Molecular and phenotypic characterisation of grapevines expressing non-vinifera PGIP encoding genes

by

Mukani Moyo

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science

at
Stellenbosch University

Institute of Wine Biotechnology, Faculty of AgriSciences

Supervisor: Prof MA Vivier

March 2011

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 04/01/2011

Summary

Plants are constantly exposed to biotic and abiotic stress inducing factors that threaten their existence. Biotic factors such as pathogens are the cause of huge yield losses to crop plants worldwide with fungal pathogens debatably constituting the worst damage. Fungal pathogens such as *Botrytis cinerea*, which has a wide host range, release cell wall degrading enzymes called endopolygalacturonases (ePGs) during plant infection. These ePGs break down the pectin component of the cell wall, thus providing an entry route, as well as nutrients for the fungus.

Plants have evolved mechanisms to counteract and suppress the action of the ePGs. This is achieved through the action of cell wall associated proteins called polygalacturonase-inhibiting proteins, PGIPs. PGIPs directly inhibit ePGs and their inhibitory action also prolongs the existence of longer chain oligogalacturonide residues which are believed to elicit a cascade of defence responses. In grapevine, a PGIP encoding gene, *VvPGIP1*, was previously isolated and characterised. VvPGIP1, as well as nine non-vinifera grapevine PGIPs have been expressed in tobacco and shown to be potent antifungal proteins that caused the transgenic tobacco to have strong resistance phenotypes against *Botrytis* in whole plant infection assays. Following on the tobacco study, two of the non-vinifera *PGIPs* were expressed in cultivars of the susceptible *Vitis vinifera*. Characterisation of the putative transgenic population showed that transgene integration was successful, the transgenes were being expressed and there were at least 29 transgenic lines with independent integration events. The transgenic lines were confirmed to have active PGIPs (transgene-derived) in their leaves. Crude protein extracts from 22 lines exhibited 100% inhibition against crude *B. cinerea* PGs (BcPGs).

The plant lines with positive transgene integration, expression, independent integration events and exhibiting 100% transgene-derived PGIP activity were further selected for whole plant and detached leaf antifungal assays where they were challenged with *B. cinerea*. The whole plant infection assay showed that expression of the non-vinifera PGIPs in *V. vinifera* promotes susceptibility to *B. cinerea*, not resistance. This surprising result could perhaps be explained by a quicker and stronger recognition between the pathogen and the host and the stronger activation of defence responses in the host. A more active hypersensitive response in the host would benefit *Botrytis* being a necrotroph. The type of lesions and the onset and speed of lesion development observed on the transgenics lines versus the wild type support this possibility. Knowledge gaps with regards to the efficiency of the ePG inhibition by the non-vinifera PGIPs during infection of grapevine tissue; the potential changes that might be caused by expressing PGIPs in a grapevine host with a native PGIP with high homology to the transgenes (including potential gene silencing) and the potential impact on defence signalling and defence responses all provides further avenues of study to elucidate this very interesting phenotype further. Overall, this study provides a comprehensively characterised population of transgenic plants that provides useful resources for *in vivo* analysis of PGIP function in defence, where the host plant harbours a native copy of the PGIP encoding gene.

Opsomming

Plante word voortdurend blootgestel aan biotiese en abiotiese faktore, wat stres veroorsaak en hul bestaan bedreig. Biotiese faktore, soos patogene, veroorsaak groot verliese in wêreldwye gewasopbrengste, met swampatogene wat moontlik die grootste skade veroorsaak. Swampatogene, soos *Botrytis cinerea,* wat ‘n wye reeks gasheerplante kan infekteer, stel selwand-afbrekende ensieme tydens plantinfeksie vry, wat as endo-poligalakturonases (ePG’s). bekend staan. Hierdie ePG’s breek die pektienkomponent van die selwand af, wat gevolglik as ‘n ingangspunt dien,asook voedingstowwe vir die swam verskaf .

Plante het meganismes ontwikkel om die aktiwiteit van hierdie ePG’s te bekamp en te onderdruk. Die aktiwiteit van die selwand-geassosieërde proteïene, genaamd poligalakturonase-inhiberende proteïene (PGIP’s), speel hier ‘n rol. PGIP’s inhibeer ePG’s direk en hul inhiberende aktiwiteit verleng ook die bestaan van langketting oligogalakturoniedresidu’s, wat blykbaar ‘n kaskade van weerstandsreaksies kan inisieer. ‘n PGIP-koderende geen, *VvPGIP1*, is voorheen uit wingerd geïsoleer en gekarakteriseer. VvPGIP1, asook nege nie-vinifera wingerd-PGIP’s is voorheen in tabak uitgedruk en bevestig as proteïene met sterk anti-swamaktiwiteit, soos bevestig deur die bevinding dat die transgeniese tabak ‘n weerstandsfenotipe teen *Botrytis* in heelplant-infeksietoetse het. Ná die tabakstudie is twee van die nie-vinifera PGIP’s uitgedruk in vatbare *V. vinifera*-kultivars. Karakterisering van die vermeende transgeniese bevolking het getoon dat die transgeen-integrasie suksesvol was, dat die transgeen uitgedruk word en dat daar ten minste 29 transgeniese lyne met onafhanklike integrasie gebeurtenisse geskep is. Daar is verder bevestig dat die transgeniese lyne aktiewe PGIP’s (transgeen-afkomstig) in hul blare het. Ongesuiwerde proteïenekstrakte van 22 lyne het 100% inhibisie teen ‘n mengsel van ongesuiwerde *B. cinerea* PGs (BcPGs) getoon.

Die plantlyne met positiewe transgeenintegrasie en -uitdrukking, asook onafhanklike integrasiegebeure en wat 100% transgeen-afkomstige PGIP-aktiwiteit getoon het, is verder aan heel-plant en verwyderde blaarswaminfeksies met *B cinerea* onderwerp. Die heelplantinfeksietoetse het getoon dat uitdrukking van nie-vinifera PGIP’s in *V. vinifera* ‘n toename, in plaas van ‘n afname, in vatbaarheid teen *B. cinerea* veroorsaak. Hierdie verbasende resultaat kan moontlik toegeskryf word aan ‘n vinniger en sterker herkenningsreaksie tussen patogeen en gasheer en die moontlike sterker stimulering van weerstandsreaksies in die gasheer. ‘n Meer aktiewe hipersensitiewe reaksie in die gasheer sal tot die voordeel van *Botrytis*, wat ‘n nektrotroof is, wees. Die tipe letsel, asook die aanvang en spoed van letselontwikkeling wat waargeneem is in transgeniese lyne teenoor die wilde-tipe ondersteun hierdie moontlikheid. Gapings in kennis ten opsigte van die doeltreffendheid van die ePG-inhibisie deur die nievinifera PGIP’s tydens infeksie van wingerdweefsel, die moontlike veranderinge (insluitend ‘n moontlike geenuitdowingseffek) wat veroorsaak kan word deur die uitdrukking van PGIP-gene in ‘n kultivar met ‘n inheemse en baie homoloë PGIP-geen, kon ‘n invloed op weerstandseine en weerstandsreaksies gehad het. Hierdie aspekte lewer verdere studiemoontlikhede om hierdie interessante fenotipe verder te verklaar.Algeheel lewer hierdie studie ‘n breedvoerig-gekarakteriseerde bevolking trangeniese plante, wat dien as nuttige hulpbronne vir *in vivo*-analise van PGIP se funksie in siekteweerstandbiedendheid, veral waar die gasheerplant ‘n inheemse kopie van die PGIP-koderende geen huisves.

This thesis is dedicated to

My parents

Biographical sketch

Mukani Moyo was born in Plumtree, Zimbabwe on the 28th of May 1982 and completed her Advanced Level studies at Founders High School in 2000. She enrolled at Midlands State University, Zimbabwe in 2001 and obtained a BSc Honours Degree in Biological Sciences, majoring in Molecular Genetics and Microbiology, in 2005. She worked as a teacher, lab technologist and research associate for different companies before enrolling at Stellenbosch University for an MSc in Wine Biotechnology.

Acknowledgements

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

- **Prof MA Vivier** for acting as supervisor, her guidance and advice throughout my studies
- **Dr Krishnan Vasanth** for performing the grapevine transformations
- **Dr JP Moore** for all the critical discussions
- **Charmaine Stander, Dr Philip Young, Dr Abre de Beer** and **Justin Lashbrooke** for all the technical guidance with the different parts of my project
- **Dan Jacobson** for the statistical analysis of my data
- **Lab colleagues** for all the discussions and ideas
- **My family** and **friends** for the unwavering support and encouragement
- **The National Research Foundation**, **Winetech**, **South African Table Grape Industry** and the **Institute for Wine Biotechnology** for financial support

Preface

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately. Chapter 3 forms part of the research that will be submitted to **Transgenic Research.**

Chapter 1	**General Introduction and Project Aims**
Chapter 2	**Literature review** PGIPs in plant defence
Chapter 3	**Research results** Expressing PGIP encoding genes from non-vinifera grapevine species in *V. vinifera* promotes susceptibility, not resistance, against *B. cinerea*
	General discussion and conclusions

I hereby declare that I was the primary contributor with respect to the experimental data presented on the the multi-author manuscript presented in Chapter 3. My supervisor, Prof. MA Vivier was involved in the conceptual development and continuous critical evaluation of the study. Dr. K. Vasanth transformed the grapevine lines that were used in the study.

Contents

Chapter 1

General introduction and project aims

1. General introduction and project aims

1.1 Introduction

Immobile organisms face the challenge of adapting to their fixed environment. Most plants are anchored in a single position by their roots and in order to endure the adverse environmental conditions that pose a challenge to their survival, they have evolved different mechanisms to survive in their habitats.

Grapevine is a perennial fruit crop of great economic importance (Thach *et al*., 2008). Biotic and abiotic stresses however affect the growth and productivity of grapevines. Pathogens such as nematodes, protozoa, bacteria, viroids, viruses, parasitic plants and fungi that attack grapevines are responsible for reduction in yields. Fungal pathogens, such as *Botrytis cinerea* which causes grey mould rot of a wide range of plant species, arguably constitute the greatest potential risk to harvested crops (Commenil *et al*., 1995; Ferreira *et al*., 2004; Kars *et al*., 2005; Egan *et al*., 2008). *Botrytis* releases numerous metabolites and enzymes such as cell wall degrading enzymes called endopolygalacturonases (ePGs) during plant host attack which macerate the pectin component of the cell wall, thus providing the fungi with an entry route and a source of nutrients for growth and proliferation (Lang *et al*., 2000; Kars, 2007; Cantu *et al*., 2008). The action of this necrotrophic phytopathogenic fungus kills plant tissue and then macerates it (ten Have *et al*., 1998).

Plants have evolved mechanisms to counteract and suppress the activity of ePGs through the action of cell wall associated proteins called polygalacturonase-inhibiting proteins (PGIPs) (Cervone *et al*., 1989; Favaron *et al*., 1997; De Lorenzo *et al*., 2002; Howell *et al*., 2005; Juge, 2006).

1.2 Polygalacturonase-Inhibiting Proteins and Plant Defence

A variety of PGIPs have been characterised from monocotyledonous and dicotyledonous plant species (Janni *et al*., 2006) and they form part of the leucine-rich repeat (LRR) protein family. The LRR motif is a highly conserved region between genes and plays a pivotal role in the recognition of molecules, such as ePGs, derived from pathogens (Mattei *et al*., 2001). PGIPs directly inhibit the action of ePGs through the formation of a complex with the fungal enzymes (Federici *et al*., 2001; Di Matteo *et al*., 2003; 2006). *In vitro* experiments have shown that the inhibition of ePGs by PGIPs also results in the accumulation of long chain pectin fragments called oligogalacturonides which act as elicitors of plant defence responses, such as the accumulation of defence gene transcripts involved in phytoalexin synthesis (Cervone *et al*., 1989; Desiderio *et al*., 1997; Aziz *et al*., 2004; Becker, 2007). PGIPs and ePGs are well studied and the availability of information on structural models, sequence variation and mutated proteins have shown that the molecular struggles between the enzymes and their inhibitors lead to some of the residues at the contact surfaces being under positive selection. Single changes in

these residues could change the ePGs-PGIPs inhibition interaction (Misas-Villamil and van der Hoorn, 2008).

Research on the involvement of PGIPs in plant defence against ePGs has shown that overexpression of *PGIP* genes in tobacco (Oelofse et al., 2006; Joubert *et al*., 2006; Joubert *et al*., 2007; Venter, 2010), pear (Sharrock *et al*., 1994; Faize *et al*., 2003), *Arabidopsis thaliana* (Ferrari *et al*., 2003; Manfredini *et al*., 2005), wheat (Janni *et al*; 2008), leek (Favaron *et al*., 1997), cabbage (Hwang *et al*., 2010), bean and tomato (Powell *et al*., 2000; Stotz *et al*., 2000) and grapevine (Aguero *et al*., 2005) results in reduced fungal susceptibility of the respective host plant species.

1.3 Polygalacturonase-Inhibiting Proteins and Grapevine

Most *Vitis vinifera* cultivars are susceptible to a wide range of fungal diseases, whereas certain non-vinifera and American grape species have been shown to be less susceptible to fungal attacks (Doster *et al*., 1985; Dai *et al*., 1995). Analysis of the *V. vinifera* genome showed that grapevine does not possess a multigene PGIP family and only contains a single gene encoding VvPGIP1. Expression of *VvPGIP1* in grapevine has been shown to be berry-specific and developmentally regulated. Low level expression is detected in the early stages of berry development that reaches a maximum at and just after véraison (the onset of ripening); whereafter expression levels diminish again towards the fully ripe stage. Induction experiments have shown that several factors such as wounding, oxidative stress, infection and the presence of elicitors overcome the tissue-specific expression pattern leading to strong and constitutive expression in all tissues tested (Joubert, 2004).

Our laboratory has previously isolated and characterised several grapevine PGIPs: *VvPGIP1* from *V. vinifera* (De Ascensao, 2001; Joubert *et al*., 2006; Joubert *et al*, 2007); as well as 37 additional grapevine *PGIPs* from wild and American-hybrid vines (Wentzel, 2005; Venter, 2010). These genes; the methods we established to study PGIP-ePG interaction *in vitro* and *in vivo*; a defined pathosystem for whole-plant infection assay of tobacco infected by *B. cinerea*; as well as our ability to genetically transform grapevine cultivars, form the resource-base for this work where we aimed to functionally characterise two non-vinifera grapevine PGIP encoding genes through expression analysis in commercial grapevine cultivars.

1.4 Rationale and scope of the study

The low susceptibility of non-vinifera and American grapevine species to fungal attack compared to their *V. vinifera* counterparts (Doster *et al*., 1985) has sparked interest in their defence pathways. Their resistance traits have been targeted in numerous breeding programmes where the aims were either to introduce useful traits into *V. vinifera* for table and wine grape production; or in rootstock breeding programmes where resistance to pathogens, pests and/or abiotic factors was the objective. These grapevine genotypes are seen as important genetic resources and increasing focus is placed on profiling the natural variation available in the wild vines and range of grapevine accessions for specific traits.

Since we have isolated and functionally characterised the *VvPGIP1* and confirmed it to be a potent antifungal gene in overexpression studies in tobacco, we used the high sequence homology between *PGIP* genes in general to isolate 37 additional grapevine *PGIP* genes from non-vinifera accessions (Wentzel *et al*., 2005) and test them for their resistance phenotypes in transgenic tobacco (Venter, 2010). All of these studies have confirmed that the non-vinifera PGIPs are even more efficient than *VvPGIP1* to protect the transgenic tobacco against *Botrytis* infection (Venter, 2010).

In this study, two of the non-vinifera PGIP encoding genes were selected for expression in *V. vinifera* cultivars. PGIP1012 and PGIP1038, isolated from *V. doaniana* Munson and *V. caribaea*, respectively, were shown to reduce lesion diameter by 33-60% against *B. cinerea* in transgenic tobacco compared to the wildtype (Venter, 2010). Overexpression constructs were mobilised into *Agrobacterium,* utilising the constitutive Cauliflower mosaic virus (CaMV) 35S promoter, to transform *V. vinifera* cultivars. Putative transgenic populations were regenerated and subjected to systematic analyses to confirm the transgenic status of the population and determine the potential PGIP-specific phenotypes displayed by the overexpressing lines.

This study should provide fully characterised transgenic grapevine lines with potentially useful and interesting phenotypes, as well as valuable information about the effectiveness of the non-vinifera PGIPs as defence genes when present at high levels in the susceptible *V. vinifera* species. This study would contribute to our understanding of the functional role of grapevine PGIPs within a grapevine host – all other studies on grapevine PGIPs thus far have used tobacco as a model system, or *in vitro* studies, to evaluate the activity and characteristics of grapevine PGIPs. The expression, if successful, will cause constantly high levels of *PGIP* expression (of the transgenes) throughout the plant body, whereas the endogenous *VvPGIP1* gene is normally only expressed during specific stages of berry development (unless induced). These transgenic vines, with a combination of the PGIPs produced from the transgenes and the endogenous *VvPGIP1* will be subjected to infection assays to evaluate the impact on the disease resistance potential in the susceptible *V. vinifera* species. The importance of this work lies partly in the fact that it represents only the second report of grapevine being engineered to overexpress PGIP. Previously, Aguero *et al.* (2005) overexpressed a pear PGIP in grapevine and confirmed it to be active in crude extracts from leaves, stems and roots against *B. cinerea*. The resulting transgenic lines were also found to be less susceptible to *B. cinerea* in a detached leaf infection assay compared to untransformed lines.

Main objective

The main aim of the study is the expression of two non-vinifera PGIP-encoding genes in *V. vinifera* cultivars and systematic genetic and phenotypic characterisation of the transgenic populations, with a specific focus on potential resistance phenotypes against *B. cinerea*.

Specific objectives and approaches of the study

1. To regenerate putative transgenic populations of *V. vinifera* cultivars expressing two non-vinifera PGIP-encoding genes.
2. To clonally multiply the putative transgenic lines and establish primary *in vitro* cultures as well as working collections of plantlets for *in vitro* and *ex vitro* experiments.
3. To genetically characterise the putative transgenic lines to identify independently transformed lines with confirmed transgene presence (PCR-analysis), transgene expression (northern blot analysis) as well as known integration patterns (Southern blot analysis).
4. To analyse the confirmed transgenic lines, in comparison with the untransformed controls, for PGIP activity against ePGs from *Botrytis*.
5. To perform infection analyses of the confirmed transgenic lines, in comparison with the untransformed controls, with *B. cinerea* to evaluate the defence phenotypes linked to the expression of the non-vinifera *PGIP*s.

1.5 References

Aguero C.B., Uratsu S.L., Greve C., Powell A.L.T., Labavitch J.M., Meredith C.P., 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.

Aziz A., Heyraud A., Lambert B. 2004. Oligogalacturonide signal transduction, induction of defence-related responses and protection of grapevine against *Botrytis cinerea*. Planta 218:767–774.

Becker J.W. 2007. Evaluation of the roles of PGIPs in plant defence responses. PhD dissertation. Stellenbosch University, Stellenbosch, Republic of South Africa.

Cantu D., Vicente A.R., Labavitch J.M., Bennet A.B., Powell A.L.T. 2008. Strangers in the matrix: plant cell walls and pathogen susceptibility. Trends Plant Sci. 13:610-617.

Cervone F., Hahn M.G., De Lorenzo G., Darvill A. 1989. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defence responses. Plant Physiol. 90:542-548.

Comménil P., Belingheri L., Sancholle M., Dehorter B. 1995. Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. Lipids 30:351-356.

Dai G.H., Andary C., Mondolot-Cosson L., Boubals D. 1995. Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera*, *V. rupestris* and *V. rotundifolia* and the downey mildew fungus *Plasmorara viticola*. Physiol. Mol. Plant Pathol. 46:177-188.

De Ascensao A. 2001. Isolation and characterisation of a polygalacturonase-inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

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

Desiderio A., Aracri B., Leckie F., Mattei B., Salvi G., Tigerlaar H., van Roekel J., Baulcombe D., Melchers L., De Lorenzo G., Cervone F. 1997. Polygalacturonase inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol. Plant-Microbe Interact. 10:852-860.

Di Matteo A., Federici l., Mattei B., Salvi G., Johnson K., Savino C., De Lorenzo G., Tsernoglou D., Cervone F. 2003. The crystal structure of polygalacturonase inhibiting protein (PGIP), a leucine-rich repeat protein involved in plant defence. PNAS 100:10124-10128.

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

Doster M.A., Schnathorst W.C. 1985. Comparative susceptibility of various grapevine cultivars to the powdery mildew fungus *Uncinula necator*. Am. J. Enol. Vitic. 36:101-104.

Egan M.J., Talbot N.J. 2008. Genomes, free radicals and plant cell invasion: recent developments in plant pathogenic fungi. Curr. Opin. Plant Biol. 11:367–372.

Faize M., Sugiyama T., Faize L., Ishii H. 2003. Polygalacturonase-inhibiting protein (PGIP) from Japanese pear: possible involvement in resistance against scab. Physiol. Mol. Plant Pathol. 63:319–327.

Favaron F., Castiglioni C., D'Ovidio C., Alghisi P. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. Physiol. Mol. Plant Pathol. 50:403-417.

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 proteins). Proc. Natl. Acad. Sci. USA 98:12425-13430.

Ferrari S., Vairo D., Ausubel F.M., Cervone F., De Lorenzo G. 2006. Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins (PGIP) are regulated co-ordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15:93-106.

Ferreira R. B., Monteiro S.S., Picarra-Perreira M.A., Teixeira A.R. 2004. Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability. Trends Biotechnol. 22:168-173.

Howell J.T., Davis M.R. 2005. Plant defence mechanisms against fungal pathogens: polygalacturonase inhibitor proteins. Can. J. Plant Pathol. 27: 5–15.

Hwang B.H., Bae H., Lim H.S., Kim K.B., Kim S.J., Im M.H., Park B.S., Kim D.S., Kim J. 2010. Overexpression of polygalacturonase-inhibiting protein 2 (*PGIP2*) of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) increased resistance to the bacterial pathogen *Pectobacterium carotovorum* ssp. *carotovorum*. Plant Cell Tiss. Org. Cult. 103:293–305.

Janni M., Di Giovanni M., Roberti S., Capodicasa C., D'Ovidio R. 2006. Characterisation of expresses *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. 113:1233–1245.

Joubert D.A. 2004. Regulation of the *Vitis vinifera pgip1* gene encoding a polygalacturonase inhibiting protein. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Joubert D.A., Slaughter A.R., Kemp G., Becker J.V.W., Krooshoof G.H., Bergmann C., Benen J., Pretorius I.S., Vivier M.A. 2006. The grapevine polygalacturonase inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgen. Res. 15:687-702.

Joubert D. A., Kars I., Wagemakers L., Bergmann C., Kemp G., Vivier M. A., Kan J. A. L. 2007. A Polygalacturonase-Inhibiting Protein from Grapevine Reduces the Symptoms of the Endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* Leaves Without Any Evidence for In Vitro Interaction. MPMI. 4:392–402.

Juge N. 2006. Plant protein inhibitors of cell wall degrading enzymes. Trends Plant Sci. 11:359-367.

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

Kars I. 2007. The role of pectin degradation in pathogenesis of Botrytis cinerea. PhD Thesis, Wageningen University, Wageningen, NL.

Lang C., Dornenburg H. 2000. Perspectives in the biological function and the technological application of polygalacturonases. Appl. Microbiol. Biotechnol. 53: 366-375.

Manfredini C., Sicilia F., Ferrari S., Pontiggia D., Salvi G., Caprari C., Lorito M., 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.

Mattei B., Bernalda M.S., Federici L., Roepstorff P., Cervone F., Boffi A. 2001. Secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (Polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. Biochem. 40:569-576.

Misas-Villamil J.C., van der Hoorn R.A.L. 2008. Enzyme-inhibitor interactions at the plant-pathogen interface. Curr. Opin. Plant Biol. 11:380-388.

Oelofse D., Dubery I. A., Meyer R., Arendse M. S., Gazendam I., Berger D. K. 2006. Apple polygalacturonase inhibiting protein1 expressed in transgenic tobacco inhibits polygalacturonases from fungal pathogens of apple and the anthracnose pathogen of lupins. Phytochemistry 67:255–263.

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

Sharrock K. R., and Labavitch J. M. 1994. Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacturonase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. Physiol. Mol. Plant Pathol. 45:305-319.

Stotz H. U., Bishop J. G., Bergmann C. W., Koch M., Albersheimp P., Darvill A G., 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.

Taylor R., Secor G. 1988. An improved diffusion assay for quantifying the polygalacturonase content of *Erwinia* culture filtrates. Phytopathology 78.

Thach L., Matz T. 2008. Wine: A Global Business. 2nd Edition. Miranda Press, Elmsford, New York.

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

Venter A. 2010. The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco. MSc thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Wentzel L. 2005. The endopolygalacturonases from *Botrytis cinerea* and their interaction with an inhibitor from grapevine. MSc Thesis, Stellenbosch University, Stellenbosch, Republic of South Africa.

Chapter 2

Literature review

PGIPs in plant defence

2. Literature review: PGIP in plant defence

2.1 Introduction

Grapevine is the world's most economically important fruit crop. Grapes and grape products such as wine, dried fruit and juice are the major export commodities in South Africa (Thach *et al*., 2008) as well as many other countries producing grapes. In 2006, South Africa was ranked the 7th largest wine producer in the world, producing an average of 3% of the world's wine. The first wine was made in the Cape in 1659 and the South African wine industry recently celebrated its 350 year anniversary in 2009. According to Wines of South Africa (WOSA), about 740 million gross litres of wine were produced in 2009 and over 400 million litres exported. Since 2003, the wine industry has been contributing at least 10% per annum to the Gross Domestic Product (GDP) of the country. Internationally, grapevine productivity is however hampered by biotic and abiotic stress-inducing factors annually (Howell, 2001). The recent shift in South Africa's climatic conditions has raised concerns on the impact that the changes in mean annual rainfall and temperatures will have on flowering and fruiting seasons, pests and disease distribution in vineyards (Mason *et al*., 1999).

Grapevine is a woody perennial plant which is susceptible to a wide variety of biotic and abiotic stresses. Huge yield losses in grapevines worldwide have been attributed partly to pathogens such as nematodes, bacteria, viruses, parasitic plants and fungi (Ferreira *et al*., 2004). Fungal pathogens cause diseases that not only result in yield loss, but also affect wine quality negatively (Egan *et al*., 2008). These fungal diseases include powdery mildew caused by *Uncinula necator* (Pearson *et al*., 1987; Gardoury *et al*., 1988; Gardoury *et al*., 2001; Rugner *et al*., 2002), eutypa dieback caused by *Eutypa lata* (Mauro *et al*., 1988; Tey-Rulh *et a*l., 1991; Molyneux *et al*., 2002; Mahoney *et al*., 2005; Camps *et al*., 2010), downey mildew caused by *Plasmopara viticola* (Dai *et al*., 1995; Gindro *et al*., 2003 Gobbin *et al*., 2005), anthracnose caused by *Elsinoe ampelina* (Magarey *et al*., 1993; Jayasankar *et al*., 2000; Yun *et al*., 2007) and grey mould rot caused by *Botrytis cinerea* (Elad *et al*., 1997; Derckel *et al*., 1999; Keller *et al*., 2003; Choquer *et al*., 2007; Williamson *et al*., 2007).

B. cinerea is a widely studied pathogenic fungus due to its broad host range. It is a necrotroph that causes tissue necrosis in its host plants (Kars *et al*., 2005). It produces numerous metabolites and enzymes such as cutinases, lipases and some cell wall degrading enzymes. These enable it to penetrate the host plant tissue (van Kan, 2005; van Kan, 2006). Triacylglycerol lipase is one of the enzymes released by *B. cinerea,* which is believed to facilitate the penetration of the wax and cuticle layer in grape berries (Commenil *et al*., 1995). After penetrating the wax and cuticle layer, the fungus is faced with the challenge of penetrating the plant's cell wall (Sarkar *et al*., 2009). This is achieved through the action of cell wall degrading enzymes called endopolygalacturonases (ePGs) which macerate the homogalacturonan component of the pectic part of the primary cell wall (Alghisi *et al*., 1995; Esquerre-Tugaye *et al*., 2000; Kars *et al*., 2004). The primary cell wall is principally made up of

cellulose, hemicellulose and pectin. The pectin network of the cell wall is composed of rhamnogalacturonans I and II and homogalacturonan, also known as polygalacturonic acid (PGA) (Perez *et al*., 2000; Ridley *et al*., 2001). The ePGs break down the pectin by depolymerisation of the homogalacturonan domain thus providing the fungus with an entry route and a source of nutrients for proliferation (Kars, 2007; Cantu *et al*., 2008). *Botrytis cinerea* possesses at least 6 isoforms of ePGs termed BcPG1, BcPG2, BcPG3, BcPG4, BcPG5 and BcPG6. Their deduced amino acid sequences vary with BcPG1 and BcPG5 being 73% identical whilst BcPG2 and BcPG3 only share 35% identity (Wubben *et al*., 1999). They also exhibit different substrate specificities as illustrated by experiments conducted on broad bean, *Arabidopsis thaliana* and tomato leaves (**Figure 1).** BcPG1 and BcPG2 showed high necrotising activity in broad bean leaves (**Figure 1a**) whilst *A. thaliana* leaves infiltrated with BcPG3, exhibited higher tissue necrosis compared to tomato leaves which showed more pronounced lesions when infiltrated with BcPG2 (**Figure 1b and 1c**) (Kars *et al*. 2005).

Plants have evolved mechanisms to counteract or suppress the damaging effects of the ePGs (Stahl *et al*., 2000; Juge, 2006). One way of achieving this is through the action of cell wall associated proteins called polygalacturonase-inhibiting proteins (PGIPs) (Gomathi *et al*., 2004; Howell *et al*., 2005). PGIPs are members of a multi-gene family and have been shown to inhibit the action of ePGs and thus reduce damage to the plant during fungal invasion (Cervone *et al*., 1990; Bergmann *et al*., 1994; Favaron *et al*., 1997; Esquerre-Tugaye *et al*., 2000; Powell *et al*., 2000; Stotz *et al*., 2000; De Lorenzo *et al*., 2001; D'Ovidio *et al*., 2004; De Lorenzo *et al*., 2002; Faize *et al*., 2003; Kemp *et al*., 2004; Aguero *et al*., 2005; Di Matteo *et al*., 2006; Federici *et al*., 2006; Oelofse *et al*., 2006; Joubert *et al*., 2006: 2007; Misas-Villamil *et al*., 2008).

In vitro experiments have shown that inhibition of ePGs by PGIP during fungal infection prolongs the existence of pectic fragments called oligogalacturonides. These molecules are believed to act as endogenous elicitors of plant defence (Aziz *et al*., 2004; Cervone *et al*. 1989; Desiderio *et al*., 1997). Oligogalacturonides have been shown *in vitro* to elicit a cascade of defence responses such as activation of protein kinase, accumulation of defence gene transcripts involved in processes such as phytoalexin synthesis, and activation of pathways involved in active oxygen species production (Esquerre-Tugaye *et al*., 2000; Poinssot *et al*., 2003; Aziz *et al*., 2004; Vorwerk *et al*., 2004; Vlot *et al*., 2008; Bolton, 2009; Hematy *et al*., 2009).

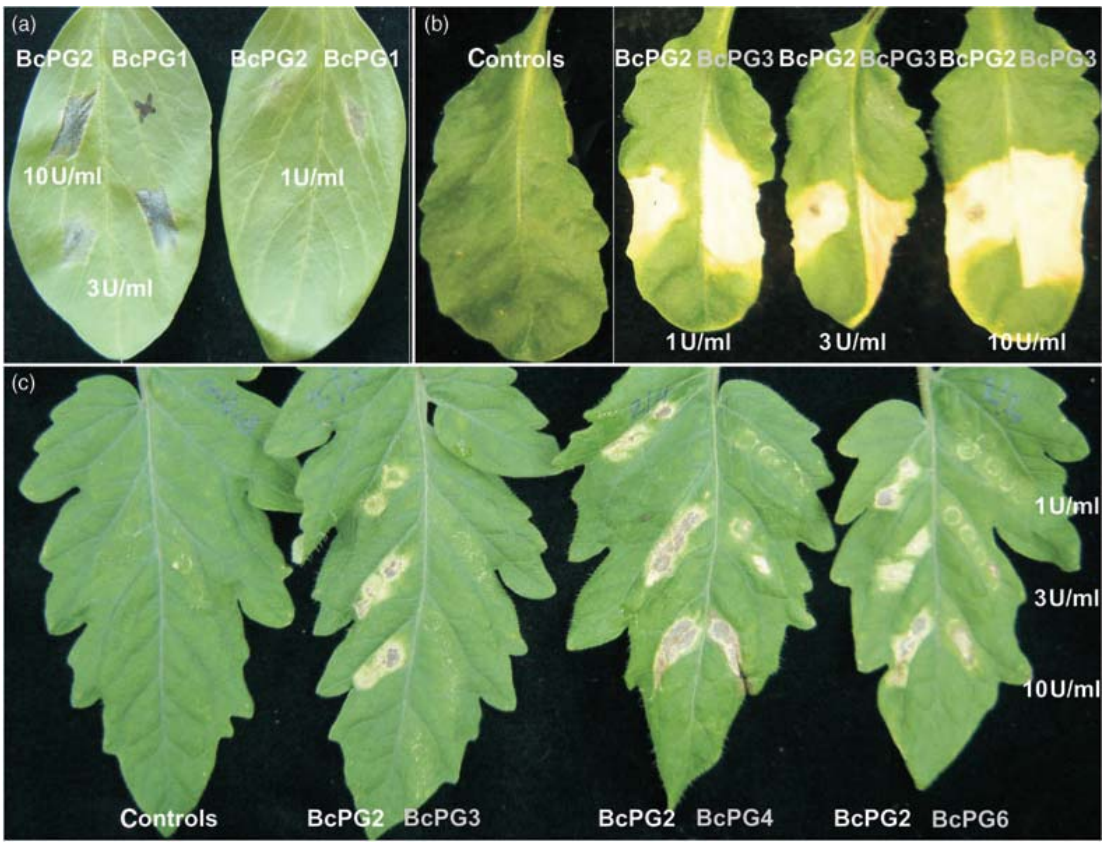


Figure 1. Necrotic symptoms on broad bean, *Arabidopsis thaliana* and tomato leaves infiltrated with *Botrytis cinerea* ePGs in a range of 1, 3 and 10U per ml as shown above where U represents enzyme activity determined with PGA as substrate before and after infiltration (**a**) BcPG1 and BcPG2 infiltrated broad bean leaves. (**b**) Control, BcPG2 and BcPG3 infiltrated *A. thaliana* leaves. (**c**) BcPG2, BcPG3, BcPG4 and BcPG6 infiltrated tomato leaves. Adopted from Kars *et al*. (2005).

A few studies have shown that PGIP is also involved in important plant processes other than disease response. These include the determination of seed protrusion during seed germination and regulation of cell wall function and architecture (Xu *et al*., 2008; Kanai *et al*., 2010). An additional role for PGIP was suggested by Becker in 2007. In a study conducted on transgenic tobacco plants overexpressing *PGIP*, an increase in lignin deposition was observed in the absence of any fungal infection (Becker, 2007). Transcriptomic and biochemical methods were used for this analysis and the increase in lignin deposition was observed in leaf and stem tissue. These findings coupled with the increase in indole-acetic acid levels observed during phytohormone profiling, led to the suggestion of a new possible role for PGIP in promoting cell wall strengthening in anticipation of infection (Becker, 2007; Alexandersson *et al*., 2010 pers. comm).

PGIP overexpression studies in numerous plant hosts such as tobacco, bean, grapevine, cabbage and tomato have resulted in reduced fungal susceptibility of the respective host plant species (Sharrock *et al*., 1994; Powell *et al*., 2000; Stotz *et al*., 2000; Faize *et al*., 2003; Aguero *et al*., 2005; Oelofse *et al*., 2005; Joubert *et al*., 2006; 2007; Hwang *et al*., 2010). The role and mechanism of PGIP in inhibiting fungal ePGs forms the main focus of this review. Structural requirements of PGIP and ePGs during enzyme-inhibitor interactions commence this review, with regulation of defence responses and PGIP overexpression studies that have elucidated the role of PGIP in plant defence also

being discussed. This review is concluded by focusing on grapevine-derived PGIP encoding genes with particular interest on non-vinifera and American grapevine species.

2.2 PGIP and ePG structures

2.2.1 PGIP: Inhibitors of fungal ePGs

Numerous PGIPs from monocotyledonous and dicotyledonous plant species have been isolated and genetically characterised. They have been shown to typically occur in complex multigene families with the members differing in substrate specificity (Frediani *et al*., 1993; Desiderio *et al*., 1997).

PGIPs are soluble glycoproteins in nature, with a molecular weight of about 40 kDa. They are part of the leucine-rich repeat (LRR) protein family which are characterised by the tandem repeat sequence xxLxLxxNxLt/sGxlPxxLxxLxxL, where L can be occupied by phenyalanine, valine and isoleucine and x can be any amino acid (Mattei *et al*., 2001). About 15% of the amino acids within the PGIP molecule consists of leucine. The LRR motif is known to be involved primarily in protein-protein interaction (Kobe and Kajava, 2001; Xu *et al*., 2009) and is flanked by 2 cysteine-rich domains (Protsenko *et al*., 2008). The first plant-specific LRR protein to be crystallised was isoform 2 of PGIP from *Phaseolus vulgaris* (PvPGIP2) (**Figure 2**), determined at 1.7-Å resolution. Single isomorphous replacement and anomalous scattering methods were used for the overall structural determination (Di Matteo *et al*., 2003).

PvPGIP2 has an elongated, curved shape with a typical (LRR protein) right-handed, superhelical fold (residues 53-289). It displays a more twisted scaffold in comparison to other LRR proteins, however. A total of 10 tandem repeats, each consisting of 24 residues, characterises the central LRR domain (Di Matteo *et al*., 2006). The residues responsible for specificity and affinity of PGIP2 are located in the B1 β-sheet, which is known to be conserved in all LRR protein structures. The B2 β-sheet, found in *P. vulgaris* PGIP is absent in many other LRR proteins and is critical for the superhelical fold of PGIP2 (Di Matteo *et al*., 2006). The variable length of the β-strands of B2 and the twisted shape of the molecule results in the distortion of this β-sheet. Hydrophobic amino acids such as leucine, occupy specific positions of the LRR repeats. These play a crucial role in the stabilisation of the overall fold and stacking of the molecule through van der Waals interactions (Di Matteo *et al*., 2006). The LRR motif in PGIP has shown high homology to other LRR proteins involved in disease or stress resistance in plants. For instance, PGIP2 LRR shows 60% homology to that of the anti-freeze protein in carrot (DcAFP) which plays an important role in plant defence under cold stress (Worrall *et al*., 1998).

The LRR motif has also been shown to play an important role in controlling cell wall architectural components such as pectin, through the regulation of cell wall function (Xu et al., 2008). Pectin-binding sites outside the LRR motif have been identified (Spadoni *et al*., 2006). It has been hypothesised that PGIP binds with pectin and PGs through overlapping regions which are not necessarily identical. Site directed mutation studies attributed this interaction to four clustered residues

of arginine and lysine within the PGIP molecule which form the pectin binding site (Spadoni *et al*., 2006). It has been hypothesised that the binding of PGIP with pectin could be a means to mask the substrate thus protecting it from hydrolysis by ePGs (Joubert *et al*., 2007). Thus subtle changes in not only the sequence of the LRR motif, but also in areas outside the LRR motif that are involved in PGIP-pectin binding, could affect the PGIP-PG interaction and ultimately the plants' response to infection (Spinelli *et al*., 2008; Misas-Villamil *et al*., 2008; Casasoli *et al*., 2009; Maulik *et al*., 2009).

2.2.2 ePGs: Structural requirements for function

Endopolygalacturonases (ePGs) are enzymes that catalyse the depolymerisation of the homogalacturonan domain of the plant cell wall during fungal attack (Kars, 2007). Research has shown that they are required for the full virulence of fungal pathogens such as *B. cinerea* (ten Have *et al*., 1998). They are among the first cell wall degrading enzymes that fungal pathogens release when they interact with the host plants' cell wall. The ePGs hydrolyse the α-1,4 linkages of the D-galacturonic acid residues (D-GalUA) found within the homogalacturonan domain (Andre-Leroux *et al*., 2009). This enzymatic hydrolysis only occurs on nonesterified galacturonic residues (Esquerre-Tugaye *et al*., 2000). The ePGs possess an active site which is utilised in the formation of reversible complexes with PGIPs during plant-pathogen interaction (Kemp *et al*., 2004).

The crystal structure of the PG from the fungus *Fusarium moniliforme* (FmPG), was determined at 1.73Å (Federici *et al*., 2001) using multiple isomorphous replacement and anomalous scattering (MIRAS) methodology and is shown in **Figure 3**. Parallel β-sheets are formed through the alignment of the β-strands of consecutive turns (**Figure 3a**). Three to five residues make up the length of the β-strands. Between β-strands, the length of the turns (T) is more variable. T1 and T2 are usually made up of only one residue, asparagine, in the α_L conformation and are very short. The H-bonding potential of asparagine is believed to be responsible for the directional changes in the polypeptide backbone. The more variable T3 turns with 3 to 24 residues, form loops which are crucial for the determination of the formation of the cleft. The putative active site is located in a deep cleft on one side of the β-helix (**Figure 3b**) (Federici *et al*., 2001). The putative active site in *Fm*PG is made up of several conserved residues, namely, Asp-191, Asp-212, Asp-213, Arg-267 and Lys-269 which are located together in a cavity within the deep cleft. The active site is pivotal to enzyme activity, as demonstrated by single and double mutations generated in the conserved sites which resulted in enzyme activity being abolished or significantly reduced in *F. moniliforme* (Federici *et al*., 2001; Raiola *et al*., 2008). It has been shown that the shapes of the active sites of ePGs from different fungal pathogens differ, as illustrated in **Figure 4,** and could possibly be responsible for the different activity levels observed during plant infection. *F. moniliforme* PG shows 44% sequence homology to *Aspergillus niger* PGII and the secondary structure elements among the two are conserved for the region both within and outside the β-helix (Sicilia *et al*., 2005).

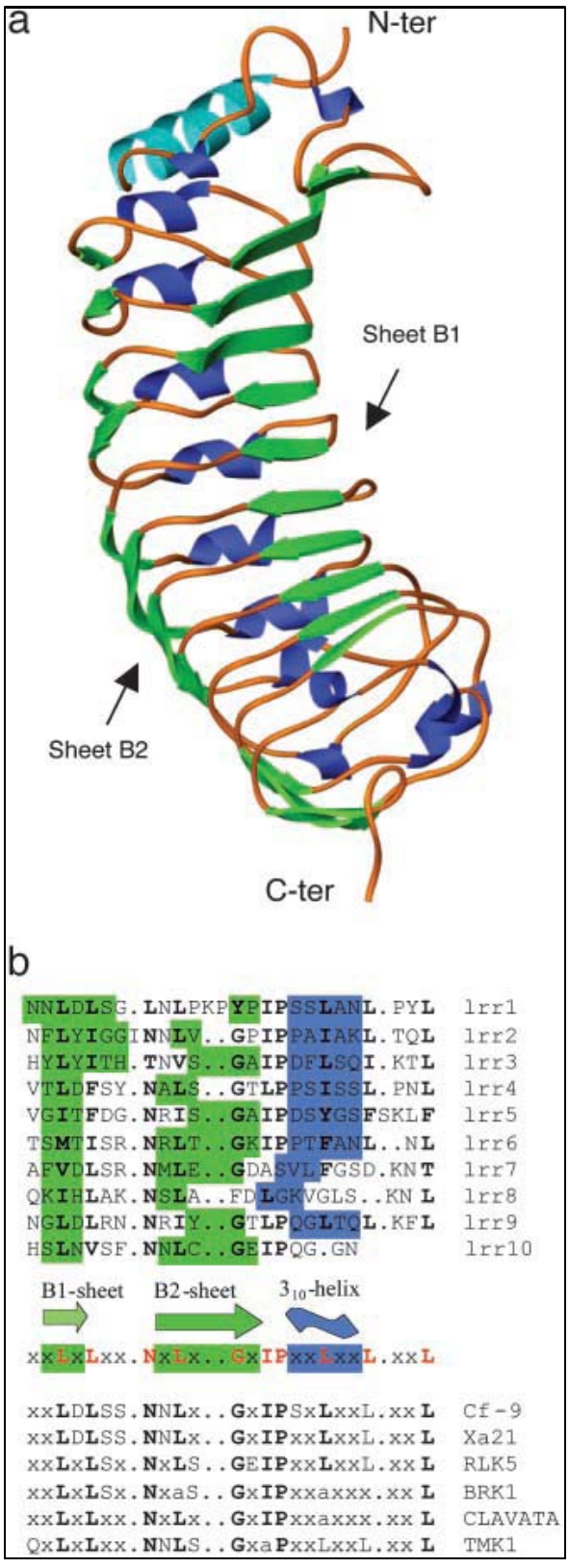


Figure 2. (a) The ribbon representation of *Phaseolus vulgaris* PGIP2 with the green colour annotating the parallel B1 and B2 sheets, the N-terminal α-helix in light blue and the 3_{10}-helices in dark blue in the central part of the LRR molecule. (b) Organisation of the secondary structure of the residues 53-289 of the PGIP2 LRR motif. Plant derived LRR sequences showing homology to PGIP2 are shown whilst the consensus sequences responsible for the formation of the secondary structure are shown in blue for the 3_{10}-helices and green for the β-sheets. Adopted from Di Matteo *et al*. (2003).

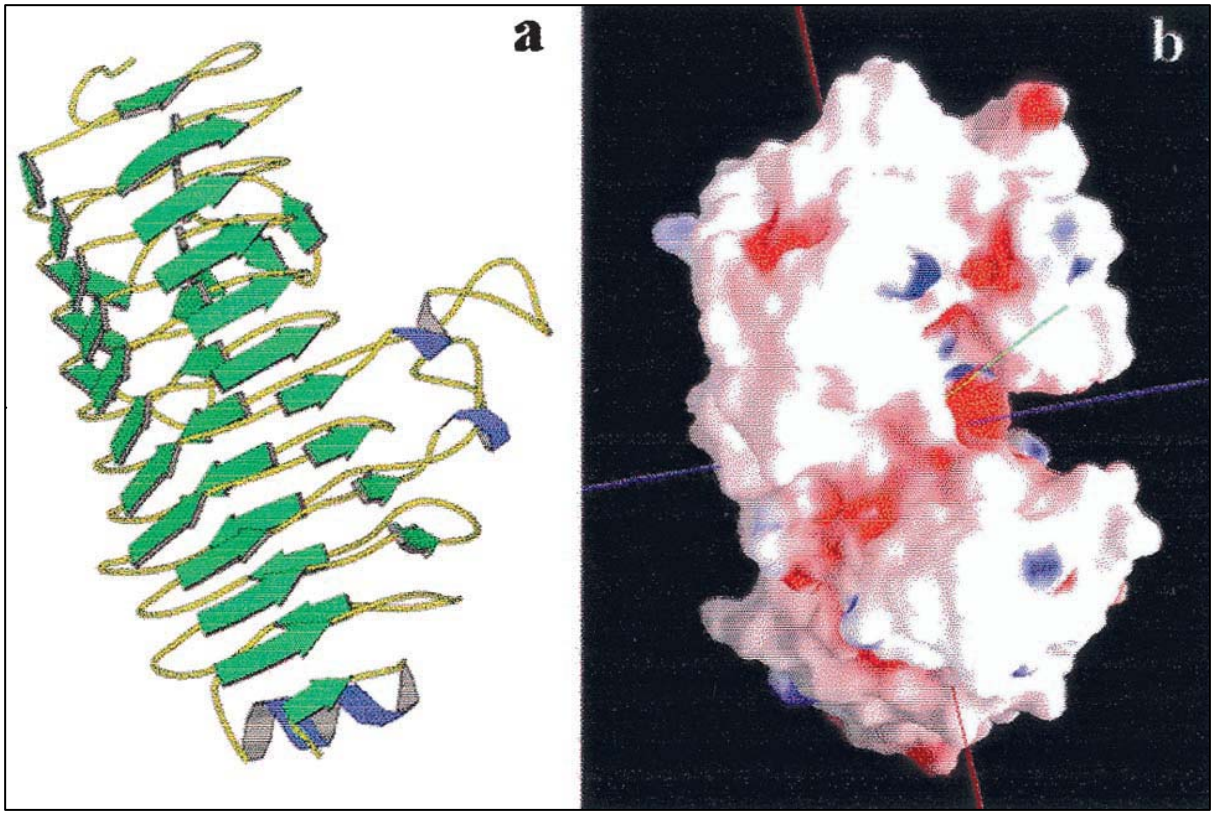


Figure 3. *Fusarium moniliforme* PG structure (**a**) MOLSCRIPT depiction of the right handed parallel β-helix. Three or four β-strands make up each coil and there are 10 coils in total. (**b**) Electrostatic potential surface demonstration, showing the possible active site. Red depicts the negative charges whilst the positive charges are depicted in blue. Adopted from Federici *et al*. (2001).

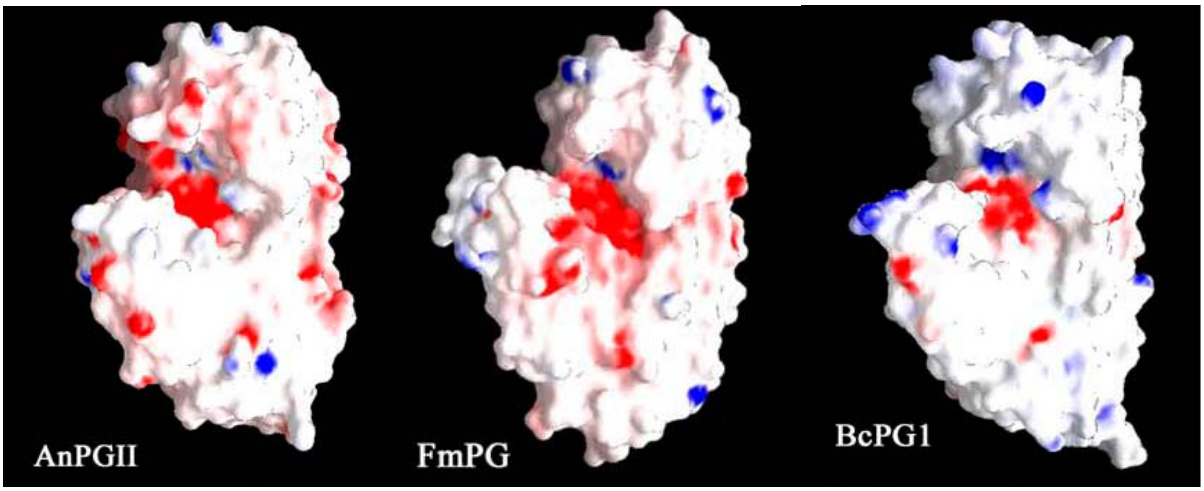


Figure 4. Electrostatic potential model of *Aspergillus niger* PGII, *Fusarium moniliforme* PG and *Botrytis cinerea* PG1 with the positive charges in blue and the negative charges in red. The illustration shows charge and molecular shape differences around the active cleft among the three PGs. Adopted from Sicilia *et al*. (2005).

2.3 ePG:PGIP interaction

In 1987, Cervone *et al*. hypothesised the role of PGIP in plant defence to be two-fold as illustrated by the model in **Figure 5**. The model illustrates the inhibitory role that PGIP is believed to play at the cell wall interface in inhibiting fungal PGs from macerating the plant tissue (Kars *et al*., 2005). Additionally, it is proposed that ePG inhibition prolongs the existence of longer chain cell wall fragments called oligogalacturonides which are believed to elicit a cascade of defence responses (Cervone *et al*., 1987; Aziz *et al*., 2004). The role of the elicitor-active oligogalacturonides was later confirmed in *in vitro* assays (Cervone *et al*., 1989).

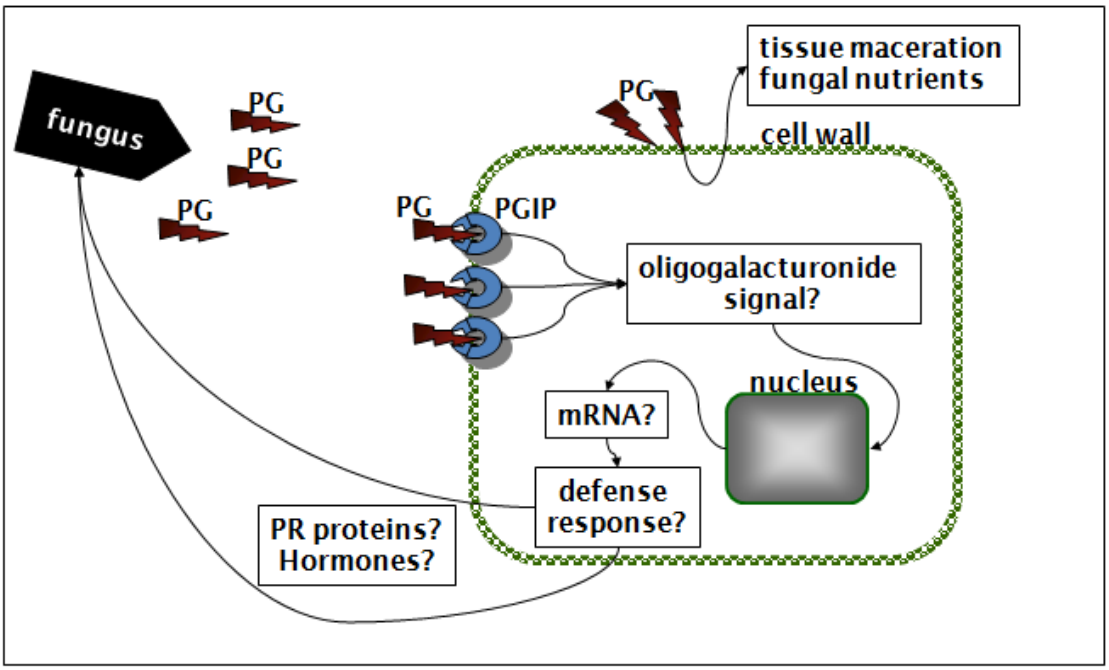


Figure 5. Current working hypothesis of PGIP-PG interaction as suggested by Cervone *et al*. (1987), illustrating the two-fold role of PGIP in plant defence. PGIP is depicted as a cell wall associated protein which directly inhibits fungal PGs. This is believed to prolong the existence of oligogalacturonides which are involved in activating defence responses.

Various plants have been shown to produce both PGs and PGIP (Ahmed *et al*., 1980). The relationship between the plants' PGIP and PGs has been shown to be mutually beneficial at certain stages of the growth cycle of the plant such as during plant growth, root elongation and fruit ripening. During fruit ripening, PGs facilitate fruit softening by contributing towards the structural changes that occur in the cell wall leading to the disassembly of pectin (Wang *et al*., 2000). Studies done on tomatoes, avocados, melons, apples, pears and kiwi fruits have all elucidated the contribution of PGs towards fruit softening during ripening (Ahmed *et al*., 1980; Crookes *et al*., 1983; Hadfield *et al*., 1998; Wang *et al*., 2000). It is also observed that the plant-derived PGIP does not appear to have any inhibitory action against its own ePGs. However, the same PGIP effectively inhibits ePGs from fungal pathogens such as *B. cinerea*. This has been hypothesised to be due to the unique structure of the endogenous plant PGs compared to those of fungal pathogens, that prevents it from associating with its own PGIP (Federici *et al*., 2001).

Studies have shown that PGIP has other important roles in plants apart from disease response. A recent study on *A. thaliana* has shown that PGIP plays an important role in determining radicle protrusion during seed germination (Kanai *et al*., 2010). Timing of radicle protrusion was investigated in seeds overexpressing *PGIP* compared to *PGIP* knockout mutants. Lower amounts in *PGIP* transcripts in the knockout mutants were shown to induce earlier radicle protrusion whilst the seeds with higher transcript levels took longer for the radicle to protrude. The degradation of pectin was also shown to be important for seed coat rupture with suppression of *PGIP*, which inhibits pectin breakdown by PGs, resulting in reduced time taken for the seeds to germinate (Kanai *et al*., 2010).

Tobacco floral nectar has been shown to play an important role in plant defence (Thornburg *et al*., 2003). This was based on a study performed on the nectar of ornamental tobacco and was found to have anti-polygalacturonase activity against BcPGs. A plate assay as described by Taylor and Secor in 1988 was used to evaluate the anti-PG nature of the nectar proteins. A zone reduction of >90% was observed when crude nectar was incubated with the BcPGs. The nectar proteins were then either precipitated with 87% ammonium sulphate or dialysed against 50 mM potassium phosphate before being incubated with BcPGs. The precipitated and dialysed proteins showed anti-PG activity leading to the hypothesis the nectar potentially contains PGIP. Furthermore, boiling the nectar led to the loss of the inhibitory activity thus confirming that the anti-PG activity observed was due to a nectar protein (Thornburg *et al*., 2003).

PGIPs are mostly present in multigene families in plant species with each member exhibiting unique substrate specificity (Janni *et al*., 2006). A recent study where two LRR protein encoding genes were isolated from tobacco, namely *NtLRR1* and *NtLRR2*, showed that the two were differentially expressed in response to the tobacco wildfire pathogen (*Pseudomonas syringae* pv. *tabaci*) and tobacco mosaic virus (TMV). *NtLRR1* was rapidly activated in response to tobacco wildfire pathogen as compared to TMV infection. On the other hand, *NtLRR2* was rapidly activated in response to TMV attack and very slowly to tobacco wildfire infection (Xu *et al*., 2009). The two genes also displayed unique subcellular localisation with *NtLRR1* transcripts being detected in abundance in stem tissue whilst *NtLRR1* was found to be localised mainly in the roots (Xu *et al*., 2009).

Fungi have also evolved different isoforms of PGs which have been shown to exhibit different levels of substrate specificity during plant infection (Favaron *et al*., 1997; Cook *et al*., 1999; Rai, 2009). For instance, the six ePGs from *B. cinerea* show different substrate specificities (Wubben *et al*., 1999). The different enzyme and inhibitor isoforms coexist though with different potentials in pathogen attack and plant defence. Infection of the plant by different fungi and also differences in level of infection activates different isoforms of PGIP that are best suited to inhibit the specific fungal PGs (Desiderio *et al*., 1997; Federici *et al*., 2006).

The fungal ePGs are inhibited by PGIPs through the formation of a bimolecular complex (Protsenko *et al*., 2008). In PGIP2 from *P. vulgaris,* the residues required for the affinity and recognition of fungal ePGs are located in the concave surface of the B1sheet (see Fig. 5) (Di Matteo *et al*., 2006). These are responsible for the formation of an irreversible complex with ePGs (Protsenko *et*

al., 2008). PGIP can also form reversible complexes with ePGs in a stoichiometric 1:1 ratio. This enzyme-substrate complex has been shown to hydrolyse homogalacturonan at a slower rate compared to unbound ePG (Kemp *et al*., 2004). The interactions of PGIPs and ePGs have been shown to be mediated by N-linked glycosylation in *Pyrus communis*. This was achieved through the characterisation of glycan heterogeneity at specific sites on the *P. communis* PGIP. All the seven predicted sites were found to be utilised during ePG-PGIP interaction (Lim *et al*., 2009)

PG inhibition, as determined by variable inhibition kinetics, can be highly competitive or non-competitive. The type of inhibition depends on the compatibility of the PG-PGIP interaction. It has also been shown in some cases that both competitive and non-competitive inhibition takes place in certain PGIP-PG interactions. (Federici *et al*., 2001; Sharrock *et al*., 2004; Di Matteo *et al*., 2006). During competitive inhibition, the PGIP binds to the active site of ePG and thus prevents it from binding to any other substrate. However, in non-competitive inhibition, PGIP binds to an allosteric site and causes conformational changes to the structure of the ePG thus reducing the affinity of the active site to any substrate (Protsenko *et al*., 2008). In some cases, PGIP actually prevents the structural changes necessary for substrate binding by attaching itself to the opposite site of the PG molecule (King *et al*., 2002).

PGIP2 from *P. vulgaris* competitively inhibits *Fm*PG from *F. moniliforme* by masking the active site and thus preventing any substrate from binding. It however exhibits a non-competitive inhibition to AnPGII as shown in **Figure 6** (Federici *et al*., 2006). Studies perfomed on the inhibition kinetics of tomato PGIP on AnPGII from *A. niger* showed a non-competitive mode of interaction (Stotz *et al*., 2000). In contrast to its inhibition mechanism to *Fm*PG, PvPGIP2 exhibits a mixed-type mode of inhibition against BcPG1 from *B. cinerea* by partially blocking the active site, thus reducing the substrate affinity (Manfredini *et al*., 2005; Sicilia *et al*., 2005).

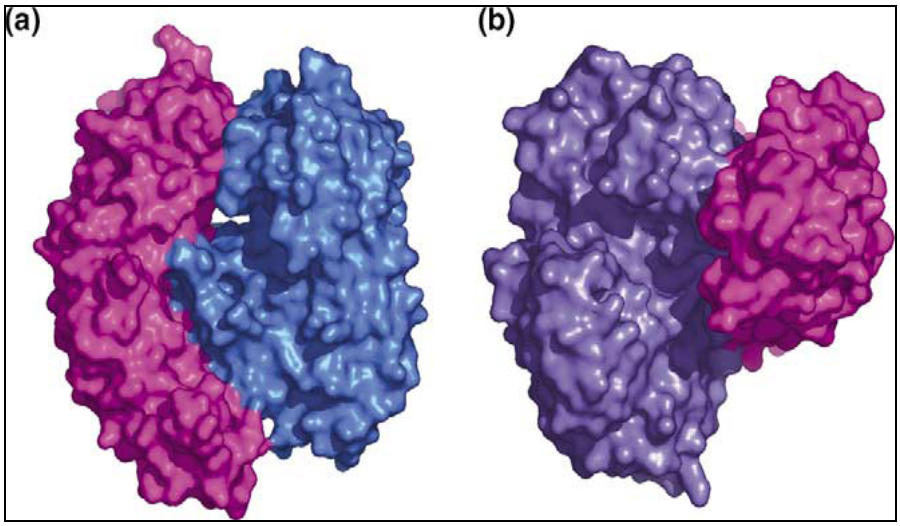


Figure 6. Docking geometry and energetic analysis of PG-PGIP interaction complex showing PvPGIP2 in purple, FmPG in light blue and AnPGII in dark blue **(a)** Competitive inhibition of FmPG by PvPGIP2. Active site cleft almost completely buried in the interaction and is not assessable to substrate **(b)** Non-competitive inhibition illustration of AnPGII by PvPGIP2. Active site is not covered and thus is left assessable to substrate. Adopted from Federici *et al*. (2006).

B. cinerea possesses at least six isoforms of PGs which display differential expression profiles (Kars *et al*., 2005). Enzyme activity assays utilising polygalacturonic acid as substrate reached optimum levels at different pH values for five of the BcPGs studied. The pH optimums were pH 4.2 for BcPG1, pH 4.5 for BcPG2 and BcPG6, pH range 3.2-4.5 for BcPG3 and pH 4.9 for BcPG4 (Kars *et al*., 2005). PGIP inhibition activity is highly pH dependent with different sources of ePGs differentially activating the PGIPs (Wubben *et al*., 2000). For example, it has been shown that the optimum pH for AnPGII:PvPGIP2 interaction is approximately 5.0 whilst AnPGII:VvPGIP1 interaction shows optimum activity at pH 4.75 (Cervone *et al*. 1987; Joubert *et al*., 2006). In a study carried out by Kemp *et al*. (2004) on *P. vulgaris* PGIP2 and five ePGs from *A. niger*, namely, PGA, PGB, PGI, PGII and PGC, it was shown that at pH 4.75 and above, PvPGIP2 either inhibits or activates the different ePGs leading to the suggestion of possibly re-naming polygalacturonase-inhibiting proteins (PGIPs) to polygalacturonase binding proteins (PGBPs) or polygalacturonase modulating proteins (PGMPs). This study was based on *in vitro* data, however, in 2007 Joubert *et al*. showed that *in vitro* and *in vivo* data does not necessarily match. They showed that VvPGIP1 strongly inhibited BcPG2 *in vivo* but no interaction was detected *in vitro*. This was hypothesised to be due to the *in vivo* environment supporting VvPGIP1 and pectin binding thus masking the substrate from the BcPG2. This was in line with the hypothesis from Spadoni *et al*. (2006), which proposes that PGIP binds with pectin and PGs through overlapping regions. It also emphasises the importance to study PGIPs *in vivo.*

2.4 PGIP inhibition studies

Numerous studies have elucidated the role of PGIP in reducing the susceptibility of the host plant to fungal attack. This has been achieved through *PGIP* gene expression analysis and overexpression studies in different plant host species including tobacco, pear, apple, tomato, *Arabidopsis*, wheat and grapevine (Benito *et al*., 1998; Powell *et al*., 2000; Atkinson *et al*., 2002; Faize *et al*., 2003; Ferrari *et al*., 2003; Tamura *et al*., 2004; Aguero *et al*., 2005; Joubert *et al*., 2006; Kortekamp, 2006; Oelofse *et al*., 2006; Gregori *et al*., 2008; Janni *et al*., 2008). This section highlights a few examples of these overexpression studies.

Gene expression studies in the Japanese pear revealed a probable involvement of PGIP in resistance against scab, a fungal disease caused by *Venturia nashicola* (Faize et al., 2003). Two pear cultivars resistant to scab and one susceptible cultivar were used for the study, namely, Kinchaku, Flemish beauty and Kousui respectively. Semi-quantitative RT-PCR results showed a high induction of the *PGIP* transcript in the resistant pear cultivars after inoculation of leaves with conidial suspensions of *V. nashiola* compared to the susceptible cultivar, Kousui (Faize et al., 2003). Despite the low levels of PG inhibition by PGIP extracts in the *in vitro* activity assays, the two resistant cultivars achieved significant levels of inhibition whilst the susceptible Kousui cultivar did not show any significant inhibition (Faize *et al*., 2003).

In an overexpression study involving tobacco plants overexpressing apple PGIP1, the transgenics showed reduced susceptibility to *Botryosphaeria obtusa*, *Diaporthe ambigua,* both

important pathogens of apple fruits, and *Colletotrichum lupini,* the pathogen that causes anthracnose on lupins (Oelofse *et al*., 2006). *Agrobacterium*-mediated transformation was used to introduce a *PGIP* gene from mature "Golden Delicious" fruit (*Malus domestica* Borkh) into tobacco plants (*Nicotiana tabacum*). Using the agarose diffusion assay, purified MgPGIP1 extracts from the transgenic tobacco plants inhibited PGs from *B. obtusa*, *D. ambigua* and *C. lupini*. MgPGIP1 however did not inhibit PGs from *A. niger*, whilst PGIP extract from the "Granny Smith" apple cultivar inhibited PGs from *A. niger* and *C. lupini*. The agarose diffusion assay coupled with Southern blotting results, led to the conclusion that there are possibly at least two active PGIPs in apple fruits with different inhibitory activity against AnPG (Oelofse *et al*., 2006).

Heterologous expression of a pear PGIP in tomato plants resulted in symptom reduction when the transgenic plants were infected with the fungal pathogen, *B. cinerea* (Powell *et al*., 2000). Cotyledon explants from tomato were transformed under the control of the constitutive CaMV 35S promoter with pear fruit PGIP, *pPGIP*, and there was accumulation of *pPGIP* all through fruit ripening and development in all tissues. Fungal infection assays were carried out on the leaves and fruit of the transgenic plants expressing *pPGIP*. A total of 5 to 6 wound sites per fruit were selected for infection using an aqueous suspension of 10^3 conidial suspensions from *B. cinerea*. A reduction in tissue maceration at the infection sites of up to 15% was observed in the transgenic fruit compared to the fruit from the untransformed plants. Detached leaf infection assays also showed a similar trend with smaller lesions observed in *pPGIP* expressing leaf material, as shown in **Figure 7** (Powell *et al*., 2000).

In a separate study, overexpression of the *pPGIP* in *V. vinifera* cvs. Thompson Seedless and Chardonnay, conferred the resultant transgenic population with reduced susceptibility to *B. cinere*a and *Xylella fastidiosa* infection (Aguero *et al*., 2005). *X. fastidiosa* is the causal agent of Pierce's disease (PD) in grapevine. The constitutive CaMV 35S promoter was utilised in the transformation of the two *V. vinifera* cultivars. The resulting putative transgenic population was screened for transgene presence and the positive lines were further evaluated for PGIP activity in leaf extracts against a crude mix of BcPGs using a semi-quantitative agarose diffusion assay. Ninety two percent of the tested lines showed inhibitory activity against the BcPGs. Detached leaf antifungal assays showed that the transgenic lines were less susceptible to *B. cinerea*, demonstrated by reduced rates of lesion expansion. Whole plant infection assays where the transgenic plants were challenged with *X. fastidiosa* bacterial suspensions, resulted in less severe PD symptoms in transgenic lines (Aguero *et al*., 2005).

Contrary to the aforementioned studies, PGIP overexpression in raspberry did not yield a resistance phenotype to fungal infection. The purified PGIP extracts failed to inhibit two exo-PGs from *B. cinerea*, bacterial endo-PGs and endopectate lyases in an enzyme activity assay. This was attributed to specific *in planta* interactions between the fungal PGs and PGIP (Johnston *et al*., 1993).

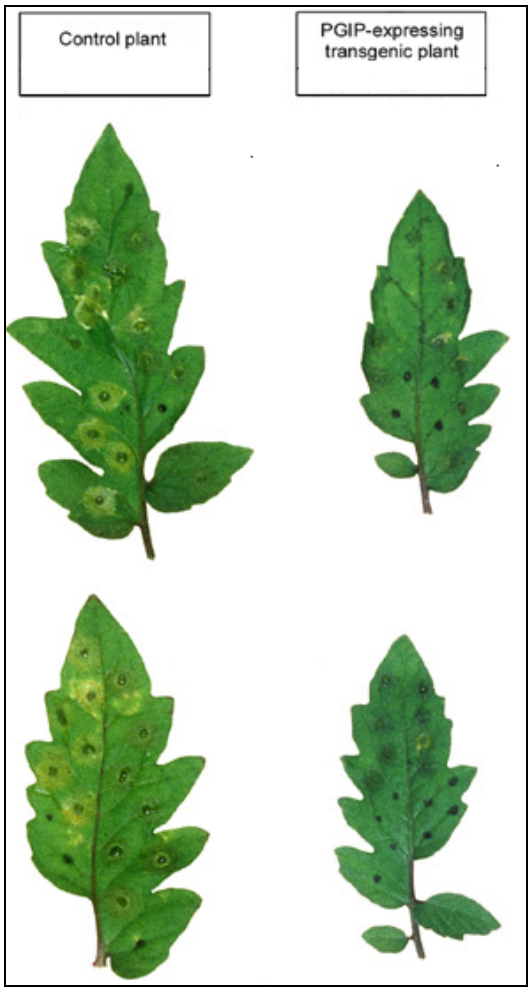


Figure 7. Colonisation of *pPGIP* expressing leaves and control leaves from tomato by *Botrytis cinerea* at 7 days post inoculation. Duplicate detached leaf infection assay lesion differences are shown. Adopted from Powell *et al*., 2000.

2.5 Grapevine-derived PGIP

2.5.1 *Vitis vinifera* PGIP

V. vinifera is the most cultivated grapevine species worldwide due to its superior quality in the production of wine, fresh table grapes and dried grapes. It is however highly susceptible to fungal attack that results in great yield losses (Vivier *et al*., 2002). The inhibitory role of the host plant's PGIP against fungal ePGs plays an important role in the plant's defence mechanism by reducing the effects of the fungal attacks (Bezier *et al*., 2002; Ferreira *et al*., 2004).

A PGIP encoding gene, *VvPGIP1*, was isolated from *V. vinifera* L. cv. Pinotage and purified protein from grapevine berries exhibited strong competitive inhibitory activity against a crude extract of BcPGs (De Ascensao, 2001). It has been shown that in grapevine, a multigene family of PGIPs is not present and thus *VvPGIP1* is the only PGIP in the entire genome. Expression of *VvPGIP1* has been shown to be highly tissue specific and developmentally regulated, only being detected in berries at and after véraison (Joubert, 2004). However Joubert (2004) further showed that expression can be strongly

induced in all tissues by wounding, infection and the presence of elicitors, amongst others. The induction thus alleviates tissue specific expression.

In a *VvPGIP1* overexpression study, tobacco was transformed using the *Agrobacterium*-mediated transformation protocol utilising the 35S CaMV promoter and nopaline synthase (NOS) terminator (Joubert *et al*., 2006). The putative transgenic population was analysed for gene presence, integration, expression and protein activity. Plant lines that were positive for these initial analyses were then acclimatised in the greenhouse together with untransformed tobacco lines for use in a whole plant *Botrytis* infection assay (**Figure 8**). The average lesion diameter from six transgenic lines was compared to the lesion diameters of the wildtype untransformed tobacco plant infected with *B. cinerea*. The transgenic plants exhibited a reduction in susceptibility to infection by the fungal pathogen as illustrated by the graph in **Figure 8**, with reduction in lesion diameter between 47 and 69% for the transgenic lines compared to the wildtype. Untransformed plants exhibited lesions which expanded rapidly from the onset of the experiment. Purified VvPGIP1 from one of the overexpressing lines was used to ascertain the inhibition profile of PGs from *A. niger* and *B. cinerea* over a wide pH range. PAHBAH reducing sugar assays showed selective inhibition of the different PGs by VvPGIP1 at varying pH optimums (Joubert *et al*., 2006). BcPG1, BcPG6, AnPGA and AnPGB were strongly inhibited by VvPGIP1 under all the conditions tested compared to BcPG3 where the inhibition was less pronounced. On the other hand, BcPG4 inhibition was highly pH dependent, only being inhibited in the lower pH ranges tested (Joubert *et al*., 2006).

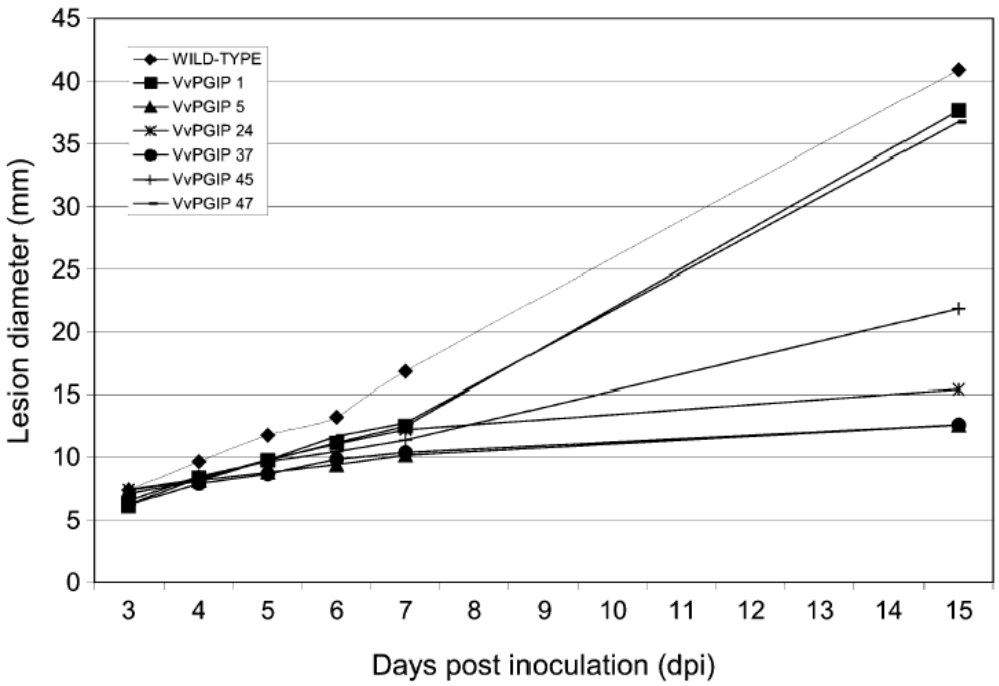


Figure 8. Whole plant infection assay showing lesion diameter on VvPGIP1 transgenic and untransformed tobacco plants infected with *Botrytis cinerea* over a 15 day period post inoculation. Measurements were taken on days 3, 4, 5, 6, 7 and 15. Adopted from Joubert *et al*. (2006).

2.5.2 Non-vinifera PGIPs

Susceptibility to fungal attack has been shown to differ in grapevine plants with non-vinifera and American grapevine species exhibiting improved resistance compared to *V. vinifera* cultivars. PGIP encoding genes were isolated and sequenced from 37 non-vinifera and American grapevine species, including rootstock material (Wentzel, 2005). Nucleotide and amino acid sequences were compared to those derived from VvPGIP1 from Pinotage. The observed total nucleotide changes ranged between 0 and 20 changes over the entire length of the gene**.** At least 95% homology was observed when the amino acid sequences from the 37 isolates were aligned with VvPGIP1. Separate alignment of the LRR domains, which play a pivotal role in the PGIP-PG interaction, showed homology of greater than 94% (Wentzel, 2005). The isolates were clustered into 14 groups according to LRR motif sequence variations and one member from each group was then randomly selected and overexpressed in tobacco lines (Venter, 2010). The putative transgenic populations from the nine successful transformations were genetically characterised and PGIP activity was also evaluated. Whole plant infection assay results showed that the non-vinifera transgenic lines displayed PGIP-specific resistance phenotypes to *Botrytis* infection compared to the untransformed wildtype, and lines overexpressing VvPGIP1, as shown by the differences in lesion development in **Figure 9 and 10**.

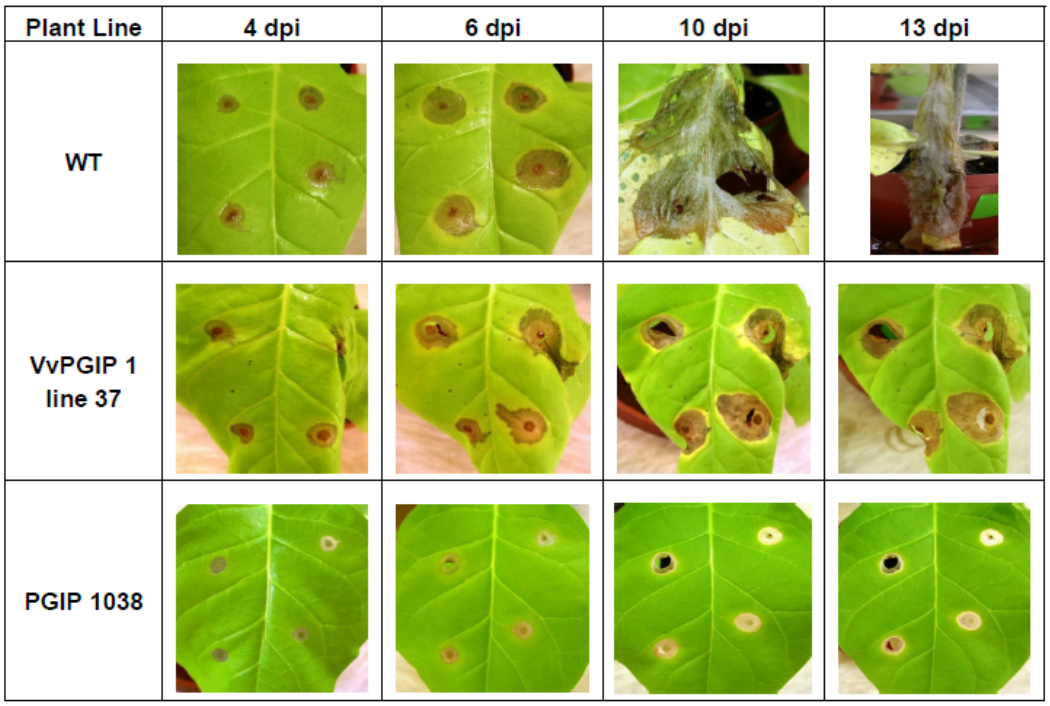


Figure 9. Whole plant infection assay in tobacco showing lesion development on the leaves of untransformed wildtype (WT), VvPGIP1 line 37 (tobacco plant overexpressing the *V. vinifera PGIP* gene) and PGIP 1038 (tobacco plant overexpressing a non-vinifera PGIP encoding gene) lines infected with *Botrytis cinerea*. Lesion development was monitored from 4 to 13 days post inoculation (dpi). WT lesions developed faster than those on the transgenic lines, followed by VvPGIP1 lines with PGIP 1038 displaying the lowest susceptibility to fungal infection. Adopted from Venter, 2010.

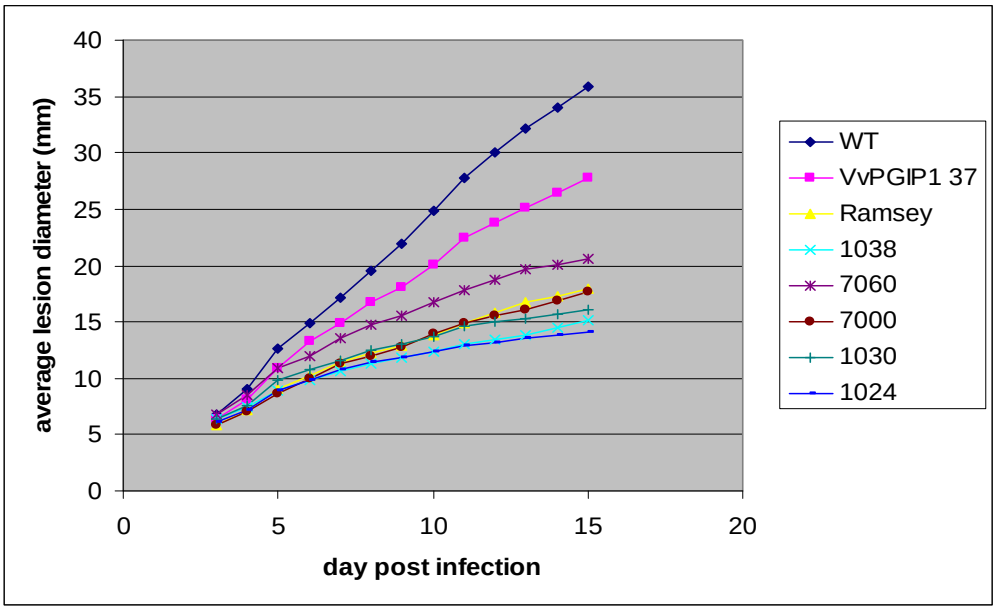


Figure 10. Lesion development over time on tobacco leaves infected with *B. cinerea* in a whole plant antifungal assay. The untransformed wildtype (WT) showed higher susceptibility to infection as observed by the larger lesion diameter during the course of the assay. The tobacco line VvPGIP1 37 overexpressing the *V. vinifera PGIP* gene also showed high susceptibility, though it performed better than the WT. The lines overexpressing *PGIP* genes from non-vinifera grapevine species (Ramsey, 1038, 7060, 7000, 1030, 1024) exhibited reduced susceptibility to *B. cinerea* infection relative to the WT and VvPGIP1 line 37. Adopted from Venter, 2010.

The reduced susceptibility of tobacco to *B. cinerea* by overexpressing the non-vinifera *PGIP* genes has led to the current study where two of the genes showing strong enhancement of resistance to fungal attack were transformed into two *Vitis vinifera* grapevine cultivars, Sultana and Redglobe (see **Chapter 3** of the thesis).

2.6 Summary

The role of PGIPs in plant defence against fungal infection has been studied in numerous plant host backgrounds. Structural determinations of PGIPs, such as PvPGIP2 from Phaseolus vulgaris and PGs such as FmPG from F. moniliforme, have played a major role in advancing the knowledge base on the mechanisms that occur during plant infection. The highly conserved LRR motif found in PGIP molecules is highly specialised for protein-protein interaction and has been shown to have high homology with other LRR proteins found in plants that play important roles in plant defence.

PGIP overexpression studies in numerous plant hosts such as *Arabidopsis*, pear, apple, tomato, tobacco and grapevine have elucidated the role of PGIP in the reduction of the plants' susceptibility to fungal attack by inhibiting ePGs from commercially important fungi such as *B. cinerea*. Other roles apart from inhibiting fungal ePGs have been attributed to PGIPs. These include determination of radicle protrusion in seeds during germination and the regulation of cell wall polymers such as pectin, which plays a major role in determining overall cell wall architecture.

In a study previously done in our environment, *VvPGIP1*, from *V. vinifera* was overexpressed in tobacco and the resulting transgenics were less susceptible to *B. cinerea* infection compared to

untransformed plants. The low susceptibility to fungal attack of some non-vinifera grapevine species prompted interest in the potential role that their PGIP encoding genes play in this regard. In a separate study, a range of the PGIP encoding genes from these non-vinifera grapevine species was also overexpressed in tobacco and the transgenics were found to be even less susceptible to *B. cinerea* infection than the *VvPGIP1* overexpressing lines. Whether these non-vinifera PGIPs will also confer reduced disease susceptibility if expressed in a *V. vinifera* background is yet to be known. This study aims to shed light on this aspect.

2.7 References

Aguero C.B., Uratsu S.L., Greve C., Powell A.L.T., Labavitch J.M., Meredith C.P., 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.

Ahmed A.E., Labavitch J.M. 1980. Cell wall metabolism in ripening fruit II. Changes in carbohydrate-degrading enzymes in ripening bartlett' pears. Plant Physiol. 65:1014-1016.

Alghisi P., Favaron F. 1995. Pectin-degrading enzymes and plant-parasite interactions. Eur. J. Plant Pathol. 101:365-375.

Andre-Leroux G., Tessier D., Bonnin E. 2009. Endopolygalacturonases reveal molecular features for processivity pattern and tolerance towards acetylated pectin. Biochim. Biophys. Acta: Proteins Proteomics 1794:5-13.

Atkinson R.G., Schroder R., Hallett I.C., Cohen D., MacRae E.A. 2002. Overexpression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involving changes in cell adhesion. Plant Physiol. 129:122–133.

Aziz A., Heyraud A., Lambert B. 2004. Oligogalacturonide signal transduction, induction of defence-related responses and protection of grapevine against *Botrytis cinerea*. Planta 218: 767–774.

Becker J.W. 2007. Evaluation of the roles of PGIPs in plant defence responses. PhD dissertation. Stellenbosch University, Stellenbosch, Republic of South Africa.

Benito E.P., ten Have A., van't Klooster J.W., van Kan J.A.L. 1998. Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. Eur. J. Plant Pathol. 104: 207–220.

Bergmann C.W., Ito Y., Singer D., Albersheim P., Darvill A.G. 2004. Polygalacturonase inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. Plant J. 5:625-634.

Bezier A., Lambert B., Baillieul F. 2002. Study of defence related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. Eur. J. Plant Pathol. 108:111-120.

Bolton M.D. 2009. Primary Metabolism and Plant Defence—Fuel for the Fire. Mol. Plant-Microbe Interact. 22:487–497.

Camps C., Kappel C., Lecomte P., Leon C., Gomes E., Coutos-Thevenot P., Delrot S. 2010. A transcriptomic study of grapevine (*Vitis vinifera* cv. Cabernet-Sauvignon) interaction with the vascular ascomycete fungus *Eutypa lata*. J. Exp. Bot. 61:1719–1737.

Cantu D., Vicente A.R., Labavitch J.M., Bennet A.B., Powell A.L.T. 2008. Strangers in the matrix: plant cell walls and pathogen susceptibility. Trends Plant Sci. 13:610-617.

Casasoli M., Federici L., Spinelli F., Di Matteo A., Vella N., Scaloni F., Fernandez-Recio J., Cervone F., De Lorenzo G. 2009. Integration of evolutionary and desolvation energy analysis identifies functional sites in a plant immunity protein. PNAS 106:7666-7671.

Cervone F., De Lorenzo G., Degra L., Salvi G., Bergami M. 1987. Purification and characterisation of a polygalacturonase inhibiting protein from *Phaseolus vulgaris* L. Plant Physiol. 85:631-637.

Cervone F., Hahn M.G., De Lorenzo G., Darvill A. 1989. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defence responses. Plant Physiol. 90:542-548.

Cervone F., De Lorenzo G., Pressey R., Darvill A.G., Albersheim P. 1990. Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? Phytochemistry 29:447-449.

Choquer M., Fournier E., Kunz C., Levis C., Pradier J.M., Simon A., Viaud M. 2007. Botrytis cinerea virulence factors:newinsights into a necrotrophic and polyphageous pathogen. FEMS Microbiol. Lett. 277:1–10

Comménil P., Belingheri L., Sancholle M., Dehorter B. 1995. Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. Lipids 30:351-356.

Cook B.J., Clay R.P., Bergmann C.W., Albersheim P., Darvill A.G. 1999. Fungal polygalacturonases exhibit different substrate degradation patterns and differ in their susceptibilities to polygalacturonase-inhibiting proteins. MPMI 12:703-711.

Crookes P., Grierson P. 1983. Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in cell wall degradation. Plant Physiol. 72:1088-1093.

Dai G. H., Andary C., Mondolot-Cosson L., Boubals D. 1995.Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera, V. rupestris* and *V. rotundifolia* and the downy mildew fungus, *Plasmopara viticola*. Physiol. Mol. Plant Pathol. 46:177-188.

De Ascensao A. 2001. Isolation and characterisation of a polygalacturonase-inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

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.

De Lorenzo G., Ferrari S. 2002. Polygalacturonase inhibiting proteins in defence against phytopathogenic fungi. Curr. Opin. Plant Biol. 5:278-285.

Derckel J.-P., Baillieul F., Manteau, S., Audran J.-C., Haye B., Lambert B., Legendre L. 1999. Differential induction of grapevine defences by two strains of *Botrytis cinerea*. Phytopathology 89:197-203.

Desiderio A., Aracri B., Leckie F., Mattei B., Salvi G., Tigerlaar H., van Roekel J., Baulcombe D., Melchers L., De Lorenzo G., Cervone F. 1997. Polygalacturonase inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol. Plant Microbe-Interact 10:852-860.

Di Matteo A., Federici l., Mattei B., Salvi G., Johnson K., Savino C., De Lorenzo G., Tsernoglou D., Cervone F. 2003. The crystal structure of polygalacturonase inhibiting protein (PGIP), a leucine-rich repeat protein involved in plant defence. PNAS 100:10124-10128.

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

D'Ovidio R., Mattei B., Robertia S., Bellincampi D. 2004. Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant–pathogen interactions. Biochim. Biophys. Acta 1696:237–244.

Egan M.J., Talbot N.J. 2008. Genomes, free radicals and plant cell invasion: recent developments in plant pathogenic fungi. Curr. Opin. Plant Biol. 11:367–372.

Elad Y. 1997. Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection. Biol. Rev 72: 381-422.

Esquerre-Tugayé M. T., Boudart G., 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.

Faize M., Sugiyama T., Faize L., Ishii H. 2003. Polygalacturonase-inhibiting protein (PGIP) from Japanese pear: possible involvement in resistance against scab. Physiol. Mol. Plant Pathol. 63:319–327.

Favaron F., Castiglioni C., D'Ovidio C., Alghisi P. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. Physiol. Mol. Plant Pathol. 50:403-417.

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 proteins). Proc. Natl. Acad. Sci. USA 98:12425-13430.

Federici L., Di Matteo A., Fernandez-Recio J., Tsernoglou D., Cervone F. 2006. Polygalacturonase inhibiting proteins: players in plant innate immunity? Trends Plant Sci. 11:65-70.

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

Ferreira R. B., Monteiro S.S., Picarra-Perreira M.A., Teixeira A.R. 2004. Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability. Trends Biotechnol. 22:168-173.

Frediani M., Cremonini R., Salvi G., Caprari C., Desiderio A., D'Ovidio R., Cervone F., and De Lorenzo G. 1993. Cytological localization of the *pgip* genes in the embryo suspensor cells of *Phaseolus vulgaris* L. Theor. Appl. Genet. 87:369-373.

Gadoury D. M., Pearson R.C. 1988. Initiation, development, dispersal and survival of cleistothecia of *Uncinula necator* in New York vineyards. Phytopathology 78:1413-1421

Gadoury, D. M., Seem, R. C., Ficke, A., Wilcox, W. F. 2001. The epidemiology of powdery mildew on Concord grapes. Phytopathology 91:948-955.

Gindro K., Pezet R, Viret O. 2003. Histological study of the responses of two *Vitis vinifera* cultivars (resistant and susceptible) to *Plasmopara viticola* infections. Plant Physiol. Biochem. 41:846-853.

Gobbin D., Jermini M., Loskill B., Pertot I., Raynal M., Gessler C. 2005. Importance of secondary inoculum of *Plasmopara viticola* to epidemics of grapevine downy mildew. Plant Pathol. 54:522–534.

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

Gregori R., Mari M., Bertolini P. , Sanudo Barajas J.A. , Tian J.B., Labavitch J.M. 2008. Reduction of *Colletotrichum acutatum* infection by a polygalacturonase inhibitor protein extracted from apple. Postharvest Biol. Technol. 48:309–313.

Hadfield K.A., Rose J.K.C., Yaver D.S., Berka R.M., Bennett A.B. 1998. Polygalacturonase Gene Expression in Ripe Melon Fruit Supports a Role for Polygalacturonase in Ripening-Associated Pectin Disassembly. Plant Physiol. 117:363–373.

Hematy K., Cherk C., Somerville S. 2009. Host-pathogen warfare at the plant cell wall. Curr. Opin. Plant Biol. 12:406-413.

Howell G.S. 2001. Sustainable grape productivity and the growth-yield relationship: A review. Am. J. Enol. Viticult. 52:165-174.

Howell J.T., Davis M.R. 2005. Plant defence mechanisms against fungal pathogens:polygalacturonase inhibitor proteins. Can. J. Plant Pathol. 27:5–15.

Hwang B.H., Bae H., Lim H.S., Kim K.B., Kim S.J., Im M.H., Park B.S., Kim D.S., Kim J. 2010. Overexpression of polygalacturonase-inhibiting protein 2 (*PGIP2*) of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) increased resistance to the bacterial pathogen *Pectobacterium carotovorum* ssp. *carotovorum*. Plant Cell Tiss. Org. Cult. 103:293–305.

Janni M., Di Giovanni M., Roberti S., Capodicasa C., D'Ovidio R. 2006. Characterisation of expresses *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. 113:1233–1245.

Janni M., Sella L., Favaron F., Blechl A.E., De Lorenzo G., D'Ovidio R. 2008. The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. MPMI 21:171-177.

Jayasankar S., Li Z., Gray D. J. 2000. In-vitro selection of *Vitis vinifera* `Chardonnay' with *Elsinoe ampelina* culture filtrate is accompanied by fungal resistance and enhanced secretion of chitinase Planta 211: 200-208.

Johnston D.J., Ramanathan V., 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. 2004. Regulation of the *Vitis vinifera pgip1* gene encoding a polygalacturonase inhibiting protein. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Joubert D.A., Slaughter A.R., Kemp G., Becker J.V.W., Krooshoof G.H., Bergmann C., Benen J., Pretorius I.S., Vivier M.A. 2006. The grapevine polygalacturonase inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. 15:687-702.

Joubert D. A., Kars I., Wagemakers L., Bergmann C., Kemp G., Vivier M. A., Kan J. A. L. 2007. A Polygalacturonase-Inhibiting Protein from Grapevine Reduces the Symptoms of the Endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* Leaves Without Any Evidence for In Vitro Interaction. MPMI 4:392–402.

Juge N. 2006. Plant protein inhibitors of cell wall degrading enzymes. Trends Plant Sci. 11:359-367

Kanai M., Nishimura M., Hayashi M. 2010. A peroxisomal ABC transporter promotes seed germination by inducing pectin degradation under the control of ABI5. Plant J. 62:936–947.

Kars I., van Kan J.A.L. 2004. Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In *Botrytis*: Biology, pathology and control. Dordrecht: Kluwer Academic Publishers pp. 99-118.

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

Kars I. 2007. The role of pectin degradation in pathogenesis of *Botrytis cinerea*. PhD Thesis, Wageningen University, Wageningen, NL.

Keller, M., Viret, O., and Cole, F. M. 2003. *Botrytis cinerea* infection in grape flowers: Defence reaction, latency, and disease expression. Phytopathology 93:316-322.

Kemp G., Stanton L., Bergmann C.W., Clay R.P., 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.W., Orlando A., Benen J.A.E., Kester H.C., 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.

Kobe B., Kajava A.V. 2001. The leucine rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. 11:725-732.

Kortekamp A. 2006. Expression analysis of defence related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiol. Biochem. 44:58-67.

Lim J-M., Aoki K., Angel P., Garrison D., King D., Tiemeyer M., Bergmann C., Wells L. 2009. Mapping glycans onto specific N-linked glycosylation sites of *Pyrus communis* PGIP redefines the interface for EPG-PGIP interactions. J. Proteome Res. 8:673-680.

Magarey R.D., Ernme R.W., Magareye P.A., Franzc P.R. 1993. Evaluation of control of grapevine anthracnose caused by *Elsinoe ampelina* by pre-infection fungicides. Australas. Plant Pathol. 22:48-52.

Mahoney N., Molyneux R . J., Smith L. R., Thomas K. Schoch T. K., Rolshausen P. E., Gubler W. D. 2005. Dying-Arm Disease in Grapevines: Diagnosis of Infection with *Eutypa lat*a by Metabolite Analysis. J. Agric. Food Chem. 53:8148-8155.

Manfredini C., Sicilia F., Ferrari S., Pontiggia D., Salvi G., Caprari C., Lorito M., 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.

Mason S.J., Waylen P.R., Mimmack G.M., Rajaratman B., Harrison J.M. 1999. Changes in the extreme rainfall events in South Africa. Climate Change 41:249-257.

Mattei B., Bernalda M.S., Federici L., Roepstorff P., Cervone F., Boffi A. 2001. Secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (Polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. Biochemistry 40:569-576.

Maulik A., Ghosh H., Basu S. 2009. Comparative study of protein-protein interaction observed in polygalacturonase-inhibiting proteins from *Phaseolus vulgaris* and *Glycine max* and polygalacturonase from *Fusarium moniliforme*. BMC Genomics 10 (Suppl 3):S19.

Mauro M. C., Vaillant V., Tey-Rulh P., Mathieu Y., Fallot J. 1988. In vitro study of the relationship between *Vitis vinifera* and *Eutypa lata* (Pers.:Fr.) Tul.I. Demonstration of toxic compounds secreted by the fungus. Am. J. Enol. Viticult. 39:200-204.

Misas-Villamil J.C., van der Hoorn R.A.L. 2008. Enzyme-inhibitor interactions at the plant-pathogen interface. Curr. Opin. Plant Biol. 11:380-388.

Molyneux R. J., Mahoney N., Bayman P., Wong R. Y., Meyer K., Irelan N. 2002. *Eutypa* Dieback in Grapevines: Differential Production of Acetylenic Phenol Metabolites by Strains of *Eutypa lata.* J. Agric. Food Chem. 50:1393-1399.

Oelofse D., Dubery I. A., Meyer R., Arendse M. S., Gazendam I., Berger D. K. 2006. Apple polygalacturonase inhibiting protein1 expressed in transgenic tobacco inhibits polygalacturonases from fungal pathogens of apple and the anthracnose pathogen of lupins. Phytochemistry 67:255–263.

Pearson R. C., Gadoury D. M. 1987. Cleistothecia, the source of primary inoculums for grape powdery mildew in Ney York. Phytopathology 77:1509-1514.

Pérez S., Mazeau K., Hervé du Penhoat C. 2000. The three-dimensional structures of the pectic polysaccharides. Plant Physiol. Biochem. 38:37−55.

Poinssot B., Vandelle E., Bentejac M., Adrian M., Levis C., Brygoo Y., Garin J., Sicilia F., Coutos-Thevenot P., Pugin A. 2003. The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defence reactions unrelated to its enzymatic activity. Mol. Plant Microbe-Interact 16:553-564.

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

Protsenko M.A., Buza N.L., Krinitsyna A.A., Bulantseva E.A., Korableva N.P. 2008. Polygalacturonase inhibiting protein is a structural component of plant cell wall. Biochemistry 73: 1053-1062.

Rai M. 2009. Advances in fungal biotechnology. I.K. International Publishing House Pvt. Limited. New Delhi, India.

Raiola A., Sella L., Castiglioni C., Balmas V., Favaron F. 2008. A single amino acid substitution in highly similar endo-PGs from *Fusarium verticillioides* and related *Fusarium* species affects PGIP inhibition. Fungal Genet. Biol. 45:776–789.

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

Rügner A., Rumbolz J., Huber B., Bleyer G., Gisi U., Kassemeyer H.-H., Guggenheim R. 2002. Formation of overwintering structures of *Uncinula necator* and colonization of grapevine under field conditions. Plant Pathol. 51:322–330.

Sarkar P., Bosneaga E., Auer M. 2009. Plant cell walls through evolution: towards a molecular understanding of their design principle. J. Exp. Bot. 60:3615-3635.

Sharrock K. R., and Labavitch J. M. 1994. Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacturonase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. Physiol. Mol. Plant Pathol. 45:305-319.

Sicilia F., Fernandez-Recio J., Caprari C., De Lorenzo G., Tsernoglou D., Cervone F., Federici L. 2005. The polygalacturonase inhibiting protein PGIP2 of *Phaseolus vulgaris* has evolved a mixed mode of inhibition of endopolygalacturonase PG1 of *Botrytis cinerea*. Plant Physiol. 139:1380-1388.

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

Spinelli F., Marrioti L., Mattei B., Salvi G., Cervone F., Caprari C. 2008. Three aspartic acid residues of polygalacturonase-inhibiting protein (PGIP) from *Phaseolus vulgaris* are critical for inhibition of *Fusarium phyllophilum* PG. Plant Biology: 1-6.

Stahl E.A., Bishop J.G. 2000. Plant–pathogen arms races at the molecular level. Curr. Opin. Plant Biol. 3:299–304.

Stotz H. U., Bishop J. G., Bergmann C. W., Koch M., Albersheimp P., Darvill A G., 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.

Tamura M., Gaob M., Tao R., Labavitch J.M., Dandekar A.M. 2004. Transformation of persimmon with a pear fruit polygalacturonase inhibiting protein (PGIP) gene. Sci. Hort. 103:19–30.

Taylor R., Secor G. 1988. An improved diffusion assay for quantifying the polygalacturonase content of Erwinia culture filtrates. Phytopathology 78

Thach L., Matz T. 2008. Wine: A Global Business. 2nd Edition. Miranda Press, Elmsford, New York.

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

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

Tey-Rulh P., Philippe I., Renaud J. M., Tsoupras G, Angelis P., Fallot J., Tabacchi R. 1991. Eutypine, a phytotoxin produced by *Eutypa lata* the causal agent of dying-arm disease of grapevine. Phytochemistry 30:471-473.

van Kan J.A.L. 2005. Infection strategies of *Botrytis cinerea*. Acta Hort. 669:77-89.

van Kan J. A. L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11:247-252.

Venter A. 2010. The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco. MSc thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Vivier M. A., Pretorius I. S. 2002. Genetically tailored grapevines for the wine industry. Trends Biotechnol. 20:472-478.

Vlot A.C., Klessig D.F., Park S. 2008. Systemic acquired resistance: the elusive signal(s). Curr. Opin. Plant Biol. 11:436–442.

Vorwerk S., Somerville S., Somerville C. 2004. The role of plant cell wall polysaccharide composition in disease resistance. Trends Plant Sci. 9:203-209.

Wang Z., MacRae E.A., Wright M.A., Bolitho K.M., Ross G.S., Atkinson R.G. 2000. Polygalacturonase gene expression in kiwifruit: relationship to fruit softening and ethylene production. Plant Mol. Biol. 42:317–328.

Wentzel L. 2005. The endopolygalacturonases from *Botrytis cinerea* and their interaction with an inhibitor from grapevine. MSc Thesis, Stellenbosch University.

Williamson B., Tudzynski B., Tudzynski P., Van Kan J.A.L. 2007. Botrytis cinerea : the cause of grey mould disease. Mol. Plant Pathol. 8:561–580.

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

Wubben J., Mulder W., ten Have A., van Kan J., Vissier J. 1999. Cloning and partial characterisation of endopolygalacturonase genes from Botrytis cinerea. Appl. Environ. Microbiol. 65:1596- 1602.

Wubben J.P., ten Have A., van Kan J.A.L., Visser J. 2000. Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. Curr. Genet. 37:152-157.

Xu S-L., Rahman A., Baskin T.I., Kieber J.J. 2008. Two leucine-rich repeat receptor kinases mediate signalling linking cell wall biosynthesis and ACC synthase in *Arabidopsis*. Plant Cell 20:3065-3079.

Xu Z., Xiong T., Ni Z., Chen X., Chen M., Li L., Gao D., Yu X., Liu P., Ma Y. 2009. Isolation and identification of two genes encoding leucine rich repeat (LRR) proteins differentially responsive to pathogen attack and salt stress in tobacco. Plant Sci. 176: 38-45.

Yun H. K., Louime C., Lu J. 2007. First Report of Anthracnose Caused by *Elsinoe ampelina* on Muscadine Grapes (*Vitis rotundifolia*) in Northern Florida. Plant Dis. 91:905-905.

Chapter 3

Research results

Expressing PGIP encoding genes from non-vinifera grapevine species in *V. vinifera* promotes susceptibility, not resistance, against *B. cinerea*

This chapter will form part of a manuscript that will be submitted to
Transgenic Research

Expressing PGIP encoding genes from non-vinifera grapevine species in *V. vinifera* promotes susceptibility, not resistance, against *B. cinerea*

Moyo M., Vasanth K. and Vivier M.A.

Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch University, Stellenbosch, 7600, RSA

3.1 Abstract

Polygalacturonase-inhibiting proteins (PGIPs) are defence related cell wall associated proteins which inhibit the endopolygalacturonases (ePGs) secreted by invading fungal pathogens. In this study two PGIP encoding genes from *Vitis doaniana* Munson and *Vitis caribaea* were functionally analysed in cultivars of *V. vinifera* by expression studies and analysis of defence phenotypes against *Botrytis cinerea*. These *PGIP* genes have previously been overexpressed in tobacco and have caused strong resistance phenotypes against *B. cinerea* in a whole plant infection assay, even stronger than the response observed for the VvPGIP1 overexpressing lines. In this study, we started off with a population of putative transgenic *V. vinifera* cv. Sultana, Redglobe and Merlot lines which were clonally multiplied resulting in the generation of 131 putative lines. However, after genetic analysis only the Redglobe populations for the two PGIPs were of a sufficient size for further evaluation. Twenty two of the Redglobe transgenic lines were shown to have the transgenes integrated, expressed and with confirmed PGIP activity in the leaves. This activity would constitute the transgene-derived PGIP activity, since the native PGIP is not expressed in leaves, unless induced. The characterised population, in comparison to the wild type (WT), was used in detached leaf and whole plant infection assays against *Botrytis cinerea*. In contrast to the results obtained in tobacco, the transgenic grapevine lines did not show an improved resistance response against *B. cinerea* in a whole plant infection assay. For both non-vinifera PGIPs, the transgenic lines were instead, more susceptible than the WT. There was also a clear difference in the onset and rate of lesion spread in the transgenic lines versus WT, with the former showing necrotic lesions quickly and an explosive speed of lesion expansion and even the formation of spores on the infected tissue. The same clear response was not seen in two separate detached leaf assays and we concluded that the induced wound response (by detaching the leaves) that would induce the native *VvPGIP1* and a compromised defence signaling response probably masked the PGIP-linked phenotype linked to the transgenes. These results highlight the importance to study and understand the *in vivo* functions of PGIPs and the transgenic lines that were generated in this study, their characterisation and the interesting phenotype provide an excellent resource to study the role of PGIPs further.

3.2 Introduction

Plants are attacked by a wide array of pathogens such as bacteria, protozoa and fungi (Ferreira *et al*., 2004). Fungal pathogens such as *Aspergillus niger* and *Botrytis cinerea* secrete numerous metabolites and enzymes that facilitate infection and colonisation of the plant host (Commenil *et al*., 1995; van Kan, 2005; Cantu *et al*., 2008). Among these are cell wall degrading enzymes (CWDE) called endopolygalacturonases (ePGs), which macerate the pectic part of the primary cell wall in plants to facilitate fungal entry and proliferation (Esquerre-Tugaye *et al*., 2000; Kars, 2007). The maceration is achieved through the hydrolysis of the non-esterified α-1,4 linkages of the D-galacturonic acid residues found within the homogalacturonan domain of the primary cell wall (Esquerre-Tugaye *et al*., 2000; Andre-Leroux *et al*., 2009).

Plants have evolved to counteract the damaging effects of ePGs. This is achieved through the inhibition of ePGs by cell wall associated proteins called polygalacturonase-inhibiting proteins (PGIPs) (Cervone *et al*., 1987; Stahl *et al*., 2000; De Lorenzo *et al*., 2001; D'Ovidio *et al*., 2004; Gomathi *et al*., 2004; Howell *et al*., 2005; Juge, 2006). PGIPs have been characterised from numerous monocotyledonous and dicotyledonous plant species and are mostly present in multi-gene families in plant species with differing substrate specificities (Janni *et al*., 2006). They are part of the leucine-rich repeat (LRR) protein family which is characterised by an LRR motif, a highly conserved region which has been shown to play a pivotal role in recognition of pathogen-derived molecules such as ePGs (Kobe and Kajava, 2001; Mattei *et al*., 2001). *In vitro* studies have shown that the inhibitory action of PGIP on ePGs prolongs the existence of longer chain pectin fragments called oligogalacturonides, molecules believed to act as endogenous elicitors of a cascade of plant defence responses such as phytoalexin synthesis (Cervone *et al*., 1989; Desiderio *et al*., 1997; Aziz *et al*., 2004).

Overexpression of PGIP encoding genes in native and heterologous plant backgrounds has further elucidated the role of PGIP in plant defence where the respective host species showed a reduction in fungal infection susceptibility (Bergmann *et al*., 1994; Sharrock *et al*., 1994; Favaron *et al*., 1997; Esquerre-Tugaye *et al*., 2000; Powell *et al*., 2000; Stotz *et al*., 2000; De Lorenzo *et al*., 2001; Faize *et al*., 2003; Kemp *et al*., 2004; Aguero *et al*., 2005; Manfredini *et al*., 2005; Oelofse *et al*., 2006; Di Matteo *et al*., 2006; Federici *et al*., 2006; Joubert *et al*., 2007; Misas-Villamil *et al*., 2008; Hwang *et al*., 2010), though an increase in resistance was not seen in raspberry against two exoPGs from *B. cinerea* (Johnson *et al*., 1993).

The first grapevine-derived PGIP encoding gene, *VvPGIP1*, was isolated and characterised by De Ascensao (2001) from the *Vitis vinifera* L. cultivar Pinotage. In grapevine, a multigene family of PGIPs is not present and the expression of *VvPGIP1* has been shown to be tissue specific and developmentally regulated (Joubert, 2004). Overexpression of *VvPGIP1* in tobacco resulted in a transgenic population with reduced susceptibility to *B. cinerea* infection (Joubert *et al*., 2006). The well known resistance of some non-vinifera and American grapevine species towards fungal attack have prompted the isolation and characterisation of a further 37 PGIP encoding genes from the

grapevine species (Wentzel, 2005). Amino acid sequence analysis showed that these PGIPs had at least 95% homology with VvPGIP1 and homology in the LRR domain of greater than 94% (Wentzel, 2005; Venter, 2010). Nine of the non-vinifera PGIPs have been overexpressed in tobacco and compared to wild type (WT) and VvPGIP1 overexpressing lines in a whole plant infection assay (Venter, 2010). The transgenic populations overexpressing grapevine PGIPs all showed reduced disease susceptibility to *B. cinerea* infection. Moreover, the non-vinifera PGIPs performed better than the VvPGIP1 overexpressing lines (Venter, 2010).

Based on these results, two of the non-vinifera PGIP encoding genes (*PGIP*1012 and *PGIP*1038 from *V. doaniana* Munson and *V. caribaea* respectively) were constitutively overexpressed in *V. vinifera*. In this study, the derived putative transgenic populations were genetically and phenotypically characterised. The putative populations were screened for transgene integration patterns as well as expression. Having identified the independently transformed lines, PGIP activity was confirmed in the lines in comparison with the untransformed WT. A preliminary pathosystem with *Botrytis* was established and used for both detached leaf and whole plant infection assays. The transgenic lines showed a pronounced increase in disease susceptibility in the whole plant infection assays, when compared to the untransformed control. This surprising result is discussed in the context of our current understanding of host and non-host defence mechanisms against necrotrophic pathogens.

3.3 Materials and Methods

3.3.1 Gene constructs and grapevine transformation

The constructs used for genetic transformation of grapevine cultivars were described in Venter (2010) and obtained from the IWBT plasmid collection. Briefly, *PGIP*1012 and *PGIP*1038 (1002 bp ORFs), using *PGIP*-specific primers 5'-GTCGACATGGAGACTTCAAAAC-3' (*Sal*I restriction site underlined) and reverse 5'-TCTAGAACTTGCAGCTCTGGAGTGGAG-3' (*Xba*I restriction site underlined), were used for amplification (Venter, 2010). The derived amplicons were then subcloned into pGEM-T-Easy vector (Promega Corporation, Madison, USA) before being transformed in *Escherichia coli* strain DH5α and sequenced for confirmation. *Sal*1 and *Xba*1 were used to cut out the resulting *PGIP* sequences from pGEM-T-Easy vector and the fragment was cloned into the *Xho*1 and *Xba*1 sites of the pART7 binary vector multiple cloning site (Gleave, 1992). The *Not*1 cassettes of the pART7 vectors containing the *PGIP* ORF were then subcloned into the pART27 expression vector and mobilised into *Agrobacterium tumefaciens* strain EHA105 (Venter, 2010). Somatic embryogenic cultures, initiated from immature anthers were obtained from the IWBT's transformation platform. *Agrobacterium*-mediated transformation of the embryogenic callus cultures (as described by Iocco *et al*. 2001) proceeded to yield putative transformed lines of V. *vinifera* cv. Sultana, Redglobe and Merlot. The putative transformed population was obtained from the grapevine transformation platform.

3.3.2 Plant growth conditions

The putative transgenic germinating embryos were cultivated in tissue culture on MS medium (Murashige and Skoog, 1962) supplemented with 10 µg/ml of 6 benzyl-aminopurine (BAP) to induce shoot formation and 100 µg/ml kanamycin to maintain selective pressure. Developing shoots were then transferred to MS medium supplemented with 2.6 µg/ml of naphthalenacetic acid (NAA) to induce root formation and 100 µg/ml kanamycin to maintain selective pressure. The fully generated plantlets, as well as the untransformed controls, were maintained on MS medium supplemented with 15 g/litre of sucrose at 26˚C with a 16 hour light and 8 hour dark photoperiod. Vegetatively propagated copies of the population was hardened off in Grodan Plugs (Grodan A/S, Denmark) supplemented with hydroponic Kompel nutrient solution (Chemicult Products Pty Limited, Camps Bay, RSA) and subsequently transferred to potting soil (Double Grow, Durbanville, South Africa). The plants were maintained at 26˚C and 65% humidity under natural light in the greenhouse, watered every third day and supplemented with Nutrisol natural organic plant food (Envirogreen (PTY) Limited, Fleuron, Braamfontein, South Africa) fortnightly. Leaf material for the genetic characterisation and protein activity assay was sampled from the *in vitro* plantlets whilst hardened off plants were the source material for the infection assays.

3.3.3 PCR and Southern blot analysis of transgenic lines

Leaf disks (0.5-0.7 cm diameter) were harvested from transgenic and WT *in vitro* plantlets using a standard paper punch. The Sigma REDExtract-N-Amp^{TM} Plant PCR Kit was used for DNA extraction and PCR screening according to the manufacturer's instructions to confirm transgene integration. Oligonucleotide primers 5'-CTCATCTTCCGCAAGCTCTCTAA-3' and 5'-GCGATCATAGGCGTCTCGCATA-3' were used to amplify a 796 bp fragment consisting of part of the *PGIP* gene and part of the CaMV 35S promoter. The primer pair was particularly designed to target amplification of the transgenic *PGIP* genes only and not the native grapevine (*VvPGIP1*) gene. The typical PCR reaction mixture consisted of 5 x PCR Ready Mix (containing Hot Start antibody for specific amplication of genomic DNA and an inert dye that acts as a tracking dye), 0.25 µM primers and 100 ng of template in 20 µl reactions. PCR cycling conditions included an initial denaturation step at 94˚C for 5 min, followed by 30 amplification cycles consisting of denaturation for 30 sec at 94˚C, annealing at 55˚C for 30 sec, elongation at 72˚C for 30 sec and a final 5 min elongation step at 72˚C.

Genomic DNA was extracted from ground leaf material obtained from *in vitro* plantlets for Southern blot analysis. The genomic DNA was RNAse treated before being restricted with *Eco*RV and separated on 0.8% (w/v) agarose gels at 20 V overnight. *Eco*RV does not digest within the *PGIP* gene but restricts once outside the 5' end of the gene, thus providing an approximation of transgene insertion events. The digested DNA was transferred to positively charged Hybond-N nylon membranes according to Sambrook *et al*. (1989). The transferred DNA was crosslinked to the membranes before using DIG Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany) for pre-hybridisation at 42˚C for 3 hours. Hybridisation was carried out using a *PGIP* labelled probe at 42˚C

for 20 hours. The *PGIP* probe was digoxigenin-labelled according to the DIG System User's Guide (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were subjected to two low stringency 30 min washes in 2 x SSC: 0.1% SDS (w/v) at room temperature followed by two high stringency 30 min washes in 0.5 x SSC : 0.1% SDS (w/v) at 68˚C. Casein blocking buffer (1% w/v casein dissolved in Maleic acid buffer) was used for blocking and antibody binding (Anti-DIG AP Fab Fragments. Roche Diagnostics GmbH, Mannheim, Germany) for 1 hour and 30 minutes respectively. Membranes were then washed twice with DIG washing buffer (Maleic acid buffer containing 3 g/litre Tween 20) at room temperature for 30 min before being equilibrated in DIG detection buffer (0.1 M Tris/HCl pH 9.5, 0.1 M NaCl) for 2-5 min. CSPD (Roche Diagnostics GmbH, Mannheim, Germany) was used as substrate for the chemiluminescent detection on nucleic acids. BstE11 digest of λ DNA was used as the marker.

3.3.4 Northern blot assays

Leaf material was harvested from *in vitro* putative transgenic and WT Redglobe plantlets and frozen in liquid nitrogen. Total RNA was extracted from the ground leaf material based on a method developed for *Eucalyptus* by Suzuki *et al*. (2003) for use in northern blot assays. Total RNA was denatured for 10 min at 68˚C and then size fractionated on a 1.2% (w/v) agarose gel containing 0.6% formaldehyde at 100 V for at least 1 hour. RNA was then transferred and cross-linked to positively charged Hybond-N nylon membranes as described by Sambrook *et al*. (1989). Pre-hybridisation was carried out at 50˚C in DIG Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany) for 2 hours followed by hybridisation at 50˚C for 20 hours in the same *PGIP* probe used in Southern Blotting. The membranes were then washed twice at room temperature in low stringency 2 x SSC: 0.1% SDS solution for 20 min followed by two washes at 68˚C in high stringency 0.2 x SSC: 0.1% SDS solution for 20 min. Blocking, antibody application and chemiluminescent detection steps were as previously described for Southern blot assays in section 3.3.3.

3.3.5 PGIP activity assays

Crude protein was extracted from transgenic and WT *in vitro* ground leaf material for use in an agarose diffusion assay (Taylor and Secor, 1988). The assay makes use of polygalacturonic acid (PGA) as substrate for polygalacturonases (PGs) and PG activity typically results in formation of a clearing zone on the agarose plate (0.8% Type II Agarose, 0.5% PGA, 50 mM NaOAc pH 5.0) in small wells. A size reduction of the clearing zones is observed when PG activity is inhibited by active PGIP being co-inoculated in the same well.

The extraction protocol entailed homogenising 1 gram of ground leaf tissue in 1 ml sodium acetate extraction buffer (50 mM NaOAc, pH 5.0, 1 M NaCl) at 4˚C for 16-20 hours. This was followed by centrifugation at 4˚C for 30 min at 10 000 rpm and the supernatants were collected. Crude ePGs 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 $MgSO_4.7H_2O$, 0.6 μM $MnSO_4.H_2O$, 25 mM

KNO_3, 30 µM $ZnSO_4.7H_2O$, 0.9 µM $CuSO_4$ and 65 µM $FeSO_4$. The spores were grown at 24˚C for 5 days with gentle shaking on a standard rotary shaker. The culture was filtered through wire mesh and centrifuged at 7 000 g for 20 min before being precipitated with 70% ethanol at -20˚C overnight. The pellet was resuspended in 50 mM sodium acetate pH 5.5.

The BCA (bicinchoninic acid) protein assay reagent (Pierce Protein Research Products, Thermo Fisher Scientific Inc, USA) was used for protein quantification according to the manufacturers' instructions. A ratio of 1:5 crude PG extract to crude PGIP extract was used in the agarose diffusion assay and all assays were done in duplicate. A boiled PGIP extract was included for each plant line to ensure that the activity observed is not due to any artefacts of the extraction procedure. The area of the clearance zones was measured using AlphaImager Imaging System Version 5.5, SpotDenso Option (Analytical and Diagnostics Products, Randburg, South Africa) and expressed as a percentage of zone reduction compared to the ePG zone without any PGIP extract added.

3.3.6 Detached leaf and whole plant antifungal assays

A *B. cinerea* strain isolated from a South African vineyard (as reported in Joubert *et al*., 2006), was utilised for the infection assays. The strain was grown on sterile apricot halves (Naturlite, Tiger Food Brands Limited, South Africa) in a dark growth chamber at 23˚C until sporulation occurred. Spores were harvested and evaluated for their viability and germination potential on 0.8% (w/v) water agar.

Greenhouse acclimatised plants were used for detached leaf and whole plant infection assays. Only the plant lines that were positive for gene presence using PCR, gene expression using northern blot assay, showed transgene integration using Southern blot assay and active protein using the agarose diffusion assay were used for the antifungal assay. Fully expanded mature leaves were selected for infection assays. Since a pathosystem with grapevine and *Botrytis* has not yet been established to follow the disease progression and optimised quantification of symptoms/lesions, both detached leaf and whole plant infection assays were performed. Initially 1000 spores per infection spot were used for a detached and whole plant infection assay. Subsequently a further detached leaf assay with 500 spores per infection spot was performed (see **Table 1** for lines used). Four leaves were infected per line for the detached leaf assay using 500 spores per infection spot (four spots per leaf) whilst two leaves were infected for the 1000 spores per infection spot assay (three spots per leaf). For the whole plant antifungal assay, four leaves per plant were infected with three infection spots per leaf. The leaves were infected on the adaxial side. Detached leaves were pre-incubated in sealed plastic containers on wetted sterile filter paper with high humidity for 24 hours prior to infection whilst whole plants were pre-incubated in perspex high humidity chambers for the same amount of time. The plastic containers and perspex chambers were maintained at room temperature under a 16/8 hour light/dark cycle prior to and throughout the course of the infection. Disease progression was monitored, for both the detached and whole plant antifungal assays, by measuring lesion diameter at 24 hour intervals from 2 days post-infection when lesions started developing.

A custom made programme was written in perl (provided by Dan Jacobson, IWBT) to parse the data, calculate the means and standard deviations and perform an all-against-all set of t-tests to determine if there were significant differences (p value < 0.05) between plant lines at each time point for each of the detached leaf (1000 and 500 spores) and whole plant infection assays. This resulted in the performance of nearly 13 000 t-tests. A graph was created to represent the statistical relationships amongst the plant lines with an edge created whenever two plant line populations were statistically indistinguishable from one another. The resulting graph was visualised in Cytoscape (Shannon *et al*., 2003; Cline *et al*., 2007).

Table 1. Summary of plant lines used for detached and whole plant antifungal assay showing the number of infected leaves per line in brackets for detached leaf assay and the number of clonal copies per line in brackets for the whole plant antifungal assay

Line	1000 spores per infection spot		500 spores per infection spot
	Detached leaf assay	Whole plant assay	Detached leaf assay
Wild type	+ (2)	+ (2)	+ (6)
R1012-1	+ (2)	+ (1)	+ (4)
R1012-13	+ (2)	+ (2)	+ (4)
R1012-15	+ (2)	+ (1)	+ (4)
R1012-16	+ (2)	+ (2)	+ (4)
R1012-24	_	_	+ (4)
R1012-28	+ (2)	+ (1)	+ (4)
R1038-2	+ (2)	+ (2)	+ (4)
R1038-15	+ (2)	+ (2)	+ (4)
R1038-17	+ (2)	+ (2)	+ (4)
R1038-35	_	_	+ (4)
R1038-38	_	_	+ (4)
R1038-39	_	_	+ (4)
R1038-43	+ (2)	+ (1)	+ (4)
R1038-45	+ (2)	+ (1)	+ (4)
R1038-46	_	_	+ (4)
R1038-48	_	_	+ (4)
R1038-51	_	_	+ (4)
R1038-52	+ (2)	+ (1)	+ (4)
R1038-53	_	_	+ (4)
R1038-56	_	_	+ (4)
R1038-57	_	_	+ (4)
R1038-59	+ (2)	+ (2)	+ (4)
R1038-60	+ (2)	+ (1)	+ (4)
R1038-61	+ (2)	+ (2)	+ (4)
R1038-62	+ (2)	+ (1)	+ (4)
R1038-66	_	_	+ (4)
R1038-67	+ (2)	+ (1)	+ (4)
R1038-68	+ (2)	+ (1)	+ (4)
R1038-69	_	+ (1)	+ (4)

+ included in the assay
- not included in the assay

3.4 Results

3.4.1 Generating a population of *V. vinifera* transgenic lines expressing non-vinifera *PGIP* genes

Two non-vinifera *PGIP* genes, *PGIP*1012 and *PGIP*1038, from *V. doaniana* Munson and *V. caribaea* respectively, cloned into the plant expression vector pART27, were transformed into the *V. vinifera* cultivars Redglobe, Sultana and Merlot using *Agrobacterium*. A summary of the putative transgenic populations generated is presented in **Table 2**. Putative transgenic populations were successfully generated for both PGIPs 1012 and 1038 in Redglobe and Sultana (designated R1012, R1038, S1012 and S1038 respectively). Only PGIP1012 putative transgenic population was obtained for Merlot and as a result, the population was not included in any further analysis. **Figure 1** shows some of the stages involved in the hardening off of a putative transgenic population.

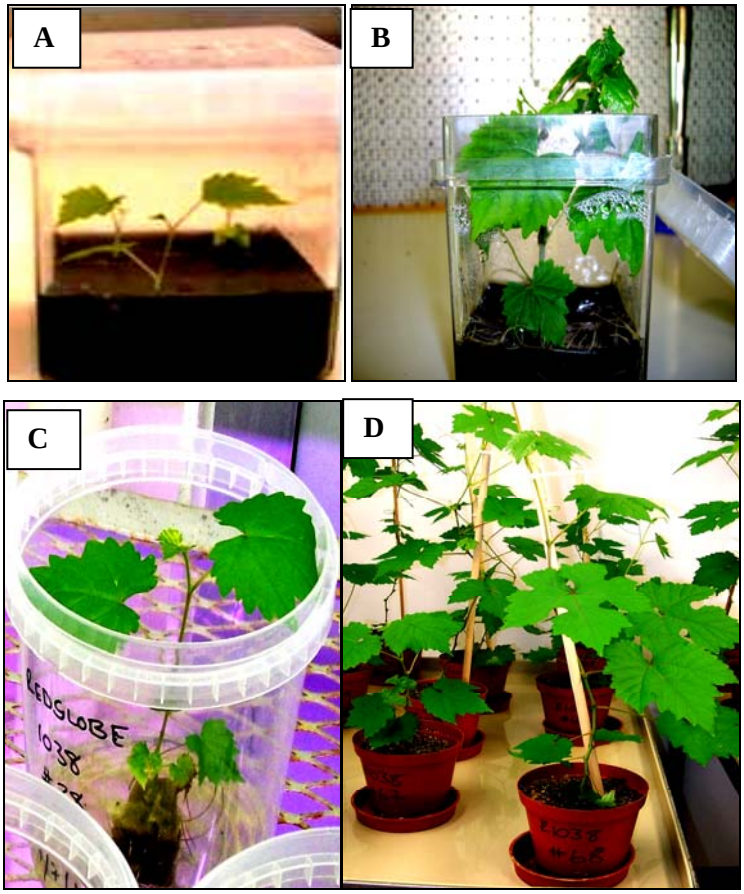


Figure 1. Stages in the hardening-off process of grapevine plants. (**A**) Germinating embryos are first placed on shooting media supplemented with kanamycin and (**B**) the shoots are then transferred to rooting media supplemented with kanamycin. (**C**) The growth points are then transferred to Grodan plugs which are kept moist and supplemented with hydroponic nutrient solution. (**D**) The whole plant is finally transferred to potting soil and fed using Nitrosol organic plant food fortnightly.

Table 2. Summary of total putative transgenic lines generated for each of the different constructs per cultivar

Cultivar	Construct	Number of putative transgenic lines generated
Redglobe	1012	12
	1038	51
Sultana	1012	17
	1038	45
Merlot	1012	6

3.4.2 Analysis of the putative transgenic population

For both *in vitro* and the hardened off putative transgenic and untransformed WT lines, no obvious phenotypic differences were observed in terms of size of plants, vegetative growth and plant architecture. The putative Redglobe and Sultana transgenic lines were screened for the presence of the transgenic *PGIP* genes with PCR, using transgene-specific primers designed for the specific amplification of only the non-vinifera *PGIP*s and not the native copy of the gene. Untransformed Redglobe and Sultana lines were used as controls. **Figure 2** shows the results obtained from screening the 12 putative Redglobe lines overexpressing PGIP1012 as a representative example. Nine out of the twelve lines tested, yielded the expected 796 bp fragment whilst 31 PGIP1038 overexpressing lines, out of the 51 lines tested, gave positive results. On the other hand, only three out of 17 Sultana lines overexpressing PGIP1012 were positive for transgene presence and eight out of 45 lines overexpressing PGIP1038 gave positive results. A summary of the results obtained is shown in **Table 3.**

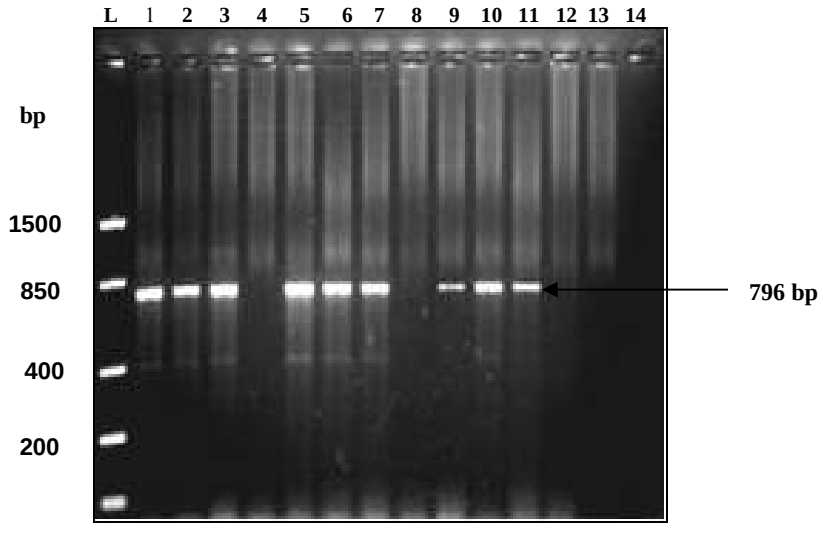


Figure 2. PCR run for R1012 including the wild type Redglobe line, which was used as a negative control. DNA was extracted from leaf discs as mentioned in section 3.3.2 and the extracts were used as template for PCR. The marker lane (L) contains the FastRuler^{TM} Low Range DNA Ladder from Fermentas (in base pairs, bp). The numbers 1 to 12 represent the putative transgenic lines tested, 13 represents the wild type negative control and 14 represents the PCR no-template water control. The wild type and the water control did not yield any PCR product, as expected.

Table 3. Summary of PCR screening results for non-vinifera *PGIP* gene presence in Redglobe and Sultana putative transgenic populations

Cultivar	Construct	Line	Transgene presence result using PCR	Cultivar	Construct	Line	Transgene presence result using PCR
Redglobe	**1012**	1	+	**Sultana**	**1012**	1	+
		5	+			14	-
		9	+			15	-
		12	-			16	+
		13	+			17	-
		15	+			18	-
		16	+			19	-
		22	-			20	-
		24	+			21	-
		27	+			22	-
		28	+			23	-
		29	-			27	+
						30	-
						31	-
						32	-
						33	-
						34	-
	1038	2	+		**1038**	1	-
		9	+			16	-
		13	+			23	-
		14	-			30	-
		15	-			31	-
		17	+			32	-
		18	-			33	-
		19	-			34	-
		23	+			36	-
		24	-			37	-
		27	-			38	-
		28	-			39	-
		30	+			40	-
		31	-			42	-
		32	-			43	-
		34	-			45	-
		35	+			46	-
		36	+			47	-
		38	+			48	-
		39	+			50	+
		40	+			52	-
		42	+			53	-
		43	+			54	-
		44	-			55	-
		45	+			56	-
		46	+			57	-
		47	-			60	-
		48	+			63	-
		49	-			65	-
		50	-			67	-
		51	+			68	+
		52	+			69	-
		53	+			71	-
		54	+			72	-
		56	+			75	+
		57	+			76	+
		58	-			77	+
		59	+			78	-
		60	+			79	-
		61	+			81	-
		62	+			82	+
		64	+			83	-
		65	+			84	+
		66	+			85	+
		67	+			86	-
		68	+			87	-
		69	+				
		70	-				
		71	-				
		72	-				
		73	-				

+ transgene present - transgene absent

Transgene expression was confirmed using Northern blot assays for the whole transgenic population. A hybridising band of 1002 bp was expected for all lines that expressed the transgene. The wild type untransformed Redglobe and Sultana lines were included as negative controls and, as expected, did not yield any bands on the blot since the native gene is not expressed in leaves unless induced. **Figure 3** shows a subset of R1038 lines tested for transgene expression. The nine PGIP1012 overexpressing Redglobe lines that yielded positive PCR results also tested positive for transgene expression whilst 29 out of the 31 PCR positive PGIP1038 overexpressing lines tested positive for transgene expression (**Table 4**). From the 17 Sultana lines overexpressing PGIP1012 screened, only the three lines that had tested positive for transgene presence also gave positive results for transgene expression. On the other hand, only four of the eight PCR positive PGIP1038 overexpressing Sultana lines were positive for transgene expression. Based on the low numbers of Sultana lines with positive transgene presence and expression, indicative of a high level of transformation escapes, this transgenic population was not utilised for any further assays. Thus only Redglobe results are presented henceforth.

Table 4. Summary of PCR results for transgene presence compared to Northern Blot assay results for transgene expression.

Cultivar	Construct	No. of lines tested	No. of lines positive for transgene presence using PCR	No. of lines positive for transgene expression using northern blot assay
Redglobe	1012	12	9	9*
	1038	51	31	29*
Sultana	1012	17	3	3
	1038	45	8	4

* Lines used for all further analyses.

A total of 30 Redglobe lines, six overexpressing PGIP1012 and 24 overexpressing PGIP1038, that tested positive for both transgene presence and expression were further analysed using Southern blot assays to confirm gene integration events and specifically the transgene copy numbers. The other eight lines were lost due to contamination. **Figure 4** shows a subset of the different integration events in individual lines from one of the R1038 runs illustrating the native PGIP band, which could be detected in the untransformed wild type and all the transgenic lines. The results summarised in **Table 5** showed that the gene copy numbers varied between lines, with some lines having a single copy of the transgene and others having up to six copies of the transgene.

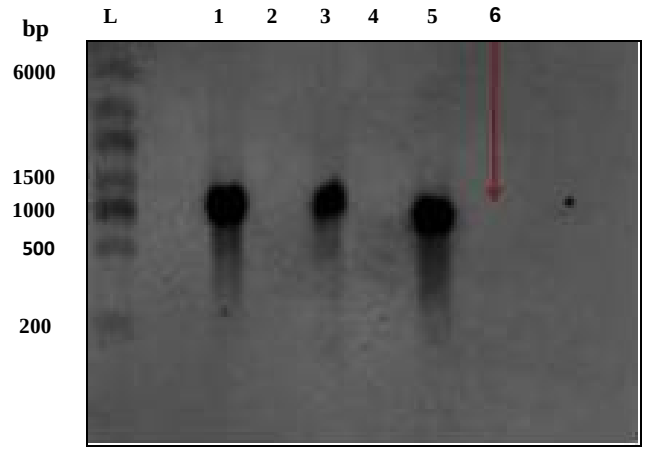


Figure 3. Northern blot assay showing a subset of five transgenic PGIP1038 Redglobe lines. The marker lane (L) contains the Riboruler^{TM} High Range RNA Ladder (in bp) and the numbers 1-5 represents the five transgenic lines tested. The WT Redglobe which was used as the negative control line is shown in lane 6.

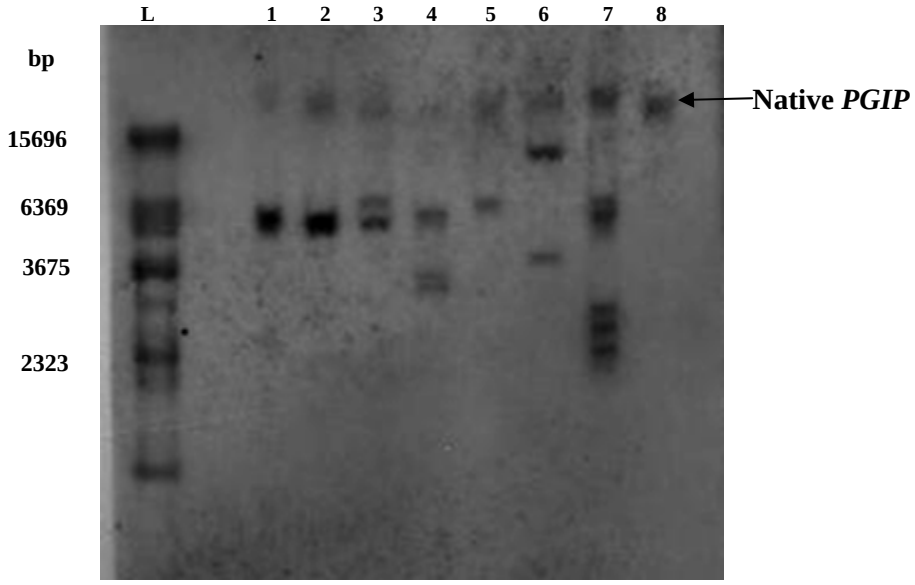


Figure 4. Southern blot assay showing a subset of seven R1038 transgenic and the wild type control. The marker lane (L) contains Lambda DNA-BstE II Digest (in bp). The numbers 1 to 7 represent the transgenic lines tested whilst lane 8 contains the wild type negative control line. The top band which is uniform for all tested lines, including the wild type, represents the native *PGIP* gene copy and was the only band obtained for the negative control on the blot.

3.4.3 PGIP activity determination of the transgenic population

All the transgenic Redglobe plant lines were then subjected to PGIP activity assays against crude PG extracts from *B. cinerea* using the agarose diffusion assay which utilises polygalacturonic acid as substrate for the ePGs (Taylor and Secor, 1988). All assays were carried out in duplicate for each sample at pH of 5.0, and they were allowed to proceed for 16 hours at 30˚C. A boiled extract was included as a control for each sample to ensure that artefacts of the extraction procedure did not influence the size of the zones. **Figure 5** shows results for a subset of seven of the tested lines, illustrating the differences in zone sizes between the untransformed wild type plant line and lines exhibiting total, partial and no inhibitory activity against a crude extract of BcPGs.

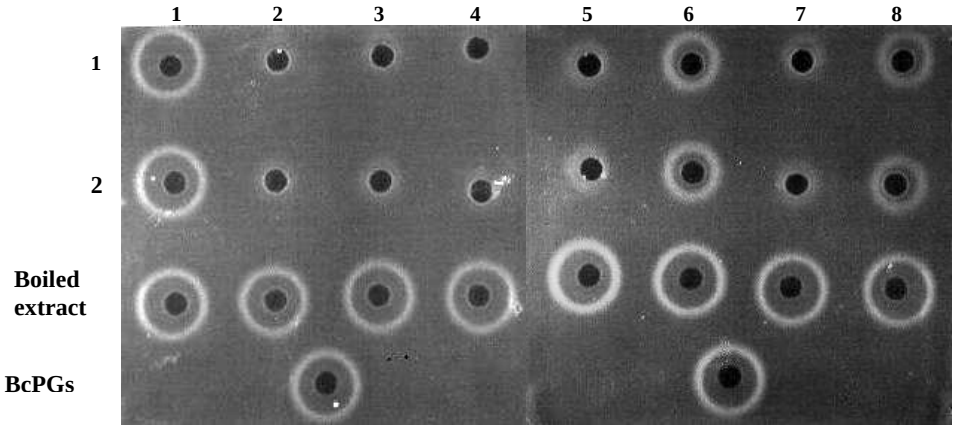


Figure 5. PGIP activity assay results for a subset of the total lines analysed using a semi-quantitative agarose diffusion assay (Taylor and Secor, 1988). Rows labelled 1 and 2 represent the duplicate assays per tested line. Column 1 contains the wild type extract which did not show any inhibitory activity against the crude PGs. Columns 2, 3, 4, 5 and 7 represent R1012-1, R1012-13, R1012-15, R1038-56 and R1038-59 transgenic lines respectively, with PGIP extracts that exhibit high inhibitory activity against the BcPGs whilst columns 6 and 8 (R1038-57 and R1038-60 respectively) only showed partial inhibition. Boiled extract for each sample was included in the assay. All zones were compared to that formed by the BcPG sample without any leaf extract added.

A summary of the combined PCR, Northern blot, Southern blot and PGIP activity assay results for all the lines is shown in **Table 5**. The data presented hereafter is for detached leaf and whole plant antifungal assays on the transgenic lines showing 100% zone reduction for the PGIP activity assay.

Table 5. Summary of analyses of transgenic populations (*PGIP* gene integration, expression, copy numbers and PGIP activity assays).

Sample	Transgene presence results using PCR	Transgene expression results using northern blot assay	%Zone reduction using Agarose Diffusion assay	Transgene copy numbers using Southern Blot assay
Wild type	-	-	0	0
R1012-1	+	+	100	3
R1012-13	+	+	100	1
R1012-15	+	+	100	3
R1012-16	+	+	100	3
R1012-24	+	+	100	6
R1012-28	+	+	100	1
R1038-2	+	+	100	1
R1038-9	+	+	100	1
R1038-15	+	+	100	2
R1038-17	+	+	100	3
R1038-35	+	+	65.0*	2
R1038-38	+	+	100	2
R1038-39	+	+	100	1
R1038-43	+	+	100	2
R1038-45	+	+	14.7*	4
R1038-46	+	+	0*	1
R1038-48	+	+	100	2
R1038-51	+	+	0*	6
R1038-52	+	+	100	4
R1038-53	+	+	47.0*	1
R1038-56	+	+	100	3
R1038-57	+	+	43.0*	3
R1038-59	+	+	100	2
R1038-60	+	+	40.0*	5
R1038-61	+	+	100	4
R1038-62	+	+	100	6
R1038-66	+	+	100	4
R1038-67	+	+	100	5
R1038-68	+	+	32.0*	1
R1038-69	+	+	100	2

* excluded from infection assays

3.4.4 Detached leaf and whole plant infection assays with *B. cinerea*

3.4.4.1 Infections of detached leaves and whole plants of grapevine: symptom development

Given the lack of a well characterised pathosystem for experimental and quantitative analysis of resistance and/or susceptible phenotypes in the host-pathogen interaction of grapevine and *B. cinerea*, the infection system is still under development in our hands. Firstly, for both the detached leaf and the whole plant infections, fully expanded mature leaves were shown to be the most suitable for infections, since younger leaves were completely consumed in the initial stages of the infection without the possibility of following lesion development over time (results not shown). The typical appearance of leaves utilised for the detached and whole plant infection assays is shown in **Figure 6A** and **B** respectively. Secondly, lesion development in the detached leaves was slower compared to that of infected leaves in the whole plant assay. For example, the average lesion diameter of all spots that had formed lesions in the detached leaves at day two post infection was 1.9 mm compared to 2.6 mm in the whole plant antifungal assay. Thirdly, for scoring purposes, two distinct types of lesions were recorded during the course of the experiments, namely, primary lesions that formed typically within 48 hours post infection and spreading lesions that expanded beyond the infection spots within 72 hours post infection (**Figure 7A** and **B** respectively). Infection spots where no lesions formed as illustrated in **Figure 7C**, were scored as an unsuccessful infection. Lesion measurements thereafter reflect active infections that were established and spreading within three days post infection.

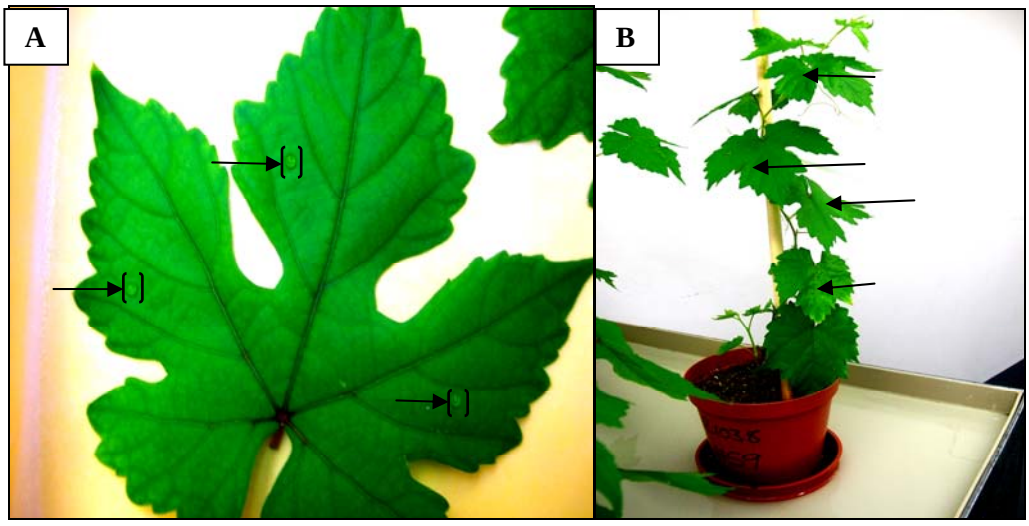


Figure 6. Typical leaf stage used for (**A**) the detached leaf assay showing the three infection spots (indicated with brackets and arrows) and (**B)** whole plant assay showing the typical plant size and leaves selected for infection. Two to four leaves were selected for infection in the detached leaf assay and four leaves, indicated by the arrows, were selected for the whole plant infection assays. For both assays, each leaf was infected with three to four spots as illustrated in (**A).**

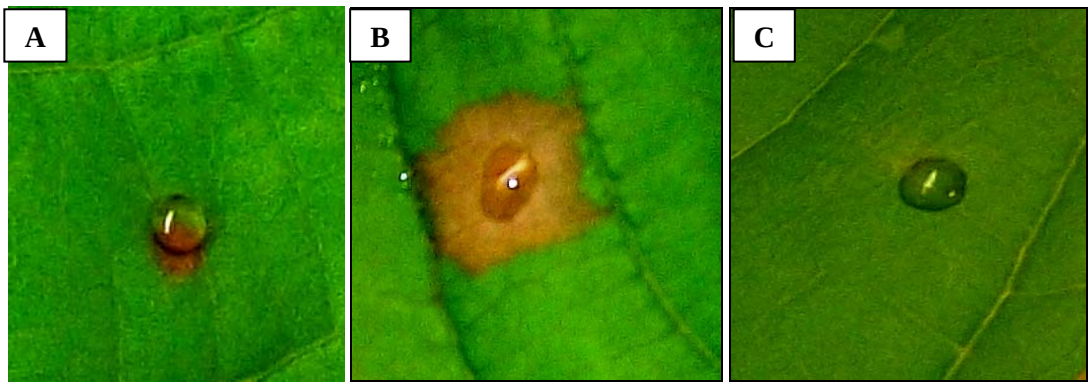


Figure 7. Examples of different types of lesions observed during the course of both the detached and the whole plant infection assays. (**A**) shows a typical primary lesion at 2 days post infection that later developed into a secondary lesion, (**B**) shows a secondary spreading lesion taken at day 3 post infection and (**C**) shows an infection spot at 6 days post infection that did not develop into a necrotic lesion. This was scored as an unsuccessful infection.

3.4.4.2 Detached leaf assays

The first detached leaf assay was conducted on a subset of transgenic and untransformed control lines (listed in **Table 1)** using 1000 *Botrytis* spores per infection spot. The infection potential of the *B. cinerea* spore suspension utilised for the infections was calculated to be 90 - 95%. Of all the infection spots, 36% had developed into primary lesions by two days post inoculation (dpi) whilst the rest developed primary lesions by three dpi. Overall, 100% of the infection spots had formed lesions at the end of the experiment, though only 85% developed into secondary spreading lesions. Lesion diameter was measured from two dpi when primary lesions started developing. The rate of lesion development was fast both in the transgenic lines and in the wild type, with the development of fungal reproductive structures on some of the plant lines appearing from four dpi. **Figure 8** shows photographs of the disease progression for a subset of the lines tested from two dpi to seven dpi. **Figures 9A and 10A** shows a summary of the growth of lesions in the transgenic lines and the wild type control. When the lesion diameters of all the spots on all the leaves per line are compared with those on the wild type, it is clear that for both R1012 and R1038 the transgenic lines do not represent a homogenous resistant or susceptible population. Instead most lines are as susceptible as the wild type, whereas a few lines show increased or decreased susceptibility. Due to the fact that the spreading lesions started flowing into one another and had variable shapes from day six post infection, day five was used to express the statistical relevance. Analysis of the lesion diameters using a custom built statistical programme which performed t-tests on all data points ($p < 0.05$) relative to each other, showed that for R1012 at five dpi, lines 1, 15 and 16 grouped with the wild type, whereas lines 13 and 28 both exhibited higher susceptibility to *B. cinerea* infection than the WT in terms of lesion growth. For R1038, most of the lines grouped with the wild type at five dpi, with only line 52 exhibiting smaller lesions, which were statistically different from the rest of the group.

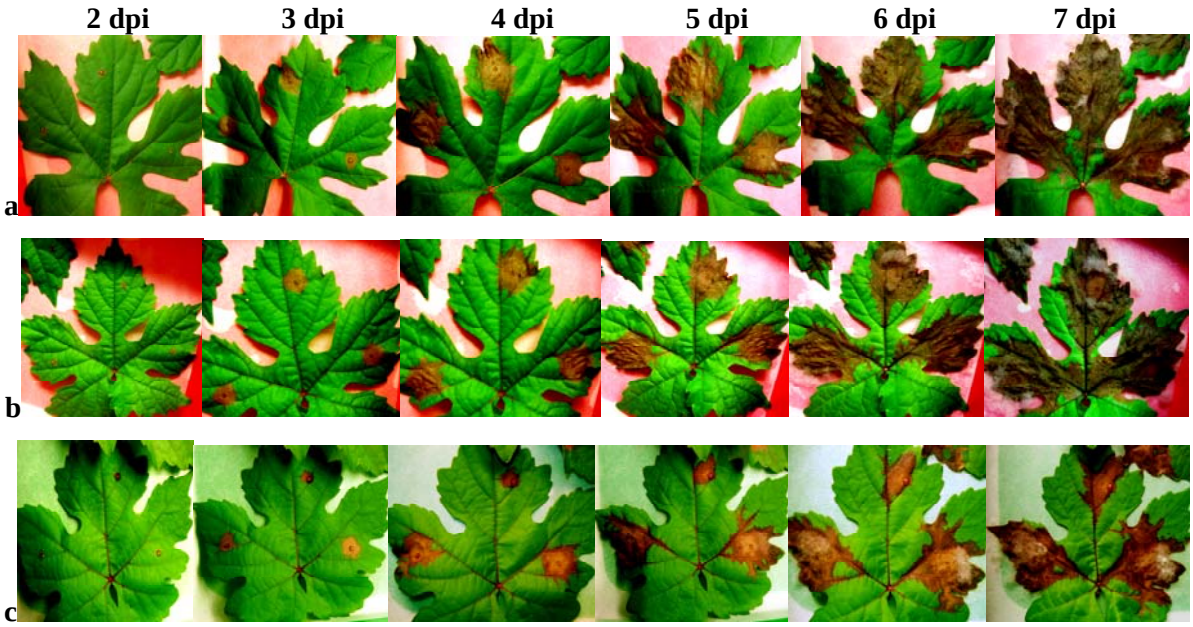
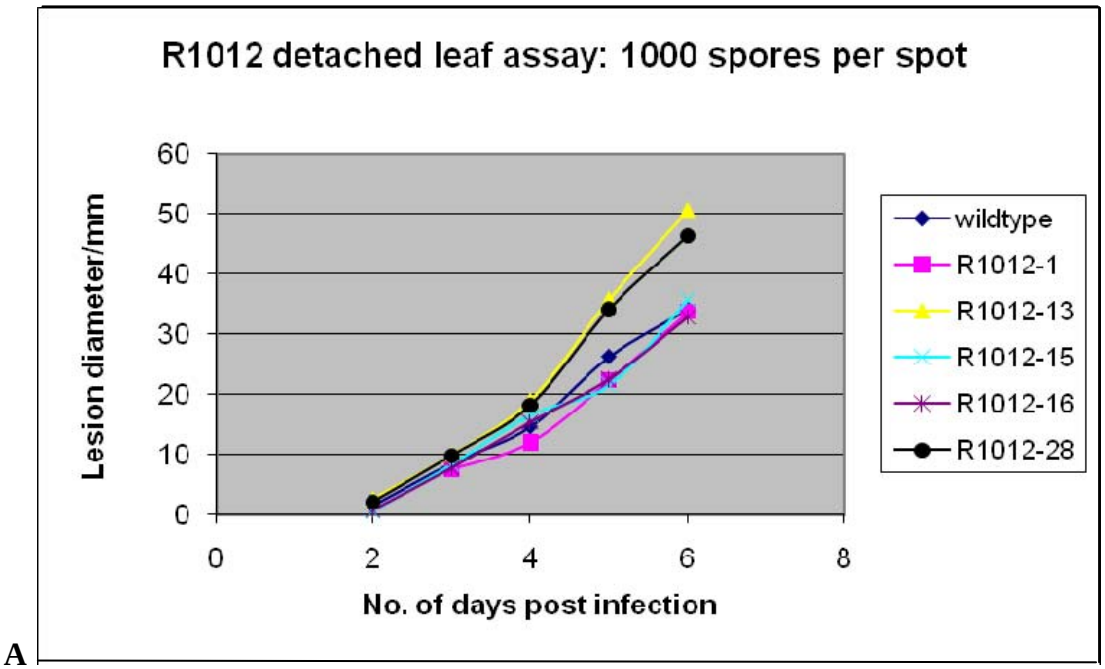
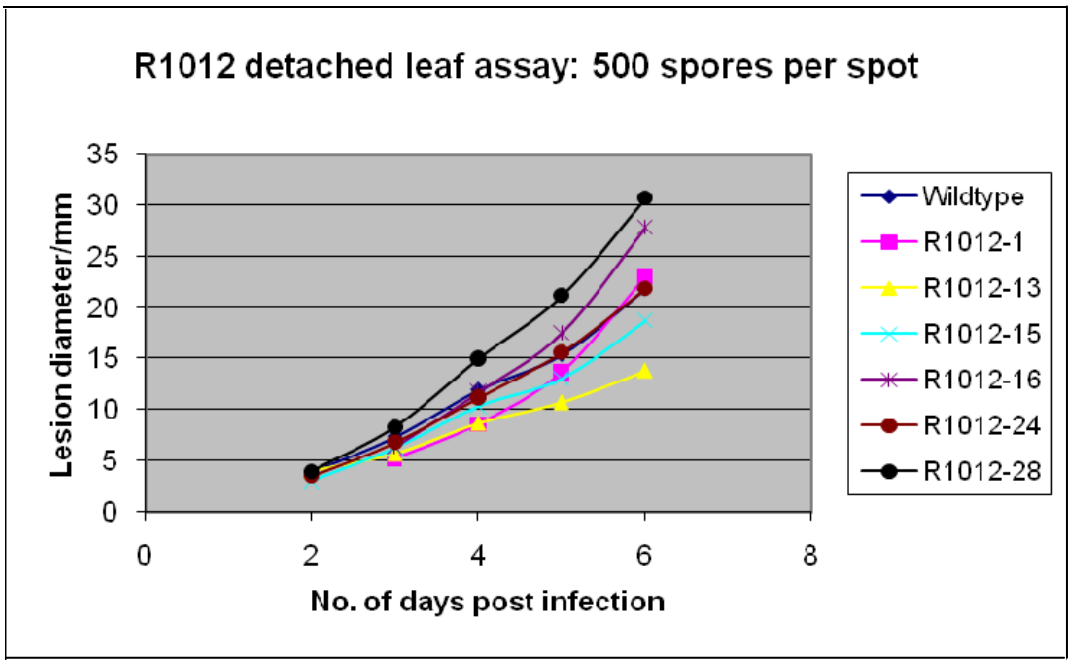


Figure 8. Detached leaf infection assay of a subset of plant lines tested showing lesion development on the leaves of (**a)** R1012 line 28, (**b)** R1038 line 67 and (**c)** WT infected with 1000 *B. cinerea* spores per infection spot from day 2 to day 7 post infections (dpi).

A second detached leaf assay was performed, using all lines in the confirmed transgenic population, and at 500 *Botrytis* spores per infection spots to evaluate whether a smaller spore load might lead to a more distinct phenotype. The number of leaves tested per line was also increased to four per line, with four infection spots each (refer to **Table 1**). Primary lesions started developing on some of the plant lines, two dpi. More than 90% of the infection spots developed into primary lesions whilst only 69% developed further into secondary spreading lesions. **Figure 9B** and **Figure 10B** show a summary of the lesion diameters of the transgenic Redglobe lines compared to the wild type. The second assay progressed slightly slower, but overall, same observations could be made as with the first assay. The transgenic population consisted of some lines grouping with the wild type and some with larger and some with smaller lesions. The pattern was in some instances comparable between the two detached leaf assays (when considering the specific response of a transgenic line versus that of the wild type), but not in all cases. For R1012, most lines again grouped with the wild type, whereas lines 16 and 28 exhibited larger lesion sizes and line 13 smaller. For R1038, 11 lines were found to be statistically different from the wild type at five dpi from which nine lines had larger lesions than the wild type, whilst two (lines 66 and 69) had significantly smaller lesions. Four lines grouped together with the wild type. In all the detached leaf assays, all the infections ended in complete maceration of the infected tissue, clearly indicating that none of the lines displayed any resistance phenotype (also refer to **Figure 8**). The only exceptions were R1038 lines 66 and 69 that showed nearly an immune response in the second detached leaf assay with 500 spores.



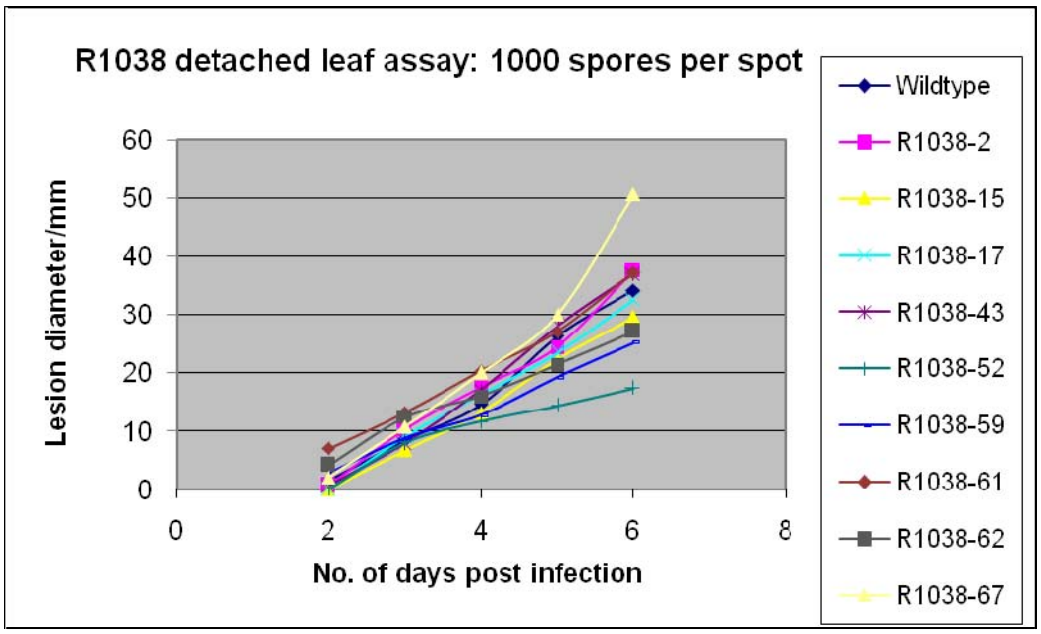
A



B

Figure 9. Lesion development on detached leaves from Redglobe lines overexpressing *PGIP*1012 (R1012) and the untransformed wild type lines infected with (**A**) 1000 *B. cinerea* spores per infection spot and (**B**) 500 spores per infection spot. Disease progression was monitored by measuring lesions from two days post infection. By day seven, about 20% of the lesions could no longer be accurately measured thus the data presented is up to day six.

A



B

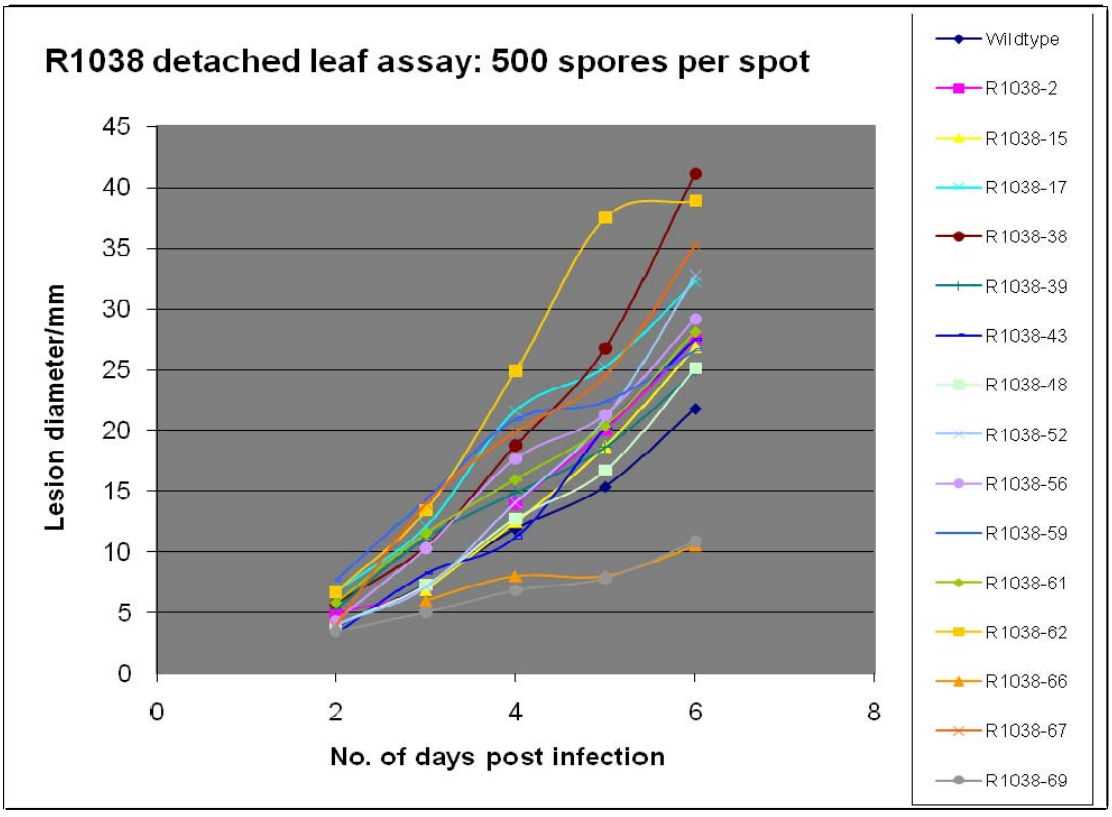


Figure 10. Lesion development on detached leaves of Redglobe lines overexpressing *PGIP*1038 (R1038) and the untransformed WT lines infected with (**A**) 1000 *B. cinerea* spores per infection spot and (**B**) 500 spores per infection spot. Disease progression was monitored by measuring lesions from two days post infection. By day seven, about 15% of the lesions could no longer be accurately measured, thus the data presented is for lesions diameter up to six days post infection.

3.4.4.3 Whole plant antifungal assay using 1000 spores per infection spot

Hardened off transgenic Redglobe plant lines were utilised for a whole plant antifungal assay against a *B. cinerea* suspension in 50% grape juice (refer to **Table 1**). The plant lines were acclimatised in the Perspex high humidity chambers for 24 hours prior to infection. At least 99% of the infection spots developed into primary lesions and ultimately secondary spreading lesions. The onset of lesion formation and the rate of lesion growth were very high in the transgenic plants compared to the untransformed wild type, as illustrated in **Figure 11**. Extensive fungal reproductive structures developed on most of the transgenic lines as early as five dpi. Accurate lesion measurements for most of the transgenic lines were no longer feasible by day six due to the spread of infection. By day seven, some of the heavily infected leaves had fallen off the plants. Comparing lesion sizes for all the spots on all the leaves per line from two dpi, it was clear that all transgenic populations were more susceptible than the wild type (**Figure 12**). Statistical analysis of the lesion diameters using a custom made statistical programme which performed t-tests on all data points ($p < 0.05$) relative to each other, showed that at five dpi for both R1012 and R1038 populations, the lesion diameter of the wild type was significantly different from the transgenic lines, (**Figure 13**) as illustrated by the Cytoscape-generated graph. From day six onwards, the transgenic lines showed increased reproductive structures of *B. cinerea* compared to the wild type (refer to **Figure 11**, 6-7 dpi). In the whole plant infection assay, as well as in the detached leaf assay, a slight difference between the necrotic lesions that formed on the R1012 versus R1038 lines was observed. The former developed slightly drier necrotic lesions than those that formed on the R1038, which typically had extremely fast and wet spreading lesions once the primary lesion has been formed.

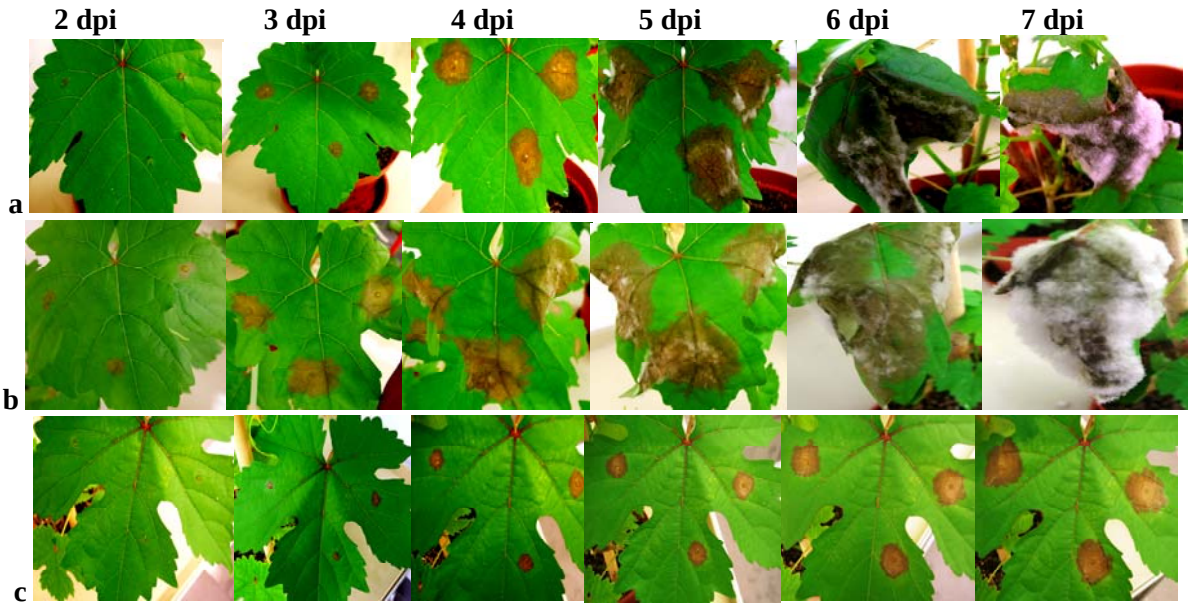
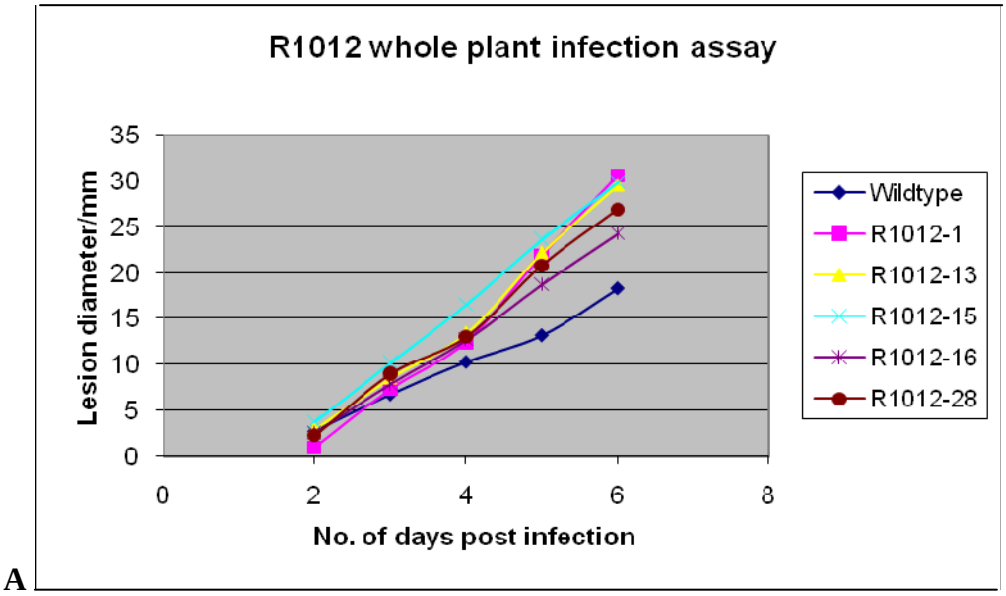
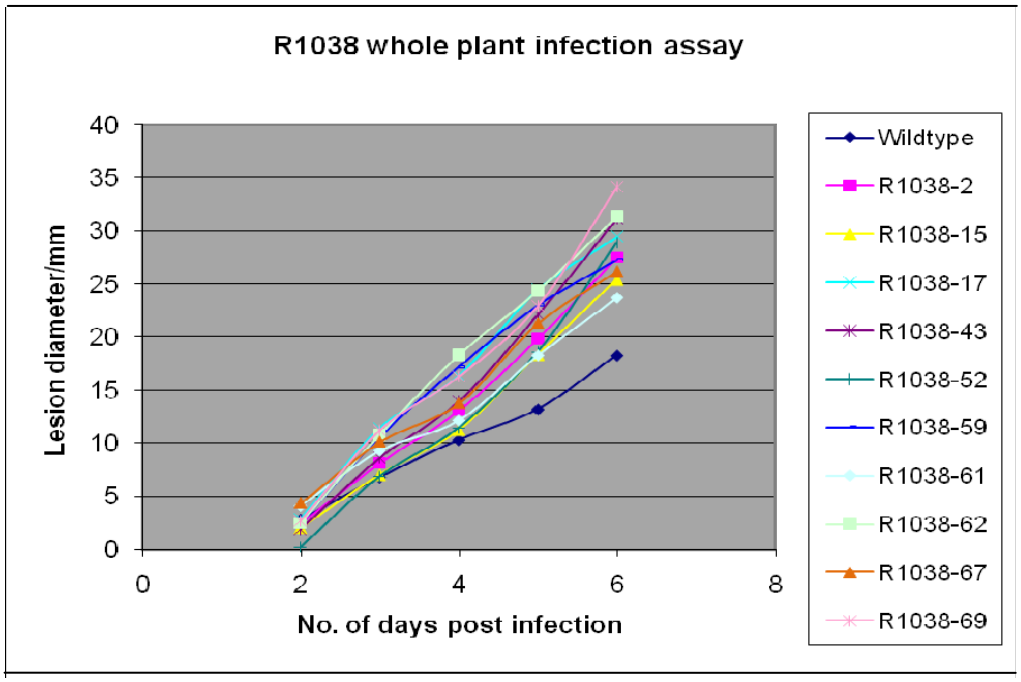


Figure 11. Lesion development of a representative subset of plant lines challenged with *B. cinerea* in a whole plant antifungal assay (1000 spores per spot). For (**a**) R1012 line 13 and (**b**) R1038 line 59, primary lesion development and spreading was faster in transgenic plants compared to the (**c**) wild type. Lesions were measured at 24 hour intervals.



A



B

Figure 12. Lesion development on whole plant Redglobe transgenic lines overexpressing (**A**) *PGIP*1012 and (**B**) *PGIP*1038. Untransformed WT lines were used as controls. All plants were challenged with 1000 *B. cinerea* spores per infection spot with four leaves being infected per plant (three infection spots per leaf). Lesions were measured at 24 hour intervals from day two post infection.

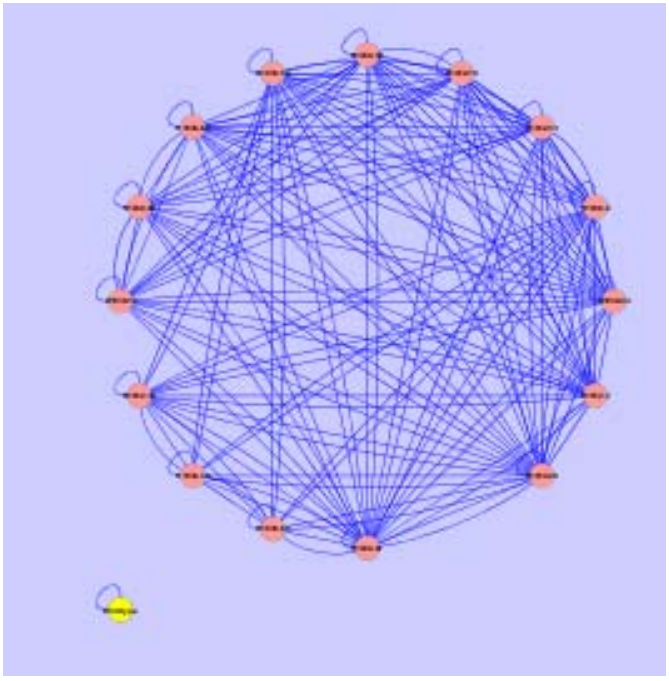


Figure 13. Cytoscape-generated graph showing the wild type (highlighted in yellow) clearly separated from the transgenic lines (highlighted in orange/dark pink) at five days post infection. The graph illustrates that at day five, the difference in lesion diameter of the wild type compared to all the transgenic lines was statistically significant at $p<0.05$.

3.5 Discussion

The role of PGIP in plant defence has been widely investigated through overexpression studies in numerous plant backgrounds. Several plant species where PGIPs were overexpressed exhibited a reduction in disease susceptibility against *B. cinerea* (Sharrock *et al*., 1994; Powell *et al*., 2000; Stotz *et al*., 2000; Faize *et al*., 2003; Aguero *et al*., 2005; Oelofse *et al*., 2006; Joubert *et al*., 2006). This has been attributed to PGIP's ability to directly inhibit fungal ePGs and also prolong the existence of longer length cell wall fragments called oligogalacturonides which have been shown, *in vitro*, to act as elicitors of numerous defence responses (Aziz *et al*., 2004; Cervone *et al*. 1989; Desiderio *et al*., 1997).

3.5.1 Transgene transcripts and PGIP activity is increased in leaves of transgenic grapevine lines overexpressing two non-vinifera PGIP encoding genes

Two non-vinifera PGIP encoding genes were expressed in *V. vinifera* cultivars and putative transgenic populations were generated and genetically and phenotypically characterised. PCR was used to screen the putative transgenic lines for transgene presence, using primers specifically designed to only amplify the transgene and not the *V. vinifera PGIP* gene. The transgene could be detected in PCR, meaning the transformation events were successful. Transgene expression was tested in leaves of *in vitro* plantlets using northern blot assays and an untransformed WT line was used as a control. As expected, no band was produced for the WT lines since the native PGIP is not expressed in grapevine leaves unless it is induced (Joubert, 2004). Independent transgene integration events were confirmed

for the transgenic plants using Southern blot assays. The native gene was detected in the WT and the same band size was uniform in all the transgenics, confirming that it was the native copy of the gene. The population had between 1-6 copies of the transgene (see **Table 5**). PGIP activity of the transgenic lines was confirmed using an agarose diffusion assay (Taylor and Secor, 1988). Crude leaf protein extracts were tested for PGIP activity against a crude mix of PGs from *Botrytis* using polygalacturonic acid as substrate for the PGs. The inhibition profile of the two non-vinifera PGIPs against the individual BcPGs is not known and it is unknown if these PGIPs are inhibiting all the BcPGs equally. It has been shown that BcPG1 and 2 are important for virulence and pathogenicity (Kars *et al*., 2005). Individual inhibition studies with the individual BcPGs will have to be performed to clarify this aspect.

Comparing all the data from the genetic analyses there does not seem to be any correlation between transgene copy number and transgene expression, or between copy number, expression and PGIP activity. The Sultana population had an excessive amount of escapes and were not part of the remainder of the study. The selective pressure might have been suboptimal during the selection and regeneration period for the Sultana population.

3.5.2 A whole plant infection assay confirms the transgenic lines to be more susceptible than the wild type when infected with *B. cinerea*

Results obtained with a whole plant infection assay revealed that all the transgenic lines were more susceptible to *B. cinerea* infection than the untransformed control. Furthermore, the infection had a quick onset and faster rate of lesion growth for the transgenic population. Despite the fact that degrees of susceptibility differed among the transgenic lines, their separation from the wild type was found to be statistically significant when t-tests were performed on the lesion diameters of each transgenic line at different time points relative to the wild type ($p < 0.05$). These findings contrast with those obtained from a previous study carried out in our environment where the same non-vinifera PGIP encoding genes were overexpressed in a tobacco model system and conferred the host with strong resistance phenotypes against *B. cinerea* (Venter, 2010).

Interactions at the host-pathogen interface during infection play a pivotal role in determining the resistance and/or susceptibility of the host to infection. The detailed inhibition-interaction of the two non-vinifera PGIPs and BcPGs has not yet been studied. It is also not known which BcPGs are predominantly responsible for the necrotising and macerating activity during early stages of infection in grapevine tissues. It is possible that a poor inhibition-interaction with the major BcPGs during grapevine infections contributes to the susceptible phenotypes but does not explain the increased susceptibility of the transgenic lines. However, it is known that subtle changes in pH could affect PGIP-PG interactions in a way that could shift inhibition of ePGs to activation (Kemp *et al*., 2004). It cannot be ruled out that the increased levels of PGIPs in the leaves could have triggered changes in the apoplast that could have led to the activation of BcPGs resulting in the transgenics exhibiting higher levels of susceptibility compared to the wild type. The presence of both the native *PGIP* and the

transgenes within the same host could have further led to the silencing of the non-vinifera *PGIPs*. These possibilities require further experimentation.

Contrary to the tobacco model system, grapevine is a natural host to *B. cinerea* and would thus have a strong recognition and hypersensitive response (HR) to *B. cinerea*. Plant defence against necrotrophs has been shown to be mediated mainly through jasmonate/ethylene (JA/ET) signalling, which works closely with the HR response (Shirasu *et al*., 2000; Liu *et al*., 2007). The HR response is one of the early plant defence responses to pathogen attack where programmed cell death of the surrounding cells is facilitated in an effort to prevent the spread of infection (Kombrik *et al*., 1995). It has been shown that a stronger HR favours *Botrytis,* whereas a weakened HR contributes to resistance against this necrotroph (van Kan, 2006). PGIPs play a pivotal role in signalling responses as shown in a study where receptor kinases that play a role in regulating cell wall function were identified in the highly conserved LRR region of *Arabidopsis* (Xu et al., 2008). An enhanced HR response in the transgenic population, brought about by more copies of the LRR motif within the same plant, could potentially result in more pronounced programmed cell death which would favour *B. cinerea* growth by providing it with food and thus increase infection in the transgenic lines. Moreover, PGIP1038 has changes in its LRR motif when compared to VvPGIP1, that could also favour recognition and triggering of defence responses (see below). The structure-function relationships of these PGIPs could provide clues regarding the interesting phenotype observed. We thus hypothesise that the whole plant infection assay results where the transgenic lines exhibited a more susceptible phenotype to fungal infection compared to the untransformed wild type could be partly due to the enhanced signalling response in the transgenic lines.

Interestingly, the same clear susceptible phenotype compared to wild type was not seen when detached leaf assays were performed. In two separate detached leaf assays the transgenic lines did not behave as a population – for both the R1012 and R1038 population, some lines grouped with the wild type, some showed a more susceptible phenotype, whereas a minority of lines showed smaller lesions compared to wild type. This assay was difficult to standardise and some lines that grouped with the wild type, or had a larger lesion size in one assay, would have a different pattern of lesion development in the next assay. It is our conclusion that the detached leaf assay results are non-conclusive and not the best method to test the effect of the overexpressed PGIPs. There are two main arguments against this assay within our study: Firstly, in the detached leaf assay, the effect of the overexpressed PGIPs is evaluated with the wound response of the plant activated and secondly, defence signalling is hampered because the leaf is removed from the rest of the plant body and the whole plant context is lost. A study done on *Arabidopsis* showed that specifically the JA/ET defence pathway is compromised in detached leaves (Liu *et al*., 2007). From the whole plant infection assay it seemed that the overexpression of the PGIPs might influence quick recognition and activation of defence signalling and this aspect could be compromised in the detached leaf assay, perhaps contributing to a less consistent phenotype. Also, in the detached leaf assay, the wound response is already activated by detaching and pre-incubating the leaves for 24 hours before the infection is

started. Apart from an activated wound response, the detached leaves would also have an activated native PGIP present by the time of infection. Expression of VvPGIP1 in grapevine has been shown to be highly tissue specific, being strictly expressed in grape berries during and after veraison (De Ascensao, 2001). Promoter and expression analysis have however shown that events such as wounding, oxidative stress, infection and presence of elicitors, amongst others, lead to the strong induction of VvPGIP1 in all tissues (Joubert, 2004). This means that due to wounding, the detached leaves in the antifungal assay had an upregulated defence response and an activated VvPGIP1 prior to infection. This could also explain the fact that the infection took longer to establish in detached leaves compared to the whole plants which did not have any prior induced defence responses. The combined influence of the wound response, the induced native PGIP, the BcPG interactions with the various PGIPs (in combination) as well as the action of the transgenes on the signalling responses is at play in these detached leaf assays and several of these individual aspects, or the combination thereof are not yet known. Given these difficulties, we consider the detached leaf assay results cautiously, since it is difficult to isolate and evaluate the defence reactions in terms of PGIP overexpression.

The non-vinifera PGIP encoding genes that were used to generate the putative transgenic population being characterised in this study, had more than 95% sequence homology to *VvPGIP1* (Wentzel, 2005). However, in a comparative study on the inhibitory activity of PGIPs from *P. vulgaris* with 99% sequence homology on *F. moniliforme* PGs, it was shown that the function and inhibitory potential of the PGIPs is not directly dependent on the degree of sequence similarity (Maulik *et al*., 2009). The amino acid sequence homology in the LRR active domain of the non-vinifera PGIPs compared to VvPGIP1 was greater than 94%. The LRR domain of PGIP1038 exhibited amino acid differences to that of VvPGIP1 (Wentzel, 2005). The LRR motif is a highly conserved region which plays a pivotal role in PGIP-PG interaction and any changes in this domain could affect PGIP-PG binding (Leckie *et al*., 1999). LRR receptor kinases were recently identified in *A. thaliana* and were shown to play a role in regulating cell wall function. Mutational changes on these kinases altered the cell wall architecture polymers such as pectin which are pivotal in plant-pathogen interactions and also affected intracellular signalling pathways (Xu *et al*., 2008). In a separate study, amino acid changes in the LRR region of *P. vulgaris* altered the inhibitory profile against *F. phyllophilum* PG (Spinelli *et al*., 2008).

It has previously been shown that the LRR motif of PGIP1012 is identical to that of VvPGIP1 (Wentzel, 2005). However, it was also observed that in the signal peptide region of the protein at position 16, VvPGIP1 has a valine but the same position in the non-vinifera PGIPs is occupied by leucine. Furthermore, in the N-terminal region of the protein at position 62, VvPGIP1 has a glycine whilst the same position is occupied by glutamate (Wentzel, 2005). Subtle changes in the sequence of plant PGIPs and amino acids have been shown to alter the specificity for inhibiting pathogenic PGs (Misas-Villamil *et al*., 2008; Casasoli *et al*., 2009; Maulik *et al*., 2009). It has also been hypothesised that PGIP binds with pectin and PGs through overlapping regions which are not necessarily identical (Spadoni *et al*., 2006). This means not only changes in the LRR motif but also in the changes in pectin

structure could alter the inhibition potential of PGIP through incompatible interaction. These structural differences between the grapevine native PGIP and the transgenes should be further studied to understand their impact on substrate specificity, inhibition interactions and ultimately defence responses.

In conclusion, *V. vinifera* cv. Redglobe lines expressing PGIP encoding genes from non-vinifera grapevine species, known for their disease resistance phenotypes, did not exhibit enhanced resistance to *B. cinerea* infection when compared to the untransformed wild type lines. Instead, in a whole plant infection analysis, these lines were more susceptible than the WT. This study provides the first report on the expression of non-vinifera PGIPs in a grapevine host. Knowledge gaps on the specific interaction dynamics of the non-vinifera PGIPs with the fungal ePGs provide an avenue for future work that could lead to the improved characterisation of the observed phenotype. The possibility of post-transcriptional gene silencing of the transgenes by the native *PGIP*, needs to be investigated further. Moreover, these transgenic lines provide an excellent system to study the *in vivo* functions of PGIPs. They constitute 'mutant' lines with PGIP-specific susceptibility phenotypes. Just as the PGIP-specific resistance phenotypes obtained in the tobacco model were useful, these lines could be useful to study the role and/or function of PGIP in defence responses in grapevine.

3.6 References

Aguero C.B., Uratsu S.L., Greve C., Powell A.L.T., Labavitch J.M., Meredith C.P., 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.

Andre-Leroux G., Tessier D., Bonnin E. 2009. Endopolygalacturonases reveal molecular features for processivity pattern and tolerance towards acetylated pectin. Biochim. Biophys. Acta: Proteins Proteomics 1794:5-13.

Aziz A., Heyraud A., Lambert B. 2004. Oligogalacturonide signal transduction, induction of defence-related responses and protection of grapevine against *Botrytis cinerea.* Planta 218: 767–774.

Bergmann C.W., Ito Y., Singer D., Albersheim P., Darvill A.G. 1994. Polygalacturonase inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. Plant J. 5:625-634.

Cantu D., Vicente A.R., Labavitch J.M., Bennet A.B., Powell A.L.T. 2008. Strangers in the matrix: plant cell walls and pathogen susceptibility. Trends Plant Sci. 13:610-617.

Casasoli M., Federici L., Spinelli F., Di Matteo A., Vella N., Scaloni F., Fernandez-Recio J., Cervone F., De Lorenzo G. 2009. Integration of evolutionary and desolvation energy analysis identifies functional sites in a plant immunity protein. PNAS 106:7666-7671.

Cervone F., De Lorenzo G., Degra L., Salvi G., Bergami M. 1987. Purification and characterisation of a polygalacturonase inhibiting protein from *Phaseolus vulgaris* L. Plant Physiol. 85:631-637.

Cervone F., Hahn M.G., De Lorenzo G., Darvill A. 1989. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defence responses. Plant Physiol. 90:542-548.

Cline M.S., Smoot M., Cerami E., Kuchinsky A., Landys N., Workman C., Christmas R., Avila-Campilo I., Creech M., Gross B., Hanspers K., Isserlin R., Kelley R., Killcoyne S., Lotia S., Maere S., Morris J., Ono K., Pavlovic V., Pico A.R., Vailaya A., Wang P-L., Adler A., Conklin B.R., Hood L., Kuiper M., Sander C., Schmulevich I., Schwikowski B.,. Warner G. J., Ideker T., Bader G.D. 2007. Integration of biological networks and gene expression data using Cytoscape. Nature Protocols 2, 2366 – 2382.

Comménil P., Belingheri L., Sancholle M., Dehorter B. 1995. Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. Lipids 30:351-356.

De Ascensao A. 2001. Isolation and characterisation of a polygalacturonase-inhibiting protein (PGIP) and its encoding gene from *Vitis vinifera* L. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

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.

Desiderio A., Aracri B., Leckie F., Mattei B., Salvi G., Tigerlaar H., van Roekel J., Baulcombe D., Melchers L., De Lorenzo G., Cervone F. 1997. Polygalacturonase inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol. Plant Microbe-Interact 10:852-860.

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

D'Ovidio R., Mattei B., Robertia S., Bellincampi D. 2004. Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant–pathogen interactions. Biochim. Biophys. Acta 1696:237–244.

Esquerre-Tugayé M. T., Boudart G., 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.

Faize M., Sugiyama T., Faize L., Ishii H. 2003. Polygalacturonase-inhibiting protein (PGIP) from Japanese pear: possible involvement in resistance against scab. Physiol. Mol. Plant Pathol. 63:319–327.

Favaron F., Castiglioni C., D'Ovidio C., Alghisi P. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. Physiol. Mol. Plant Pathol. 50:403-417.

Federici L., Di Matteo A., Fernandez-Recio J., Tsernoglou D., Cervone F. 2006. Polygalacturonase inhibiting proteins: players in plant innate immunity? Trends Plant Sci. 11:65-70.

Ferreira R. B., Monteiro S.S., Picarra-Perreira M.A., Teixeira A.R. 2004. Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability. Trends Biotechnol. 22:168-173.

Franks T., He D.G., Thomas M.R. 1998. Regeneration of transgenic *Vitis vinifera* L. Sultana plants: genotypic and phenotypic analysis. Mol Breeding 4:321–333.

Gleave A.P. 1992. A versatile binary vector system with a T-DNA organisational structure condusive to efficient integration of cloned DNA into a plant genome. Plant Mol. Biol. 20:1203-1207

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

Howell J.T., Davis M.R. 2005. Plant defence mechanisms against fungal pathogens:polygalacturonase inhibitor proteins. Can. J. Plant Pathol. 27:5–15.

Hwang B.H., Bae H., Lim H.S., Kim K.B., Kim S.J., Im M.H., Park B.S., Kim D.S., Kim J. 2010. Overexpression of polygalacturonase-inhibiting protein 2 (*PGIP2*) of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) increased resistance to the bacterial pathogen *Pectobacterium carotovorum* ssp. *carotovorum*. Plant Cell Tiss. Org. Cult. 103:293–305.

Iocco P., Franks T., Thomas M.R. 2001. Genetic transformation of major wine grape cultivars of *Vitis vinifera* L. Transgenic Res. 10:105-112.

Janni M., Di Giovanni M., Roberti S., Capodicasa C., D'Ovidio R. 2006. Characterisation of expresses *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. 113:1233–1245.

Johnston D.J., Ramanathan V., 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. 2004. Regulation of the *Vitis vinifera pgip1* gene encoding a polygalacturonase inhibiting protein. PhD Thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Joubert D.A., Slaughter A.R., Kemp G., Becker J.V.W., Krooshoof G.H., Bergmann C., Benen J., Pretorius I.S., Vivier M.A. 2006. The grapevine polygalacturonase inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. 15:687-702.

Joubert D. A., Kars I., Wagemakers L., Bergmann C., Kemp G., Vivier M. A., Kan J. A. L. 2007. A Polygalacturonase-Inhibiting Protein from Grapevine Reduces the Symptoms of the Endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* Leaves Without Any Evidence for In Vitro Interaction. MPMI 4:392–402.

Juge N. 2006. Plant protein inhibitors of cell wall degrading enzymes. Trends Plant Sci. 11:359-367

Janni M., Di Giovanni M., Roberti S., Capodicasa C., D'Ovidio R. 2006. Characterisation of expresses *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. 113:1233–1245.

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

Kars I. 2007. The role of pectin degradation in pathogenesis of *Botrytis cinerea*. PhD Thesis, Wageningen University, Wageningen, NL.

Kemp G., Stanton L., Bergmann C.W., Clay R.P., Albersheim P., Darvill A. 2004. Polygalacturonase inhibiting proteins can function as activators of polygalacturonase. Mol. Plant Microbe-Interact. 17:888-894.

Kobe B., Kajava A.V. 2001. The leucine rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. 11:725-732.

Kombrink E., Somssich I.E. 1995. Defence responses of plants to pathogens. Advances in Bot. Res. 21:1-34.

Leckie F., Mattei B., Capodicasa C., Hemmings A., Nuss L., Aracri B., De Lorenzo G., 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.

Liu G., Kennedy R., Greenshields D.L., Peng G., Forseille L., Selvaraj G., Wei Y. 2007. Detached and attached Arabidopsis leaf assays reveal distinctive defence responses against hemibiotropic *Colletotrichum* spp. MPMI 20:1308-1319.

Manfredini C., Sicilia F., Ferrari S., Pontiggia D., Salvi G., Caprari C., Lorito M., 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.

Mattei B., Bernalda M.S., Federici L., Roepstorff P., Cervone F., Boffi A. 2001. Secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (Polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. Biochemistry 40:569-576.

Maulik A., Ghosh H., Basu S. 2009. Comparative study of protein-protein interaction observed in polygalacturonase-inhibiting proteins from *Phaseolus vulgaris* and *Glycine max* and polygalacturonase from *Fusarium moniliforme*. BMC Genomics 10 (Suppl 3):S19.

Misas-Villamil J.C., van der Hoorn R.A.L. 2008. Enzyme-inhibitor interactions at the plant-pathogen interface. Curr. Opin. Plant Biol. 11:380-388.

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

Oelofse D., Dubery I. A., Meyer R., Arendse M. S., Gazendam I., Berger D. K. 2006. Apple polygalacturonase inhibiting protein1 expressed in transgenic tobacco inhibits polygalacturonases from fungal pathogens of apple and the anthracnose pathogen of lupins. Phytochemistry 67:255–263.

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

Sambrook J., Fritsch E., Maniatis T. 1989. Molecular cloning: a laboratory manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shannon P., Markiel A., Ozier O., Baliga N.S., Wang J.T., Ramage D., Amin N., Schwikowski B., Ideker T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Research 13:2498-504.

Sharrock K. R., and Labavitch J. M. 1994. Polygalacturonase inhibitors of Bartlett pear fruits: differential effects on *Botrytis cinerea* polygalacturonase isozymes, and influence on products of fungal hydrolysis of pear cell walls and on ethylene induction in cell culture. Physiol. Mol. Plant Pathol. 45:305-319.

Shirasu K., Schulze-Lefert P. 2000. Regulators of cell death in disease resistance. Plant Mol. Biol. 44:371-385.

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

Spinelli F., Marrioti L., Mattei B., Salvi G., Cervone F., Caprari C. 2008. Three aspartic acid residues of polygalacturonase-inhibiting protein (PGIP) from *Phaseolus vulgaris* are critical for inhibition of *Fusarium phyllophilum* PG. Plant Biology: 1-6.

Stahl E.A., Bishop J.G. 2000. Plant–pathogen arms races at the molecular level. Curr. Opin. Plant Biol. 3:299–304.

Stotz H. U., Bishop J. G., Bergmann C. W., Koch M., Albersheimp P., Darvill A G., 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.

Suzuki Y., Hibino T., Kawazu T., Wada T., Kihara T., Koyama H. 2003. Extraction of total RNA from leaves of Eucalyptus and other woody and herbaceous plants using sodium isoascorbate. Biotechniques 34:988-993.

Taylor R., Secor G. 1988. An improved diffusion assay for quantifying the polygalacturonase content of Erwinia culture filtrates. Phytopathology 78

van Kan J.A.L. 2005. Infection strategies of *Botrytis cinerea*. Acta Hort. 669:77-89.

van Kan J. A. L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11:247-252.

Venter A. 2010. The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco. MSc thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Wentzel L. 2005. The endopolygalacturonases from *Botrytis cinerea* and their interaction with an inhibitor from grapevine. MSc Thesis, Stellenbosch University.

Xu S-L., Rahman A., Baskin T.I., Kieber J.J. 2008. Two leucine-rich repeat receptor kinases mediate signalling linking cell wall biosynthesis and ACC synthase in *Arabidopsis*. Plant Cell 20:3065-3079.

Chapter 4

General discussion and conclusions

4. General discussion and conclusions

4.1 General discussion

Since the advent of farming, farmers all over the world have tried and tested different plant breeding techniques in an effort to improve the agronomic characteristics of crops. These desirable characteristics include resistance to drought, salt stress, weeds, pests and diseases. Genetic engineering of crops with these sought-after characteristics has great potential in reducing yield losses of economically important crops such as grapevine.

Grapevine is a perennial fruit crop which is attacked by a wide array of pathogens such as bacteria, viruses, parasitic plants and fungi, with the latter arguably constituting the greatest potential to yield losses (Ferreira *et al*., 2004). Fungal pathogens such as *Botrytis cinerea*, release cell wall degrading enzymes called endopolygalacturonases (ePGs) during infection which break down the pectin component of the plants' cell wall (Kars *et al*., 2005; van Kan, 2005; 2006). In defence, plants inhibit these ePGs through the action of polygalacturonase-inhibiting proteins (PGIPs) (Cervone *et al*., 1989; Esquerre-Tugaye *et al*., 2000; De Lorenzo *et al*., 2001; D'Ovidio *et al*., 2004; Howell *et al*., 2005; Juge, 2006). Overexpression of PGIPs in numerous plant hosts, including grapevine, has resulted in enhanced disease resistance phenotypes, although in raspberry, this trait was not observed (Johnston *et al*., 1993; Favaron *et al*., 1997; Powell *et al*., 2000; Faize *et al*., 2003; Ferrari *et al*., 2003; Aguero *et al*., 2005; Oelofse *et al*., 2006; Hwang *et al*., 2010).

In our environment, overexpression of the PGIP encoding gene from grapevine *Vitis vinifera*, *VvPGIP1*, in tobacco resulted in reduced susceptibility to *B. cinerea* infection compared to the untransformed wild type (Joubert *et al*., 2006). The low susceptibility to fungal attack of some American and non-vinifera grapevine species sparked interests in the role that their PGIP encoding genes play in this regard (Wentzel *et al*., 2005). Overexpression of some of the non-vinifera *PGIP* genes in tobacco conferred the transgenic plants with improved resistance against *B. cinerea* when compared to the untransformed wild type (Venter *et al*., 2010). This led to the subsequent expression of two non-vinifera *PGIP*s in *V. vinifera*. In this study, the generated putative transgenic population was genetically and phenotypically characterised with regards to the potential resistance phenotypes in an effort to expand on the current knowledge base on PGIP and plant defence.

4.2 Results obtained against stated objectives

The main aim of the study was functional analysis of two non-vinifera PGIP encoding genes in cultivars of *V. vinifera*. All aims as stated in Chapter 1 were reached. A population of putative transgenic plants expressing non-vinifera PGIP encoding genes was successfully generated for *V. vinifera* cv. Redglobe, Sultana and Merlot. The lines were clonally multiplied under tissue culture conditions to establish a working population of plants. A total of 131 putative transgenic lines were generated. Copies of these putative transgenics were then hardened off for *ex vitro* experiments.

Ground leaf material from the *in vitro* population was utilised to genetically characterise the plant lines.

Transgene presence was confirmed through PCR for 51 of the putative transgenic lines whilst 45 lines were further shown to express the transgene through northern blot assays. A selection of 38 transgenic lines was then further analysed for independent gene integration events using Southern blots and these were confirmed for 29 of the lines. Further analysis of the transgenics for PGIP activity using an agarose diffusion assay, revealed that 22 of the lines exhibited 100% inhibition of crude BcPGs in comparison to the untransformed wild type line. There was no correlation between transgene copy numbers and PGIP activity in the transgenic lines. These 22 transgenic lines which had previously tested positive for transgene presence, expression and had proven to be independent copies, were then utilised for detached leaf and whole plant infection assays where they were challenged with *B. cinerea*. Disease progression was monitored and compared to the untransformed wild type. The detached leaf assay did not give a clear indication of the defence phenotypes linked to the expression of the non-vinifera PGIPs. However, the whole plant infection assay showed that the transgenic lines had increased susceptibility to *B. cinerea* infection compared to the untransformed wild type.

4.3 Major findings of the study and their relevance

Expression of non-vinifera PGIP encoding genes in *V. vinifera* resulted in enhanced susceptibility against *B. cinerea*. Since the inhibition interactions of the non-vinifera PGIPs and the BcPGs has not yet been studied before and is thus not known, it is possible that a poor inhibition-interaction occurs with the major BcPGs responsible for maceration of grapevine material. This aspect will need further study to elucidate. Since it has been hypothesised that PGIP binds with pectin and PGs in overlapping regions (Spadoni *et al*., 2006), the structural differences between the native and transgenic PGIPs within and outside the LRR motif could have affected the inhibition profile of the non-vinifera PGIPs (Leckie *et al*., 1999). These structural differences could have additionally triggered changes in the apoplast that would have led to the activation of the BcPGs, instead of inhibition, in the transgenic lines (Kemp *et al*., 2004). The presence of non-vinifera PGIPs and the native PGIP in a *V. vinifera* host could have resulted in negative interactions that would affect the inhibition profile of the transgenic lines. These interactions could have subsequently led to post-transcriptional gene silencing of the non-vinifera PGIPs. Moreover, an enhanced HR response could have been brought about by numerous copies of PGIP within the same host. This enhanced programming of cell death could have favoured *B. cinerea* since it is a necrotroph, thus increasing infection in the transgenic lines (van Kan, 2006). These possibilities need to be further studied to attempt and clarify the susceptible phenotype observed. The results obtained in this study add to the PGIP knowledge base on the complexity of PGIP-PG interactions in heterologous overexpression studies, where the host also harbours a native *PGIP* gene.

The contrasting results obtained in tobacco overexpression studies with these same PGIPs (Joubert *et al*., 2006; 2007; Venter 2010) also provides an interesting model to evaluate the effect of

the PGIPs in a non-host plant (tobacco) to *Botrytis* versus a natural host (grapevine) of this pathogen. The results obtained in this study suggest that in a natural host, the overexpressed PGIPs might have amplified the pathogen recognition ability, or improved the signalling ability that could ultimately favour *Botrytis* since it thrives on dying tissue.

The surprising phenotype is the only report (to our knowledge) where overexpression of a PGIP has caused an increase in susceptibility against a fungal pathogen. These lines will also be evaluated against other types of pests and pathogens to determine whether the observed phenotype is strictly linked to *Botrytis* infection, or not.

4.4 Conclusion and future work

Subtle changes in the amino acids sequences of the PGIP protein molecule have been shown to cause drastic changes in the inhibitory profiles of the resulting protein (Maulik *et al*., 2009). The effect of the changes in amino acids in the non-vinifera PGIPs, compared to VvPGIP1, to the inhibitory profile still needs to be established. A well established pathosystem for grapevine infections is still at preliminary stages. Infection conditions need to be optimised for the non-vinifera PGIP:BcPG interactions in a *V.vinifera* host before conclusive deductions can be made from the obtained results. This could be coupled with profiling analyses on these lines to provide valuable insights to understanding the changes in gene expression that underlie these phenotypes. The accurate profiling of PGIP gene expression with qRT-PCR during infection could shed light on the possibility of the native PGIP silencing the non-vinifera PGIPs in a *V. vinifera* host. The *in vivo* functions of PGIPs can be studied using the transgenic lines to further understand the role of PGIP in defence responses. A sequenced grapevine genome and the availability of advanced technologies for this important fruit crop provide ample scope to understand the genetic basis of the observed phenotypes and disease resistance and susceptibility in *Vitis* spp. better.

4.5 References

Aguero C.B., Uratsu S.L., Greve C., Powell A.L.T., Labavitch J.M., Meredith C.P., 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. 1989. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defence responses. Plant Physiol. 90:542-548.

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.

D'Ovidio R., Mattei B., Robertia S., Bellincampi D. 2004. Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant–pathogen interactions. Biochim. Biophys. Acta 1696:237–244.

Esquerre-Tugayé M. T., Boudart G., 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.

Faize M., Sugiyama T., Faize L., Ishii H. 2003. Polygalacturonase-inhibiting protein (PGIP) from Japanese pear: possible involvement in resistance against scab. Physiol. Mol. Plant Pathol. 63:319–327.

Favaron F., Castiglioni C., D'Ovidio C., Alghisi P. 1997. Polygalacturonase inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. Physiol. Mol. Plant Pathol. 50:403-417.

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

Ferreira R. B., Monteiro S.S., Picarra-Perreira M.A., Teixeira A.R. 2004. Engineering grapevine for increased resistance to fungal pathogens without compromising wine stability. Trends Biotechnol. 22:168-173.

Howell J.T., Davis M.R. 2005. Plant defence mechanisms against fungal pathogens:polygalacturonase inhibitor proteins. Can. J. Plant Pathol. 27:5–15.

Hwang B.H., Bae H., Lim H.S., Kim K.B., Kim S.J., Im M.H., Park B.S., Kim D.S., Kim J. 2010. Overexpression of polygalacturonase-inhibiting protein 2 (*PGIP2*) of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) increased resistance to the bacterial pathogen *Pectobacterium carotovorum* ssp. *carotovorum*. Plant Cell Tiss. Org. Cult. 103:293–305.

Joubert D.A., Slaughter A.R., Kemp G., Becker J.V.W., Krooshoof G.H., Bergmann C., Benen J., Pretorius I.S., Vivier M.A. 2006. The grapevine polygalacturonase inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res. 15:687-702.

Johnston D.J., Ramanathan V., 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.

Juge N. 2006. Plant protein inhibitors of cell wall degrading enzymes. Trends Plant Sci. 11:359-367

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

Kemp G., Stanton L., Bergmann C.W., Clay R.P., Albersheim P., Darvill A. 2004. Polygalacturonase inhibiting proteins can function as activators of polygalacturonase. Mol. Plant Microbe-Interact. 17:888-894.

Leckie F., Mattei B., Capodicasa C., Hemmings A., Nuss L., Aracri B., De Lorenzo G., 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.

Maulik A., Ghosh H., Basu S. 2009. Comparative study of protein-protein interaction observed in polygalacturonase-inhibiting proteins from *Phaseolus vulgaris* and *Glycine max* and polygalacturonase from *Fusarium moniliforme*. BMC Genomics 10 (Suppl 3):S19.

Oelofse D., Dubery I. A., Meyer R., Arendse M. S., Gazendam I., Berger D. K. 2006. Apple polygalacturonase inhibiting protein1 expressed in transgenic tobacco inhibits polygalacturonases from fungal pathogens of apple and the anthracnose pathogen of lupins. Phytochemistry 67:255–263.

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

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

van Kan J.A.L. 2005. Infection strategies of *Botrytis cinerea*. Acta Hort. 669:77-89.

van Kan J. A. L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11:247-252.

Venter A. 2010. The functional analysis of *Vitaceae* polygalacturonase-inhibiting protein (PGIP) encoding genes overexpressed in tobacco. MSc thesis. Stellenbosch University, Stellenbosch, Republic of South Africa.

Wentzel L. 2005. The endopolygalacturonases from *Botrytis cinerea* and their interaction with an inhibitor from grapevine. MSc Thesis, Stellenbosch University