dyes Cy3 and Cy5. The pooled reference (WT) and test cDNAs (VvPGIP 37 or VvPGIP 45) were hybridized to TIGR 10K potato microarray (version 3) slides. Three microarray slides in total were hybridized: two slides using VvPGIP 37 and WT, with a dye swap included to account for dye bias; and one slide using VvPGIP 45 as test and WT as reference.

A total of 52 genes were found to be differentially expressed by Significance Analysis of Microarrays (SAM) from the three slides (Full set of genes in Addendum A to Chapter 4). Genes found to be differentially expressed were all downregulated in the two transgenic lines as compared to WT. All genes exhibiting downregulation greater than 1.5-fold (Log_2 = 0.58) are shown in Table 4.1. The SAM parameters were adjusted to obtain a maximum of 5% falsely discovered genes. Gene annotations highlighted in bold share high homology and encode for xyloglucan endotransglucosylase/hydrolases (XTHs). Due to the nature of cross-species cDNA microarrays, genes may hybridize to spots with which they share high homology. BLAST analysis (Altschul et al., 1997) of the sequence annotated as Brassinosteroid Regulated Protein BRU1 (the cDNA with the highest degree of downregulation) revealed that it shared the highest homology with tobacco *XTH* (Genbank Acc AB017025.1 and D86730). Primers for use in quantitative Real-Time PCR with this gene were subsequently obtained to verify the relative expression levels in a larger set of transgenic plants.

Table 4.1. Genes found to be differentially expressed between the untransformed control and *Vvpgip1*-transformed lines. Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) was performed in the TM4 software suite (Saeed et al., 2003), available from TIGR. The SAM delta value was adjusted to obtain a maximum of 5% falsely discovered genes. Annotations from the TIGR 10k potato array ver3 in bold share homology and are genes encoding xyloglucan endotransglycosylases.

Annotation	Mean (Log_2) Relative expression	VvPGIP 37	VvPGIP 37 (dye swap)	VvPGIP 45
Brassinosteroid-regulated protein BRU1 precursor {*Glycine max*}	-1.296	-1.335	-1.125	-1.428
Q8LNW9 Hypothetical protein	-1.044	-0.864	-1.235	-1.034
Q9FZ05 Xyloglucan endotransglycosylase LeXET2	-1.013	-1.010	-1.088	-0.940
Q8S902 Syringolide-induced protein 19-1-5	-0.958	-0.980	-0.828	-1.066
Xyloglucan endo-1,4-beta-D-glucanase precursor clone tXET-B2 tomato	-0.941	-0.918	-0.882	-1.025
Q9LLC2 Xyloglucan endotransglycosylase XET2 EC_2.4.1.207 Q8S902 Syringolide-induced protein 19-1-5	-0.921	-0.837	-0.822	-1.104
AAQ54533 Putative DnaJ protein Fragment	-0.908	-0.865	-0.902	-0.956
Q9M9N3 T17B22.17 protein	-0.887	-1.159	-0.721	-0.782
Q94JV1 At1g69340/F10D13.28 AAQ54533 Putative DnaJ protein fragment	-0.880	-0.852	-0.970	-0.818
Pyruvate kinase isozyme A chloroplast precursor {*Nicotiana tabacum*} EC 2.7.1.40	-0.820	-0.994	-0.551	-0.916
Plasma membrane protein – common tobacco {*Nicotiana tabacum*}	-0.785	-0.765	-0.791	-0.800
Q7YDN0 ATP synthase 6	-0.785	-0.873	-0.755	-0.727
ADP ATP carrier protein mitochondrial precursor ADP/ATP translocase	-0.780	-0.957	-0.557	-0.827
Q9M517 TMV-induced protein	-0.778	-0.598	-0.874	-0.864
Plasma membrane protein- common tobacco {*Nicotiana tabacum*}	-0.740	-0.675	-0.937	-0.610
hypothetical protein F19C24.7 imported – *Arabidopsis thaliana*	-0.738	-0.760	-0.658	-0.797
Q8I430 Serine protease belonging to subtilisin family putative	-0.733	-0.791	-0.600	-0.808
Remorin pp34 {*Solanum tuberosum* }	-0.675	-0.76	-0.712	-0.554
ADP ATP carrier protein mitochondrial precursor ADP/ATP translocase	-0.664	-0.729	-0.548	-0.715
Translationally controlled tumor protein homolog TCTP P23 {*Solanum tuberosum*}	-0.662	-0.750	-0.646	-0.589
Q9LGP3 P0684C01.2 protein P0698A04.25 protein	-0.659	-0.731	-0.415	-0.833
Histidine decarboxylase HDC TOM92 {*Lycopersicon esculentum*}	-0.640	-0.455	-0.674	-0.791
Hypothetical protein predicted by GeneMark.hmm etc {*Oryza sativa* japonica cultivar-group}	-0.622	-0.632	-0.470	-0.765
Q93ZG1 At1g23060/T26J12 16 CAB38630 SPAC3G9.01 protein	-0.617	-0.754	-0.459	-0.640
Q93Z81 At3g51860/ORF11Ca2+/H+-exchanging protein – mung bean {*Vigna radiata*}	-0.610	-0.741	-0.635	-0.454
Xyloglucan endo-1,4-beta-D-glucanase precursor clone_tXET-B2- tomato Q84UH3 Putative ascorbate peroxidase	-0.608	-0.615	-0.616	-0.595
Q8GTJ0 Xyloglucan endotransglycosylase	-0.602	-0.431	-0.652	-0.723
AT3g56200/F18O21 160 {*Arabidopsis thaliana*}	-0.587	-0.685	-0.428	-0.648

The transcriptomic data from the independent transgenic lines was also screened for overlapping pathways affected by *Vvpgip1* overexpression. Thus, genes differentially expressed in the individual lines, but acting in the same pathway, were identified. It was found that genes encoding proteins which function in the lignin biosynthetic pathway were affected in both lines, but different genes were differentially regulated in the two lines. In line VvPGIP 37, the gene encoding cinnamyl alcohol dehydrogenase (*CAD*) was upregulated 1.5-fold whereas in VvPGIP 45, the gene encoding lignin-forming peroxidase was upregulated 2-fold. These two genes encode the proteins which catalyze the last two reactions in the formation of lignin in plants. The deposition of lignin in the transgenic lines and wild-type was investigated further.

4.4.2 Quantitative Real-Time PCR

Relative expression levels (relative to untransformed WT) of the gene encoding *Nicotiana tabacum XTH* (Genbank Accession AB017025) are shown in Figure 4.1. Expression of the gene was downregulated in the transgenic lines that were subjected to transcriptome analysis. The level of downregulation corresponds to the levels observed from microarray analyses. Expression analyses from an independent experiment confirmed the downregulation of the *XTH* gene in both transgenic lines used in the study (Fig 4.1A), as well as in additional transgenic lines that statistically clustered with the PGIP-specific resistant lines in the study of Joubert et al. (2006) (Fig 4.1B). One of these lines (VvPGIP 1) did not exhibit the observed downregulation of *XTH* and instead had similar *XTH* levels as the wild type (Fig 4.1B). This line was shown to be as susceptible to *Botrytis* infection as the WT (Joubert et al. 2006). However, line VvPGIP 47, also exhibiting susceptibility levels comparable to the WT, showed downregulation of this gene to levels comparable to the more resistant transgenic lines (VvPGIP 24, VvPGIP 37 and VvPGIP 45) (Joubert et al., 2006). Additional members of the tobacco XTH family are currently being assessed for differential expression.

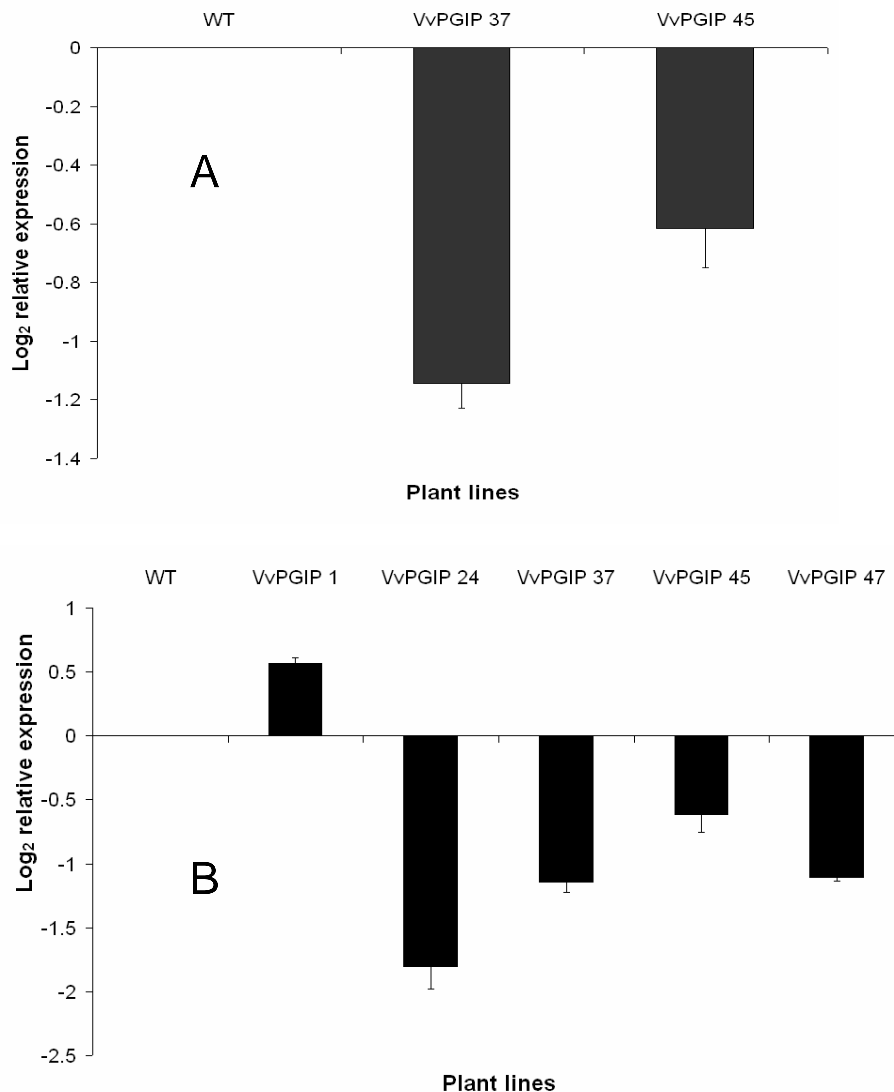


Figure 4.1. (**A)** Expression of *Nicotiana tabacum* xyloglucan endotransglycosylase (Genbank Acc AB017025.1) in *Vvpgip1* transgenic lines 37 and 45 relative to the untransformed control (WT). *XTH* expression was downregulated in both transgenic lines showing significant reductions in susceptibility to infection by *Botrytis cinerea* (Joubert et al., 2006). **(B)** Determination of the relative expression of *XTH* in a larger pool of transgenic lines. *XTH* was downregulated in all transgenic lines except VvPGIP 1. Relative gene expression was calculated following Real-Time quantitative PCR analysis using the LightCycler instrument. The mean relative expression of four replicates per plant line is shown with standard deviation. The results were confirmed in an independent experiment.

4.4.3 Determination of Lignin Content

Histochemical analysis of the two *Vvpgip1*-plant lines analyzed revealed that these two lines had increased lignin content compared to the WT. Potassium permanganate staining of thin leaf sections indicated an increased deposition of lignin in the cell walls of the transgenic lines (Fig. 4.2A). Figure 4.2B shows the phloroglucinol stained secondary xylem in tobacco stem tissue of the same plant lines. As with Figure 4.2A, an increased lignin deposition in the two transgenic lines, as compared to WT, can clearly be observed. Similar results were obtained when

more transgenic lines with the resistance phenotype was analyzed for lignin content (results not shown).

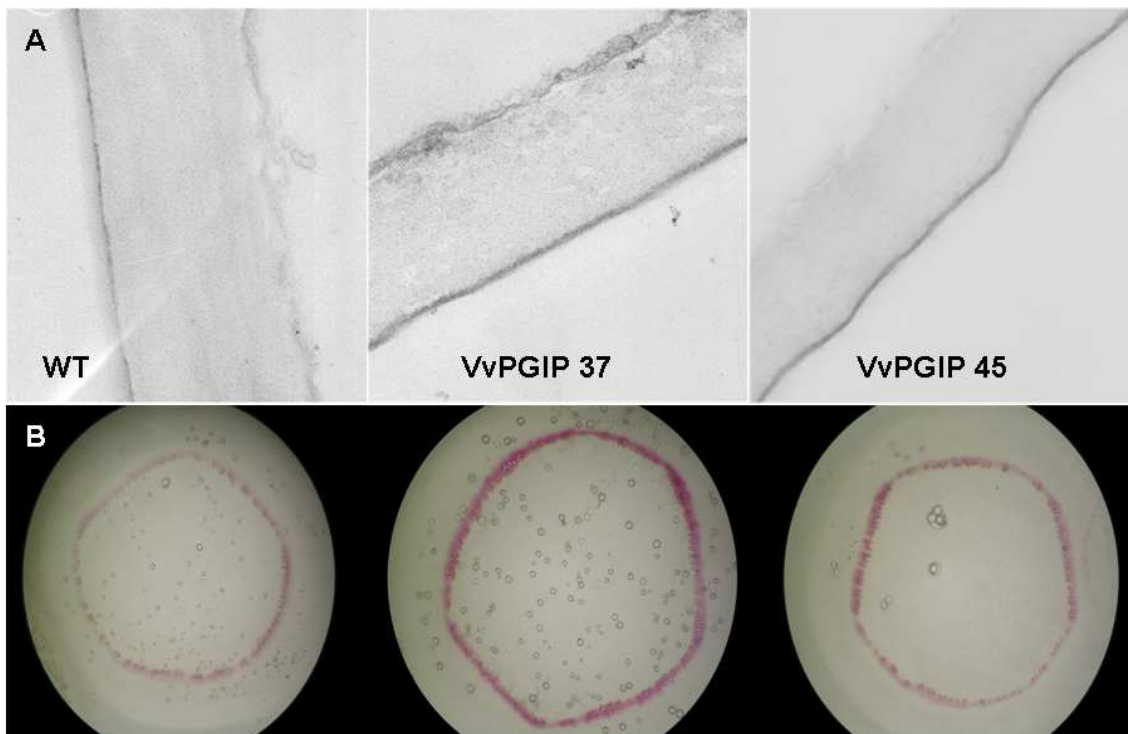


Figure 4.2. Leaf **(A)** and stem **(B)** sections of untransformed (WT) and transgenic lines (VvPGIP 37 and 45) expressing the *Vvpgip1* transgene. **(A)** Leaf sections were stained with potassium permanganate according to Fromm et al. (2003). Lignin in the cell walls stain darker in the transgenic lines. **(B)** Stem sections were hand cut with a razor and stained with a solution of phloroglucinol according to Ruzin (1999). The intensity of the pink-red stain indicates increased lignin deposition in the secondary xylem. Stem sections were taken from the same internode of plants of the same growth stage; leaves for embedding and sectioning were from the same position on plants of the same age (6-8 leaf stage).

Absolute quantitation of lignin content in the lines was also performed. The lignin content of the leaf material of lines WT, VvPGIP 37 and VvPGIP 45 were quantified using the acetyl bromide method as described by De Ascensao and Dubery (2003). The lignin content of these lines is shown in Figure 4.3, represented as the percentage of the alcohol insoluble residue (AIR) (cell wall material). Statistical analyses (range tests) of the lignin content of these lines were performed. At 0.95 confidence intervals, using Duncan's Range test, the WT and VvPGIP37 represented a homogeneous group, but VvPGIP 37 also grouped with VvPGIP 45, a line consistently exhibiting increased lignin content. At 0.90 confidence intervals, the WT was separable from the two transgenic lines in both Duncan's and Newman-Kuels Range tests. These results indicate that increased amounts of lignin may be deposited in the cell walls of leaf tissue of the transgenic lines. With independent assays, it was found that the transgenic lines consistently exhibited increased lignin deposition, although the variability of the assay remains unexpectedly high, which hampers statistical analyses. Refinement of the method is currently in progress.

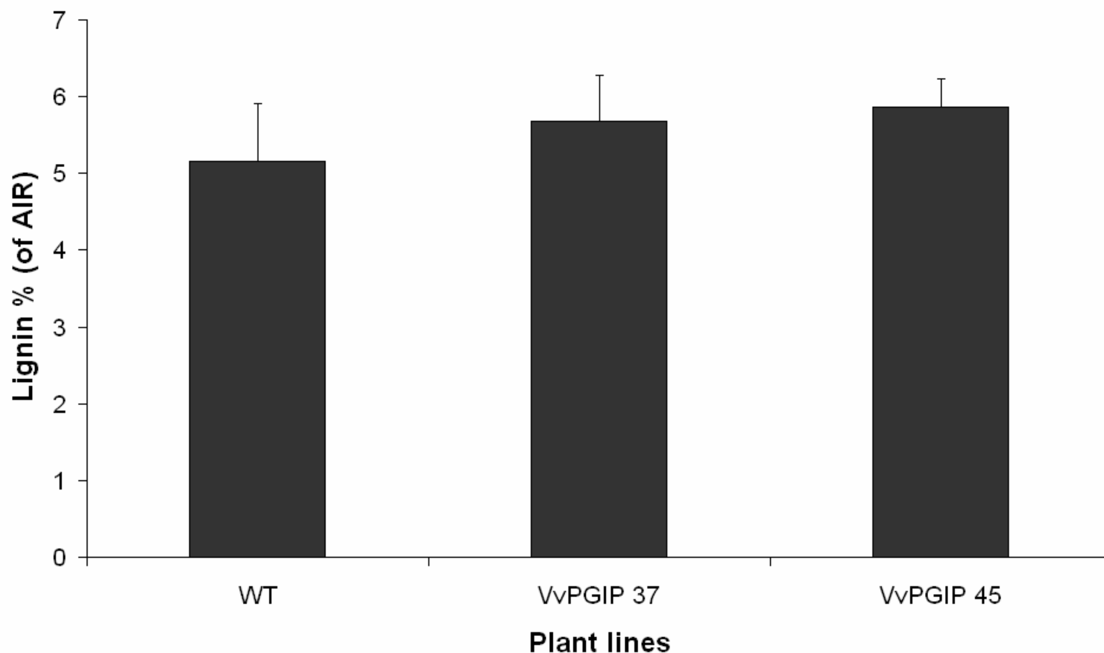


Figure 4.3. Percentage lignin content in the untransformed control (WT) and transgenic lines VvPGIP 37 and VvPGIP 45. The lignin content is expressed as the percentage contained in the alcohol-insoluble residue (AIR), consisting of cell wall material. Statistical analyses (Range tests) could separate the WT from the transgenic lines, but at a confidence interval of 0.90. Refinement of the method, to minimize intra- and interassay variability, is currently underway. Lignin content was determined with the acetyl bromide lignin method, as described by De Ascensao and Dubery (2003). Graphs show the mean of eight replicates.

4.4.4 Simultaneous Analysis of Phytohormones Using GC/MS

Total pools of free (unmethylated and methylated) salicylic acid (SA) and indole-acetic acid (IAA) from untransformed and transgenic lines VvPGIP 37 and VvPGIP 45, represented as their corresponding methyl esters, are shown in Figure 4.4. The jasmonic acid (JA) content of the plant lines was also measured but no JA was detected under the assay conditions. Transgenic lines VvPGIP 37 and VvPGIP 45 (WT) exhibited higher levels of IAA, which was statistically separable in range tests at 0.95 confidence intervals (Duncan's Range Test, STATISTICA 6, Statsoft Inc., Tulsa, OK, USA) from the untransformed WT line. IAA levels inversely correlate to the relative expression of the *XTH* gene (Figure 4.1) in the transgenic lines: the transgenic lines VvPGIP 37 and VvPGIP 45 with higher IAA levels, exhibited downregulation of this gene relative to the WT. SA levels were generally lower in the transgenic lines compared to WT, which was confirmed in an independent experiment. Also, in all transgenic lines the ratio of SA:IAA was higher compared to the WT. The results show the mean of five replicate measurements with standard deviation.

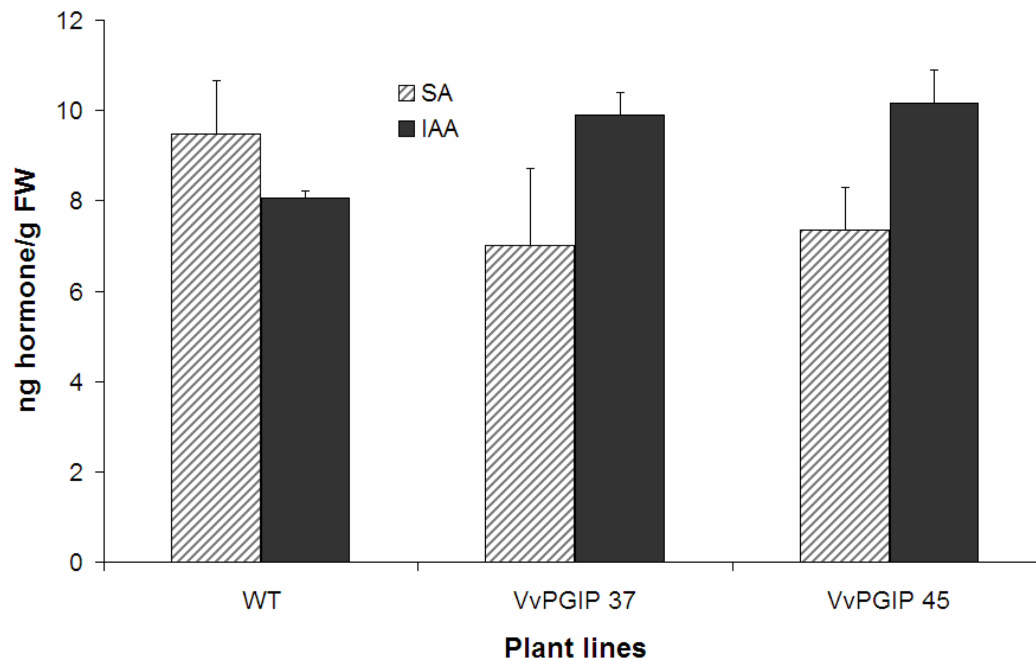


Figure 4.4. Analysis of the phytohormone content of untransformed wild-type and two transgenic lines transformed with the *Vvpgip1* gene. Salicylic acid (SA), indole-acetic acid (IAA), jasmonic acid and abscisic acid were measured as their corresponding methyl esters. The total pool of SA (hatched bars) present in plants as free and methylated forms were analyzed. IAA levels (solid bars) were statistically separable by Duncan's range test in two groups: the untransformed wild-type (WT) had lower levels of IAA; the two transgenic lines (VvPGIP 37 and VvPGIP 45) exhibited higher IAA levels. These IAA levels inversely correlate to *XTH* expression (Figure 4.1). No jasmonic acid (total) was detected in the samples under the analysis conditions. The results show the mean of five replicate measurements with standard deviation.

4.5 Discussion

Polygalacturonase-inhibiting proteins (PGIPs) provide transgenic plants overexpressing their encoding genes with a degree of protection from pathogenesis by *B. cinerea* (Powell et al., 2000, Ferrari et al., 2003, Aguero et al., 2005, Manfredini et al., 2005). Also, *Arabidopsis* plants with antisense *pgip* expression (Ferrari et al., 2006) show increased susceptibility towards infection with *Botrytis*. Raspberries (Johnston et al., 1993) and pear fruit (Abu-Goukh et al., 1983) show correlations between PGIP levels and fungal susceptibility, as do bean hypocotyls of increasing age (Salvi et al., 1990). Clearly, accumulation of appreciable levels of PGIP before the onset of pathogen infection contributes significantly to the outcome of the host-pathogen interaction.

Two transgenic lines, considered to have a PGIP-specific resistance phenotype (Joubert et al., 2006, Chapter 3 of this dissertation), were analyzed for transcriptomic and hormonal differences as compared to an untransformed wild-type (WT) line. These analyses were performed on healthy plants, without inducing the resistance phenotype (without *Botrytis* infection) for the elucidation of the possible *in planta* roles of these proteins towards mediating these resistance phenotypes.

Results from the transcriptomic analyses indicated a number of genes were differentially expressed between the PGIP-accumulating lines and the WT. When considering the two transgenic lines as a *Vvpgip1*-genotype**,** several genes involved in cell wall metabolism were found to be downregulated in the *Vvpgip1*-overexpressing lines. Several annotations reveal that the gene encoding tobacco xyloglucan endotransglycosylase (XET or XTH) was downregulated in these lines, and this was confirmed with quantitative Real-Time PCR. Analyzing the expression of the two independent transgenic lines, it was revealed that two genes encoding proteins catalyzing the last two steps in the formation of lignin was upregulated in the two PGIP-accumulating lines. These genes encode cinnamyl-alcohol dehydrogenase and lignin-forming peroxidase. Biochemical methods indicated that increased lignin was deposited in the cell walls of the transgenic lines. Also, hormonal profiling showed that the levels of IAA were increased in the transgenic lines, as compared to a slight decrease of SA for the two lines. These alterations in the PGIP-specific resistant lines are discussed in detail below.

Tobacco *XTH* is downregulated in PGIP-accumulating lines

Quantitative Real-Time PCR confirmed that tobacco *XTH* is downregulated in all the transgenic lines exhibiting the PGIP-specific resistance phenotype. These results indicate that in the PGIP-accumulating lines, the metabolism of cell wall xyloglucan may be altered. In tobacco plants downregulation of tobacco *XTH* (*Nt*XET-1) lead to reduced XET activity in the leaves. This resulted in a shift towards xyloglucan with a higher molecular weight, resembling that of older leaves (Herbers et al. 2001). The authors noted that the cell walls may be strengthened by the reduced turnover and hydrolysis of xyloglucan and it was suggested that the resultant wall strengthening may hold implications for plant-pathogen interactions. This is plausible since xyloglucan plays a central role in the structure of plant cell walls. It is the most abundant hemicellulose in dicotyledonous plants and cross-links cellulose microfibrils (McNeil et al., 1984; Carpita and Gibeaut, 1993). XTH enzymes also form part of important mechanisms for regulation of cell wall strength, extensibility and tissue integrity (Saladié et al., 2006).

These results indicate that *XTH* downregulation may contribute, at least in part, to the observed decrease in *Botrytis* susceptibility. Downregulation of this gene was also observed in VvPGIP 47, which did not exhibit an appreciable decrease in susceptibility to *Botrytis* infection at the whole-plant level. This line, together with VvPGIP 1, is currently being assessed in detail to elucidate the factors contributing to their susceptible phenotype, despite the production of active PGIP (measured by ePG inhibition in Joubert et al., 2006). Additional factors, including the increased lignin deposition and induced defenses (Becker et al., Chapter 5 of this dissertation) may also contribute significantly to the observed decrease in disease susceptibility. Infecting tobacco plants downregulated in *XTH* expression (Herbers et al. 2001) will

no doubt shed more light on the role of this gene in modulating cell wall metabolism, as it relates to fungal pathogenesis.

Xyloglucan endotransglycosylase activity increases during kiwifruit ripening (Redgwell and Fry, 1993) and expression of *Vitis vinifera XET1* is steadily increased following the *véraison* stage of grape berry ripening, and reaches a maximum at the fully ripe stage (Nunan et al., 2001). It is during this time when berry softening occurs. Interestingly, *VvXET1* expression in grape berries is inversely correlated to *Vvpgip1* expression (De Ascensao, 2001). Grape *pgip* expression reaches a maximum at *véraison* and steadily declines until grape berries reach the fully ripe stage. In transgenic tobacco plants overexpressing *Vvpgip1*, downregulation of the tobacco *XTH* gene is observed. This inversely correlated expression remains interesting and will be investigated in further detail.

VvPGIP-accumulating lines show an increased lignin content

Transcriptomic and biochemical methods strongly suggest the increased deposition of lignin in the transgenic lines (Figure 4.2a, 4.2b and Figure 4.3). In both leaf and stem tissue, with two different methods, was this increased deposition observed. Absolute quantitation of lignin content (Figure 4.3) in the plant lines consistently showed that the two transgenic lines had increased lignin deposited in their cell walls, although these differences were only statistically significant at confidence intervals of 0.90 in Range tests. At 0.95 confidence intervals, VvPGIP 37 grouped with both the WT and VvPGIP 45, indicating at least that VvPGIP 45 showed statistically separable lignin content as compared to WT. Refinement of this quantitation method is currently underway to conclusively support evidence from transcriptomic analyses and biochemical methods. The consequence of increased lignin content in the cell walls of the PGIP-specific resistant lines will no doubt impact positively on plant defense. The role of lignin in plant defense is well documented (Nicholson and Hammerschmidt, 1992; Walter, 1992). Lignin is largely resistant to degradation by microorganisms and cell walls with increased lignin content provide the plant with an effective physical barrier against phytopathogens (Ride, 1983).

VvPGIP transgenic lines show an altered phytohormone profile

Transgenic leaf material exhibited slightly lower levels of salicylic acid (SA) (Figure 4.4). High endogenous levels of SA are reported to protect rice from both biotic and abiotic stress (Yang et al., 2004). However, the SA levels reported in these plants were between 5000 and 30000 $ng.g^{-1}$ fresh weight, far exceeding both the basal and infected levels of SA observed in tobacco or *Arabidopsis*. The levels observed in the untransformed and transgenic lines were deemed too low for any significant impact by salicylic acid, such as the anti-oxidative role played by endogenous SA in rice (Yang et al., 2004).

Indole-acetic acid (IAA) levels (Figure 4.4) could be statistically separable (Duncan's range test, STATISTICA software) in two groups with high confidence: the

transgenic lines VvPGIP 37 and 45, which had higher levels of IAA, grouped together; while the WT, with lower levels, fell into another group.

Previous work indicates that increased IAA content in the transgenic lines may result in the regulation of *XTH* and the deposition of lignin (Sitbon et al., 1992; Sitbon et al. 1999; Catalá et al. 2001). VvPGIP 37 and VvPGIP 45, with higher IAA levels, show relative downregulation of the *XTH* gene. This is in agreement with the work of Catalá et al. (2001), where downregulation of a tomato *XTH* gene (*LeXET2*) by auxin was reported. Also, transgenic tobacco lines overproducing IAA exhibited increased lignin content and altered lignin composition (Sitbon et al., 1992). Sitbon et al. (1999) suggested that the increased lignin deposition may have resulted from increased peroxidase activity, brought about by increased IAA levels. In transgenic line VvPGIP 45, the line which consistently has the highest levels of IAA, upregulation of a lignin-forming peroxidase is observed. This line also shows an increased deposition of lignin in histochemical assays (Figure 4.2a and 4.2b) and with the acetyl bromide lignin quantitative method (Figure 4.3). However, whether these changes are observed as a result of constitutive accumulation of PGIP influencing IAA, remains to be seen. Another distinct possibility could be that the PGIP-associated cell wall metabolism alterations could possibly lead to an altered IAA content. Whichever hypothesis may hold true, will only be known after additional experimental work. It could be envisaged that analysis of plants unable to accumulate IAA, but high levels of PGIP, may provide some insight to this argument.

Taken together, these results show that transgenic lines overexpressing *Vvpgip1* have altered cell wall metabolism when compared to their untransformed counterparts, even in the absence of pathogen infection. Both the downregulation of the tobacco *XTH* gene and the increased deposition of lignin may contribute to the reduced *Botrytis* susceptibility in these PGIP-specific resistant lines. The current working hypothesis of PGIP in plant defense involves the inhibition of ePGs and therefore the limitation of tissue maceration and necrosis. Also, following inhibition of ePG by PGIP, it is hypothesized that the lifetime of molecules with elicitor activity towards the activation of plant defenses are extended (Cervone et al., 1989). However, since all the analyses reported in this work was performed under conditions where the pathogen was absent, neither the inhibition of ePG nor the extension of the lifetime of oligogalacturonides was involved. Therefore, an additional role for PGIP in the contribution to plant defenses is proposed. PGIP may directly influence, whether by virtue of its structural features or its integration in the cell wall; defense responses in the plant leading to the strengthening of cell walls. These reinforced cell walls are thus “primed” before pathogen ingress and contributes to the observed decrease in disease susceptibility observed in lines accumulating high levels of PGIP. Recently it has been shown that a rice PGIP regulated floral organ number in rice (Jang et al., 2003). This observation, together with the work presented in this manuscript, is shedding light on the *in planta* role of PGIP, in addition to the inhibition of ePGs and the subsequent signaling following the interaction of the proteins.

4.6 References

Abu-Goukh, A.A., Strand, L.L., and Labavitch, J.M. 1983. Development-related changes in decay susceptibility and polygalacturonase inhibitor content of “Bartlett” pear fruit. Physiol. Plant Pathol. 23: 101-109.

Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce’s disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pathol. 6: 43-51.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Carpita, N.C., and Gibeaut, D.M. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the wall during growth. Plant J. 3: 1-30.

Catalá, C., Rose, J.K.C., York, W.S., Albersheim, P., Darvill, A.G., and Bennett, A.B. 2001. Characterization of a tomato xyloglucan endotransglycosylase gene that is down-regulated by auxin in etiolated hypocotyls. Plant Physiol. 127: 1180-1192.

Cervone, F. Hahn, M.G., De Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. 90: 542-548.

De Lorenzo, G., D’Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu. Rev. Phytopath. 39: 313-335.

De Lorenzo, G., and Ferrari, S. 2002. Polygalacturonase-inhibiting proteins in defence against phytopathogenic fungi. Curr. Opin. Plant Biol. 5: 295-299.

De Ascensao, A.R.F.D.C. 2001. Isolation and characterization of a polygalacturonase-inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L. Ph.D. Thesis, Stellenbosch University, Stellenbosch, RSA.

De Ascensao, A.R.F.D.C., and Dubery, I.A. 2003. Soluble and wall-bound phenolics and phenolic polymers in *Musa acuminata* roots exposed to elicitors from *Fusarium oxysporum* f.sp. *cubense*. Phytochemistry 63: 679-686.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. 2003. *Arabidopsis* polygalacturonase-inhibiting proteins (PGIP) are regulated by different signal transduction pathways during fungal infection. Plant Cell 15: 93-106.

Ferrari, S., Galletti, R., Vairo, D., Cervone, F., and De Lorenzo, G. 2006. Antisense expression of the *Arabidopsis thaliana AtPGIP1* gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. Mol. Plant Microbe Interact. 8: 931-936.

Fromm, J., Rockel, B., Lautner, S., Windeisen, E., and Wanner, G. 2003. Lignin distribution in wood cell walls determined by TEM and backscattered SEM techniques. J. Struct. Biol. 143: 77-84.

Herbers, K. Lorences, E.P., Barrachina, C., and Sonnewald, U. 2001. Functional characterization of *Nicotiana tabacum* xyloglucan endotransglycosylase (*Nt*XET-1): Generation of transgenic tobacco plants and changes in cell wall xyloglucan. Planta 212: 279-287.

Isshiki, A., Akimitsu, K., Yamamoto, M., and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Mol. Plant Microbe Interact. 14: 749-757.

Jang, S., Lee, B., Kim, C., Kim, S.J., Yim, J., Han, J.J., Lee, S., Kim, S.R., and An, G. 2003. The OsFOR1 gene encodes a polygalacturonase-inhibiting protein (PGIP) that regulates floral organ number in rice. Plant Mol. Biol. 53: 357-369.

Johnston, D.J., Ramanathan, V., and Williamson, B. 1993. A protein from immature raspberry fruits which inhibits endopolygalacturonases from *Botrytis cinerea* and other micro-organisms. J. Exp. Bot. 44: 971-976.

Joubert, D.A., Slaughter, A.R., Kemp, G., Becker, J.V.W., Krooshof, G.H., Bergmann, C., Benen, J., Pretorius, I.S., and Vivier, M.A. 2006. The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. DOI 10.1007/s11248-006-9019-1.

Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and van Kan, J.A.L. 2007. A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *B. cinerea* in *N. benthamiana* leaves without any evidence for *in vitro* interaction. Mol. Plant Microbe Interact. (in print).

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and van Kan, J.A. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 43: 213-225.

Li, R., Rimmer, R., Buchwalt, L., Sharpe, A.G., Seguin-Swartz, G., and Hegedus, D.D. 2004. Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of *endo*-and *exo*-polygalacturonases expressed during the saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

Manfredini, C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M., and De Lorenzo, G. 2005. Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol. Mol. Plant Pathol. 67: 108-115.

McNeil, M., Darvill, A.G., Fry, S.C., and Albersheim, P. 1984. Structure and function of the primary cell walls of plants. Annu. Rev. Biochem. 53: 625-663.

Nicholson, R.L., and Hammerschmidt, R. 1992. Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. 30: 369-389.

Nunan, K.J., Davies, C., Robinson, S.P., and Fincher, G.B. 2001. Expression patterns of cell-wall modifying enzymes during grape berry ripening. Planta 214: 257-264.

Oeser, B., Heidrich, P.M., Muller, U., Tudzynski, P., and Tenberge, K.B. 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. Fungal Genetic. Biol. 36: 176-186.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: 2002-2007.

Powell, A.L., van Kan, J.A., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. 13: 942-950.

Ramakers, C., Ruijter, J.M., Lekanne Deprez, R.H., and Moorman, A.F.M. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339: 62-66.

Redgwell, R.J., and Fry, S.C. 1993. Xyloglucan endotransglycosylase activity increases during kiwifruit (*Actinidia deliciosa*) ripening. Implications for fruit softening. Plant Physiol. 103: 1399-1406.

Ride, J.P. 1983. Cell walls and other structural barriers in defense. Pp 215-236. In: Biochemical Plant Pathology. Ed. J.A. Callow, Wiley Publishers, Chichester, UK.

Rodriguez-Palenzuela, P., Burr., T.J., and Collmer, A. 1991. Polygalacturonase is a virulence factor in *Agrobacterium tumefaciens* biovar 3. J. Bacteriol. 173: 6547-6552.

Rose, J.K.C., Braam, J., Fry, S.C., and Nishitani, K. 2002. The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature. Plant Cell Physiol. 43: 1421-1435.

Ruzin, S.E. 1999. Plant microtechnique and microscopy. Oxford University Press, New York.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., and Quackenbush, J. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34:374-378.

Saladié, M., Rose, J.K.C., Cosgrove, D.J., and Catalá, C. 2006. Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymic action. Plant J. 47: 282-295.

Salvi, G., Giarrizzo, F., De Lorenzo, G., and Cervone, F. 1990. A polygalacturonase-inhibiting protein in the flowers of *Phaseolus vulgaris* L. J. Plant Physiol. 136: 513-518.

Schmelz, E.A., Engelberth, J., Tumlinson, J.H., Block, A., and Alborn, H.T. 2004. The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. Plant J. 39: 790-808.

Shieh, M.T., Brown, R.L., Whitehead, M.P., Cary, J.W., Cotty, P.J., Cleveland, T.E., and Dean, R.A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63: 3548-352.

Sitbon, F., Hennion, S., Sundberg, B., Little, C.H.A., Olsson, O., and Sandberg, G. 1992. Transgenic tobacco plants coexpressing the *Agrobacteium tumefaciens iaaM* and *iaaH* genes display altered growth and indole-acetic acid metabolism. Plant Physiol. 99: 1062-1069.

Sitbon, F., Hennion, S., Anthony Little, C.H., and Sundberg, B. 1999. Enhanced ethylene production and peroxidase activity in IAA-overproducing tobacco plants with increased lignin content and altered lignin deposition. Plant Sci. 141: 165-173.

Spadoni, S., Zabotina, O., Di Matteo, A., Mikkelsen, J.D., Cervone, F., De Lorenzo, G., Mattei, B., and Bellincampi, D. 2006. Polygalacturonase-inhibiting protein interacts with

pectin through a binding site formed by four clustered residues of arginine and lysine. Plant Physiol. 141: 557-564.

Tans-Kersten, J., Huang, H., and Allen, C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J. Bacteriol. 183: 3597-3605.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant Microbe Interact. 11: 1009-1016.

Tusher, V.G., Tibshirani, R., and Chu, G. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA 98: 5116-5121.

Vaquerizas, J.M., Dopazo, J., and Díaz-Uriarte, R. 2004. DNMAD: web-based Diagnosis and Normalization for MicroArray Data. Bioinformatics 20: 3656-3658; doi:10.1093/bioinformatics/bth401.

Walter, M.H. 1992. Regulation of lignification in defense. Pp 327-352. In: Plant gene research. Genes involved in plant defense. Eds. T.P. Boiler and F. Meins, Springer-Verlag, Vienna.

Yang., Y., Qi, M., and Mei, C. 2004. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stresses. Plant J. 40: 909-919.

4.7 Acknowledgements

This work was supported by funding from the National Research Foundation (NRF), the Wine Industry Network of Expertise and Technology (Winetech), the Deciduous Fruit Producers Trust (DFPT) and the THRIP fund.

ADDENDUM A

Table A1. Genes found to be differentially expressed between the untransformed control and *Vvpgip1*-transformed lines. Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) was performed in the TM4 software suite (Saeed et al., 2003), available from TIGR. The SAM delta value was adjusted to obtain a maximum of 5% falsely discovered genes.

Annotation	Mean (Log_2) Relative expression	VvPGIP 37	VvPGIP 37 (dye swap)	VvPGIP 45
Brassinosteroid-regulated protein BRU1 precursor. {*Glycine max*}	-1.296	-1.335	-1.125	-1.428
Q8LNW9 Hypothetical protein	-1.04433	-0.864	-1.235	-1.034
Q9FZ05 Xyloglucan endotransglycosylase LeXET2	-1.01267	-1.01	-1.088	-0.94
Q8S902 Syringolide-induced protein 19-1-5	-0.958	-0.98	-0.828	-1.066
xyloglucan endo-1,4-beta-D-glucanase precursor clone tXET-B2 tomato	-0.94167	-0.918	-0.882	-1.025
Q9LLC2 Xyloglucan endotransglycosylase XET2 EC 2.4.1.207 Q8S902 Syringolide-induced protein 19-1-5	-0.921	-0.837	-0.822	-1.104
AAQ54533 Putative DnaJ protein Fragment	-0.90767	-0.865	-0.902	-0.956
Q9M9N3 T17B22.17 protein	-0.88733	-1.159	-0.721	-0.782
Q94JV1 At1g69340/F10D13.28 AAQ54533 Putative DnaJ protein Fragment	-0.88	-0.852	-0.97	-0.818
Pyruvate kinase isozyme A chloroplast precursor {*Nicotiana tabacum*} EC 2.7.1.40 EC 2.7.1.40	-0.82033	-0.994	-0.551	-0.916
plasma membrane protein – common tobacco {*Nicotiana tabacum*}	-0.78533	-0.765	-0.791	-0.8
Q7YDN0 ATP synthase 6	-0.785	-0.873	-0.755	-0.727
ADP ATP carrier protein mitochondrial precursor ADP/ATP translocase	-0.78033	-0.957	-0.557	-0.827
Q9M517 TMV-induced protein I	-0.77867	-0.598	-0.874	-0.864
plasma membrane protein – common tobacco {*Nicotiana tabacum*}	-0.74067	-0.675	-0.937	-0.61
hypothetical protein F19C24.7 imported –*Arabidopsis thaliana*	-0.73833	-0.76	-0.658	-0.797
Q8I430 Serine protease belonging to subtilisin family putative	-0.733	-0.791	-0.6	-0.808
Remorin pp34 {*Solanum tuberosum*}	-0.67533	-0.76	-0.712	-0.554
ADP ATP carrier protein mitochondrial precursor ADP/ATP translocase	-0.664	-0.729	-0.548	-0.715
Translationally controlled tumor protein homolog TCTP P23 {*Solanum tuberosum*}	-0.66167	-0.75	-0.646	-0.589
Q9LGP3 P0684C01.2 protein P0698A04.25 protein	-0.65967	-0.731	-0.415	-0.833
Histidine decarboxylase HDC TOM92 {*Lycopersicon esculentum*}	-0.64	-0.455	-0.674	-0.791
hypothetical protein predicted by GeneMark.hmm etc. {*Oryza sativa* japonica cultivar-group}	-0.62233	-0.632	-0.47	-0.765
Q93ZG1 At1g23060/T26J12 16 CAB38630 SPAC3G9.01 protein	-0.61767	-0.754	-0.459	-0.64
Q93Z81 At3g51860/ORF11Ca2+/H+-exchanging protein - mung bean {*Vigna radiata*}	-0.61	-0.741	-0.635	-0.454
xyloglucan endo-1,4-beta-D-glucanase precursor clone tXET-B2 –tomato Q84UH3 Putative ascorbate peroxidase	-0.60867	-0.615	-0.616	-0.595
Q8GTJ0 Xyloglucan endotransglycosylase	-0.602	-0.431	-0.652	-0.723

AT3g56200/F18O21 160 {*Arabidopsis thaliana*}	-0.587	-0.685	-0.428	-0.648
Q9FXW9 Pectin methylesterase-like protein	-0.57467	-0.622	-0.408	-0.694
protein F28K20.13 imported – *Arabidopsis thaliana*	-0.57267	-0.571	-0.717	-0.43
Q9FFT6 Translation initiation factor-like protein AT5g54940/MBG8 21	-0.569	-0.484	-0.649	-0.574
Q9AR56 Putative membrane protein	-0.568	-0.738	-0.525	-0.441
hypothetical protein T6K21.80 – *Arabidopsis thaliana*	-0.56767	-0.608	-0.607	-0.488
Q9FFT6 Translation initiation factor-like protein AT5g54940/MBG8 21	-0.565	-0.524	-0.615	-0.556
Q9XGF3 Major intrinsic protein 2	-0.565	-0.622	-0.524	-0.549
Probable xyloglucan endotransglucosylase/hydrolase 1 precursor LeXTH1.	-0.55867	-0.497	-0.676	-0.503
AAR21295 Bacterial spot disease resistance protein 4	-0.552	-0.531	-0.527	-0.598
Q8W582 At1g53570/F22G10 18	-0.549	-0.666	-0.449	-0.532
Q9M624Putative water channel protein	-0.548	-0.534	-0.545	-0.565
Q8H9B8 Low temperature and salt responsive protein	-0.54333	-0.59	-0.609	-0.431
BAD10188 Putative transcription factor RAU1	-0.54133	-0.458	-0.456	-0.71
Q9FNI3 Similarity to RAV2-like DNA-binding protein	-0.52767	-0.412	-0.522	-0.649
AT3g60900/T4C21 310 {*Arabidopsis thaliana*}	-0.51633	-0.38	-0.609	-0.56
Q9XEY5 Nt-iaa28 deduced protein	-0.51033	-0.424	-0.54	-0.567
Q9XGF3 Major intrinsic protein 2	-0.48933	-0.545	-0.363	-0.56
trehalose-6-phosphate phosphatase homolog T1P17.20 – *Arabidopsis thaliana*	-0.48667	-0.549	-0.491	-0.42
Q7PFG6 ENSANGP00000024712	-0.452	-0.44	-0.412	-0.504
Q94JV2 AT3g27050/MOJ10 14	-0.45	-0.477	-0.371	-0.502
Q8H9B8 Low temperature and salt responsive protein	-0.40933	-0.427	-0.448	-0.353
hypothetical protein L73G19.50 – Arabidopsis thaliana unknown protein {*Oryza sativa* japonica cultivar-group}	-0.37433	-0.382	-0.37	-0.371
Q8GY29 Putative receptor protein kinase	-0.36133	-0.334	-0.375	-0.375
Q93W77 AT4g01940/T7B11 20 NFU1 protein	-0.36033	-0.332	-0.371	-0.378

RESEARCH RESULTS

Transgenic tobacco plants with a PGIP-specific resistance phenotype show increased induction of jasmonate synthesis and *des1* expression in the local response following *Botrytis cinerea* infection

John V.W. Becker[1], Katherine J. Denby[2,3] and Melané A. Vivier[1]

[1]Institute for Wine Biotechnology, Department of Viticulture and Oenology, Faculty of AgriSciences, Stellenbosch University, Stellenbosch, South Africa
[2]Department of Molecular and Cell Biology, University of Cape Town, Private Bag, Rondebosch, Cape Town, South Africa
[3]Warwick HRI and Warwick Systems Biology, University of Warwick, Wellesbourne, Warwick, CV35 9EF, United Kingdom (current address)

5.1 Abstract

Transgenic plants accumulating polygalacturonase-inhibiting proteins (PGIPs) generally show resistance phenotypes against phytopathogenic fungi that utilize enodpolygalacturonases (ePGs) for their infection and subsequent colonization of host tissue. PGIPs, as a result of their inhibition of fungal ePGs, are believed to prolong the existence of molecules capable of eliciting plant defense responses, termed oligogalacturonides. *In vitro* evidence has supported this hypothesis, but *in vivo* experimentation as well as data to elucidate the specific mechanisms and pathways involved are lacking. In this study characterized *Vvpgip1* transgenic tobacco lines, exhibiting a PGIP-specific resistance phenotype, were utilized to elucidate the affected pathways which may contribute to the observed resistance in the transgenic lines following *B. cinerea* infection. These lines were also recently shown to exhibit altered cell wall metabolism under non-infecting conditions. The alterations observed point to cell wall strengthening in response to the constitutively expressed PGIP, providing the first report of a possible role(s) of PGIP that is not linked to the PGIP-ePG interaction. Here the transgenic lines were compared to an untransformed control in transcriptomic and hormonal analyses, following *Botrytis* inoculation. The analysis focused on the early defense-related responses at the point of infection and immediately surrounding areas and therefore provides data on the local acquired resistance. Transcriptomic analysis revealed that *Botrytis*-responsive genes were upregulated in all backgrounds, confirming a successful infection/induction response. Statistical analysis of the transcriptomic data revealed limited differential expression between the infected wild-type (WT) and transgenic plants at the various time points, but a gene involved in the formation of the antifungal divinyl ethers colneleic and colnelenic acid, was upregulated more than two-fold in the transgenic lines when compared to the WT following infection. These two acids have been shown to have potent inhibitory effects on *Botrytis* spore germination. The gene encodes tobacco divinyl ether synthase (*des1*) and its differential expression was confirmed using Real-Time quantitative PCR. Also, hormonal profiling of the locally infected leaf tissue of the plant lines revealed a greater induction of the synthesis of the defense-related hormones jasmonic acid and salicylic acid in the transgenic lines. Jasmonic acid also accumulated to higher levels at 24 hours post-inoculation. These hormones have been shown to be important for the restriction of *B. cinerea* at the site of infection. These results

suggest that in transgenic lines accumulating PGIP; and exhibiting resistance phenotypes, the induction of important defense-related pathways are induced to a greater extent at the site of infection and the surrounding tissues. This work is the first to report the differential induction of defense-related pathways in any of the transgenic backgrounds where *pgip* was overexpressed. These data corroborate the role for PGIP in defense signaling and significantly adds to our understanding of the role of PGIP in plant-pathogen interactions.

5.2 Introduction

The role of polygalacturonase-inhibiting proteins (PGIPs) in plant defense has clearly been demonstrated with strategies involving overexpression of genes encoding PGIP (Powell et al., 2000, Ferrari et al., 2003a, Aguero et al., 2005, Manfredini et al., 2005), and antisense expression (Ferrari et al., 2006). In all cases, reduced susceptibility towards the fungal pathogen was observed in the plant lines with a higher PGIP content. These proteins have the ability to specifically and effectively inhibit fungal endopolygalacturonases (ePGs), enzymes that cleave the α-1,4 glycosidic linkages between the galacturonic acid residues (De Lorenzo et al., 2001) of homogalacturonan in the plant cell wall. ePGs are secreted soon after infection. Hence, ePG inhibition by PGIP limits the cell wall degradation and tissue maceration observed when ePGs continue their action unhindered. Apart from their role in host colonization, ePGs also play a role in fungal (and bacterial) pathogenicity (Rodriguez-Palenzuela et al., 1991; Shieh et al., 1997; ten Have et al., 1998; Isshiki et al., 2001; Tans-Kersten et al., 2001; Oeser et al., 2002; Li et al., 2004; Kars et al., 2005).

The inhibition of ePG by PGIP is believed to increase the lifetime of elicitor-active molecules (oligogalacturonides), fragments generated from the digestion of plant cell wall components, with the ability to induce plant defense responses (Cervone et al., 1989). In view of the fact that these experiments were performed under *in vitro* conditions (Cervone et al., 1989) and the fact that exogenous application of these molecules can elicit a range of defense responses in different plant species (reviewed in Ridley et al. 2001), evidence is yet to emerge to elucidate their role following ePG inhibition by PGIP *in planta*. Similarly, the affected pathways following this inhibition which may contribute to reduced fungal susceptibility remain to be elucidated.

Transgenic tobacco plants overexpressing a grapevine PGIP encoding gene, *Vvpgip1,* were recently shown to be significantly less susceptible to *Botrytis cinerea* infection in detached leaf and whole-plant infection assays (Joubert et al., 2006). In these lines, PGIP activity, ePG inhibition and decreased susceptibility to *B. cinerea* could be correlated. Thus, these lines are believed to exhibit a PGIP-specific resistance phenotype (Joubert et al., 2006). For these lines, it could be shown that alterations to their cell wall metabolism, positively influencing cell wall strengthening, may contribute to the observed resistance phenotypes (Becker et al., Chapter 4 of this work). Since these results were obtained in healthy, uninfected plants, it points to

a possible role for PGIP in activating some of the basic defense-related processes in plants without the involvement of the PGIP-ePG inhibition interaction (Becker et al., Chapter 4 of this work).

These lines provided the ideal resource to study downstream events following pathogen ingress, comparing lines accumulating high levels of PGIP and their untransformed counterparts. Transcriptomic and phytohormone analyses were performed on *Botrytis cinerea* infected leaf material of these lines at different time points, and compared to the untransformed wild-type line. The analyses were focused on elucidating the local acquired resistance responses (LAR) and involved analyses of tissues in and around the infection and primary lesion sites.

5.3 Materials and Methods

5.3.1 Plant Material and Growth Conditions

Wild-type and transgenic *Nicotiana tabacum* SR1 (Petit Havana) plants were grown in a soil and peat moss (Jiffy Products International AS, Norway) mixture in a growth room at 24ºC and 55% relative humidity. The two transgenic lines utilized (VvPGIP 37 and VvPGIP 45) showed significant reductions in susceptibility towards *Botrytis cinerea* infection at the detached leaf and whole-plant level, which correlated to PGIP activity and ePG inhibition (Joubert et al., 2006). These lines are therefore considered to exhibit a PGIP-specific resistant phenotype. A light intensity of 120 $\mu mol.m^2.s^{-1}$ was maintained for the 16 h light photoperiod. Leaf material of tobacco plants collected for RNA extraction and phytohormone quantification was flash frozen in liquid nitrogen and stored at -80°C.

5.3.2 Fungal Inoculation on Tobacco Plants

Botrytis cinerea pathogenic cultures were grown as described in Joubert et al. (2006). Plants were placed in transparent Perspex humidity chambers at 100% relative humidity and a light intensity of 120 $\mu mol.m^2.s^{-1}$, for a 16 h period, after which 5 µL of a *B. cinerea* spore suspension (1 x 10^3 spores in a 50% grape juice medium) was spotted on the adaxial surface of the leaves, without wounding or detachment. Three leaves of six to eight week old plants (WT, VvPGIP 37 and VvPGIP 45) were inoculated with four spots per leaf. Infections were performed on leaf positions three to five in the plant lines. Infections were allowed to progress at 23°C for 0 (harvested immediately following spore inoculation) 24 and 48 h for microarray experiments; 0, 18, 24 and 30 h for Real-Time PCR and 0, 18, 24, 30 and 40 h for phytohormone profiling. At these time points the tissue immediately surrounding, and including the infection spots (15 mm diameter) were harvested and flash frozen in liquid nitrogen.
An independent infection experiment was performed to validate Real-Time PCR and phytohormone profiling (results not shown).

5.3.3 Microarray Analyses

5.3.3.1 RNA Extraction

RNA was extracted from leaf tissue of the untransformed control, line VvPGIP 37 and VvPGIP 45 using a sodium perchlorate-based method. An extraction buffer consisting of 5 M sodium perchlorate, 0.3 M Tris-HCl (pH 8.3), 8.5% polyvinylpolypyrollidone (PVPP), 2.0% PEG 4000, 1.0% β-mercapto-ethanol and 1.0% SDS was added to plant material finely ground in liquid nitrogen. The tissue was allowed to thaw in this buffer and shaken for 30 min at room temperature. Following centrifugation, the supernatant was passed through a syringe plugged with cotton wool to remove insoluble reagents and plant debris. Several phenol/chloroform extractions were performed before precipitating the RNA with 2.5 M LiCl at -20°C overnight. The pellet was washed with 70% ethanol and the resuspended RNA was purified using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). RNA was quantified using the Nanodrop (Wilmington, DE, USA).

5.3.3.2 cDNA Synthesis and Hybridization

Two cDNA synthesis reactions of 25 µg each were set up for each test or reference RNA sample in a total of 30 µL. Before denaturation at 70ºC for 10 min, 2 µL of oligo d(T) primers (500 µg/mL) were added. Following denaturation, first strand buffer and DTT were added according to the manufacturer's recommendation (InVitrogen, Carlsbad, CA, USA). A 2:3 (aa-dUTP:dTTP) aminoallyl-dNTP (Ambion, Austin, TX, USA) mix was added to a 1X concentration (0.5 mM each of dATP, dCTP and dGTP; 0.2 mM aa-dUTP and 0.3 mM dTTP) before incubation at 46ºC for 2 min, after which 200 U of SuperScript III Reverse Transcriptase (InVitrogen, Carlsbad, CA, USA) was added. The same amount of enzyme was added following incubation for 4 h at 46ºC, after which cDNA synthesis proceeded overnight.

RNA was hydrolyzed by the addition of 10 µL each of 1 M NaOH and 0.5 M EDTA solutions and incubated at 65ºC for 15 min. To neutralize the pH, 10 µL of a 1 M HCl solution was added following hydrolysis. Unincorporated aminoallyl dUTP and free amines were removed by purification using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The eluted cDNA was quantified using a Nanodrop (Wilmington, DE, USA) and similar quantities of test and reference cDNA were dried down to volumes of less than 1 µL in a vacuum drier. Five microliters of a 0.1 M Na_2CO_3 buffer, pH 9.0, was added to the cDNA, mixed well, and incubated at 37ºC for 10 min. Cy3 or Cy5 (4.5 µL) (Amersham Biosciences, Buckinghamshire, UK) ester was added and the coupling allowed to proceed for 1 h in the dark at room temperature. Uncoupled dyes were removed by purification with the RNeasy Mini Kit. Probe labeling with Cy-esters were estimated by measuring the absorbance at 550nm and 650nm on a Nanodrop and calculating the fold of incorporation of dye.

Similar amounts of labeled probe were hybridized to the microarray slides. Slides were prehybridized with a solution containing 5X SSC (from 20X SSC: 3 M NaCl, 1.5 M sodium citrate, pH 7.0), 0.1% SDS and 1% BSA, prewarmed to 42ºC. The solution was pipetted under lifterslips (Erie Scientific, Portsmouth, NH, USA) and the slides incubated at 42ºC for at least 30 min. Following prehybridization, the slides were washed in five washes of deionized water and finally briefly submerged in ethanol before centrifugation for 5 min at 1000 rpm. The combined Cy-labelled probes (28 µL) were mixed with 30 µL of a 2X hybridization buffer (50% formamide, 5X SSC and 0.2% SDS), to which was added 1 µL each of COT1 DNA (1 µg/µL) and poly(A)-DNA (12 µg/µL), for a total volume of 60 µL. The probes were denatured at 90ºC for 3 min, and subsequently applied to the slide under lifterslips. The microarray slides were enclosed in ArrayIt (Telechem International, Sunnyvale, CA, USA) hybridization chambers and submersed in a heated water bath and incubated for 16 h at 42ºC. The slides were successively washed in low stringency (2X SSC, 0.5% SDS; heated to 55ºC), medium stringency (0.5X SSC) and high stringency (0.05X SSC) wash buffers for 5 min each. Prior to centrifugation (5 min, 1000 rpm) the slides were briefly submerged in ethanol. Scanning was performed with an Axon GenePix 4000A scanner (Molecular Devices, Sunnyvale, CA, USA). Photomultiplier tube (PMT) settings for each channel were optimized during prescans to obtain equal output from the red and green channels and to minimize the amount of saturated spots, before proceeding to full scans. GenePix results (gpr) files were generated using GenePix 5.1 (Molecular Devices, Sunnyvale, CA, USA) software, without normalization.

5.3.3.3 Microarray Data Analysis

The results files were normalized with DNMAD (Vaquerizas et al., 2004) using print-tip loess without background subtraction. Inconsistent replicates were removed using the preprocessor. The normalized values were subsequently downloaded and analyzed with the Multiexperiment Viewer (MeV) in the TM4 software suite (Saeed et al., 2003). T-tests, Hierarchical Clustering (HCL, Eisen et al., 1998), Analysis of Variance (ANOVA, Zar et al., 1999) and Pavlidis Template Matching (PTM, Pavlidis and Noble, 2001) were performed. HCL, ANOVA and PTM were utilized to screen the microarray data for genes that were expressed differently in WT and the transformed transgenic lines at either 24 or 48 hours post-inoculation.

5.3.4 Quantitative Real-Time PCR

RNA for quantitative PCR analyses was extracted in a similar fashion to the protocol utilized for extracting RNA for microarray analyses. A microgram of RNA was utilized for each cDNA synthesis reaction, using SuperScript III Reverse Transcriptase according to the manufacturer's specifications. Both oligo d(T) and random primers were added to ensure full length cDNA was obtained. Real-Time PCR analysis was

performed using the LightCycler Instrument (Roche Diagnostics GmbH, Mannheim, Germany). cDNA from each sample was amplified using the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). Primer sequences used for amplifying tobacco actin (*ACT*, GenBank Acc AB158612) and divinyl ether synthase 1 (*des1*, GenBank Acc AF070976) were: *ACT* forward 5'-GTCCCTATACGCCAGT-3' and reverse 5'-ACATCGCGGACAATTT-3', priming a 216 bp actin fragment, and *des1* forward 5'-AGGAAGCTGTACATAACATACTTT -3' and reverse 5'- GCAAGTCGAGCGTGTAGA-3', amplifying a 124 bp *des1* fragment. cDNA amplification conditions were as follows: denaturation at 95ºC for 10 min, followed by 45 cycles of denaturation, 95ºC for 10 s; primer annealing at 58ºC for 10 s and primer extension at 72ºC for 8 s, during which a single data acquisition per cycle was performed. A melting curve analysis was performed at the end of the 45 cycles by raising the temperature from 65ºC to 95ºC, increasing the temperature by 0.1ºC per second in continuous acquisition mode. PCR efficiencies for each sample were calculated using LinRegPCR, software described in Ramakers et al., (2003). Fluorescence per cycle of each sample is exported from which the efficiencies of individual samples were calculated. These efficiencies were used to calculate relative expression in a mathematical model described by Pfaffl (2001).

5.3.5 Simultaneous Analysis of Phytohormones Using GC/MS

5.3.5.1 Chemicals

Salicylic acid, indole-3-acetic acid, methyl jasmonate, *o*-anisic acid, trimethylsilyldiazomethane, hexane and 1-propanol were purchased from Sigma-Aldrich (Steinheim, Germany). Dichloromethane and acetic acid were purchased from BDH (Poole, BH15 TD, England). Hydrochloric acid was obtained from Saarchem (Wadeville, South Africa). Water was deionized using a MilliQ water purification system (MilliQ, Billerica, MA, USA).

5.3.5.2 Sample Preparation for Vapor Phase Extraction

Leaf tissue flash frozen in liquid nitrogen was extracted according to the method of Schmelz et al. (2004), with some modifications. Approximately 100 mg of locally infected leaf tissue was ground to a fine powder in eppendorf tubes prior to the addition of extraction solvent (n-propanol/water/HCl) and internal standard (*o*-anisic acid), of which 30 ng per sample was added. Samples were vortexed to ensure homogenization prior to partitioning with dichloromethane. For the conversion of phytohormone acids to their corresponding methyl esters, the organic phase (dichloromethane/propanol) was derivatized in 4 mL glass vials for 30 minutes using 4 µL of a 2 M trimethylsilyldiazomethane solution in hexane. The activity of

derivatization agent was subsequently quenched with 4 µL of a 2 M acetic acid solution, also in hexane.

5.3.5.3 Vapor Phase Extraction

Extraction of the derivatized organic phase proceeded according to Schmelz et al. (2004), with the exception that commercially available Super Q filters were purchased from Analytical Research Systems, Inc., (Gainesville, FL, USA). Briefly, the derivatized sample was evaporated at 70ºC and passed through a Super Q filter under a N_2 flow of 500 mL/min. To ensure complete vaporization of less volatile compounds the vial was subsequently heated to 200ºC for 2 min while passing the vapour through the filter. The analytes were eluted from the Super Q filter with 150 µL CH_2Cl_2 and analysed by EI-GC/MS as described below.

5.3.5.4 Instrumentation

A Trace Gas Chromatograph (GC) (ThermoFinningan, Milan, Italy) coupled to a Trace Mass Spectrometer (MS) (Thermo MassLab, Manchester, UK) was used for all analyses. GC/MS conditions were amended from that described by Schmelz et al. (2004). Briefly, 2 µL of the dichloromethane eluent was injected in the split/splitless injector of the GC, operated in the splitless mode (purge time 3.5 min., 50 mL/min.) at 280ºC. Compounds were separated on a Factor Four VF5-MS capillary column (Varian, Palo Alto, CA, USA) with dimensions 30 m l. x 0.25 mm i.d. x 0,25 um f.t. Flow of the carrier gas (Helium) through the column was 0.7 mL/min. in the constant flow mode. The oven program used was 40ºC, hold 1 min., ramp 15ºC/min to 250ºC, hold 5 min., ramp 20ºC/min. and hold 2 min. In order to avoid carryover a post-run was performed after each analysis at 280ºC under a head-pressure of 300 kPa. The temperature of the MS interface was kept at 280ºC and the source at 200ºC. The MS-detector was operated in Electron Impact (EI) mode at 70 eV and Selected Ion Monitoring (SIM) mode. The electron multiplier voltage was set at 500 V.

Three carboxylic acid methyl ester analytes were detected and quantified using SIM with retention times and ion mass to charge ratios (m/z) as follows: methyl salicylate (8.39 min., m/z 92, 120, 152); methyl jasmonate (12.38 min., m/z 83) and methyl indole-3-acetate (13.81 min., m/z 130). The internal standard, *o*-anisic acid methyl ester, was eluted at 9.70 min. with m/z 92, 120 and 152.

For quantitation the internal standard method was used. Calibration curves were constructed for each analyte over the range from 2-200 $ng.mL^{-1}$. The regression equations and their correlation co-efficients obtained for SA, MeJA and IAA are detailed respectively: $y=0.0370x+0.2511$ ($r^2=0.9924$), $y=0.0087x+0.0303$ ($r^2=0.9954$) and $y=0.0120x+0.0238$ ($r^2=0.9964$). The limit of quantification (LOQ) was established to be 2 pg for all analytes. Statistical analysis was performed using STATISTICA 6 software (Statsoft Inc., Tulsa, OK, USA).

5.4 Results

The transgenic lines used in this study overexpressed a grapevine PGIP encoding gene *Vvpgip1*, and were chosen for their reduced susceptibility towards *Botrytis cinerea*. Their resistance phenotypes could be correlated to PGIP expression, activity and ePG inhibition (Joubert et al., 2006; Chapter 3 of this work) and are thus described as PGIP-specific resistance phenotypes. These lines were used in transcriptomic and hormone profiling experiments to understand the *in planta* reactions to constitutive levels of PGIP and the possible role of PGIP in inducing the observed defense responses. The transgenic lines were shown to have altered cell wall metabolism, pointing to strengthened walls, even in the absence of infection or any defense induction (Becker et al., Chapter 4 of this work). In this study, these lines were transcriptionally and hormonally profiled subsequent to *Botrytis* infection. The site of infection and immediately surrounding tissues were used to evaluate the LAR phase of the defense response of the untransformed plants and their *Vvpgip1*-overexpressing counterparts.

5.4.1 Microarray Analyses

RNA from locally infected leaf material of *Botrytis*-infected wild-type (WT) and transgenic lines VvPGIP 37 and VvPGIP 45 was extracted and the integrity visualized on 1.2% formaldehyde gels (results not shown). No degradation of RNA was visible in any of the samples. The purity of the samples was further checked on a NanoDrop and deemed satisfactory (absorbance ratios of 260/230 and 260/280 >2). cDNA synthesis from a total of 50 µg of RNA yielded more than a microgram of cDNA, and equal amounts of reference and test cDNA were indirectly coupled to the dyes Cy3 and Cy5. The pooled reference and test cDNAs were hybridized to TIGR 10K potato microarray (version 3) slides.

Since the uninfected transgenic lines show differential expression compared to uninfected WT (Chapter 4, this work); the infected WT, VvPGIP 37 and VvPGIP 45 at either time point 24 or 48 hours post-inoculation (hpi) (as test) were hybridized to their respective uninfected (time 0 hpi) (as reference) counterparts. For 24 hour analyses, two WT and two transgenic slides (VvPGIP 37 and VvPGIP 45) were hybridized; for 48 hour analyses; six slides in total were hybridized (two each of WT, VvPGIP 37 and VvPGIP 45).

Transcriptomic analysis of the WT confirmed that the plants mounted a *Botrytis*-specific response. A t-test performed on WT samples at 24 and 48 hpi, revealed that 727 of 7229 genes (9%), were differentially expressed as compared to WT at 0 hpi ($p<0.01$) (results not shown). The *p*-value was based on t-distribution and was calculated using only alpha. Table 5.1 shows a selection of known *Botrytis*-responsive genes that were obtained from this t-test. Genes in bold function in the jasmonate or ethylene pathway and show a larger response at 24 hpi than 48 hpi.

Table 5.1. Result of t-test performed on wild-type (WT) samples at 24 and 48 h post-inoculation, showing the Log_2 transformed relative expression of genes responsive to *Botrytis cinerea* infection. Table shows a selection of known *Botrytis* responsive genes. A total of 727 genes from 7229 (9%) were differentially expressed at $p<0.01$.

Annotation	24 h Mean Log_2 expression	48 h Mean Log_2 expression
Proteinase inhibitor type II CEVI57 precursor {*Lycopersicon esculentum*}	3.32	3.51
Proteinase inhibitor II {*Solanum tuberosum*}	2.54	2.69
Q8W2K6 Proteinase inhibitor IIa	2.12	2.74
Tomato 5-LOX	2.46	1.85
pathogenesis-related protein 1b– common tobacco {*Nicotiana tabacum*}	1.64	2.27
Q43834 Class II chitinase EC 3.2.1.14	1.97	1.92
AAH01460 KIAA0731 Pathogenesis-related protein P2 precursor. {*Lycopersicon esculentum*}	1.89	1.79
1-aminocyclopropane-1-carboxylate synthase 1A – tomato {*Lycopersicon esculentum*}	2.05	1.36
Q9AWB7 Phospholipase PLDb1	2.09	1.20
Probable glutathione S-transferase Auxin induced protein PGNT35/PCNT111 {*Nicotiana tabacum*}	1.96	1.16
S-adenosylmethionine synthetase 3	1.73	1.30
S-adenosylmethionine synthetase 1	1.81	1.22
Q9ZWP2 1-aminocyclopropane-1-carboxylate oxidase	1.76	1.15
Q43190 Lipoxygenase EC 1.13.11.12	1.44	1.01
Q84L58 1-aminocyclopropane-1-carboxylic acid oxidase	1.46	0.98

HCL (average and complete linkage clustering) performed on all 24 hpi samples (WT and transgenic lines) revealed that samples processed on the same day clustered together (data not shown). HCL of the 48 hpi samples (data not shown) clustered the WT lines together, and transgenic lines whose hybridizations were performed on the same day (i.e. VvPGIP 37 and VvPGIP45).

Results of ANOVA analysis ($p<0.01$) of 48 hpi samples are shown in Table 5.2. The gene annotations are ranked in the order of the largest difference between the groups. This analysis revealed that 58 genes were differentially expressed between the three groups. The groups were divided according to plant line (WT, VvPGIP 37 and VvPGIP 45). The annotation Cytochrome P450 (row 1) reveals the largest expression difference among the groups (Log_2 difference of 0.76), while the remaining annotations show very little difference. These results indicate that large expression differences between the WT and either transgenic line at 48 hpi were not discernible.

Table 5.2. ANOVA analysis of 48 hpi microarray samples. Genes were differentially expressed between the groups at $p<0.01$. Gene annotations are ranked according to the differences between the groups- from the largest to the smallest gene expression difference. These results indicate that large expression differences between WT and the transgenic lines are not apparent at 48 hpi.

Annotation	WT mean (Log_2)	VvPGIP 37 mean (Log_2)	VvPGIP 45 mean (Log_2)	*p* value
Q9SWE3 Cytochrome P450	2.17	1.41	1.40	0.00183
Q9LV03 NADH-dependent glutamate synthase	-0.32	0.12	0.17	0.00233
Q7YDN0 ATP synthase 6	0.41	0.39	0.86	0.00762
Q9SLK2 F20D21.14 protein At1g54320/F20D21 50	0.42	-0.02	0.07	0.00793
60S RIBOSOMAL PROTEIN L7A protein *Arabidopsis thaliana*	0.48	0.12	0.15	0.00165
Phosphoprotein phosphatase catalytic beta chain alfalfa {*Medicago sativa* }	-0.14	-0.49	-0.28	0.00728
Probable ARP2/3 protein complex subunit p41 imported *Arabidopsis thaliana*	-0.21	0.12	0.07	0.00027
Q8RVG0 Protein phosphatase 2C	0.07	-0.22	-0.20	0.00563
Q7PFG6 ENSANGP00000024712	-0.34	-0.16	-0.03	0.00318
AAQ10954 Zinc finger protein	0.27	0.18	-0.03	0.00619
Probable complex I intermediate-associated protein 30 {*Arabidopsis thaliana*}	-0.11	0.20	0.07	0.00393
Glycogen starch synthase chloroplast precursor GBSSII Granule-bound starch synthase II	0.21	-0.02	0.28	0.00626
ADP-ribosylation factor {*Oryza sativa* japonica cultivar-group}	0.32	0.03	0.11	0.00474
Q8Y304 Probable UDP-N-acetylglucosamine pyrophosphorylase protein EC 2.7.7.23	0.08	-0.12	-0.21	0.00586
Q9SM52 NBS-LRR protein Q9LKQ0 RGC1 Fragment	0.21	-0.05	0.20	0.00023
Q9XI44 F9L1.19 protein Q9XI44 F9L1.19 protein	-0.17	-0.44	-0.25	0.00035
Hypothetical protein At2g22500 imported *Arabidopsis thaliana*	0.19	-0.05	-0.05	0.00577
beta-fructofuranosidase precursor soluble carrot {*Daucus carota*}	-0.18	-0.28	-0.01	0.00441
Q9FTL7 Putative WRKY DNA binding protein	0.36	0.09	0.21	0.00313
Q9LIV7 Hypothetical protein	0.11	-0.02	-0.14	0.00796
Probable bHLH transcription factor imported *Arabidopsis thaliana*	0.01	0.23	0.06	0.00415
AAR99175 ATP synthase F0 subunit 6	0.13	0.09	-0.09	0.00429
Hypothetical protein M3E9.120 *Arabidopsis thaliana*	0.01	0.03	-0.18	0.00942
Q940Y6 AT4g27600/T29A15 90	-0.17	0.05	-0.07	0.00976
T1K7.9 protein *Arabidopsis thaliana*	-0.18	-0.16	0.02	0.00460
Hypothetical protein T20K18.100 *Arabidopsis thaliana*	-0.07	0.15	0.06	0.00393
Q95DV2 RNA polymerase beta subunit DNA-directed RNA polymerase beta chain Fragment	-0.13	0.02	0.08	0.00070
Nuclear ribonuclease Z RNase Z tRNA 3 endonuclease Zinc phosphodiesterase ELAC Fragment	-0.10	0.06	-0.14	0.00994
Q94AW9 At2g25350/F13B15.1 Expressed protein	-0.15	-0.14	0.03	0.00108
Probable adenylate kinase 2 chloroplast precursor ATP-AMP transphosphorylase	0.06	0.03	-0.13	0.00074
Q8H349 OJ1720 F04.5 protein	-0.13	-0.10	0.05	0.00221
AT3g52300/T25B15 70 {*Arabidopsis thaliana*}	0.11	0.28	0.28	0.00933
Q9LNH1 F21D18.9	0.07	0.18	0.26	0.00808
Miraculin homolog root-knot nematode-induced tomato {*Lycopersicon esculentum*}	-0.19	-0.12	0.00	0.00741
Q8LMV3 Putative reverse transcriptase	-0.06	0.00	0.12	0.00945
Q83A54 Hypothetical protein	0.05	0.22	0.19	0.00393
Dihydrodipicolinate synthase chloroplast precursor DHDPS {*Nicotiana_tabacum*}	-0.07	0.10	0.03	0.00664
Protein Ser/Thr protein kinase homolog imported *Arabidopsis thaliana*	0.23	0.11	0.09	0.00383
O23169 Selenium-binding protein homolog	-0.04	0.10	0.08	0.00627
Hypothetical protein At2g04340 imported *Arabidopsis thaliana*	-0.12	0.02	0.01	0.00084

Hypothetical protein T19P19.80 *Arabidopsis thaliana*	-0.05	-0.18	-0.05	0.00597
At5g39890 {*Arabidopsis thaliana*}	0.15	0.11	0.00	0.00874
Q8LGU6 HOBBIT protein	-0.10	0.05	-0.02	0.00522
Q9LRX7 Phosphatidylinositol/phosphatidylcholine transfer protein-like	-0.11	-0.02	0.04	0.00635
Q8H0H2 Chromomethylase-like protein	0.21	0.26	0.35	0.00651
Thioredoxin M-type 3 chloroplast precursor TRX-M3 {*Arabidopsis thaliana*}	-0.09	0.02	0.03	0.00608
Glutamate synthase ferredoxin clone C 35 – common tobacco fragment	-0.14	-0.25	-0.13	0.00579
Hypothetical protein F1C12.90 *Arabidopsis thaliana*	0.02	0.09	0.15	0.00757
At5g28040 {*Arabidopsis thaliana*} unknown protein 41916-40546	0.11	0.01	-0.01	0.00385
Unknown protein {*Lycopersicon chilense*} Q84UV4 CSN3 Fragment	-0.01	-0.02	0.09	0.00449
Q9FGK8 Similarity to unknown protein	0.04	0.14	0.05	0.00366
F3H9.12 protein *Arabidopsis thaliana*	-0.07	0.03	-0.03	0.00844
Cleavage and polyadenylation specificity factor 100 kDa subunit CPSF100 kDa subunit	-0.02	-0.13	-0.06	0.00242
At2g03430 {*Arabidopsis thaliana*}	-0.03	0.00	0.06	0.00779
Q8LFP0 Chalcone isomerase putative	-0.11	-0.03	-0.04	0.00730
O23055 YUP8H12.27 protein O23055	0.08	0.16	0.11	0.00701
AAN85383 Resistance protein Fragment	0.03	0.10	0.03	0.00525
Q9LQK7 F5D14.28 protein	0.00	0.05	-0.01	0.00765

PTM was performed on both 24 and 48 hpi samples. The templates used were either for expression up in WT and down in the transgenic lines; or down in the transgenic lines and up in WT. The results of this analysis are shown in Table 5.3. These differences are significant at $p<0.01$. A large number of genes fitted the template at either time point, although the expression differences between WT and the transgenic lines were less pronounced. When applying a two-fold cutoff (Log_2 =1), only one gene emerged from the PTM analysis. This was at 24 hpi, using the expression template WT down, transgenic up. The annotation of this gene is divinyl ether synthase (*des1*). This gene is involved in the formation of the divinyl ether oxylipins colneleic and colnelenic acid from linoleic and linolenic acid, respectively.

At 48 hpi, the annotation Cytochrome P450 was identified, as previously from ANOVA analysis (Table 5.2). However, applying the two-fold cutoff ruled this gene out of further analyses.

Table 5.3. Result of PTM analysis of the 24 and 48 hpi microarray samples. A large number of genes fitted the respective templates, although the expression difference between WT and transgenic lines were not very pronounced. A gene encoding divinyl ether synthase (*des1*) was identified from the 24 hpi analysis which fitted the Log_2 expression cutoff of 1 between WT and transgenic lines.

Samples	Template	Numer of Genes fitting template	Largest expression difference (Log_2)	Genes fitting Log_2=1 cutoff
24 hpi	WT↓ transgenic↑	19	1.00	1
	WT↑ transgenic↓	25	0.65	0
48 hpi	WT↓ transgenic↑	55	0.48	0
	WT↑ transgenic↓	34	0.76	0

5.4.2 Quantitative Real-Time PCR

Leaf tissue of the wild-type (WT) and the two transgenic lines VvPGIP 37 and VvPGIP 45 were infected with *Botrytis cinerea* spores and locally infected material harvested and flash-frozen at 0, 18, 24 and 30 hpi. This material was used for cDNA synthesis to verify expression differences obtained from microarray analyses. Relative expression differences between the WT and transgenic lines at the indicated hpi are shown in Figure 5.1. The results show the mean relative expression of VvPGIP 37 and VvPGIP 45 compared to WT, of four replicates per plant line with standard deviation. At 24 hpi, the relative expression of the tobacco *des1* gene in the transgenic lines was already slightly upregulated; at 30 hpi, the expression in VvPGIP 37 and VvPGIP 45 was approximately 2.5 fold higher compared to that of the WT. Infection progression was most likely slower in this experiment since the upregulation of *des1* in the same order (2 fold upregulation) as the microarray experiment occurs at 30 hpi; compared to 24 hpi in transcriptomic analyses. Expression of the *des1* gene was also upregulated in the WT following *Botrytis cinerea* infection, but to a lesser extent as mentioned. The seemingly low relative expression level in the two transgenic lines at 0 hpi can be attributed to the very low level of *des1* expression before *Botrytis* infection takes place (Fammartino et al., 2006). The expression of the *des1* gene was normalized using the tobacco actin gene.

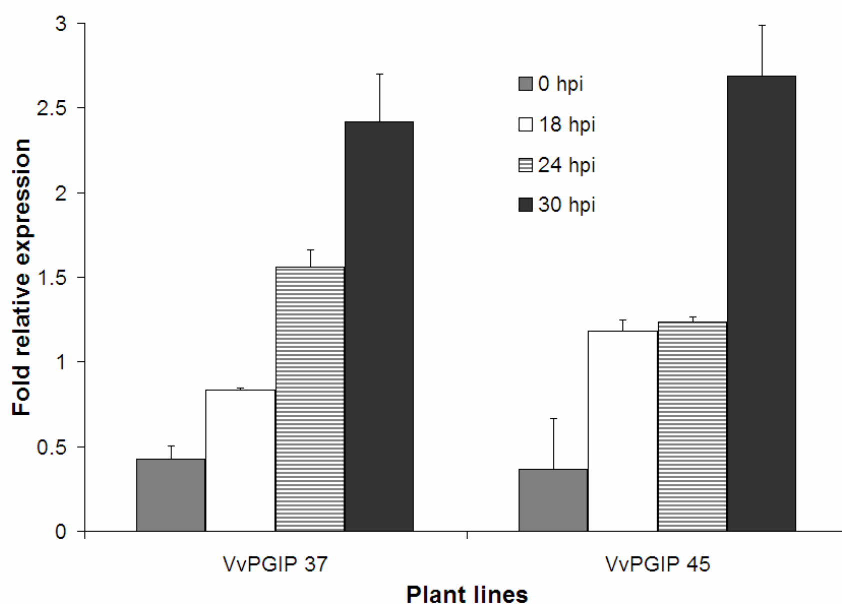


Figure 5.1. Expression of the *Nicotiana tabacum* divinyl ether synthase 1 (*des1*) gene in locally infected leaf material of two *Vvpgip1*-transformed transgenic lines (VvPGIP 37 and VvPGIP 45) relative to the untransformed wild-type, following *Botrytis cinerea* inoculation for 0, 18, 24 and 30 hours. Transcripts were also upregulated in the untransformed wild-type. The expression in the transgenic lines shows an approximate 2.5 fold increase of the expression of the *des1* gene at 30 hours post-inoculation (hpi). The relative low level of expression in the transgenic lines at time 0 can be attributed to the very low transcript abundance of *des1* pre-infection (Fammartino et al., 2006). Relative gene expression was calculated following Real-Time Quantitative PCR analysis using the LightCycler instrument. The mean relative expression of four replicates per plant line is shown with standard deviation. These results were confirmed in an independent experiment.

5.4.3 Simultaneous Analysis of Phytohormones Using GC/MS

Locally infected leaf tissue of the untransformed WT, VvPGIP 37 and 45 was analyzed for their phytohormone profile following *B. cinerea* infection at 0, 18, 24, 30 and 40 hours post-inoculation. Figure 5.2 shows the total jasmonic acid (JA) (free and methylated) (Figure 5.2A), salicylic acid (SA) (Figure 5.2B) as well as the indole-acetic acid (IAA) (Figure 5.2C) content in the locally infected leaf tissues. Conjugated hormones were not measured. The graphs show the phytohormone profile determined from at least three replicates per infected time point per plant line, with standard deviation.

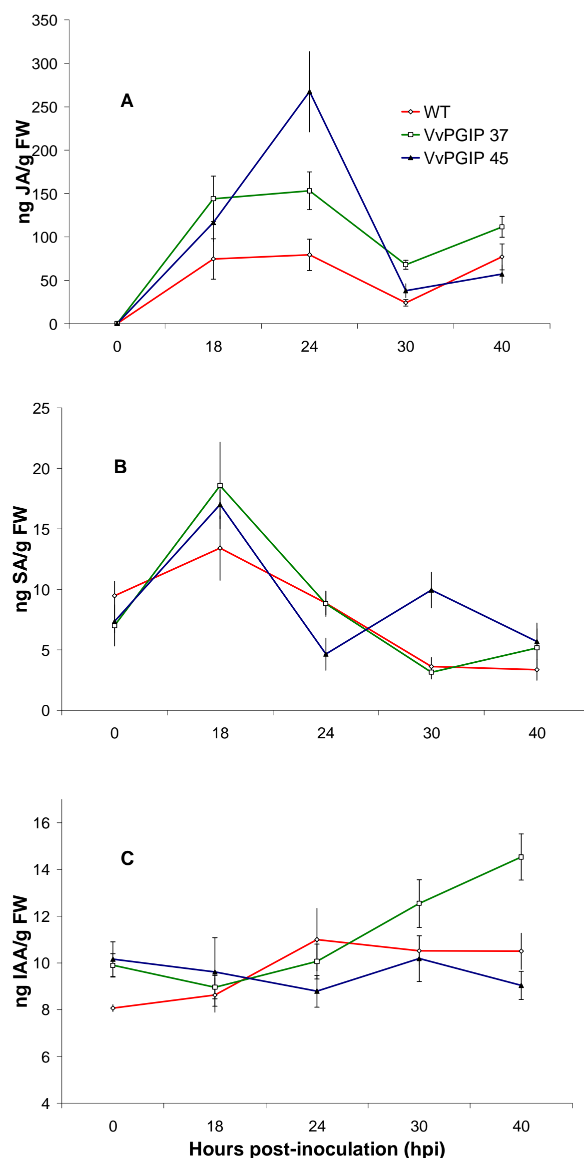


Figure 5.2. Analysis of the phytohormone profile of the untransformed wild-type (WT) and transgenic lines VvPGIP 37 and VvPGIP 45 following *Botrytis cinerea* infection. Phytohormones were quantified according to the method of Schmelz et al. (2004). The hormones jasmonic acid (JA) (A), salicylic acid (SA) (B), and indole-acetic acid (IAA) (C) were measured as their corresponding methyl esters. Thus, the pool of free and esterified SA, JA and IAA were measured. The conjugated hormone levels were not determined. Figure indicates the means of at least three replicates per time point, with standard deviation.

The JA content of the locally infected WT and transgenic plant lines (Figure 5.2A), undetectable at 0 hpi, increased following incubation for 18 h with *Botrytis*. However, in the transgenic lines VvPGIP 37 and VvPGIP 45, this induction was more pronounced. In WT, JA accumulated to only 75 ng/g FW (standard deviation shown in Fig 2) after 18 hpi and reached a maximum of 79 ng/g FW after 24 hpi. This is in contrast to VvPGIP 37 and VvPGIP 45, accumulating to 143 and 116 ng/g FW after 18 hpi, respectively. After 24 hpi, this was even more pronounced, as VvPGIP 37 reached levels of 153 ng/g FW and VvPGIP 45 267 ng/g FW; which totals to almost double and more than triple the amount observed for WT. The increased induction of JA for VvPGIP 45 and VvPGIP 37 was confirmed in a separate infection experiment in which JA content was quantified. In this experiment, VvPGIP 45 again accumulated more JA than VvPGIP 37.

Similarly, differential induction of the synthesis of salicylic acid (SA) was observed between the WT and the two transgenic lines (Figure 5.2B). Even though the levels of SA at 0 hpi were considerably lower in the two transgenic lines than in WT, they accumulated to slightly higher levels (not statistically significant) in the *Vvpgip1*-overexpressing lines at 18 hpi (standard deviation shown in Fig 2). In the latter plant lines, levels increased from seven to between 17 and 18 ng/g FW at 18 hpi, a net increase of 10 ng/g FW. For WT, this increase was from nine to only 13 ng/g FW during the same period. Although the accumulated SA amounts were not significantly different, the transgenic lines showed a stronger induction of SA synthesis following the early stages of *Botrytis* infection, which was significant in statistical analyses. Repeated measures ANOVA was utilized to show that the induction of SA was stronger in transgenic lines. The *p*-values for SA induction (difference between time 0 and 18 hpi) for WT was 0.990723, indicating no significant induction, while the two transgenic lines (VvPGIP 37 p=0.000152; VvPGIP 45 p=0.000174) revealed inductions with much higher statistical confidence. Similar results were obtained in an independent infection assay in which SA content was quantified. Curiously, a “secondary” SA response after 24 hpi can be seen for VvPGIP 45. In an independent infection assay, this “secondary” SA response was also observed for VvPGIP 37.

Clear differential patterns of IAA content in the plant lines (Figure 5.2C) were less obvious to distinguish. Although the transgenic lines clearly had higher levels of IAA at 0 hpi, differences in accumulation patterns were not clearly discernable between WT and *Vvpgip1*-expressing lines.

Independent infection experiments confirmed the results observed for JA and SA.

5.5 Discussion

Polygalacturonase-inhibiting proteins (PGIPs) are involved in plant defense by inhibiting the action of fungal macerating enzymes (endo-polygalacturonases, ePGs)

required for the virulence of several phytopathogenic fungi and bacteria (De Lorenzo et al., 2001); thereby limiting their destructive potential. Also, Cervone et al. (1989) have shown *in vitro* that the inhibition of ePGs in the presence of polygalacturonic acid may lead to the prolonged existence of molecules capable of eliciting plant defense responses. Several plant species overexpressing *pgip* genes have been found to be less susceptible to infection by the necrotrophic pathogen *B. cinerea* (Powell et al., 2000, Ferrari et al., 2003a, Aguero et al., 2005, Manfredini et al., 2005; Joubert et al., 2006). While it has been shown that the heterologously expressed PGIPs inhibit fungal polygalacturonases from *Botrytis* (Manfredini et al., 2005, Joubert et al., 2006), differential elicitation of plant defense responses between wild-type (WT) and lines accumulating PGIP is yet to be shown.

Tobacco overexpressing the grapevine *Vvpgip1* gene has been shown to render a PGIP-specific defense response against *Botrytis* (Joubert et al., 2006). The PGIP-specific resistant lines were used as resources to study the role(s) of PGIP. Following from a previous study where transcriptomic and hormonal analysis of these lines were performed without *Botrytis* infection, this work profiled the transgenic lines in comparison with the WT control subsequent to *Botrytis* infection. The analysis focused on the local response and spanned the first 48 hpi.

Differential expression analysis identifies a gene involved in the synthesis of a potent antifungal compound

TIGR 10K potato microarray slides were used to screen for genes differentially regulated between WT and those accumulating VvPGIP to a high degree. *Vvpgip1*-overexpressing tobacco lines did not exhibit differential gene expression in a large number of genes at the local response within the first 48 hpi (as analyzed by ANOVA and Pavlidis Template Matching). PTM (Pavlidis and Noble, 2001) revealed the gene encoding divinyl ether synthase 1 (*des1*) to be induced to a level two-fold higher in the transgenic lines compared to WT. This differential expression was confirmed by independent infections and quantitative Real-Time PCR (Figure 5.1). This gene acts in the 9-LOX branch of the lipoxygenase (oxylipin) pathway, specifically the branch responsible for the formation of the oxylipins colneleic and colnelenic acid in Solanaceous plant species.

In potato, the accumulation of the two divinyl ether fatty acids colneleic and colnelenic acid is observed following infection by the late-blight pathogen *Phytophtora infestans* (Weber et al., 1999). The accumulation of these compounds occurs more rapidly in the less susceptible cultivar Matilda, as compared to the more susceptible Bintje cultivar. Also, these authors could show that the divinyl ethers accumulated during the incompatible interaction of *Nicotiana tabacum* with tobacco mosaic virus. Comparing partially and highly compatible tomato-*Phytophtora infestans* interactions, Smart et al. (2003) were able to show that partial resistance was not dependent on differential upregulation of pathogenesis-related (PR) gene expression. However, a much stronger induction of the tomato divinyl ether synthase

gene could be observed at 48 hours post-inoculation (hpi) in the partially compatible interaction. Colneleic and colnelenic acid also show potent inhibitory effects on *B. cinerea* spore germination (Prost et al., 2005). Fammartino et al. (2006) reported that these divinyl ethers modulated the outcome of the tobacco-*P. parasitica* interaction, and provided evidence that the 9-LOX divinyl ethers participate in the local response to pathogen attack, where these antimicrobial acids are formed at the site of infection, rather than in a systemic fashion. These lines of evidence strongly support the notion that the upregulation of divinyl ether synthase, and the synthesis of its oxylipins contribute significantly to resistance against fungal pathogens at the site of infection.

In this work, the quantification of the antifungal compounds colneleic and colnelenic acid was also attempted. Although induction of the synthesis of colneleic acid could be observed (result not shown), absolute quantification of this compound in the tobacco lines requires some optimization of the GC-MS method, and is currently in progress. Weber et al. (1997) reported the existence of an "oxylipin signature", referring to the complex mix of signals of the numerous compounds derived from the oxylipin pathway. Measurements of JA and two related oxylipin compounds revealed different "signatures" in different species and tissues following wounding. Hause et al. (2000) could show distinct oxylipin signatures in tomato flowers. It could be proposed that *Vvpgip1*-overexpressing plants may produce their own distinct "oxylipin signature" following *Botrytis* infection, which may contribute to the observed decrease in disease susceptibility. However, additional data on the oxylipin content of these plants are needed to strengthen this argument.

More jasmonates are present in the PGIP-specific resistant lines at the local response sites after *Botrytis* infection

The lipoxygenase pathway also leads to the formation of JA and MeJA, although via a different branch, the 13-LOX branch. In the two transgenic lines, induction of the synthesis of the pool of jasmonates (JA + MeJA) was affected (Figure 5.2). In the transgenic lines, jasmonates accumulated to much higher levels at 24 hpi. Jasmonic acid and methyl jasmonate are known to affect a myriad of functions in plants, from plant developmental processes and responding to environmental stresses, to activating plant defense responses following herbivory, insect attack and pathogen ingress (reviewed in Cheong and Do Choi, 2003; Devoto and Turner, 2003; Farmer et al., 2003). Seo et al., (2001) has shown that increased resistance to *Botrytis* was obtained by overexpressing the gene converting JA to MeJA, which may then induce defense responses (Devoto and Turner, 2003). Thomma et al. (1998, 1999) reported that, in *Arabidopsis*, JA and ethylene have roles in the mediation of the local resistance to *Botrytis cinerea*.

It is tempting to speculate that the observed increased *des1* expression was a result of JA signaling. The delay between JA accumulation and *des1* upregulation and the fact that *des1* expression in VvPGIP 45 is higher (Figure 5.1), seems to

support this notion. Also, VvPGIP 45, which consistently accumulates more JA, was found to have higher levels of colneleic acid 40 hpi in initial experiments (result not shown). However, as mentioned before, the analytical method for the quantification of colneleic acid requires some optimization before absolute levels of this compound can be reported.

Data from *Phytophtora* infected tomato and potato, two Solanaceous species closely related to tobacco, does not seem to support the notion that *des1* is induced by jasmonates. For tomato (Smart et al., 2003), induction of *des1* expression is not accompanied by induction of *Pin2* expression, a JA responsive gene. Also, Weber et al. (1999) reported that formation of the 9-LOX divinyl ether oxylipins in potato was independent of 13-LOX activity, responsible for the formation of jasmonates. The levels of JA were comparable in control and *Phytophtora*-infected tissues three days after infection. Whether tobacco *des1* expression is upregulated by jasmonates remains to be seen. Regarding the currently available data, it seems more plausible that in transgenic plants overexpressing *Vvpgip1*, the lipoxygenase pathway seems to be affected. Both branches of this pathway, the 9-LOX, leading to the formation of, amongst others, divinyl ether oxylipins, and the 13-LOX pathway, leading to the formation of jasmonates, are affected in *Vvpgip1* overexpressing lines following *Botrytis* infection.

This is the only work to date which has examined the defense-related phytohormone content of plant lines overexpressing a *pgip* gene. The stronger induction of SA synthesis observed in the transgenic lines may also be involved in the reduced susceptibility to *Botrytis*. The role of SA in defense against this fungus has been demonstrated in tomato (Díaz et al., 2002) and *Arabidopsis* (Ferrari et al., 2003b). Partial resistance against *Botrytis* was observed in tomato following exogenous application of SA (Díaz et al., 2002). Ferrari et al. (2003b) were able to show that SA was involved in localized resistance to *Botrytis* on *A. thaliana* plants.

Conclusions

The work presented in this manuscript shows that plants, overexpressing a *Vitis vinifera pgip*, and showing significant decreased susceptibility to *Botrytis* infection, show differential expression of a defense-related gene (*des1*) and the profile of its defense-related phytohormone content is altered at the level of the local response. These changes, together with the cell wall strengthening prior to infection that was previously shown, may all contribute to the observed decrease in disease susceptibility in VvPGIP plants. The number of genes differentially affected by microarray analyses was surprisingly low concerning that JA was upregulated earlier and to a greater extent (Figure 5.2). Additional microarrays at different time points would no doubt shed light on additional jasmonate regulated genes differentially regulated between WT and transgenic lines. This type of analysis on systemic tissue could also identify systemic-response genes upregulated by increased levels of jasmonates.

HCL indicated the 24 hpi arrays clustered by date, rather than genotype. This might be a reflection of the small number of changes between the untransformed and *Vvpgip1*-transgenics, but more replicates at this time point could be helpful. The strategy for the current work, however, was to use the arrays as a screen to obtain genes or pathways for further analysis and this was successful.

This work represents the first data to emerge following the proposal of Cervone et al. (1989) that PGIPs may prolong the life of elicitor-active oligogalacturonides, following the action of fungal macerating enzymes on plant cell wall constituents. Their *in vitro* work suggested that plant defenses may then be upregulated as a result of these defense-related signaling molecules. This study corroborates the role of PGIP in defense signaling and although the presence and role of elicitors have not been studied here, the downstream effects of the signaling were observed. The lipoxygenase pathway was affected in the transgenic lines at the local response, suggesting that in transgenic lines accumulating PGIP, this pathway may be regulated following *Botrytis* infection. Detailed analysis of the pathway and its products are envisaged.

5.6 References

Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pathol. 6: 43-51.

Cervone, F. Hahn, M.G., De Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. 90: 542-548.

Cheong, J-J., and Do Choi, Y. 2003. Methyl jasmonate as a vital substance in plants. Trends Genet. 19: 409-413.

Cimerman, A., Reignault, P., Levis, C., and Boccara, M. 2003. Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme1* reduces virulence on several host plants. Mol. Plant Microbe Interact. 16: 360-367.

De Lorenzo, G., D'Ovidio, R., and Cervone, F. 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu. Rev. Phytopath. 39: 313-335.

Devoto, A., and Turner, J.G. 2003. Regulation of jasmonate-mediated plant responses in Arabidopsis. Ann. Bot. 92: 329-337.

Díaz, J., ten Have, A., and van Kan, J.A.L. 2002. The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. Plant Physiol. 129: 1341-1351.

Eisen, M.B., Spellman, P.T. Brown, P.O., and Botstein, V. 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA 95: 14863-14868.

Fammartino, A., Cardinale, F., Göbel, C., Mène-Saffrané, L., Fournier, J., Feussner, I., and Esquerré-Tugayé, M-T. 2006. Characterisation of a divinyl ether biosynthetic pathway specifically associated with pathogenesis in *Nicotiana tabacum*. Plant Physiol. DOI: 10.1104/pp.106.087304.

Farmer, E.E, Alméras, E., and Krishnamurthy, V. 2003. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr. Opin. Plant Biol. 6: 372-378.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. 2003a. *Arabidopsis* polygalacturonase-inhibiting proteins (PGIP) are regulated by different signal transduction pathways during fungal infection. Plant Cell 15: 93-106.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. 2003b. *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4.* Plant J. 35: 193-205.

Ferrari, S., Galletti, R., Vairo, D., Cervone, F., and De Lorenzo, G. 2006. Antisense expression of the *Arabidopsis thaliana AtPGIP1* gene reduces polygalacturonase-inhibiting protein accumulation and enhances susceptibility to *Botrytis cinerea*. Mol. Plant Microbe Interact. 8: 931-936.

Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J., and Wasternack, C. 2000. Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J. 24: 113-126.

Isshiki, A., Akimitsu, K., Yamamoto, M., and Yamamoto, H. 2001. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Mol. Plant Microbe Interact. 14: 749-757.

Joubert, D.A., Slaughter, A.R., Kemp, G., Becker, J.V.W., Krooshof, G.H., Bergmann, C., Benen, J., Pretorius, I.S., and Vivier, M.A. 2006. The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. DOI 10.1007/s11248-006-9019-1.

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and van Kan, J.A. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 43: 213-225.

Li, R., Rimmer, R., Buchwalt, L., Sharpe, A.G., Seguin-Swartz, G., and Hegedus, D.D. 2004. Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of *endo*-and *exo*-polygalacturonases expressed during the saprophytic and parasitic modes. Fungal Genet. Biol. 41: 754-765.

Manfredini, C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M., and De Lorenzo, G. 2005. Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol. Mol. Plant Pathol. 67: 108-115.

Oeser, B., Heidrich, P.M., Muller, U., Tudzynski, P., and Tenberge, K.B. 2002. Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea*/rye interaction. Fungal Genetic. Biol. 36: 176-186.

Pavlidis, P., and Noble, W.S. 2001. Analysis of strain and regional variation in gene expression in mouse brain. Genome Biology 2:research0042.1-0042.15

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29: 2002-2007.

Powell, A.L., van Kan, J.A., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. 13, 942-950.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M.J., Kift, N., Carbonne, F., Griffiths, G., Esquerré-Tugayé, M-T., Rosahl, S., Castresana, C., Hamberg, M., and Fournier, J. 2005. Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol. 139: 1902-1913.

Ramakers, C., Ruijter, J.M., Lekanne Deprez, R.H., and Moorman, A.F.M. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339: 62-66.

Ridley, B.L., O'Neill, M.A., and Mohnen, D. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57: 929-967.

Rodriguez-Palenzuela, P., Burr., T.J., and Collmer, A. 1991. Polygalacturonase is a virulence factor in Agrobacterium tumefaciens biovar 3. J. Bacteriol. 173: 6547-6552.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., and Quackenbush, J. 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 34: 374-378.

Schmelz, E.A., Engelberth, J., Tumlinson, J.H., Block, A., and Alborn, H.T. 2004. The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. Plant J. 39: 790-808.

Seo, H.S., Song, J.T., Cheong, J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D. 2001. Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. USA 98: 4788–4793.

Shieh, M.T., Brown, R.L., Whitehead, M.P., Cary, J.W., Cotty, P.J., Cleveland, T.E., and Dean, R.A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63: 3548-352.

Smart, C.D., Myers, K.L., Restrepo, S., Martin, G.B., and Fry, W.E. 2003. Partial resistance of tomato to *Phytophtora infestans* is not dependent upon ethylene, jasmonic acid or salicylic acid signaling pathways. Mol. Plant Microbe Interact. 16: 141-148.

Tans-Kersten, J., Huang, H., and Allen, C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J. Bacteriol. 183: 3597-3605.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant Microbe Interact. 11: 1009-1016.

Thomma, B.P., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA 95: 15107-15111.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. 1999. Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. 121: 1093-1102.

Vaquerizas, J.M., Dopazo, J., and Díaz-Uriarte, R. 2004. DNMAD: web-based Diagnosis and Normalization for MicroArray Data. Bioinformatics 20: 3656-3658; doi:10.1093/bioinformatics/bth401.

Weber, H., Vick, B.A., and Farmer, E.E. 1997. Dinor-oxo-phytodienoic acid: A new hexadecanoid signal in the jasmonate family. Proc. Natl. Acad. Sci. USA 94: 10473-10478.

Weber, H., Chételat, A., Caldelari, D., and Farmer, E.E. 1999. Divinyl ether fatty acid synthesis in late-blight diseased potato leaves. Plant Cell 11: 485-493.

Zar, J.H. 1999. Biostatistical Analysis. 4th ed. Prentice Hall, NJ.

5.7 Acknowledgements

This work was supported by funding from the South African National Research Foundation (NRF), the Wine Industry Network of Expertise and Technology (Winetech), the Deciduous Fruit Producer's Trust (DFPT) and the THRIP fund.

GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion and Conclusions

In the agricultural sector, intensive efforts by producers and researchers alike have been invested in managing biotic stress factors associated with the decline in quality of cultivated crops. Current means of managing the organisms associated with this decline have been met with limited success and high cost. The management of microorganisms by means of chemical control has several drawbacks. Pathogen resistance to a range of chemical pesticides is a harsh reality (Gerhardson, 2002), and concerns regarding the health hazards chemical pesticides are posing to humans are on the rise (Barr et al., 2004). These factors have contributed to the opposition of consumers to the extensive use of chemicals on crops aimed at human consumption. Biological control involves the introduction of organisms antagonistic to spoilage microbes, and may involve a reduction in the dependency on chemicals. However, even after years of intensive research this method is reliant on several factors for satisfactory pest control (Gerhardson, 2002). The more traditional field of selection and breeding for resistance also suffers from drawbacks. This method is time-consuming and the resultant progeny rarely harbor both the resistance and quality traits initially bred for. A method for obtaining resistance traits in elite cultivars without compromising quality, has not only been proposed, but utilized for crop improvement. The use of genetic engineering, with the unmatched potential for the improvement of cultivated crops, for several outcomes, has already resulted in numerous field trials and crops to market.

Genetic engineering has especially been useful as a tool for increasing tolerance towards fungal pathogens in a diverse range of plants, including the most economically important fruit crop worldwide- grape. Fungal pathogens of grapevine include the biotrophic pathogens *Uncinula necator* and *Plasmopara viticola*, and the devastating necrotroph *Botrytis cinerea*. Tolerance towards the latter broad host range pathogen has been obtained in two *Vitis vinifera* cultivars, Chardonnay and Thompson Seedless, by genetic transformation of a single gene, in a proof of concept study. This gene encodes a polygalacturonase-inhibiting protein (PGIP) and was isolated from pear (Aguero et al., 2005). This grape species (*V. vinifera*) is considered extremely susceptible to grape fungal pathogens and extensive grape breeding efforts are yet to yield a resistant cultivar.

Similarly, in a number of backgrounds where *pgip* genes were expressed at high levels, was reduced susceptibility towards *B. cinerea* observed (Powell et al., 2000; Ferrari et al., 2003; Manfredini et al., 2005). Currently, *in vitro* evidence suggests that these reductions in disease susceptibility, in PGIP-accumulating genotypes, are concerted in two ways. Firstly, inhibition of endopolygalacturonases (ePGs), required for the virulence of several phytopathogenic fungi, directly limits plant tissue maceration and necrosis which is synonymous with the unhindered

action of these enzymes. The importance of ePG inhibition is further illustrated by the fact that overexpression of a gene encoding a PGIP with weak inhibitory activity against *Botrytis* ePGs, did not provide the plant with tolerance towards the pathogen (Manfredini et al., 2005). Additionally, *in vitro* evidence suggests that, following the inhibition of fungal ePGs by PGIP, the half-life of molecules (oligogalacturonides) which activate plant defense responses, are prolonged (Cervone et al, 1989). These molecules are formed as a result of the digestion of cell wall fragments, and soon disappear when ePGs continue their action. Thus, in lines overexpressing *pgips* and accumulating PGIP, protection might be afforded on two fronts. Firstly, PGIP limits tissue damage; subsequently, plant defenses are activated which lead to the arrest of fungal growth and pathogenesis.

At the Institute for Wine Biotechnology, one of the research initiatives entails the study of plant-pathogen interactions, specifically utilizing the PGIP-ePG interaction as a model. To this end, a gene encoding *V. vinifera* PGIP (*Vvpgip1*) was isolated and transformed into *Nicotiana tabacum* (Joubert et al., 2006), for the elucidation of its role in reducing fungal susceptibility in the transgenic lines. The effectiveness of VvPGIP1 against ePGs from *Botrytis* has also been studied in *in vitro* inhibition assays, as well as in novel *in vivo* analysis (Joubert et al. 2007). The PGIP-ePG interaction model as well as the transgenic resources mentioned above were utilized in this study to further expand on the current knowledge of PGIP and its *in planta* role in plant defense.

Transgenic tobacco overexpressing *Vvpgip1* are suitable resources to study the possible *in planta* roles of PGIP

Transgenic plants overexpressing *Vvpgip1* were convincingly shown to be significantly less susceptible to infection by *B. cinerea*. The infection assays included both detached leaf and time-course, whole-plant infection assays. This work is the first to report a whole-plant infection assay which confirms the antifungal nature of PGIP. These results are in agreement with those obtained by previous authors (Powell et al., 2000; Ferrari et al., 2003; Manfredini et al., 2005), where overexpression of *pgip* genes resulted in increased tolerance to *Botrytis* infection. In the tobacco lines, overexpression, protein activity and ePG inhibition could be correlated and the lines were thus regarded as PGIP-specific resistant lines. The purified VvPGIP was shown to inhibit BcPG1, an enzyme required for the full virulence of *B. cinerea* (ten Have et al., 1998; Kars et al., 2005). Thus, these results confirmed that VvPGIP, by inhibiting an ePG from *Botrytis*, contributes, at least in part, to the decreased fungal susceptibility of the transgenic tobacco plants. These results are in agreement with current knowledge regarding the role of PGIP in plant defense and confirm the antifungal nature of VvPGIP. The possible *in planta* roles of PGIP were further investigated.

Tobacco lines accumulating VvPGIP show alterations to cell wall metabolism

Untransformed and VvPGIP tobacco lines exhibiting the resistant phenotype were analyzed for transcriptomic and hormonal differences. These analyses were performed without inducing the resistance phenotype, i.e. before fungal inoculation. From these analyses, it could be shown that the VvPGIP-accumulating lines exhibited alterations to their cell-wall related metabolism. The changes which were observed can be linked to the strengthening of plant cell walls (Figure 6.1A). These changes are usually observed following pathogen attack and suggest that in the PGIP-specific resistant lines, their cell walls exhibit characteristics of those observed after plant defense responses are activated. Changes in cell wall metabolism included increases in lignin deposition and downregulation of *XTH* expression, which may result in the production of xyloglucan of a higher molecular weight. Both these alterations contribute to a strengthened cell wall. Also, higher levels of indole-acetic acid were found in the transgenic lines. Whether IAA levels influence lignin deposition and *XTH* expression in these lines remains to be seen. Thus, cell walls of the PGIP-specific tobacco lines are considered to be "primed". Considering that no ePGs were involved in these analyses, and hence no oligogalacturonide signaling could take place, this work adds new information to the current knowledge of PGIP in plant defense. To date, the inhibition of ePGs and the subsequent oligogalacturonide signaling represented our knowledge on the role of PGIPs in plant defense. Following the data presented in this work, it is clear that an additional role for PGIP, in contributing to "primed" defenses of plants, may be postulated. PGIP may directly influence plant defense responses- whether by means of its structural features or interaction with cell wall components.

Following *Botrytis cinerea* infection, differential regulation of the lipoxygenase pathway, at the localized response, is observed in the transgenic lines

Transcriptomic and phytohormone analyses of locally infected leaf tissue were performed on untransformed and PGIP-specific resistant lines following *Botrytis* infection. Following transcriptomic analyses, upregulation of *Botrytis*-responsive genes could be observed in both genotypes. Although limited differences were observed between the genotypes following *Botrytis* inoculation, a plant defense pathway, differentially induced in the local resistance response in the transgenic lines, was identified (Figure 6.1B). The transcriptomic analyses, utilized as a screen, were thus considered successful. Both branches of the lipoxygenase (LOX) pathway were affected- the 9-LOX and 13-LOX branches were induced to a greater extent in the transgenic lines. For the 9-LOX pathway, differential expression for the gene encoding divinyl ether synthase (*des1*) was reported. This enzyme is involved in the formation of the antifungal divinyl ethers colneleic and colnelenic acid that have been shown to have potent inhibitory effects on *Botrytis* spore germination (Prost et al., 2005). Additionally, a greater induction of the synthesis of jasmonate, a defense related hormone, was observed in the transgenic lines. This hormone is synthesized

via the 13-LOX pathway. The increased induction of the synthesis of the pool of jasmonates has been shown to be important for the mediation of local resistance to *Botrytis* (Thomma et al., 1998, 1999). Taken together, these results are the first to provide evidence and to corroborate and expand on the hypothesis of defense signaling put forward by Cervone et al. (1989). Although the contribution of oligogalacturonides was not elucidated in this study, it could be shown that in PGIP-accumulating plants, differential induction of at least one important plant defense-related pathway is observed in the local response to *Botrytis*. The data presented in this dissertation provide the first elucidation of differential regulation of a defense-related pathway between PGIP-specific resistant lines and their untransformed counterparts. The contribution to the current understanding of the role of PGIP in plant defense is outlined in Figure 6.1.

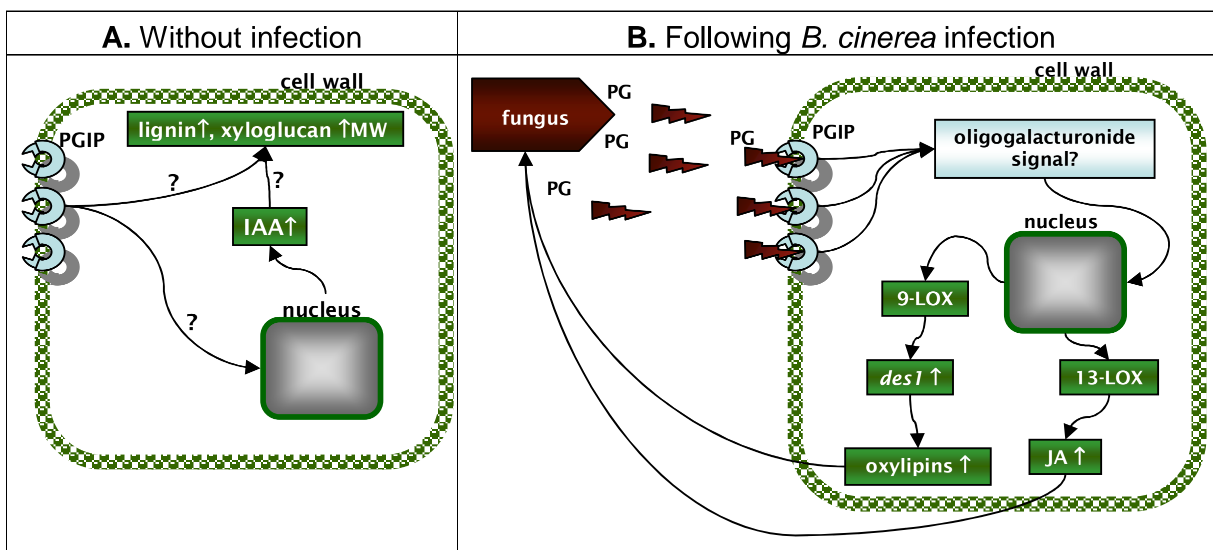


Figure 6.1. The current understanding of the role of PGIP in plant defense responses. New data regarding the role of PGIP, presented in this manuscript (indicated in green boxes) builds on existing knowledge (indicated in blue boxes). **(A)** Briefly, before pathogen ingress, PGIP may "prime" the cell wall, leading to a reinforced wall usually associated with defense responses. Following transcriptomic and hormonal analysis of PGIP-specific resistant lines, it was found that PGIP in these lines cause altered cell wall metabolism, even in the absence of pathogen infection. These results indicate a novel role for PGIP, which may in turn contribute to the reduced disease susceptibility observed in the transgenic lines. **(B)** Following inhibition of fungal ePG by PGIP, the signaling cascade (presumably by oligogalacturonides) results in the upregulation of a gene encoding divinyl ether synthase, which leads to the formation of antifungal divinyl ether oxylipins. Also, signaling results in an increase in the synthesis of the defense related hormone jasmonic acid and its methyl ester, methyl jasmonate. This work proposes a new role for PGIP in reducing susceptibility to *Botrytis* infection and expands on the hypothesis of Cervone et al. (1989).

Differential induction of the synthesis of salicylic acid (SA) was also observed between the WT and transgenic lines. The role of SA towards reduced susceptibility will be investigated in further detail, as induction differences for SA were less pronounced than JA in the transgenic lines.

Perspectives

In this dissertation, evidence was provided for novel roles for PGIP in plant defense. Also, evidence was put forward elucidating the downstream pathways affected in PGIP-accumulating plants, providing the first evidence of defense-related pathways differentially affected in the localized response and possibly contributing to locally decreased disease susceptibility to *Botrytis*. The contribution of each of these pathways to the overall reduction in susceptibility of the PGIP-specific resistant lines needs to be clarified. Additionally, systemic tissue, distal to the infection, needs to be analyzed to gain a full perspective on the affected pathways in the transgenic lines. For the contribution of the "priming" phenomenon, evaluation of the role of both the lignin biosynthetic pathway and downregulation of *XTH* will be further investigated to ascertain the contribution of each to the plants' reduced susceptibility. Tobacco plants accumulating divinyl ether oxylipins could be utilized in the same manner. Antifungal assays on all these genetic backgrounds will no doubt shed light on the contribution of each of these mechanisms to the decreased disease susceptibility in *Vvpgip1*-overexpressing tobacco lines. Additionally, it is envisaged that mutant lines (eg. *Arabidopsis*), unable to accumulate jasmonates, but overexpressing *pgip*, may be utilized to shed some light on the contribution of this hormone to the localized response to *Botrytis* in PGIP-accumulating lines.

In the analyses following *Botrytis* infection, transcriptomic differences were obtained for leaf tissue responding to localized pathogen ingress, within the first 48 hpi. Undoubtedly, analyzing tissue distal to the infection site in a similar manner will shed more light on the PGIP resistance phenotype. This is of particular importance, concerning the differential involvement of a defense-related hormone which is known to be involved in long-range signaling (reviewed in Cheong and Do Choi, 2003).

The transcriptomic analyses utilized microarray slides from the closely related plant species *Solanum tuberosum*. The purpose of these analyses was to screen for genes or pathways involved in the decreased susceptibility of PGIP-accumulating lines. These analyses successfully identified differentially regulated genes and pathways in the transgenic lines tested, which were corroborated by Real-Time expression analyses or biochemical methods. This approach (cross-species hybridization) has also been successfully employed by Schmidt et al. (2005).

The work presented in this dissertation will be expanded to include grapevine plants overexpressing a *pgip* gene(s). The same strategy could be followed for analyzing the *in planta* role(s) of PGIP in this woody plant species, i.e. transcriptional and hormonal differences. Also, in such transgenic plants the tolerance towards the biotrophic pathogens of grape may be evaluated. This strategy (PGIP overexpression) could be particularly useful in grapevine, as resistance to mildew fungi has been reported to be achieved by priming plants with elicitors which activate cell wall processes, particularly the increased deposition of lignin (Hamiduzzaman et al., 2005).

Potential benefit of the study to the grapevine industry

The work presented in this dissertation sheds light on the mechanisms by which an antifungal protein from grapevine may exert its action. This protein has been convincingly shown to have antifungal benefits for the host plant when expressed at high levels in a transgenic background. Similarly, expression of a pear PGIP in grapevine has resulted in tolerance to a necrotrophic grape pathogen and reduced titer of a bacterium which is the causal agent of Pierce's disease.

Efforts to produce noble grape varieties expressing this protein to high levels, by means of biotechnology or traditional breeding should prove advantageous for the production of grapes with a reduced disease burden. Similarly, this work has pointed to the mechanisms by which this protein decreases fungal susceptibility. This may be advantageous when considering host-pathogen interactions and the evolution of pathogens to curb host defenses. It has been shown that grape PGIP not only inhibits fungal polygalacturonases, but also triggers defense responses in plants both before and following fungal attack. Plant susceptibility is therefore decreased in multiple ways. Mutation of the pathogen is therefore unlikely to result in such plants having increased susceptibility, as the case may be with chemical control agents. Furthermore, the generally less susceptible grapevine relatives may be screened for more potent inhibitors of fungal polygalacturonases and ultimately whether they activate plant defense responses to a greater extent.

Also, additional candidate genes for use in biotechnology or breeding programs for the reduced susceptibility to fungal pathogens have been identified.

6.2 References

Aguero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P., and Dandekar, A.M. 2005. Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Pathol. 6: 43-51.

Barr, D.B., Bravo, R., Weerasekera, G., Caltabiano, L.M., Whitehead, R.D., Olsson, A. O., Caudill, S.P., Schober, S.E., Pirkle, J.L., Sampson, E.J., Jackson, R.J., and Needham, L.L. 2004. Concentrations of dialkyl phosphate metabolites of organophosphorus pesticides in the U.S. population. Environ. Health Perspect. 112: 186-200.

Cervone, F. Hahn, M.G., De Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. 90: 542-548.

Cheong, J-J., and Do Choi, Y. 2003. Methyl jasmonate as a vital substance in plants. Trends Genet. 19: 409-413.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. 2003. *Arabidopsis* polygalacturonase-inhibiting proteins (PGIP) are regulated by different signal transduction pathways during fungal infection. Plant Cell 15: 93-106.

Gerhardson, B. 2002. Biological substitutes for pesticides. Trends Biotechnol. 20: 338-343.

Hamiduzzaman, M.M., Jakab, G., Barnavon, L., Neuhaus, J.M., and Mauch-Mani, B. 2005. beta-Aminobutyric acid-induced resistance against downy mildew in grapevine acts through the potentiation of callose formation and jasmonic acid signaling. Mol. Plant Microbe Interact. 18: 819-829.

Joubert, D.A., Slaughter, A.R., Kemp, G., Becker, J.V.W., Krooshof, G.H., Bergmann, C., Benen, J., Pretorius, I.S., and Vivier, M.A. 2006. The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. DOI 10.1007/s11248-006-9019-1.

Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and van Kan, J.A.L. 2007. A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *B. cinerea* in *N. benthamiana* leaves without any evidence for *in vitro* interaction. Mol. Plant Microbe Interact. (in print).

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and van Kan, J.A. 2005. Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. 43: 213-225.

Manfredini, C., Sicilia, F., Ferrari, S., Pontiggia, D., Salvi, G., Caprari, C., Lorito, M., and De Lorenzo, G. 2005. Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol. Mol. Plant Pathol. 67: 108-115.

Powell, A.L., van Kan, J.A., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M. 2000. Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant Microbe Interact. 13: 942-950.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M.J., Kift, N., Carbonne, F., Griffiths, G., Esquerré-Tugayé, M-T., Rosahl, S., Castresana, C., Hamberg, M., and Fournier, J. 2005. Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol. 139: 1902-1913.

Schmidt, D.D., Voelckel, C., Hartl, M., Schmidt, S., and Baldwin, I.T. 2005. Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. Plant Physiol. 138: 1763-1773.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant Microbe Interact. 11: 1009-1016.

Thomma, B.P., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA 95: 15107-15111.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. 1999. Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. 121: 1093-1102.