The interaction between *Vitis vinifera* and fungal pathogens: A molecular approach using characterized grapevine mutants

by

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Dissertation presented for the degree of
Doctor of Philosophy (Agricultural Sciences)

at
Stellenbosch University
Institute for Wine Biotechnology, Faculty of AgriSciences

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March 2017

Declaration

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Summary

The commercially cultivated grapevine species, *Vitis vinifera*, is highly susceptible to a wide range of pathogens and pests which include the fungus, *Botrytis cinerea*. During infection of a wide range of hosts, *B. cinerea* utilises a combination of cell wall degrading enzymes, phytotoxins and metabolites (amongst others) to facilitate entry into host cells, killing them in the process. Being a necrotroph, *B. cinerea* feeds off the dead cells and continues to proliferate. One of the lines of defence utilised by plants is through the action of cell wall associated polygalacturonase inhibiting proteins (PGIPs) whose roles include inhibiting the activity of *B. cinerea* endopolygalacturonases (BcPGs), prolonging the existence of longer chain cell wall fragments involved in signalling and priming the plant prior to infection.

The defence roles of grapevine *pgip* encoding genes (*Vvipgip1* from *V. vinifera* and non-*vinifera pgip*s from wild vines) were previously elucidated in tobacco overexpression studies where they increased resistance to a hyper-virulent *B. cinerea* strain isolated from grapes. However, overexpressing two of the non-*vinifera pgip*s in *V. vinifera* conferred the transgenic population with hyper-susceptibility to the same *B. cinerea* grape strain. This study aimed to comprehensively investigate the basis of the hyper-susceptible phenotype displayed by asking and answering important questions regarding the ability of the non-*vinifera* PGIPs to interact with and inhibit *Botrytis* ePGs on the one hand and on the other hand also investigate potentially other (non-ePG inhibition related) functions of the grapevine PGIPs.

In silico structural docking simulations of grapevine PGIPs (VviPGIP1 and the two non-*vinifera* PGIPs) against BcPGs from the grape strain and two other *B. cinerea* strains (included for comparison) were conducted to gain an understanding of the inhibition interactions from a structural perspective. The predicted PGIP-BcPG interactions were highly *B. cinerea* strain specific with subtle PGIP genotype specificity. This prompted infection of the transgenic grapevine population with a different *B. cinerea* strain (B05.10) and the results complemented the *in silico* docking simulations. The transgenic grapevines did not display hyper-susceptibility to B05.10, indicating that it was a strain specific response. Transgenic tobacco with the same genes overexpressed, on the other hand, displayed increased resistance irrespective of *B. cinerea* strain used. The phenotype displayed by transgenic grapevine to *B. cinerea* grape strain infection was thus considered both host and strain specific. Moreover, when *B. cinerea* mutants in ePGs and galacturonic acid metabolism were used in infection analyses on these grapevine and tobacco populations, both host specific virulence factors and potential recognition/decoy factors could be identified. These results all confirm the importance of the specific host and pathogen and the resulting phenotype and makes it clear that interactome studies would be the most insightful in studying infection/defence. Interestingly, the transgenic grapevine population displayed partial resistance to a biotrophic pathogen, specifically in blocking initial penetration of the pathogen, indicating that the PGIP overexpression could have modulated pre-formed defences in a possible priming mechanism.

Further analysis of the transgenic grapevine population confirmed that both the native and transgenic *pgip*s were expressed during infection and active proteins, which effectively inhibited BcPGs, was produced. However, prior to infection, transgenic grapevine leaves displayed a reduction in abundance of cell wall components associated with cell wall strengthening, indicating potential weakened cell walls. Additionally, they emitted significantly lower levels of defence-related sesquiterpenes compared to the controls during *B. cinerea* grape strain infection. These findings were suggestive of changes in metabolic processes, brought about by overexpressing non-*vinifera pgip*s in *V. vinifera* background, which favoured the pathogen over the host during infection. Thus to build on this, a whole transcriptomic study to investigate the strain specific infection strategy together with the host specific defence strategy as a dynamic interaction was conducted during the early stages of infection.

B. cinerea grape strain expressed significantly higher levels of genes involved in phytotoxin synthesis on transgenic plants compared to the controls, at the local infection site. On the other hand, the transgenic plants expressed significantly lower levels of defence-related genes, also at the local infection site. Taken together, the findings of this study challenge our current understanding of the roles of PGIPs in plant defence

during *B. cinerea* infection. It points towards the possibility that grapevine PGIPs in their native backgrounds are not primarily linked to the classical PGIP-PG fungal inhibition interactions. It also provides insight that the hyper-virulent grape strain possibly optimised mechanisms to use the plant's defence mechanism against itself and even modulate the host-responses in its favour. The host- and pathogen specific reactions observed in this study strongly highlights the impact that the choice of host-pathogen pairing has on defining defence phenotypes. Future studies should consider strain and host specific responses and interactome approaches would be valuable to that effect. This study successfully characterised the hyper-susceptible phenotype as set out initially, but also provided several new insights as well as new testable hypothesis that can lead to further studies.

Opsomming

Vitis vinifera, die kommersieel-verboude wingerdstokspesie, is hoogs vatbaar vir 'n wye verskeidenheid peste en patogene, insluitend die swam *Botrytis cinerea*. Tydens infeksie van 'n wye verskeidenheid gashere, gebruik *B. cinerea* onder andere 'n kombinasie van selwandafbrekingsensieme, fitotoksiene en metaboliete om toegang tot gasheerselle te fasiliteer, ‘n proses wat ook lei tot die selle se afsterwe. As 'n nekrotroof, voed *B. cinerea* op hierdie dooie selle en so versprei die infeksie dan verder. Een van die verdedigingslinies wat deur plante ontplooi word, is met behulp van selwand-geassosieerde poligalakturonase-inhiberende proteïene (PGIPs) wie se rolle beperking van die aktiwiteit van *B. cinerea* endopoligalakturonases (BcPGs) insluit, en sodoende die teenwoordigheid van langer-ketting selwandfragmente verleng: laasgenoemde is betrokke by sein-herleiding tydens infeksie en ook die voorbereiding op weerstand nog voor infeksie plaasvind (“priming”).

Die verdedigingsrolle van druifplant *pgip* gene (*Vvipgip1* van *V. vinifera* en nie-*vinifera pgips* van wilde druifspesies) is voorheen uitgeklaar in ooruitdrukkingstudies in tabak waar hulle verhoogde weerstand veroorsaak het teen 'n hipervirulente *B. cinerea* ras wat van druiwe geïsoleer is. Intereeasnt genoeg het die ooruitdrukking van twee van die nie-*vinifera pgips* in *V. vinifera* gelei tot hipersensitiwiteit van die transgeniese bevolking toe dit met die druifafkomstige *B. cinerea* ras geinokuleer is. In hierdie studie het word gepoog om omvattend ondersoek in te stel wat die grondslag van die hipervatbare(sensitiewe) fenotipe is, deur sleutelvrae te vra en te beantwoord met betrekking tot die vermoë van die nie-*vinifera* PGIPs om interaksies te hê met BcPGs en hul aktiwiteit te beperk, en om ander moontlike funksies, wat nie verband hou met aktiwiteitsbeperking van BcPGs nie, te ondersoek met betrekking tot druifplant PGIPs.

Rekenaargebaseerde strukturele koppelingsimulasies van druifplant PGIPs (VviPGIP1 en die twee nie-*vinifera* PGIPs) teenoor BcPGs van die druifafkomstige rasen twee ander *B. cinerea* rasse (ingesluit ter vergelyking) is uitgevoer om, vanuit ‘n struktuuroogpunt, 'n begrip van die aktiwiteitsbeperking-interaksies te verkry. Die voorspelde PGIP-BcPG interaksies was hoogs spesifiek tot die *B. cinerea* ras, maar PGIP spesifisiteit was subtiel beïnvloed deur genotipe. Daarvolgens is die transgeniese wingerdstokbevolking met ‘n ander *B. cinerea* ras (B05.10) infekteer en die resultate het die rekenaargebaseerde simulasies ondersteun. Die transgeniese druifplante het nie hipervatbaarheid teenoor die B05.10 ras vertoon nie, wat daarop dui dat dit 'n rasspesifieke reaksie was. Transgeniese tabak met dieselfde gene ooruitgedruk, aan die ander kant, vertoon verhoogde weerstand ongeag die *B. cinerea* stam. Die fenotipe van transgeniese druifplante wat waargeneem was tydens *B. cinerea* infeksie word daarom beskou as beide gasheer- en rasspesifiek. Verder, toe *B. cinerea* mutante in BcPGs en galakturonaatmetabolisme gebruik was tydens infeksiestudies op hierdie druifplant- en tabakbevolkings, kon beide gasheer-spesifieke virulensiefaktore en moontlike erkennings- of verwarringsfaktore geïdentifiseer word. Hierdie resultate bevestig tesame die belangrike invloed van die spesifieke gasheer en patogeen op die gevolglike fenotipe en maak dit duidelik dat studies wat die interaksie van beide komponente insluit die mees insiggewende benadering sou wees in die bestudering van infeksie en verdediging. Dis interessant dat die transgeniese druifplante gedeeltelik weerstandbiedend was teen ‘n biotrofiese patogeen, en met ‘n meganisme wat die aanvanklike kolonisasie/indringing van die patogeen keer. Die dui daarop dat PGIP ooruitdrukking moontlik vooraf-gevormde verdedigingsmeganismes kon beïnvloed.

Verdere ontleding van die transgeniese druifplante het bevestig dat beide die inheemse en transgeniese *pgips* uitgedruk was tydens infeksie en aktiewe proteïene, wat effektief BcPG aktiwiteit kon beperk, geproduseer was. Maar voor infeksie vertoon transgeniese wingerdblare minder selwandkomponente wat verband hou met selwandversterking, wat moontlik verswakte selwande aandui. Daarbenewens, in vergelyking met die kontrole, laat hulle aansienlik laer vlakke van verdedigingsverwante seskwiterpene vry tydens infeksie met die druifafkomstige ras van *B. cinerea*. Hierdie bevindings dui aan dat daar moontlik veranderinge in metaboliese prosesse was, veroorsaak deur die ooruitdrukking van nie-*vinifera pgips* in die *V. vinifera* konteks, wat tydens infeksie meer gunstig was vir die patogeen as die gasheer. Om voort te bou op die bevinding is ‘n geheeltranskriptoomstudie geloods om die rasspesifieke infeksiestrategie tesame met

die gasheerspesifieke verdedigingstrategie as ‘n dinamiese interaksie te ondersoek tydens die vroeë infeksiefases.

B. cinerea druifafkomstige ras het op transgeniese plante, in vergelyking met die kontrole, by die plaaslike infeksiesone aansienlik hoër vlakke van gene wat betrokke is in fitotoksiensintese uitgedruk. Aan die ander kant, het die transgeniese plante aansienlik laer vlakke van verdedigingsverwante gene uitgedruk, ook by die plaaslike infeksiesone. Tesame daag die bevindinge van hierdie studie ons huidige begrip van die rolle van PGIPs in plantverdedigingsreaksies tydens *B. cinerea* infeksie uit. Dit dui op die moontlikheid dat druifplant PGIPs in hul inheemse konteks dalk nie primêr gekoppel kan word aan die klassieke PGIP-PG swambeperkende interaksies nie. Dit bied verder ook insig dat die hipervirulente druifafkomstige ras moontlik strategieë optimeer het om die gasheer se verdedigingsmeganismes teen die gasheer te gebruik en selfs die gasheer-reaksies in sy guns te moduleer. Die gasheer- en patogeenspesifieke reaksies waargeneem in hierdie studie beklemtoon ten sterkste die impak wat die keuse van die gasheer-patogeen paring het op die omskrywing van verdedigingsfenotipes. Toekomstige studies behoort ras- en gasheerspesifisiteit in ga te neem en interaksiestudies is veral waardevol wees in hierdie opsig. Hierdie studie het, soos aanvanklik beplan, suksesvol die hipervatbare fenotipe omskryf, maar het ook tot nuwe insigte, asook nuwe toetsbare hipotese gelei wat verdere studies kan ondersteun.

This dissertation is dedicated to

My kondie Luke Kweya Okoba

Biographical sketch

Mukani Moyo was born in Plumtree, Zimbabwe on 28 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 in 2009, which she completed in 2011. She then continued with her studies and enrolled for a PhD 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
- **Prof Hugh Patterton** for the guidance with the structural modelling work
- **Dr JAL van Kan** for kindly providing the *Botrytis* mutant strains
- **Ralph Jones** for performing the mutant infections
- **Dr Jay Belli Kullan** for the collaborative work on the interactome project
- **Prof Mario Pezzotti** for the collaborative work on the AGP chapter
- **Dr Dan Jacobson** for the bioinformatics support with the grapevine AGP family
- **Dr John Moore**, **Dr Phillip Young**, **Dr Fanny Buffetto** and **Dr Hans Eyeghe-Bickong** for all the critical discussions
- **Carin Basson** for always being willing to listen and assist with analysis ideas
- **Lab colleagues** for all the discussions and ideas
- **My husband Luke Kweya Okoba** for keeping me smiling through all the storms
- **My family and friends** for the unwavering support and encouragement
- **To God** who has brought me this far and continues to bless me
- **The National Research Foundation**, **Winetech**, and the **Institute for Wine Biotechnology** for financial support

Preface

This dissertation is presented as a compilation of seven chapters and an addendum chapter. Each chapter is introduced separately and is written according to the style of the South African Journal of Enology and Viticulture (SAJEV).

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Grapevine defence against mildews and grey mould rot
Chapter 3	**Research results** Interaction of grapevine polygalacturonase inhibiting proteins (PGIPs) and *Botrytis cinerea* endopolygalacturonases (BcPGs): An *in silico* study
Chapter 4	**Research results** Non-*vinifera pgip*s as defence genes against *Botrytis cinerea* B05.10 and *Erysiphe necator* in tobacco and grapevine
Chapter 5	**Research results** Evaluating the hyper-susceptible phenotype of transgenic grapevine overexpressing non-*vinifera pgip*s to *Botrytis cinerea* grape strain infection further
Chapter 6	**Research results** A first overview analysis of a Grapevine-*Botrytis* leaf interactome study using a transgenic PGIP and control population
Chapter 7	**General Discussion and Conclusions**
Addendum A to Chapter 5	Genome-wide identification, classification and expression analysis of arabinogalactan proteins in *Vitis vinifera*

I hereby declare that I was a co-contributor to the multi-author manuscript presented in Chapter 4. My contribution involved the infection of the grapevine population with *Erysiphe necator* as well as supervising of the mutant infections, which were conducted by Mr Ralph E.S. Jones (an intern from Cardiff University). I was the primary contributor with respect to the experimental data presented in Research Chapters 3, 5 and 6. Prof. Hugh Patterton was involved in the conceptual planning and adaptation of *in silico* methods presented in Chapter 3. Dr Hans Eyeghe-Bickong assisted with the GC-MS analysis presented in Chapter 5. Dr Fanny Buffetto assisted with the interpretation of cell wall data presented in Chapter 5. The work presented in Chapter 6 is the collaborative effort between myself and Dr Jay Belli Kullan. All raw data was compiled, analysed and interpreted for the thesis by me. My supervisor Prof MA Vivier was involved in the conceptual development of the study and continuous critical evaluation of the results and research in general.

Table of Contents

Chapter 1

Introduction and project aims

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The geographic distribution of plants is primarily determined by their ability to adapt to external environmental conditions. The major limiting factors are abiotic and include temperature, water, light and soil composition (Chapin *et al*., 1987). There are a number of plants with high levels of genetic variability which possess the ability to adapt to diverse climates and soils. Grapevine is one of them and this attribute is responsible for its cultivation in over 60 countries in all continents except for Antarctica (Bisson *et al*., 2002). It is a fruit crop of high economic value, with *Vitis vinifera* being the species of choice cultivated in all commercial vineyards due to its superior grape quality. Unfortunately current *V. vinifera* cultivars' adaptability does not extend to biotic stress, a trait which has been attributed to domestication, human selection for quality factors and vegetative propagation practices which prevented the species from evolving with its natural pathogens (Myles *et al*., 2011). The economically important cultivars of this species are thus highly susceptible to a range of pests and pathogens with fungi, arguably causing the most extensive pre- and post-harvest damage in commercial vineyards globally.

The main grapevine fungal diseases that have been widely studied include grey mould rot, as well as downy and powdery mildew (Wong *et al*., 2001; Williamson *et al*., 2007; Boso *et al*., 2014). The symptoms associated with each disease are distinct and in most cases irreversible. Downy and powdery mildew are both caused by biotrophs (*Plasmopara viticola* and *Erysiphe necator* respectively) which require live host tissue for growth and proliferation (Gadoury and Pearson, 1991; Wong *et al*., 2001). Both fungi attack green, above-ground grapevine organs such as leaves and berries (Gadoury *et al*., 2001; Kennelly *et al*., 2007). Host defence mechanisms against biotrophs usually include programmed cell death around the infection site which deprives the fungi of essential nutrients required for further growth and reproduction (Glazebrook, 2005).

The causal agent for grey mould rot is a necrotroph called *Botrytis cinerea* which is active over a wide temperature and geographical range (van Kan, 2006; Fillinger and Elad, 2016). Interestingly, *B. cinerea* infection can lead to either grey mould rot which is associated with huge yield losses or noble rot which is associated with the production of high value botrytised wines. Under moderate to cool weather with alternating humid and dry conditions, *B. cinerea* infection results in penetration of the plants' cell wall and conidium germination, although the proliferation of fungal spores is limited. This results in pores forming on the berry skins, causing water to evaporate from the inside. The berries then shrivel and high levels of aroma and flavour compounds accumulate (Blanco-Ulate *et al*., 2015). The resulting berries are then utilised to make highly viscous dessert wines of exceptional quality (Nelson & Amerine, 1956; Spellman, 1999). Interestingly, a noble rot *B. cinerea* BcDW1 strain has been sequenced and no genetic differences were found when its genome was compared to that of grey mould rot strains (Blanco-Ulate *et al*., 2013).

Unlike noble rot, grey mould rot is highly destructive. It attacks above-ground tissues such as leaves, berries and young buds leading to irreversible damage (van Kan, 2006). The genomic sequences of three *B.*

cinerea strains (B05.10, T4 and BcDW1) have been assembled and are publicly available for studying the pathogen further (Amselem *et al*., 2011; Staats and van Kan, 2012; Blanco-Ulate *et al*., 2013). During infection, *B. cinerea* secretes an array of metabolites and enzymes in an attempt to breach the hosts' surface. One of the most well studied pathogenesis-associated enzyme classes is cell wall degrading endopolygalacturonases (ePGs) called BcPGs in *B. cinerea*. At least six BcPG isoforms have been identified in the *B. cinerea* genome, with BcPG1 and 2 being the major virulence factors (ten Have *et al*. 1998; Kars *et al*., 2005; Nakajima and Akutsu, 2014).

BcPGs hydrolyse the α-1,4 linkages of the D-galacturonic acid residues within the homogalacturonan component of the hosts' primary cell wall (Andre-Leroux *et al*., 2009). D-galacturonic acid is the main source of nutrients for *B. cinerea* and knock-out mutants in fungal genes involved in its hydrolysis show reduced virulence on several plant hosts (Zhang and Van Kan, 2013). This maceration of the pectin component of the plants' cell wall leads to successful penetration and death of underlying epidermal cells. *B. cinerea* then utilises the dead tissue as a source of nutrients leading to proliferation of the infection (van Kan, 2006; Andre-Leroux *et al*., 2009). Thus any host defence mechanism involving programmed cell death would substantially benefit *B. cinerea* (Govrin and Levine, 2000; Glazebrook, 2005; Spoel *et al*., 2007).

Grapevine responses during *B. cinerea* infection, according to current literature, involve both preformed and inducible defence mechanisms. Preformed defences include physical barriers to fungal entry such as leaf hairs, cuticles, epicuticular wax layers and the cell walls (Maganu & Paolocci, 2013). They also include constitutively produced antimicrobial compounds such as volatile organic compounds (VOCs) (Lucker *et al*., 2004). These defence attributes are usually influenced by berry developmental stage and thus contribute towards ontogenic resistance (Kretschmer *et al*., 2007; Deytieux-Belleau *et al*., 2009). Collectively, preformed defences are geared to suppress fungal growth during the initial stages of infection, giving the plant ample time to recognise the pathogen and induce the next stages of defence.

Inducible responses on the other hand, are only activated in the presence of an infection. These include the upregulation of genes encoding pathogenesis related (PR) proteins, phytoalexins, antimicrobial VOCs and cell wall strengthening compounds (Timperio *et al*., 2012; Golshani *et al*., 2015). Sesquiterpenes such as α-copaene and β-caryophyllene have been identified as inducible VOCs during *B. cinerea* infection on tomato leaves and shown to possess antifungal activity by inhibiting spore germination and hyphal growth (Thelen *et al*., 2005; Zhang *et al*., 2008). Lignin, suberin, callose and arabinogalactan proteins (AGPs) have been shown to play a role in physically reinforcing the plants' cell wall during fungal infections (De Leeuw, 1985). AGPs are heavily glycosylated proteoglycans which exhibit high levels of developmental regulation and are also induced in response to both biotic and abiotic stress (Deepak *et al*., 2010; Pereira *et al*., 2015). They are suggested to contribute towards cell wall strengthening through self-aggregation and formation of cross-links with pectin (Tan *et al*., 2013; Hijazi *et al*., 2014).

Despite possessing all these preformed and inducible defence mechanisms, to date, all cultivated *V. vinifera* varieties are known to be highly susceptible to *B. cinerea*. However, varying degrees of resistance

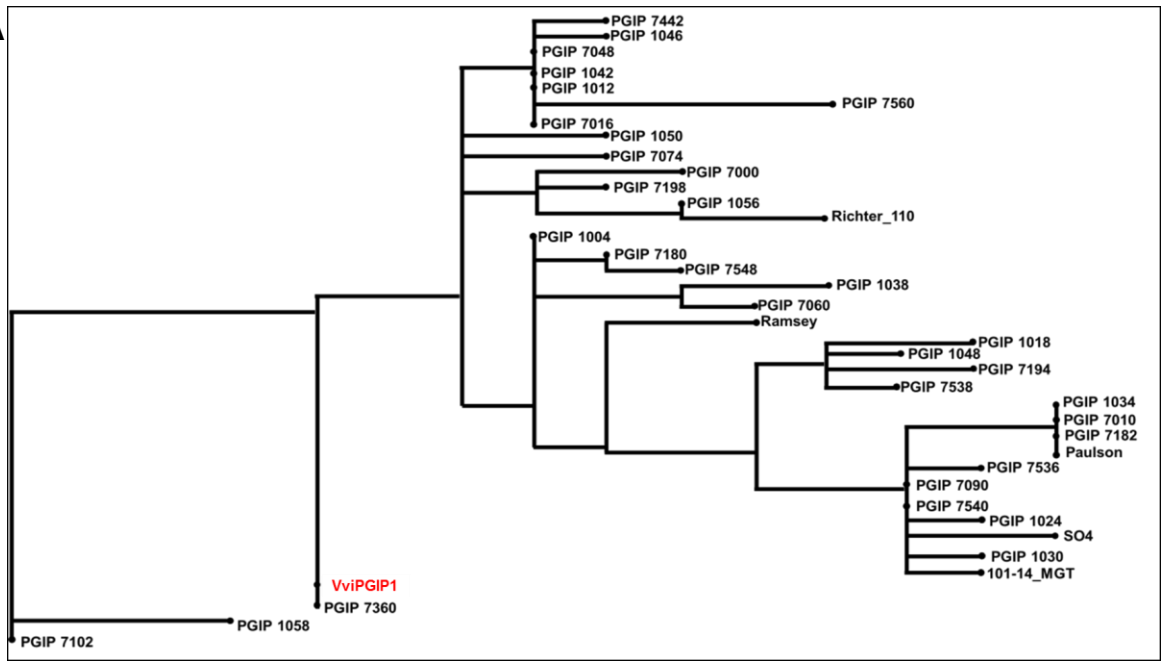
against infection have been detected in numerous American wild vines and *Vitis* hybrids (non-*vinifera* species) such as *V. labrusca* (Gabler *et al*., 2003). Several studies have attributed these inherent resistance traits exhibited by the wild vines against an array of fungal pathogens to the presence and expression of various genes and proteins (Dalbo *et al*., 2001; Pauquet *et al*., 2001; Welter *et al*., 2007; Hoffmann *et al*., 2008; Bellin *et al*., 2009; Ramming *et al*., 2011). One group of proteins, whose defence roles have been studied in many plant species and more recently in grapevine, are the polygalacturonase inhibiting proteins (PGIPs) (recently reviewed by Kalunke *et al*., 2015). They are cell wall associated proteins which form part of the leucine rich repeat (LRR) family. PGIPs' main described functions are linked to their inhibitory effects against ePGs from fungi, bacteria and even insect pests (De Lorenzo and Ferrari, 2002). The focus of this study is PGIPs from grapevines and their functions and roles in the plant body and in defence.

1.2 GRAPEVINE PGIPs: *what we know*

The first grapevine PGIP encoding gene, *Vvipgip1*, was isolated from *V. vinifera* L. cv. Pinotage (De Ascensao, 2001). Expression analysis showed that it is developmentally regulated and tissue specific, only being detected in roots and berries at and just after véraison (Joubert, 2004). However, *B. cinerea* infection, wounding, osmotic stress and presence of elicitors results in upregulation of *Vvipgip1* in all grapevine tissues (Joubert *et al*., 2013). The role of *Vvipgip1* in defence was confirmed in a tobacco overexpression study where the transgenic population exhibited reduced susceptibility when infected with a hyper-virulent *B. cinerea* grape strain (Joubert *et al*., 2006). Using *in vitro* and *in planta* assays, the resistance phenotype was attributed to VviPGIP1 effectively inhibiting the virulence factors, BcPG1 and BcPG2 (Joubert *et al*., 2006; 2007).

Further studies revealed subtle cell wall and hormone changes in the transgenic tobacco population overexpressing *Vvipgip1* prior to any infection (Alexandersson *et al*., 2011; Nguema-Ona *et al*., 2013). The increased lignin and indole-acetic acid levels together with remodelling of the cellulose-xyloglucan network in transgenic tobacco leaves prior to infection, were indicative of a primed state suggesting that the presence of VviPGIP1 enhanced the ability of tobacco to mobilise pathogen induced defence responses. This would consequently lead to an enhanced and accelerated response when infection actually occurred. These findings supported previous work which elucidated that PGIPs display additional roles other than the direct inhibition of fungal ePGs (Spadoni *et al*., 2006; Xu *et al*., 2008; Kanai *et al*., 2010). Therefore, the transgenic populations' primed state combined with VviPGIP1's ability to effectively inhibit *B. cinerea*'s two main virulence factors could explain the resistance phenotype exhibited by the transgenic tobacco population. Thirty seven grapevine PGIPs were isolated from a number of grapevine accessions (**Figure 1**) and shown to share high sequence identity (Wentzel, 2005).

A



B

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1030	95.8	98.2	98.2	98.5	98.5	97.3	96.4	99.1	99.1	99.1	99.1	99.1	99.1	99.4	100																						
7536	95.8	98.2	98.2	98.8	98.5	97.3	96.4	99.1	99.1	99.1	99.1	99.1	99.1	99.4	99.4	100																					
7540	96.1	98.5	98.5	98.8	98.8	97.6	96.7	99.4	99.4	99.4	99.4	99.4	99.4	99.7	99.7	99.7	100																				
7090	96.1	98.5	98.5	98.8	98.8	97.6	96.7	99.4	99.4	99.4	99.4	99.4	99.4	99.7	99.7	99.7	100	100																			
7000	97	97.3	97	97.3	97.3	98.2	97.3	97.3	97.3	97.9	97.9	97.9	97.9	97.6	97.6	97.6	97.9	97.9	100																		
7198	97.3	97.6	97.3	97.6	97.6	97.9	97	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	99.1	100																	
Richter	97	98.2	98.2	98.5	98.5	97.3	96.4	97.9	97.9	98.5	98.5	98.5	98.5	98.2	98.2	98.2	98.5	98.5	98.2	98.5	100																
1056	97	98.2	98.2	98.5	98.5	97.6	96.7	97.9	97.9	98.5	98.5	98.5	98.5	98.2	98.2	98.2	98.5	98.5	98.8	99.1	99.4	100															
1038	97	97.9	97.6	97.9	97.9	97.9	97	98.5	98.5	98.5	98.5	98.5	98.5	98.8	98.8	98.8	99.1	99.1	98.2	97.9	97.6	97.6	100														
Ramsey	96.7	98.5	98.2	98.5	98.5	97.6	96.7	97.6	97.6	97.9	97.9	97.9	97.9	97.9	97.9	97.9	98.2	98.2	97.9	98.2	97.9	97.9	98.5	100													
7060	96.7	97.6	97.3	97.6	97.6	98.2	97.3	98.2	98.2	98.8	98.8	98.8	98.8	98.5	98.5	98.5	98.8	98.8	99.1	98.2	97.9	97.9	99.1	98.2	100												
Vvipgip1	97.3	97.6	97.3	97.6	97.6	98.8	97.9	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	98.5	98.8	98.5	98.5	97.9	98.2	98.2	100											
7360	97.3	97.6	97.3	97.6	97.6	98.8	97.9	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	98.5	98.8	98.5	98.5	97.9	98.2	98.2	100	100										
7074	97.3	97.6	97.3	97.6	97.6	97.6	96.7	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	98.5	98.8	98.5	98.5	97.9	98.2	98.2	98.8	98.8	100									
1050	97.3	97.6	97.3	97.6	97.6	97.6	96.7	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	98.5	98.8	98.5	98.5	97.9	98.2	98.2	98.8	98.8	98.8	100								
1004	97.6	98.5	98.2	98.5	98.5	98.5	97.6	97.9	97.9	98.5	98.5	98.5	98.5	98.2	98.2	98.2	98.5	98.5	98.8	99.1	98.8	98.8	98.8	99.1	99.1	99.1	99.1	99.1	99.1	100							
7548	97	97.9	97.6	97.9	97.9	97.9	97	97.3	97.3	97.9	97.9	97.9	97.9	97.6	97.6	97.6	97.9	97.9	98.2	98.5	98.2	98.2	98.2	98.5	98.5	98.5	98.5	98.5	98.5	99.4	100						
7180	97.3	98.2	97.9	98.2	98.2	98.2	97.3	97.6	97.6	98.2	98.2	98.2	98.2	97.9	97.9	97.9	98.2	98.2	98.5	98.8	98.5	98.5	98.5	98.8	98.8	98.8	98.8	98.8	98.8	99.7	99.7	100					
1046	97.9	97.6	97.3	97.6	97.6	97.6	96.7	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	98.5	98.8	98.5	98.5	98.5	98.2	98.2	98.8	98.8	98.8	98.8	99.1	98.5	98.8	100				
7442	97.9	97.6	97.3	97.6	97.6	97.6	96.7	97	97	97.6	97.6	97.6	97.6	97.3	97.3	97.3	97.6	97.6	98.5	98.8	98.5	98.5	98.5	98.2	98.2	98.8	98.8	98.8	98.8	99.1	98.5	98.8	99.4	100			
1012	98.2	97.9	97.6	97.9	97.9	97.9	97	97.3	97.3	97.9	97.9	97.9	97.9	97.6	97.6	97.6	97.9	97.9	98.8	99.1	98.8	98.8	98.8	98.5	98.5	99.1	99.1	99.1	99.1	99.4	98.8	99.1	99.7	99.7	100		
1042	98.2	97.9	97.6	97.9	97.9	97.9	97	97.3	97.3	97.9	97.9	97.9	97.9	97.6	97.6	97.6	97.9	97.9	98.8	99.1	98.8	98.8	98.8	98.5	98.5	99.1	99.1	99.1	99.1	99.4	98.8	99.1	99.7	99.7	100	100	
7016	98.2	97.9	97.6	97.9	97.9	97.9	97	97.3	97.3	97.9	97.9	97.9	97.9	97.6	97.6	97.6	97.9	97.9	98.8	99.1	98.8	98.8	98.8	98.5	98.5	99.1	99.1	99.1	99.1	99.4	98.8	99.1	99.7	99.7	100	100	100
7048	98.2	97.9	97.6	97.9	97.9	97.9	97	97.3	97.3	97.9	97.9	97.9	97.9	97.6	97.6	97.6	97.9	97.9	98.8	99.1	98.8	98.8	98.8	98.5	98.5	99.1	99.1	99.1	99.1	99.4	98.8	99.1	99.7	99.7	100	100	100

Figure 1. (**A**) Phylogenetic tree of the full-length amino acid sequences of the non-*vinifera* PGIPs. VviPGIP1 was included for comparison. The tree was constructed using PhyML (Guindon *et al*., 2005). (**B**) Percentage similarity matrix of non-*vinifera* PGIPs with VviPGIP1 created using MUSCLE software (Edgar, 2004). Adapted from Wentzel, 2005.

The potential defence roles of these homologous PGIPs isolated from other *Vitis* species were also investigated through overexpression in tobacco. The resulting transgenic populations were even more resistant to infection by the *B. cinerea* grape strain compared to VviPGIP1 plant lines as shown in **Figure 2** (Venter, 2010).

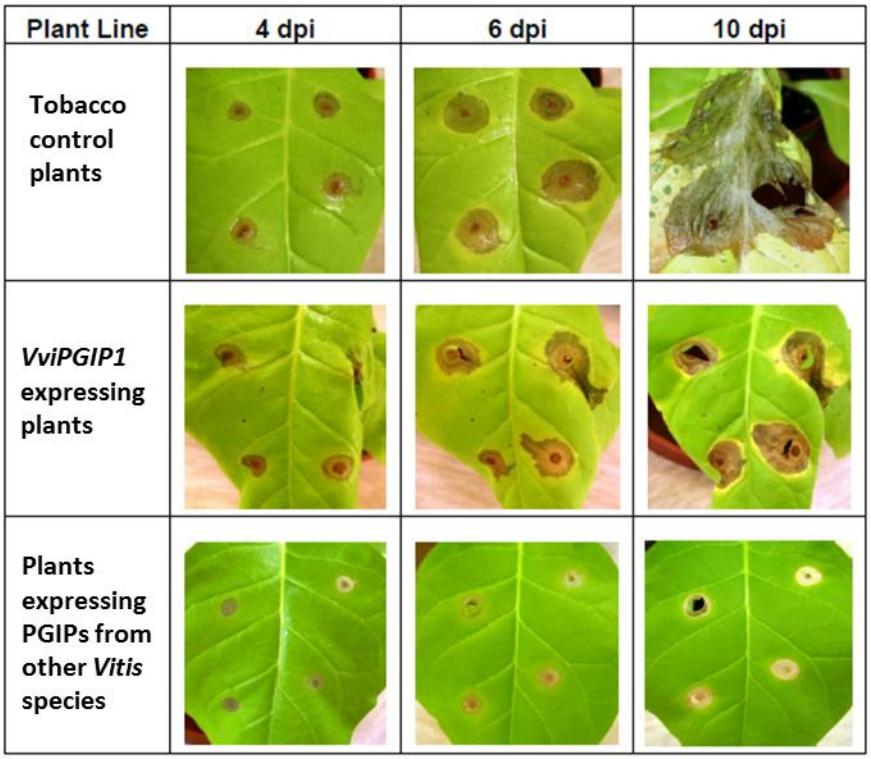

Figure 2. Lesion development on tobacco leaves infected with *B. cinerea* from 4 to 10 days post infection (dpi). Transgenic plants expressing *Vvipgip1* showed reduced susceptibility compared to the control plants whilst transgenic plants expressing PGIPs from other *Vitis* species (a selection of 14 genes from homologous groups based on LRR domain sequence alignments: *pgip*1004, *pgip*1012, *pgip*1018, *pgip*1024, *pgip*1030, *pgip*1034, *pgip*1038, *pgip*1056, *pgip*1058, *pgip*7000, *pgip*7060, *pgip*7194, *pgip*7538 and *Ramsey*) showed an even higher degree of reduced susceptibility. Adopted from Venter, 2010.

These results prompted further analysis in the native host and two of the wild vine PGIPs displaying strong defence phenotypes in transgenic tobacco were then selected for overexpression in *V. vinifera*. These were PGIP1012 (from *V. x doaniana* Munson, a hybrid of *V. mustangensis* and *V. acerifolia*) and PGIP1038 (from *V. caribaea*), referred to as non-*vinifera* PGIPs henceforth. Comprehensive genetic and initial phenotypic characterisation of the putative *V. vinifera* transgenic population revealed that the transgenes were successfully introduced and expressed in the transgenic materials and *in vitro* protein activity of the PGIPs against crude extracts of BcPGs were confirmed (Moyo, 2011). Surprisingly, when the transgenic population was challenged with the same *B. cinerea* grape strain utilised for the tobacco antifungal assays (Venter, 2010), it displayed enhanced susceptibility to the pathogen, leading to explosive growth of the pathogen and yielding a hyper-susceptible phenotype on the transgenic grapevines, compared to the untransformed controls (**Figure 3**, taken from Moyo, 2011). Although there are other examples where overexpression of PGIPs did not lead to enhanced resistance (Desiderio *et al*., 1997; Janni *et al*., 2013), enhanced susceptibility has not yet been reported according to our knowledge and literature searches.

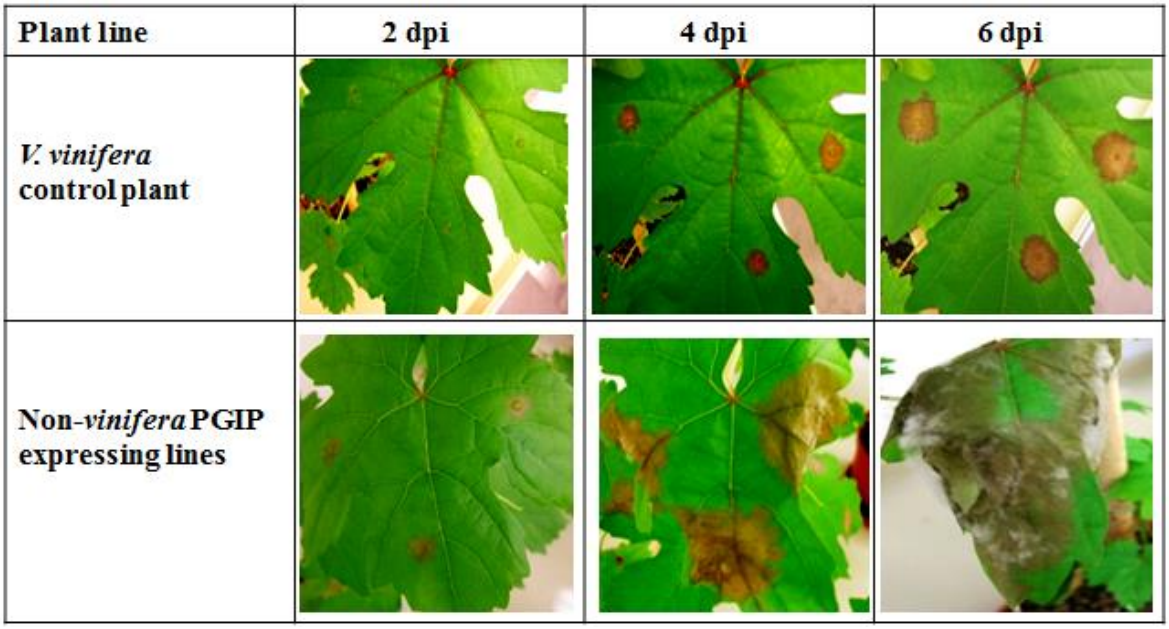

Figure 3. Lesion development on *V. vinifera* leaves infected with a hypervirulent *B. cinerea* strain isolated from grapes from to 2 to 6 days post infection (dpi). Transgenic plants expressing non-*vinifera* PGIPs showed enhanced susceptibility compared to the untransformed controls (Moyo, 2011).

1.3 SCOPE AND OBJECTIVES OF THE STUDY

The starting point for this study was thus the unexpected and very strong (hyper)susceptible phenotype observed when grapevine (*V. vinifera*) was transformed with two PGIP encoding genes, originating from a hybrid and a *Vitis* species, and infected with a hyper-virulent *B. cinerea* strain. The genetic and initial phenotypic characterisation of the transgenic population was comprehensively discussed in the MSc thesis of Ms Moyo (Moyo, 2011) and the unanswered questions of that study sparked the current study.

In this study, we hypothesise that the phenotype observed was not linked to the inability of the grapevine PGIPs to inhibit Botrytis ePGs, but rather linked to the non-ePG functions of PGIPs that influenced host plant defence mechanisms in a way that benefitted the pathogen. The following research questions were formulated to systematically test the PGIP-ePG inhibition interactions and the host-pathogen interaction that lead to the phenotype:

1. What is the structure-function relationship between grapevine PGIPs and *B. cinerea* BcPGs?
2. How does the transgenic grapevine population react to infection by a different *B. cinerea* strain and/or *Botrytis* mutants, as well as another fungal species?
3. Are non-*vinifera* PGIPs able to effectively inhibit BcPGs? How do their inhibition profiles compare to VviPGIP1?
4. Does post transcriptional gene silencing of either the native *Vvipgip1* or transgenic non-*vinifera* *pgip*s play a role in the hyper-susceptible phenotype?

5. Were there changes in the cell walls of the transgenic leaves prior to infection as a result of PGIP overexpression? If so, could these changes potentially benefit *B. cinerea* during infection and in what way?
6. Did the transgenic grapevine population exhibit signs of altered volatile organic compound (VOC) emissions? If so, what could be the potential impact on plant defence against *B. cinerea* infection?
7. What can we learn from an interactome study between transgenic grapevine and the *B. cinerea* grape strain? Are there any differences in defence strategies between transgenic and control plants during infection?

The existing and previously characterised different tobacco and grapevine transgenic populations, as well as tools and techniques to follow the interactions between hosts and pathogen and PGIP and ligands (ePGs) were available resources to this study. The outcomes of the research aimed to address these questions are presented as follows in this thesis:

Chapter 3 (addresses Question 1)

- Modelling the putative protein structures of VviPGIP1 and non-*vinifera* PGIPs together with BcPGs from three *B. cinerea* strains (B05.10, SAS56 and grape-strain);
- Conducting docking simulations of all PGIP-BcPG complexes.

Chapter 4 (addresses Question 2)

- Characterisation of transgenic grapevine populations' defence phenotype against *B. cinerea* B05.10 strain and knock out mutants in genes involved in pectin degradation and galacturonic acid catabolism;
- Characterisation of the transgenic grapevine populations' response to *E. necator* infection, a biotrophic fungus.

Chapter 5 (addresses Questions 3, 4, 5 and 6)

- Profiling the inhibition interaction of non-*vinifera* grapevine PGIPs against BcPGs using *in vitro* and *in planta* platforms.
- Determining the expression patterns of *Vvipgip1* and the non-*vinifera* PGIPs in transgenic grapevine leaves during *B. cinerea* infection.
- Profiling cell wall composition of uninfected transgenic grapevine leaves using CoMPP (Comprehensive Microarray Polymer Profiling).
- Profiling defence-related sesquiterpene emission profiles of transgenic leaves during *B. cinerea* infection.
- An addendum on the characterisation of the grapevine AGP family is also provided

Chapter 6 (addresses Question 7 by presenting preliminary results of an interactome study)

- Profiling the grapevine-*Botrytis* interactome using RNAseq during the early stages of infection

The research chapters presented in this thesis follow a literature review (presented in Chapter 2), whereas the major outcomes, insights and new questions from this study are contextualised in Chapter 7 of this thesis.

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Chapter 2

Literature review

Grapevine defence against mildews and grey mould rot

This review will be submitted to the **South African Journal of Enology and Viticulture** (SAJEV)

GRAPEVINE DEFENCE AGAINST MILDEWS AND GREY MOULD ROT

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2.1 INTRODUCTION

Grapevine (genus *Vitis*) is a high-value fruit crop consisting of at least 60 species differing primarily in type and quality of grapes produced (Bouquet, 2011). These species differ in their degree of susceptibility to invading pathogens and adaptation to diverse environmental conditions (Boso and Kassemeyer, 2008). Of all the *Vitis* species, the most widely cultivated in the global wine industry is the fully domesticated European grape *Vitis vinifera*, favoured for its fruit quality traits. Its grapes are used to produce wine, brandy, grape juice and also consumed fresh, or dried to make raisins (Bisson *et al*., 2002; This *et al*., 2006; Terral *et al*., 2010). *V. vinifera* encompasses a wide range of cultivated varieties; some are widely planted and renowned international cultivars such as Merlot, Cabernet Sauvignon, Shiraz, Sauvignon Blanc and Chardonnay, whereas autochthonous varieties are planted and promoted mostly regionally in certain areas/countries (Sefc *et al*., 2000; Almadanim *et al*., 2007). Irrespective of where cultivars and clones of *V. vinifera* are planted, they display significant susceptibility to pathogens and pests such as bacteria, nematodes, parasites, phytoplasma, viruses, insects and fungi. These infectious organisms cause destructive grapevine diseases like anthracnose, phylloxera, Pierce's disease, downy and powdery mildew, grey mould rot, *Eutypa* dieback, leafroll and black rot amongst others (Granett *et al*., 1998; Wong *et al*., 2001; Hopkins and Purcell, 2002; Williamson *et al*., 2007; Boso *et al*., 2014; Molitor and Beyer, 2014; Barros *et al*., 2015; Montero *et al*., 2016). These and other infections limit the cultivation success and profitability of grapevines in commercial vineyards and the costly (both in monetary and negative environmental impacts) control strategies are not sustainable.

Numerous factors are known to contribute to the overall susceptibility of any *Vitis* species to infection (Kummuang *et al*., 1996). These include vineyard location, age of infected plant, extent of competition from other invading micro-organisms, pathogen genotype and density, presence of pruning wounds and phenological stage of berry or bud development (Rumbolz and Gubler, 2005; Eskalen *et al*., 2007; Cadle-Davidson, 2008; Gadoury *et al*., 2012). The prevailing weather conditions are also crucial in determining the success rate of some infections since certain pathogens are only active under specific temperature or humidity conditions. Thus, susceptibility of *Vitis* to invading pathogens is a complex trait with numerous interconnected processes and environmental conditions influencing the phenotype.

Of all *Vitis* pathogens, fungi arguably cause the most general damage to commercial vineyards worldwide. From an economic point of view, the most important fungal pathogens to the global wine

industry are often listed as *Plasmopara viticola* (cause for downy mildew), *Erysiphe necator* (cause for powdery mildew) and *Botrytis cinerea* (cause for grey mould rot). In an attempt to minimise damage to grapevine caused by these fungal pathogens, various breeding programs were initiated in the early 20th century with the sole purpose of developing *Vitis* hybrids with more commercially beneficial resistance traits. For example, crosses between *V. vinifera*, which is highly susceptible to *E. necator*, and a resistant species, *V. rupestris*, resulted in a resistant hybrid, Royalty (Doster and Schnathorst, 1985). Also, *V. longii* is highly susceptible to *E. necator* but when it is crossed with *V. mustangensis*, the resulting hybrid, *V. doaniana* Munson, was found to be highly resistant to powdery mildew (Staudt, 1997; Cadle-Davidson, 2008).

Unfortunately these conventional hybridisation techniques take long and some of the hybrids produce grapes of unacceptably poor quality. “Next generation breeding” which proposes the combined use of advanced genetic, bioinformatic and next generation sequencing (NGS) technologies to develop new hybrids for crop improvement, is a promising avenue (Barabaschi *et al*., 2016). Some of the advances in bioinformatics include the development of software pipelines such as HetMappS and AmpSeq, for the genetic mapping of highly heterozygous species (Hyma *et al*., 2015; Yang *et al*., 2016). Breeding programmes assisted with marker technology, such as introgressive hybridisation, are a commonly utilised strategy, given the increasing understanding of the genetic determinants of defence (Harrison and Larson, 2014; Dangl *et al*., 2015). An excellent example of this new generation breeding programmes is the USDA-NIFA VitisGen project involving 11 institutions, and using a combination of molecular approaches to accelerate grape cultivar improvement (Cadle-Davidson *et al*., 2015).

Molecular approaches such as defence-related gene overexpression studies provide an alternative avenue for enhancing grapevine resistance against fungal infections in commercially cultivated varieties (Dry *et al*., 2010). For example, overexpression of chitinase genes in *V. vinifera* resulted in significantly higher levels of resistance against *P. viticola* (Nookaraju & Agrawal, 2012). Products from these potentially promising approaches would currently not be acceptable to the international wine industries mainly due to general resistance against genetically modified crops and thus the primary means of disease management involves the application of preventative and curative fungicides numerous times during the growing season (Madden *et al*., 2000; Gessler *et al*., 2011). Alternative cultural practises such as canopy management are also being utilised in an effort to reduce the incidence of fungal infections; unfortunately this is usually not enough (Lemut *et al*., 2011). Numerous studies have identified biological agents to control fungal pathogens (Falk *et al*., 1995; English-Loeb *et al*., 1999; Crisp *et al*., 2006), however, implementing such measures on a large-scale often proves problematic.

Detailed understanding of how the fungal pathogens infect grapevine and consequent plant defence strategies is supportive of more targeted control mechanisms. Thus, this review will give a brief overview of the different infection strategies employed by *P. viticola*, *E. necator* and *B. cinerea* during grapevine infections and the resulting disease symptoms. Recent and comprehensive summaries in this regards also include reviews from Gadoury *et al*., 2012; Gessler *et al*., 2011; and Williamson *et al*., 2007; Fillinger and

Elad, 2016). **Table 1** summarises the main characteristic features of the pathogens and the diseases they cause that will form part of this review. The main focus will be on summarising our current knowledge on pre-formed and induced grapevine defence responses to downy mildew, powdery mildew and grey mould rot infections at the phenotypic level. Detailed reviews on the underlying defence signalling responses are available from Sharon *et al*., 2007; Bari and Jones 2009; Verhage *et al*., 2010; Derksen *et al*., 2013; Windram and Denby, 2015; Lu *et al*., 2016; and Pandey *et al*., 2016.

Table 1. A summary of some of the characteristics which differentiate the most economically important grapevine fungal diseases.

	Downy mildew	**Powdery mildew**	**Grey mould rot**	**Noble rot**
Causal organism	*P. viticola*	*E. necator*	*B. cinerea*	*B. cinerea*
Feeding lifestyle	Biotroph	Biotroph	Necrotroph	Necrotroph
Optimum temperature for spore germination	18-22°C	26°C	~20°C	20-25°C
Optimum humidity for spore germination	≥98%	≥85%	≥93%	85-95% followed by a drop to below 60%
Mode of host entry	Through stomata	Ruptures cell wall using appresoria	Degrades cell wall using cell wall degrading enzymes	Degrades cell wall using cell wall degrading enzymes
Ontogenic resistance	Increases towards berry ripening	Increases towards berry ripening	Decreases towards berry ripening	Decreases towards berry ripening
Visual symptoms on leaves	Pale yellow oil spots	Whitish-grey powdery appearance	Brown necrotic lesions	Brown necrotic lesions
Visual symptoms on berries	White berries turn dull-grey and red berries turn pinkish-red	Whitish-grey powdery appearance	Brown necrotic lesions with fungal growth on surface	Berry shrinking without visible fungal growth

2.2 DOWNY MILDEW

All cultivated *V. vinifera* varieties are susceptible to the fungal disease which is caused by the heterothallic oomycete *P. viticola* (Wong *et al*., 2001). However, there are non-*vinifera* grapevine species which are either moderately or completely resistant to the fungus, probably due to their longer co-evolution with the pathogen (Boso *et al*., 2014; Liu *et al*., 2015). These include *V. riparia*, *V. rotundifolia*, *V. amurensis* and *Muscadinia rotundifolia*. Numerous factors contributing to this resistance phenotype have been identified. Some are anatomical whilst others involve defence gene induction leading to disruption of the infection cycle of the pathogen (Bosso and Kassemeyer, 2008; Cadle-Davidson, 2008). A draft genome sequence for *P. viticola* was recently released, providing a valuable platform to study the pathogen further and its interaction with grapevine on a molecular level through transcriptomic analyses (Dussert *et al*., 2016).

2.2.1 *P. viticola* life cycle and disease symptom development

P. viticola in an obligate biotroph which reproduces sexually to produce oospores. The oospores germinate optimally around 18-22°C to form sporangia which in turn releases infectious flagellate zoospores. The zoospores penetrate the host plant through the stomata, shed the flagellum and encyst. Germ tubes emerge from each spore, grow into the substomatal cavity and dilate into infection vesicles. Host chemotactic attractants are believed to control the release of zoospores from sporangia and the morphogenesis of germ tubes and primary vesicles (Kiefer *et al*., 2002; Jurges *et al*., 2009). Primary hyphae emerges from the infection vesicles and develops into mycelia which grow intercellularly to produce haustoria that penetrate the host cell walls. Secondary infections occur when masses of sporangia produced from sporangiophores are dispersed by wind currents and rain splash. The infection is then initiated under weather conditions favourable to support *P. viticola* growth and development, and in the absence of fungicide protection (Kiefer *et al*., 2002).

In susceptible cultivars, the fungus infects all green tissues including berries, shoots, tendrils, inflorescences and leaves. Heavy leaf infections usually result in leaf curling and defoliation. Young inflorescences can also be infected before they reach flowering stage without any visually detectable symptoms. The green fruit later turns light brown to purple, shrivel and detach easily, resulting in huge crop losses. The disease is most prevalent in areas with high rainfall and humidity and the fungus is active under a wide range of temperatures (Kennelly *et al*., 2007). The typical visual symptoms of the disease are irregular pale yellow oil spots on the upper parts of leaves, appearing 5-7 days after infection, depending on temperature and humidity (**Fig. 1A**). The colour of the spots however, differs according to grape variety, with white varieties exhibiting yellow spots whilst some red varieties develop red-brown spots. These later develop to form the characteristic white fungal growths on the underside of the leaves and any other infected organs (**Figs. 1B, C** and **D**).

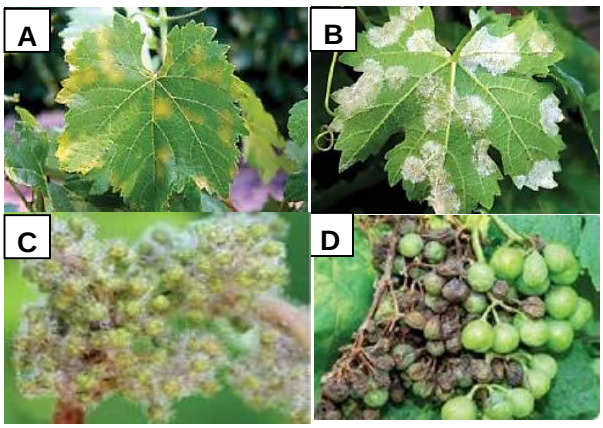

Figure 1. Typical downy mildew symptoms on (**A**) the upper grapevine leaf surface showing yellow oil spots, (**B**) underside of a grapevine leaf showing cotton-like white fungal growth, (**C**) infected grape cluster after berry set, (**D**) a fully developed table grape cluster showing heavy infection (*Images adopted from Wineland Media, 2015*).

2.2.2 Grapevine defence against downy mildew

During *P. viticola* attack, grapevine plants employ numerous defence mechanisms in an effort to reduce the amount of damage caused by the invading organism. Some forms of defence are pre-formed whilst others are induced during infection. The promptness and intensity at which these defence responses are mounted determine whether a grapevine species is classified as susceptible or resistant to downy mildew. The environment in which the plant-pathogen interaction occurs also has a strong influence on the progression of the pathogen from one stage of its infection cycle to the next. Thus susceptible grapevine cultivars either lack or have delayed resistance responses to *P. viticola* under numerous environmental conditions (Kennelly *et al*., 2007; Boso *et al*., 2014). This partial host resistance is believed to enable ample time for the pathogen to adapt and increase aggressiveness. Over time, this trend encourages the development of more virulent fungal strains (Delmas *et al*., 2016).

2.2.2.1 Pre-formed defence responses

Since the fungal zoospores penetrate the host plant through the stomata, there were some speculations in the past that leaves with higher numbers of stomata would naturally be more susceptible to *P. viticola.* The stomatal openings would provide the fungus with direct access to the mesophyll layer thus facilitating quicker and more efficient infection. However, research has shown that stomatal density is a genetic trait in grapevine species with most of them ranging between 140 and 300/mm^2. *Muscadinia rotundifolia*, known to be highly resistant to *P. viticola*, has stomatal density reaching 400/mm^2 (Shiraishi *et al*., 1996). Thus it seems unlikely that stomatal density alone determines resistance levels of grapevine to *P. viticola*.

Other physical defence mechanisms against *P. viticola* have been identified. These include pre-formed passive defences such as differences in leaf characteristics. Highly susceptible grapevine cultivars were found to possess thick spongy mesophyll layers whilst the more resistant varieties had thin and compact mesophyll layers (Alonso-Villaverde *et al*., 2011). These thin, compact mesophyll layers are believed to somehow impede or significantly retard the intercellular growth of the mycelium during the infection life

cycle of *P. viticola*. This would in turn reduce the number of haustoria formed, thus greatly reducing the degree of infection inflicted by the invading fungal pathogen on the plant host. Moreover, it would allow the host plant ample time to establish other active defence responses which are induced by infection.

Other forms of pre-formed defence involve the constitutive expression of major defence genes and accumulation of antimicrobial compounds in resistant grapevine cultivars prior to infection (Figueiredo *et al*., 2008). Gene expression analysis has shown that genes encoding polygalacturonase inhibiting proteins (PGIPs), pathogenesis related (PR) proteins and genes involved in the phenylpropanoid metabolism pathway such as PR2, PR4, PGIP and LDOX are constitutively expressed in higher levels in resistant grapevine cultivars like *V. riparia* cv. Gloire de Montpellier and Regent (a hybrid cross between Diana and Chambourcin varieties) whilst susceptible cultivars like *V. vinifera* cv. Riesling and Trincadeira display very low levels prior to infection (Kortekamp, 2006; Figueiredo *et al*., 2012). Furthermore, resistant varieties have been shown to constitutively express compounds with antimicrobial properties which represent chemical barriers to *P. viticola* attack. These include inositol, alanine, glutamate, glutamine and caffeic acid (Figueiredo *et al*., 2008). This suggests that the resistant cultivars possess a primed defence response against *P. viticola* infection compared to susceptible varieties. Age-related or ontogenic resistance has also been reported, with mature berries being less susceptible to infection compared to young berries (Kennelly *et al*., 2005).

2.2.2.2 Induced defence responses

In addition to pre-formed defence mechanisms against *P. vitivola*, other studies have shown that the resistance phenotypes of some grapevine species is based on the kinetics and amplitude of gene induction during infection (Polesani *et al*., 2010; Figueiredo *et al*., 2012). These induced defence genes encode for PR proteins and enzymes involved in jasmonate biosynthesis, hypersensitive defence responses and signal transduction pathways (Glazebrook, 2005; Spoel *et al*., 2007). The high susceptibility of *V. vinifera* to *P. viticola* compared to the resistant *V. riparia* was thus attributed to the former cultivars' weaker and slower defence responses during infection, possibly due to lack of a primed state (Polesani *et al*., 2010).

Although all cultivars of *V. vinifera* are susceptible to downy mildew, there is a small level of genetic resistance with Trincadeira and Carbernet Sauvignon being less susceptible when compared to Pinot Noir and Riesling. Significantly reduced length of hyphae and number of haustoria was seen on Cabernet Sauvignon during the course of these infection trials (Unger *et al*., 2007; Boso and Kassemeyer, 2008; Boso *et al*., 2011; Figueiredo *et al*., 2012; Yu *et al*., 2012). Furthermore, there were differences in degree of necrosis, suggesting an enhanced defence phenotype involving the hypersensitive response (HR) causing programmed cell death (PCD) at the site of infection. PCD is one of the primary mechanisms of induced defence responses in plants during fungal infections. Gene expression analysis has shown that the first signs of induced grapevine defence response are triggered when haustoria enter the mesophyll cells. Thus haustoria play a crucial role in inducing HR during *P. viticola* infection (Diez-Navajas *et al*., 2008). The

localised necrosis is associated with reduction in fungal performance, causing less severe symptom development over time (Bellin *et al*., 2009).

Currently, a total of 14 Quantitative Trait Loci (QTL) have been identified from various grapevine species and shown to play important roles in resistance against *P. viticola*. These include *Rpv1* (Resistance to *P. viticola* 1) from *M. rotundifolia*, *Rpv3* and *Rpv14* from *V. vinifera* and *Rpv8, Rpv10* and *Rpv12* from *V. amurensis* (Merdinoglu *et al*., 2003; Bellin *et al*., 2009; Schwander *et al*., 2010; Blasi *et al*., 2011; Venuti *et al*., 2013; Ochssner *et al*., 2016). Some are currently being introduced into cultivated grapevines through introgressive hybridisation and marker assisted gene pyramiding or stacking of two or more resistance loci within the same cultivar to reduce the chances of *P. viticola* breaking down the hosts' resistance barrier (Katula-Debreceni *et al*., 2010; Harrison and Larson, 2014).

Though the mechanisms of resistance employed by the QTL are not yet fully understood, *Rpv3* was shown to possess the ability to not only initiate HR within the first 48 hours post infection, but also quantitatively reduce *P. viticola* performance through reduced rate of fungal sporulation (Bellin *et al*., 2009). The findings complemented the work by Pezet *et al*., 2004 which showed that the resistant grapevine cultivars utilised HR for defence against *P. viticola* whilst the highly susceptible ones did not. This trait was attributed to the high production of ε- and δ-viniferin in resistant cultivars during infection. These compounds are highly toxic to *P. viticola* compared to resveratrol and piceid which are produced in higher concentrations in the susceptible cultivars (Pezet *et al*., 2004; Gindro *et al*., 2006). Furthermore, enhanced grapevine resistance phenotypes against downy mildew were strongly correlated with callose synthesis and deposition around the stomata, at the sites of haustoria formation and around invading hyphae. Callose acts as a physical cell wall reinforcement, effectively reducing the rate of fungal penetration during secondary infection of neighbouring stoma thus limiting nutrient exchange (Gindro *et al*., 2006; Palmieri *et al*., 2012; Toffolatti *et al*., 2012; Yu *et al*., 2012; Liu *et al*., 2015).

Transcriptional changes in the highly susceptible grapevines compared to resistant cultivars further elucidated the differences in defence gene induction during *P. viticola* infection (Figueiredo *et al*., 2012). The resistant cultivars had upregulated signal transduction components, LRR receptor-like proteins, specific resistance (*R)* genes, PR-10, subtilisin-like protease, chitinase-like proteins, lipid metabolism and chalcone synthase genes. The kinetics of the defence-related gene induction during *P. viticola* infection explained the higher disease resistance capability of some cultivars compared to their susceptible counterparts (Figueiredo *et al*., 2012).

2.3 POWDERY MILDEW

From a global perspective, *E. necator* (syn. *Uncinula necator*) is the most economically important fungal pathogen in commercial vineyards. It is a heterothallic pathogen which causes a disease known as powdery mildew in cultivated grapevine varieties (Gadoury and Pearson, 1991). To date, none of the commercially cultivated *V. vinifera* varieties are resistant to *E. necator*. However, there are some wild grape species like *M.*

rotundifolia, *V. riparia*, *V. rupestris* and *V. aestivalis* with high levels of resistance though not much is known about the mechanism behind this phenotype. As for downy mildew, the long co-evolution of these resistant species with *E. necator* plays a significant role in the development of resistance (Doster and Schnathorst, 1985; Staudt, 1997).

2.3.1 *E. necator* life cycle and disease symptom development

E. necator is a highly virulent obligate biotroph that obtains nutrients for growth and reproduction from feeding on live plant host tissue. During infection, the fungal spore, called a conidium, lands on the plant tissue and germinates, forming an infection structure called an appresorium. The appresorium possesses a penetration peg to pierce the cuticle and rupture the plants' cell wall to gain access to the contents of the cells. The fungus then forms a specialised globular or elongated feeding structure called the primary haustorium to absorb nutrients from the host cells. Secondary hyphae then develops and branches out across the plant surface, forming more appresoria and haustoria. This can result in explosive multiplication of the fungus ultimately leading to the colonisation of the entire infected organ (Gadoury & Pearson, 1990; Heintz & Blaich, 1990).

The fungus grows optimally at a relative humidity of approximately 85% (Carroll & Wilcox, 2003). It survives under a wide range of temperatures, although 26°C is the optimum temperature for sporulation to occur. Fungal activity is inhibited by long term exposure to high temperatures of up to 40°C and ultraviolet-B (UV-B) radiation. The radiation causes a reduction in conidium germination, appresorium formation and colony development whilst conidia are killed at 40°C (Willocquet *et al*., 1996; Austin & Wilcox, 2012). In susceptible cultivars, the pathogen infects all green tissues including berries, young buds, leaves, shoots and stems. Heavily infected leaves usually senesce and develop chlorotic or necrotic spots leading to premature defoliation. This reduces net photosynthesis and debilitates the vines leading to retarded berry ripening, berry crack and ultimately poor wine quality. Furthermore, cracking or splitting of berries associated with *E. necator* infections have been positively correlated with an increase in other opportunistic infestations from spoilage microbes and insects resulting in further yield losses (Rumbolz & Gubler, 2005; Gadoury *et al*., 2001; 2007).

The characteristic visual symptom of powdery mildew is a dusty, whitish-grey, powdery appearance caused by the presence of fungal mycelia and conidia on the surface of infected leaves and grape berries (**Fig. 2**). Infected grapevine stems also exhibit white fungal growth which later develop to form reddish, dark brown to black spotty necrotic lesions (**Fig. 2C)**.

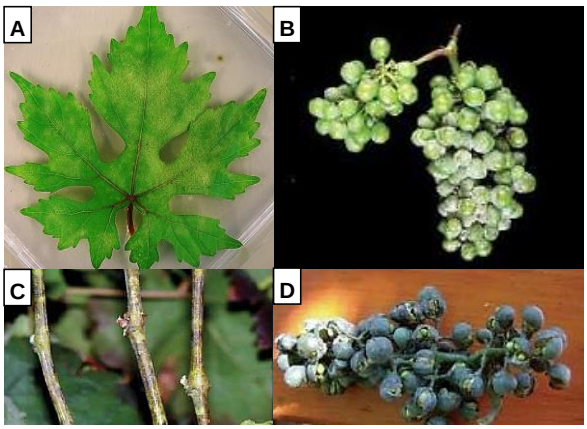

Figure 2. Typical powdery mildew symptoms on (**A**) upper side of a grapevine leaf showing the characteristic whitish powdery appearance, (**B**) grape bunch also showing whitish powdery appearance, (**C**) grapevine canes showing dark infection patches, (**D**) infected bunch showing berry crack which predisposes them to attack by other pathogens (*Images* **B, C** *and* **D** *were adopted from Wineland Media, 2015).*

2.3.2 Grapevine defence against powdery mildew

Numerous factors have been shown to strongly influence the degree of host susceptibility to *E. necator*. These include pathogen inoculum density and virulence, temperature, humidity, grapevine cultivar, plant organ and its developmental stage (Doster & Schnathorst, 1985; Staudt, 1997; Carroll & Wilcox, 2003; Austin & Wilcox, 2012).

2.3.2.1 Pre-formed defence responses

The degree of susceptibility of any grapevine species, including *V. vinifera*, to *E. necator* is not uniform throughout the plants' development. There exists ontogenic resistance of the grapevine host to *E. necator* with reduction in level of susceptibility as different tissues age. Generally, grape berries have been shown to exhibit high levels of susceptibility during pre-véraison stages, but acquire ontogenic resistance during ripening (Chellemi & Marois, 1992; Ficke *et al*., 2002; Gadoury *et al.*, 2003). This ontological resistance is accompanied by a decline in penetration of berries by germinating fungal spores, increase in hyphal death within colonies, significant reduction in formation of haustoria, retarded development of secondary hyphae and a marked increase in the latent period between germination and sporulation (Gadoury *et al*., 2003). Though ontogenic resistance does not lead to immunity, it significantly slows and eventually stops powdery mildew development on grapevine tissues. Resistant cultivars like *V. riparia* acquire ontological resistance at an earlier phenological stage compared to the susceptible *V. vinifera*, thus limiting disease severity more effectively with a potential to even escape infection (Ficke *et al*., 2003).

One of the preformed defences which have been connected to ontological resistance is cuticle thickness. The cuticle provides one of the first potential physical barriers that *E. necator* encounters during grapevine infection. Successful penetration of the cuticle by the appresorium is required in order for the fungus to gain access to the plants' nutrients for growth and reproduction. Initial studies showed that cuticle thickness in leaves and berries was positively correlated with tissue age and degree of resistance to *E. necator* infection (Heintz & Blaich, 1990). However, further studies have shown that some susceptible young

grapes possess thicker cuticles compared to older and resistant grape berries, indicating that other factors also contribute to ontological resistance (Ficke *et al*., 2004). Induction of defence genes during infection seems to play a more direct role in grapevine defence against powdery mildew.

2.3.2.2 Induced defence responses

To date, at least seven loci responsible for resistance traits against *E. necator* in grapevine have been identified (Qui *et al*., 2015). Expression of genes found at these loci results in plant responses involving necrosis of the fungal appresorium within epidermal cells, necrosis of the host cells at the site of infection or elicitation of further defence responses (Feechan *et al*., 2011). One such locus is *Run1* (Resistance to *Uncinular necator* 1) located on chromosome 12 of the grapevine genome (Pauquet *et al*., 2001). *Run1* is present in the resistant grapevine cultivar *M. rotundifolia* but absent in the widely cultivated *V. vinifera.* Sequencing of the *Run1* locus led to the identification of the *MrRun1* gene, which when transformed into highly susceptible cultivars such as *V. vinifera*, conferred the host plants with strong powdery mildew resistance. MrRUN1 protein is suggested to play a possible role in disease resistance signalling (Feechan *et al*., 2013).

In 2011, Riaz *et al*., identified two allelic variants of powdery mildew resistance loci, located on chromosome 18 of the grapevine genome named *Run2.1* and *Run2.2* from two different *M. rotundifolia* cultivars. Preliminary studies showed that the defence mechanism attributed to these loci was also PCD of host tissue leading to arrested fungal development. Other resistance loci that have been identified include *Ren1* located on chromosome 13 (Hoffmann *et al*., 2008), *Ren 2* located on chromosome 14 (Dalbo *et al*., 2001), *Ren3* located on chromosome 15 (Welter *et al*., 2007), *Ren4* located on chromosome 18 (Ramming *et al*., 2011), *Ren5* located on chromosome 14 (Blanc *et al*., 2012), *Ren6* located on chromosome 9 and *Ren7* located on chromosome 19 (Pap *et al*., 2016). The *Ren1* locus is the only one which occurs naturally in the commercial grapevine cultivar *V. vinifera*. *Ren1, Ren4* and *Ren6* were shown to control *E. necator* colonisation by restricting fungal growth and also elevating levels of PCD in infected cells, leading to complete resistance (Hoffman *et al*., 2008; Ramming *et al*., 2011; Pap *et al*., 2016). On the other hand, *Ren2, Ren3, Ren5* and *Ren7* only confer partial resistance to the invading fungus (Dalbo *et al*., 2001; Welter *et al*., 2007; Blanc *et al*., 2012; Pap *et al*., 2016).

Synthesis of PR proteins is another defence strategy employed by grapevine to combat powdery mildew infections. These include chitinase, *β*-1,3-glucanase, osmotin and thaumatin-like proteins. Some PR proteins are involved in preformed defences whilst others are induced by infection, wounding and the presence of elicitors. The speed at which these defence proteins are produced by the host plant, the levels to which they accumulate coupled with their cellular localisation relative to the invading fungi plays a crucial role in determining their efficiency in limiting degree of fungal infection. Osmotin and thaumatin-like proteins accumulate to high levels in ripening berries without any fungal infection, suggesting that they might also be involved in ontological resistance (Tattersall *et al*., 1997; Pocock *et al*., 2000). Chitinase, *β*-1,3-glucanase, osmotin and thaumatin-like proteins are induced in leaves and berries infected with *E. necator*

(Jacobs *et al*., 1999; Monteiro *et al*., 2003; Fung *et al*., 2008; Fekete *et al*., 2009). Resistant grapevine cultivars were shown to consistently exhibit higher levels of chitinase and *β*-1,3-glucanase activity during infection compared to the more susceptible cultivars. Purified extracts of the PR proteins were shown to possess antifungal activity against *E. necator* by inhibiting germ tube growth, more-so when the extracts were combined. This suggests that the PR proteins act synergistically in the plant host to combat powdery mildew infection (Giannakis *et al*., 1998; Jacobs *et al*., 1999). Other studies have shown that overexpression of chitinase genes in *V. vinifera* results in enhanced resistance against *E. necator* (Yamamoto *et al*., 2000; Rubio *et al*., 2015). This further elucidates the role that these defence proteins play in protecting grapevine against powdery mildew.

Another group of antimicrobial metabolites which are induced by *E. necator* infection in grapevine plants are phytoalexins such as stilbenes. *Trans*-resveratrol, *trans*-piceid, viniferins and pterostilbene are examples of stilbenes that have been identified in *Vitis* (Chong *et al*., 2009). They inhibit fungal growth and proliferation through their fungitoxic activity, acting as chemical shields against *E. necator*. They accumulate to high levels in grapevine leaves and berries infected with powdery mildew (Romero-Perez *et al*., 2001; Fung *et al*., 2008; Schnee *et al*., 2008; Dai *et al*., 2012). Furthermore, stilbenes accumulate to high levels in the presence of elicitors of defence mechanisms such as methyl jasmonate and salicylic acid (Belhadj *et al*., 2006; Jiao *et al*., 2016). Interestingly stilbene synthase, a key enzyme in stilbene biosynthesis and modification, increases in berry skin post véraison reaching very high levels at harvest in the absence of *E. necator* infection (Dai *et al*., 2012), suggesting that it could contribute to the ontological resistance.

2.4 GREY MOULD ROT

B. cinerea is the causal agent for grey mould rot disease which affects commercial vineyards worldwide leading to huge losses in yield and grape quality. It is a filamentous necrotroph, feeding on dead plant tissue for its growth and proliferation (van Kan, 2006; Williamson *et al*., 2007; Tudzynski & Kokkelink, 2008). To date, none of the commercially cultivated *V. vinifera* species have been shown to possess complete resistance to *B. cinerea* although some wild vines like *V. labrusca* exhibit very high levels of resistance (Gabler *et al*., 2003; Wan *et al*., 2015). This has prompted strong industrial interest in this fungal pathogen and has led to *B. cinerea* being the most extensively studied necrotroph, making it an ideal model organism for research in fungi-plant interactions (Elad *et al*., 2016). Many strains and/or isolates have been identified from different plant sources and geographical regions. Some have been sequenced, assembled, annotated and are currently available as useful resources to study the pathogen further (**Table 2**).

Table 2. List of some common *Botrytis* strains utilised in plant infection studies.

***B. cinerea* strain/isolate**	**Description/virulence**	**Origin**	**Reference**
B05.10[a]	Haploid derivative of SAS56	Germany	Amselem *et al*., 2011
T4[a]	Lower virulence than B05.10	France	Amselem *et al*., 2011
BcDW1[a]	Virulent strain	California	Blanco-Ulate *et al*., 2013
SAS56	Virulent mono-ascospore strain	Italy	Wubben *et al*., 1999
Grape	Hyper-virulent	South Africa	Joubert *et al*., 2006

[a]full genome has been sequenced

2.4.1 *B. cinerea* life cycle and disease symptom development

B. cinerea infections cause devastating pre- and post-harvest decay in commercial vineyards globally. Fungal spores, called conidia, are largely generated as primary sources of infection within the plant and then disseminated by wind currents or water to other healthy tissues. Conidia can remain dormant on numerous grapevine tissues for prolonged periods without any visible symptoms, only completing the life cycle when conditions are more favourable for growth and reproduction. Infection of grapevine tissue by *B. cinerea* can also be opportunistic whereby penetration is through wound sites or open stoma. It can also act as a saprophyte on senescent and dead plant material (Williamson *et al*., 2007; van Kan *et al*., 2014).

Under favourable conditions, upon landing on receptive plant tissue, the conidium attaches to the surface and germinates forming germ tubes. These then develop an appresorium which possesses a penetration peg. Unlike *E. necator* which uses physical pressure from the penetration peg to rupture the plants' surface, *B. cinerea* is believed to utilise a different strategy since the appresorium lacks a sealing septum. Through the penetration peg, it secretes numerous metabolites, toxins and cell wall degrading enzymes which include cutinases, lipases, proteases and pectinases in order to breach the host surface (Alghisi *et al*., 1995).

One of the well-studied pectinases is endopolygalacturonase (ePG). *B. cinerea* possesses at least six ePG isoforms (BcPG1, BcPG2, BcPG3, BcPG4, BcPG5 and BcPG6) with differential regulation and substrate specificities (Kars *et al*., 2005). BcPG sequences from more than 30 *Botrytis* strains have been sequenced and they provide a valuable resource for evaluating strain specific interactions with characterised plant defence proteins using bioinformatic docking simulations (Rowe and Kliebenstein, 2007; Wubben *et al*., 1999). BcPG1 and 2 are generally considered as the main virulence factors though a study by Blanco-Ulate *et al*. (2014) showed that conditions in the host cell wall matrix influence *B. cinerea*'s infection strategy. For example, both BcPG1 and 2 were expressed during tomato infection, but BcPG2 was not expressed during grape berry infection.

BcPGs are responsible for hydrolysing the α-1,4 linkages of the D-galacturonic acid residues within the homogalacturonan component of the primary cell wall (Andre-Leroux *et al*., 2009). D-galacturonic acid

is the most abundant pectin component in the plant cell wall and the main source of nutrients for *B. cinerea* (Zhang *et al*., 2011). The breakdown of D-galacturonic acid leads to successful penetration of host cells and death of the underlying epidermal cells resulting in primary lesion formation. Being a necrotroph, *B. cinerea* utilises the dead tissue as a nutrient source for further growth and proliferation. This causes the lesion to expand as more host tissue is macerated and the abundance of nutrients to the invading pathogen encourages it to sporulate and spread further (Kars *et al*., 2005; van Kan, 2006; Williamson *et al*., 2007). Knock-out mutants in *Botrytis* genes involved in galacturonic acid catabolism show reduced virulence on tobacco and *Arabidopsis* (Zhang and Van Kan 2013).

Botrytis mutant analysis of pathogenicity factors has provided valuable insights on the dynamics of both host and fungal strain specificity during infection. BcPG1 knockout mutants from B05.10 strain exhibited reduced virulence on apple and tomato plants whilst BcPG2 mutants showed reduced virulence on tomato and broad bean (ten Have *et al*., 1998; Kars *et al*., 2005). B05.10, SAS56 and T4 deletion mutants in a gene encoding for botrydial, a phytotoxin released by *B. cinerea* during plant infection, exhibited strain-specific infection profiles when tested against bean and tomato (Colmenares *et al*., 2002; Siewers *et al*., 2005). Deletion of *Bcpme1*, a pectin methylesterase gene in strain Bd90 caused reduced virulence in apple fruits, grapevine and *Arabidopsis* leaves (Valette-Collet *et al*., 2003). However, deletion of the same gene in B05.10 did not reduce virulence in tomato and grapevine leaves (Kars *et al*., 2005).

B. cinerea also makes use of small regulatory RNAs (sRNAs) with the ability to suppress host immunity (Weiberg *et al*., 2013; 2014; Knip *et al*., 2014; Weiberg and Jin, 2015). These pathogen-derived effectors are induced during infection and are believed to function by either blocking the ability of the host to recognise the pathogen on the surface or they suppress signalling pathways upon entering infected host cells (Weiberg *et al*., 2013; Wang *et al*., 2015). Interestingly, *B. cinerea* has also been shown to secrete compounds which elicit host defence responses such as the oxidative burst, triggering programmed cell death which ultimately benefits the necrotrophic lifestyle of the invading pathogen (Govrin *et al*., 2006). Thus apart from utilising a wide array of enzymes and metabolites to breach host barriers during infection, *Botrytis* is also able to control some host defence responses by either blocking them or inducing them.

Despite being active over a wide temperature and geographical range, including cold conditions, *B. cinerea* conidia germinate optimally at around 20°C, relative humidity of >93% and still air (Williamson *et al*., 2007). The fungus infects mainly above ground grapevine organs such as leaves, berries, buds, flowers and shoots. Infection trends and severity can be tissue specific as evidenced by differences in defence gene induction on, for example, infected berries versus leaves (Bezier *et al*., 2002). Depending on the prevailing environmental conditions such as level of humidity, *B. cinerea* infections can be quiescent, restricted or aggressive. The most common visual symptom of grey mould rot in grapevine is either localised infections or spreading necrotic lesions (Williamson *et al*., 2007).

Under favourable conditions, grey masses of conidia typically develop on infected grapevine surfaces (**Fig. 3**). This is due to *B. cinerea* decomposing plant biomass and converting it to fungal mass (Elad, 1997). Infected leaves develop brown necrotic lesions which also have the potential to spread,

ultimately leading to the entire leaf being covered in fungal mass resulting in defoliation (**Figs. 3C** and **D**). Infected flower clusters, buds and young shoots dry and fall off. Wines made from grape berries infected with *B. cinerea* typically exhibit off-flavours due to high levels of oxidation, poor colour and aroma development and they are often difficult to ferment and clarify.

However, not all *B. cinerea* infections result in grey mould rot. Under specific climatic conditions which slow down the rotting process, the fungus causes noble rot in certain wine grape cultivars. Infection causes pores to develop on the grape skins and under moderate to cool temperatures these result in water evaporating from inside the fruit. Thus humidity should be high enough to enable successful conidia germination, but it should be followed by drier and warmer weather coupled with optimum air velocity to encourage evaporation of berry water to occur. Imbalances in the alternating weather conditions can easily result in *B. cinerea* overgrowth and ultimately huge yield losses. The shrivelled wine grape berries of a noble rot infection (**Fig. 3B**) have high levels of tartaric acid, sugars and other soluble solids. These changes are also associated with an increase in anthocyanins, terpenes and fatty acid aroma precursors (Blanco-Ulate *et al*., 2015). At late-harvest, the botrytised grapes are utilised to produce viscous dessert wines of exceptional quality (Nelson & Amerine, 1956; Spellman, 1999). The genome sequence of *B. cinerea* strain BcDW1, isolated from *V. vinifera* cv. Sémillon and used in the production of late harvest Dolce Wines (Oakville, California) is currently available (Blanco-Ulate *et al*., 2013) and provides a useful resource to study noble rot infections further.

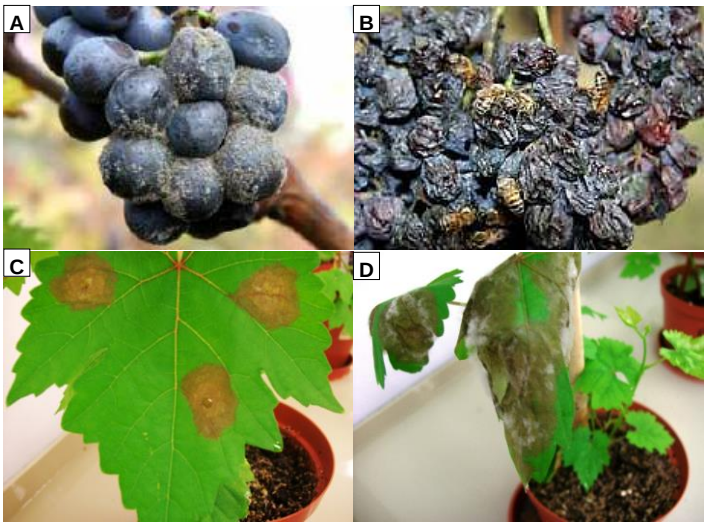

Figure 3. Typical *B. cinerea* infection symptoms on (**A**) grape bunch showing fungal mass on grape surfaces, (**B**) grape bunch with honey bees, a potential indicator for noble rot, (**C**) grapevine leaf showing brown necrotic lesions, (**D**) heavily infected leaf showing leaf curling and fungal mass on surface just before defoliation occurs (*Images* **A** *and* **B** *were adopted from Wineland Media, 2015).*

2.4.2 Grapevine defence against grey mould rot

The *Arabidopsis-Botrytis* interaction is one of the most well defined host-pathogen systems, providing a base on which other plant-*Botrytis* interactions are built upon (Windram *et al*., 2012). Time-series transcriptional

analysis of *Arabidopsis* leaves infected with *B. cinerea* revealed that defence-related gene expression profiles changed within the first 48 hours post infection. Most of the changes were observed before disease symptoms were clearly visible, in a small time window within the first 24 hours post infection (Windram *et al*., 2012). This time frame corresponds to penetration of host tissue by *B. cinerea* during infection (Van Kan, 2006; Williamson *et al*., 2007). Furthermore, the defence responses were shown to override the circadian clock which has been shown to influence susceptibility of *Arabidopsis* to *B. cinerea* infections (Windram *et al*., 2012; Ingle *et al*., 2015).

The small size and short generation time with large number of offspring have favoured *A. thaliana* as a model host plant compared to woody perennials like grapevine (Meinke *et al*., 1998). These features have contributed towards the high throughput generation of knockout mutants and investigation of *in planta* roles of numerous genes and metabolites (AbuQamar *et al*., 2006). Recent advances with the sequencing of the grapevine genome has enabled researchers to translate defence-related mechanisms reported in *A. thaliana* to the grapevine-*Botrytis* system (Jaillon *et al*., 2007; Windram *et al*., 2012; Blanco-Ulate *et al*., 2014; Kelloniemi *et al*., 2015).

During *B. cinerea* infection, the first lines of defence that grapevine utilises are pre-formed. These defence mechanisms give the host plant time to sense infection signals and respond by inducing defence pathways that help fight the invading pathogen (Elad, 1997; Glazebrook, 2005; Spoel *et al*., 2007; Hematy *et al*., 2009). These two defence mechanisms need to work hand in hand in order to significantly limit the amount of damage inflicted by the fungus (**Fig. 4**). A delayed or weak response from the plant host could result in irreversible damage (Muganu & Paolocci, 2013).

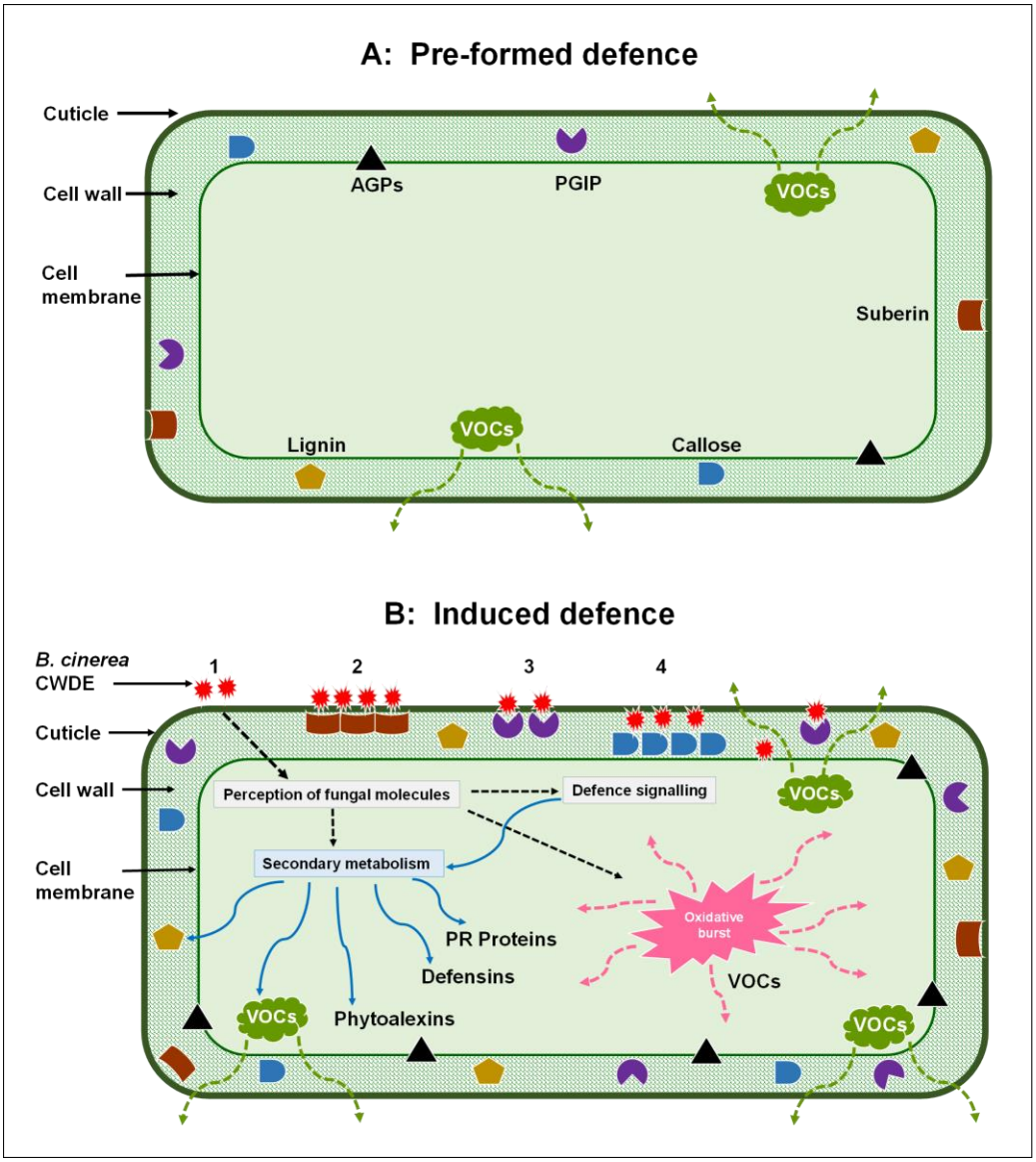

Figure 4. An overview of defence responses in plants during *B. cinerea* infection. (**A**) Pre-formed defence employs mainly physical barriers to pathogen entry such as the cuticle, cell wall and cell membrane. Furthermore, there are basal levels of compounds involved in cell wall strengthening such as lignin, callose, suberin and arabinogalactan proteins (AGPs). Low levels of cell wall associated PGIPs (polygalacturonase inhibiting proteins) are also involved in pre-formed defence through priming. Emission of some defence related VOCs (volatile organic compounds) such as sesquiterpenes have been identified prior to *B. cinerea* infections. (**B**) Induced defence is associated with a substantial increase in cell wall strengthening at the site of infection and around the whole plant organ. (**1**) During host attack, *B. cinerea* releases CWDE (cell wall degrading enzymes) and the cuticle is one of the first lines of physical defence. Upon recognition of *B. cinerea* molecules on the host surface, a cascade of events including defence signalling, oxidative burst and induction of secondary metabolism occurs in plant cells. This results in the accumulation of defence-related phytoalexins, PR proteins, emission of VOCs, increased lignification, amongst others. (**2**) Suberin deposition increases at the site of infection, forming a protective physical barrier that blocks *B. cinerea* appresoria from entering the cells and releasing CWDE. (**3**) An increase in PGIPs in the cell wall is associated with the inhibition of *B. cinerea* endopolygalacturonases (BcPGs). (**4**) Callose forms a protective plug between *B. cinerea* and the plasma membrane, blocking diffusion of fungal toxins and CWDE.

2.4.2.1 Pre-formed defences

Constitutive or pre-formed defences against *B. cinerea* infection are active within the plant preceding any infection and they either inhibit fungal entry or suppress infection during the initial phases. They are chiefly genetic traits though they can also be influenced by the surrounding environment. They largely involve pre-formed antifungal compounds, as well as physiological and/or anatomical features of grapevine tissues. These include presence of preformed phenolic compounds, number and length of leaf hairs, stomata/pore density, number of epidermal cell layers, berry skin and cuticle thickness (Sarig *et al*., 1998; Maganu & Paolocci, 2013).

The number and length of leaf hairs, called trichomes and bristles, on any grapevine leaf is a genetic trait which can be used for taxonomic purposes. It differs according to grapevine species or cultivar, though it can also be strongly influenced by the prevailing environmental conditions. Leaf hairs mainly contribute towards physical defence against fungal invasion, though some plants possess glandular secreting trichomes which play a significant role in chemical defence through the secretion of antifungal exudates. The general consensus is that leaf hairs play a dual role in plant defence; they hinder contact between the spore and the plant surface but can also promote adhesion of fungal spores to the leaf structure (Lazniewska *et al*., 2012). The density of leaf hairs also affects the water retention capacity of the leaves (Levin 1973; Wagner *et al*., 2004). This is an important trait since *B. cinerea* requires a hydrophobic surface in order to successfully adhere to any plant tissue (Doss *et al*., 1993). Thus resistant grapevine cultivars possess leaves with leaf hair densities that effectively hinder contact with *B. cinerea* whilst providing an unfavourable environment for adhesion.

The developmental stage of the grapevine plant influences some forms of pre-formed defence. Grape berries exhibit age-related resistance to *B. cinerea* infection with young, green berries being highly resistant to the pathogen whilst mature, ripening berries show higher levels of susceptibility. There are, however, cultivar differences reported for this trend (Kretschmer *et al*., 2007; Deytieux-Belleau *et al*., 2009; Kelloniemi *et al*., 2015). Grey mould disease symptoms rapidly progress between véraison and berry ripening. This can be partly attributed to the thickness and rigidity of the berry skin which reduces as the berry ripens due to cell wall disassembly (Cantu *et al*., 2008). The reduction is positively correlated with a decrease in number and thickness of both the epidermal and external hypodermal cell layers. The thinner skin possibly makes it easier for *B. cinerea* to penetrate the berry during infection and access nutrients (Sarig *et al*., 1998; Gabler *et al*., 2003).

There is evidence to suggest that the cuticle and the epicuticular wax layers also play a significant role in decreased susceptibility of green berries to *B. cinerea*. The two layers shield plant tissue from the harsh climatic environment also acting as physical barriers to invading pathogens. The wax possibly inhibits spore attachment and germination thus significantly limiting degree of infection (Blakeman & Sztejnberg, 1973; Vorwerk *et al*., 2004). As grape berries ripen the density of the cuticle and epicuticular wax layer decreases. This predisposes the fruits to infections from *B. cinerea* and could thus also contribute towards the

age-related susceptibility phenotype observed (Commenil *et al*., 1997; Gabler *et al*., 2003; Rogiers *et al*., 2004; Vorwerk *et al*., 2004; Deytieux-Belleau *et al*., 2009; Hematy *et al*., 2009).

The micro-climatic condition around grape berries is another factor that has a strong influence on characteristics that influence level of susceptibility to *B. cinerea* infections. For example, grape cluster morphology affects the fruit microclimate and the development of the epicuticular wax layer at the points of contact with neighbouring berries (Marois *et al*., 1986; Vail and Marois, 1991). Compact clusters result in high humidity, temperature and poor air circulation around individual grape berries. This creates optimum conditions for *B. cinerea* germination and proliferation. The contact surfaces lack wax platelets, have lower cuticle content and are characterised by perforations or micro-fissures (Fermaud *et al*., 2001; Gabler *et al*., 2003). All these factors are positively correlated with an increase in susceptibility to *B. cinerea*. Moreover, the cuticle and the epicuticular wax layer exude phenolic antifungal compounds onto the berry skin which have been associated with an increase in resistance against *B. cinerea* infection. The compounds include pterostilbene, cyanidins, cathechin and caftaric acid with known antimicrobial activities which affect spore germination and mycelial growth. This makes the contact surfaces with lower cuticle and wax content even more susceptible to infection. Interestingly, the antifungal compounds are exuded in higher amounts in young resistant berries compared to mature susceptible berries, further supporting their potential role in defence against *B. cinerea* (Padgett & Morrison, 1990; Vail & Marois, 1991; Osbourn, 1996; Dixon, 2001; Wittstock & Gershenzon, 2002; Conrath *et al*., 2006; Ahuja *et al*., 2012).

Apart from the anatomical pre-formed defences, grapevine also emits pre-formed volatile organic compounds (VOCs), such as sesquiterpenes, with strong antifungal activity against *B. cinerea* (Tsao and Zhou, 2000; Aranega-Bou *et al*., 2014). There is evidence that the emission of VOCs is developmentally regulated with low levels being detected in flowers at anthesis and at the early onset of fruit development. However, these levels significantly increase to high levels in grape berries at late ripening/pre-harvest stage where they are speculated to contribute towards enhancing flavour and aroma during wine making (Lucker *et al*., 2004). One of these sesquiterpenes, β-caryophyllene, was also emitted by tomato leaves and shown to strongly inhibit *B. cinerea* spore germination and hyphal growth (Zhang *et al*., 2008). Other studies have shown that some VOC emissions are strongly induced under stress conditions such as exposure to UV-B radiation in grape berries and *B. cinerea* infection in tomato leaves, further suggesting roles in defence (Thelen *et al*., 2005; Gil *et al*., 2013).

Additionally, grapevine can utilise preformed antifungal metabolites called phytoanticipins against *B. cinerea* infection (Van Etten *et al*., 1994). These compounds are present in plant cells prior to infection or can be produced from pre-existing compounds without any active defence response on the plants' part. They are toxic to *B. cinerea*, inhibiting fungal germination and growth (Van Baarlen *et al*., 2007). However, despite possessing a wide array of pre-formed defence mechanisms against *B. cinerea* infection, commercial grapevine cultivars remain highly susceptible to grey mould rot infections. A combination of pre-formed and induced defence responses are therefore likely to be required to significantly reduce the degree of damage that the fungus inflicts on commercial vineyards worldwide.

2.4.2.2 Induced defence responses

During *B. cinerea* infection, grapevine plants react by triggering a spectrum of defence pathways which results in enhancement of physical barriers, accumulation of defence compounds and elicitation of further defence responses (Jones & Dangl, 2006). Several studies have focused on identifying genes which are upregulated in response to *B. cinerea* infection (Hammond-Kosack & Jones, 1996). Though there are general trends in plant response against fungal infections, there are differences which are observed in some defence pathways depending on factors such as grapevine cultivar, developmental stage, plant organ, genotype of the *B. cinerea* strain, inoculum density and prevailing environmental conditions such as humidity and temperature (Kretschmer *et al*., 2007).

Numerous *B. cinerea* strains have been studied and shown to differ in virulence against grapevine. Infections typically lead to strain specific symptom development as was observed when *V. vinifera* L. cv. Chardonnay leaves were infected with T4 and T8 *B. cinerea* strains. T8 was more aggressive resulting in larger spreading lesions and induction of distinct defence responses compared to T4 (Derckel *et al*., 1999). These responses included changes in cell wall architecture, production of reactive oxygen species, phytoalexin biosynthesis and the production of defence proteins such as PR proteins and PGIPs (Bezier *et al*., 2002; Aziz *et al*., 2004).

(i). Physical reinforcements

One of the initial forms of induced defence against fungal attack involves the reinforcement of the physical barriers to fungal entry (Miedes *et al*., 2014). Compounds that have been studied and shown to contribute in this aspect include callose, lignin, suberin and arabinogalacturonan proteins (De Leeuw, 1985).

Callose deposition

Callose is a linear sugar polymer of β-1,3-D-glucan and has been shown to accumulate in numerous plant tissues during fungal infection. Callose deposition is mediated by callose synthases and has been associated with induced defence responses against *B. cinerea* in plants such as bean, tomato and *Arabidopsis* (Garcia-Arenal & Sagasta, 1977; De Leeuw, 1985; Ton & Mauch-Mani, 2004). It has also been associated with the ontogenic resistance of véraison berries compared to mature berries against *B. cinerea* infection (Kelloniemi *et al*., 2015). Asselbergh and Hofte (2007) described callose deposition in plants infected with *B. cinerea* as induced basal defence response.

Callose forms plugs called papillae at the infection site, sealing tissue wounds and forming a protective interface between the pathogen and the plasma membrane. It can also be deposited in the stomatal region, further blocking penetration of *B. cinerea* through natural openings. This significantly limits interchange of nutrients between the invading pathogen and the host plant, whilst also blocking the diffusion of toxins and cell wall degrading enzymes to host cells. The ultimate result is the slowing down of the colonisation of the plant by the pathogen or halting the process altogether. Some researchers however,

suggest that the papillae also blocks the diffusion of plant defence compounds from the host to the invading fungus thus giving ample time for the pathogen to mount other attack mechanisms and, in some cases, degrade callose (Conrath *et al*., 2002; Hulten *et al*., 2006; Luna *et al*., 2011). Despite the proposed dual role, callose deposition remains one of the first lines of defence that plants employ when attacked by *B. cinerea*.

Lignin deposition

Lignification is another induced defence response which plays a role in making the plant cell more impermeable to fungal invasion (Moura *et al*., 2010). Lignin is a complex aromatic polymer of phenylpropanoid compounds. It is developmentally deposited in secondary plant cell walls where it provides structural integrity particularly in primary growth and strength of the stem. It also waterproofs the cell wall, an attribute which enables the vascular system to transport water and solutes (Boerjan *et al*., 2003). Numerous studies have shown that lignin accumulates to higher levels in response to infection or mechanical damage as evidenced by an increase in levels of enzymes involved in lignin biosynthesis (Denness *et al*., 2011).

One of the enzymes, phenylalanine ammonia lyase (PAL), is considered a marker for plant defence responses against pathogen attack. It has been shown to accumulate in grapevine leaves and berries infected with *B. cinerea*. (Bezier *et al*., 2002; Aziz *et al*., 2004; Protel-Aziz *et al*., 2006; Agudelo-Romero *et al*., 2015). Lignification improves cell wall fortification, retarding or preventing the invading pathogen from accessing the host nutrients or depositing toxins thus limiting lesion expansion. This process alters the suitability of the cell wall as a substrate to the invading pathogen leading to pathogen starvation (Miedes *et al*., 2014). There is evidence that *B. cinerea* can degrade lignin using enzymes such as laccase, however, the process is relatively slow and it thus gives ample time for the host plant to mount other defence strategies (Nun *et al*., 1988; Slomczynski *et al*., 1995; Baldrian, 2006).

Suberisation

Suberin is a cell-wall associated biopolymer found in both aerial and below ground plant organs (reviewed by Vishwanath *et al*., 2015). Suberin deposition, termed suberisation, is mainly associated with cell wall strength and water retention during plant growth and development. It is also a pathogen induced defence response in numerous plant species where it forms a physical antimicrobial barrier to the invading pathogen and any other opportunistic infections (Bernards, 2002). Grape berries infected with *B. cinerea* exhibit induced accumulation of suberin in the early stages of infection. Suberin accumulates around the fungal appresorium thus preventing cell wall degrading enzymes and toxins from entering and damaging the plant cell wall (Coertze *et al*., 2002). Rapid suberisation also prevents water loss through the injured and exposed tissues (Pollard *et al*., 2008).

Arabinogalactan proteins (AGPs)

AGPs are heavily glycosylated proteoglycans belonging to the hydroxyproline-rich glycoprotein (HRGP) family (reviewed by Pereira *et al*., 2015). They are mainly found in the cell wall, plasma membrane, apoplastic space and secretions of terrestrial and aquatic plants (Herman and Lamb, 1992; Showalter, 2001). They have been detected in numerous plant species where they exhibit high levels of developmental regulation, being implicated in various growth and development processes such as cell division and expansion, pollen germination, xylem differentiation and somatic embryogenesis (Pereira *et al*., 2015). In grapevine, AGP epitopes increase in abundance in Cabernet Sauvignon and Crimson seedless berry cell walls during ripening, also suggesting developmental regulation (Moore *et al*., 2014).

AGPs have been implicated in induced defence responses against both biotic and abiotic stress in numerous plant species (Deepak *et al*., 2010). They are upregulated in response to salt stress in tobacco (Lamport *et al*., 2006), heat shock in cabbage (Yang *et al*., 2006), cold stress in cotton (Gong *et al*., 2012) and phylloxera infection in grapevine roots (Du *et al*., 2014). They are believed to play a major role in defence mechanisms involving cell wall fortification (Deepak *et al*., 2010). For example, AtAGP31 from *Arabidopsis* was found to exist cross-linked to arabinoxylan and pectin in the cell wall matrix (Hijazi *et al*., 2014). This is thought to result in the formation of a dense interconnected network which is more difficult for fungal pathogens such as *B. cinerea* to penetrate. The network is also believed to play a role in masking pectin, thus protecting it from pectinases such as BcPGs (Tan *et al*., 2013). Interestingly, there is also evidence of AGPs forming aggregates through self-binding, further contributing towards cell wall strengthening (Hijazi *et al*., 2014).

Since these functional studies were conducted in *Arabidopsis*, it is not known if grapevine AGPs behave similarly under different stress conditions, especially *B. cinerea* infection. To date, functional studies on grapevine AGPs have not been reported. In fact, very limited information currently exists on members of this gene family in grapevine.

(ii). The oxidative burst

One of the early defence mechanisms induced in plants infected with fungal pathogens involves the hypersensitive response (HR) which triggers rapid accumulation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2), in a process called the oxidative burst (Doke *et al*., 1996; O'Brien *et al*., 2012). The production of superoxide and H_2O_2 occurs mainly through the NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase system and the levels to which they accumulate after fungal infection is host specific (Doke *et al*., 1996; Papadakis *et al*., 1999). Accumulation of these compounds is believed to be directly toxic to the invading pathogen, altering its redox status. However, there is evidence that H_2O_2, on its own, does not induce oxidative stress in *B. cinerea* during plant infection (Temme & Tudzynski, 2009). The researchers suggested that toxicity is only achieved by a combination of H_2O_2, superoxide and nitric oxide (NO) working together and not independent of each other.

Accumulation of ROS also plays a crucial role in signalling the induction of other cellular protection and defence genes and the oxidative cross-linking of cell wall proteins. Superoxide and H_2O_2 work synergistically with NO in signalling processes (Levine *et al*., 1994; Delledonne et al., 1998: 2001; Bolwell, 1999; Yoshioka *et al*., 2009). Release of H_2O_2 is induced by numerous elicitors which include lipids, proteins, ozone and oligosaccharides (Baillieul *et al*., 1995; Sandermann *et al*., 1998; Buhot *et al*., 2001; Aziz *et al*., 2004; Garcia-Brugger *et al*., 2006; Repka, 2006). It is accompanied by the up-regulation of known defence related genes and stimulation of PAL, PGIP and chitinase activity (Aziz *et al*., 2004; Asselbergh *et al*., 2007). ROS accumulation also triggers localised PCD (Lamb and Dixon, 1997).

PCD of infected tissue typically leads to the restriction of lesion expansion and limited pathogen proliferation when plants are infected with a biotroph like *E. necator* (Garcia-Brugger *et al*., 2006; O'Brien *et al*., 2012). However, for a necrotroph like *B. cinerea*, which has developed mechanisms to protect itself from the toxic effects of ROS, localised PCD is actually beneficial for growth since the fungus can utilise the dead plant tissue. Elevated generation of ROS which occurs mainly during cuticle penetration and lesion formation has been shown to result in enhanced *B. cinerea* growth (Govrin & Levine, 2000; Mayer *et al*., 2001; Wan *et al*., 2015). Interestingly, studies have shown that *B. cinerea* utilises ROS as part of its infection machinery using a superoxide dismutase gene called BcSOD1 (Rolke *et al*., 2004). In a separate study, a *B. cinerea* elicitor for ROS was found in intercellular fluid of infected *Arabidopsis* leaves and shown to induce cell death in other plants (Govrin *et al*., 2006). Furthermore, one of the main virulence factors, BcPG1 released during infection, has been shown to induce the production of ROS by the host plant (ten Have *et al*., 1998; Poinssot *et al*., 2003). Thus the benefits of this defence mechanism to a grapevine host being attacked by *B. cinerea*, depends on the rate at which ROS elicits other defence responses such as synthesis of PR proteins and phytoalexins (Verhagen *et al*., 2011).

(iii). Synthesis of phytoalexins

Phytoalexins are antimicrobial metabolites of low molecular weight with biological activity against an array of phytopathogens, including fungi. Their production in plants is induced by responses to various forms of stress such as UV-irradiation, injury and fungal infections (Douillet-Breuil *et al*., 1999; Keller *et al*., 2000). They exhibit antimicrobial and antioxidative properties, acting as toxins towards the invading fungal pathogens, ultimately inhibiting fungal growth (Langcake and Pryce, 1976:1977; Hammerschmidt, 1999). Their level of accumulation in different plant tissues depends on numerous factors including the prevailing environmental conditions, vineyard management practices, grape variety, berry developmental stage and the invading fungal strain (Jeandet *et al*., 1991; Bavaresco *et al*., 1997; Bais *et al*., 2000; Kretschmer *et al*., 2007; Timperio *et al*., 2012). They are involved in the ontogenic resistance of grape berries against fungal infections. Highest levels of accumulation have been detected in young, green berries, yet the levels decrease as the berry ripens and total sugar concentration increases. This has been speculated to contribute significantly towards the higher susceptibility of ripe berries to *B. cinerea* compared to green, unripe berries

(Jeandet *et al*., 1991; Bavaresco *et al*., 1997; Kelloniemi *et al*., 2015). This line of thought is supported by the strong antifungal activity of the phytoalexins *in vitro* against *B. cinerea* growth (Langcake, 1981).

In grapevine, the induced phytoalexin response to infection or stress involves upregulation of stilbene synthase and production of the stilbene molecules *trans*-resveratrol (3,5,4'-trihydroxystilbene), pterostilbenes, α-viniferins and ε- viniferins (Pryce and Langcake, 1977; Langcake *et al*., 1979; Langcake, 1981; Liswidowati *et al*., 1991; Bavaresco *et al*., 1997; Mohamed *et al*., 2007; Agudelo-Romero *et al*., 2015). Resveratrol is the most widely studied and has been shown to be highly toxic to *B. cinerea* conidia. It inhibits germination of fungal spores, disrupts the plasma membrane and alters the mitochondrial structure (Adrian & Jeandet, 2012). It is thus not surprising that over-expression of stilbene synthase, a key enzyme in stilbene biosynthesis, in grapevine results in reduced susceptibility of the host to *B. cinerea* (Dabauza *et al*., 2015).

B. cinerea infection of grapevine leaves, shoots and flowers leads to the accumulation of both *trans*-resveratrol and ε-viniferin in considerable amounts (Timperio *et al*., 2012; Verhagen *et al*., 2011). The other grapevine phytoalexins with known antifungal tendencies, α-viniferin and pterostilbene, also accumulate in grape berries during *B. cinerea* infection, albeit in lower concentrations compared to *trans*-resveratrol and ε-viniferin (Langcake *et al*., 1979; Langcake, 1981; Bavaresco *et al*., 1997). A comparison of phytoalexin accumulation in grape berries infected with *B. cinerea* showed that resistant cultivars had faster accumulation and higher levels of *trans*-resveratrol and ε-viniferin around the infection site compared to the susceptible *V. vinifera*, further suggesting a possible contribution of the phytoalexins to the observed defence phenotypes (Bavaresco *et al*., 1997). Thus the speed and intensity with which phytoalexins accumulate can be considered as reliable indicators of the plants' resistance status.

Interestingly, using stilbene oxidases or laccases, *B. cinerea* is capable of biotransforming resveratrol and pterostilbene into a number of oxidised metabolites which are less toxic to the fungus. This enables the fungus to circumvent the plant defence mechanism thus enabling it to create an environment which is conducive for its own growth and reproduction at the detriment of the host plant (Breuil *et al*., 1998; Pezet, 1998; Pezet *et al*., 1991; Cichewicz *et al*., 2000; Nakajima & Akutsu, 2014). The ability of *B. cinerea* to oxidise phytoalexins is strain specific and is crucial to the pathogenicity of the fungus on grapevine hosts (Sbaghi *et al*., 1996). On the other hand, young grape berries were shown to release potent stilbene oxidase inhibitors which suppress the activity of *B. cinerea* laccases (Goetz *et al*., 1999). This suggests that the low susceptibility of young berries to *B. cinerea* is enhanced by their ability to cripple a crucial pathogenicity factor of the invading pathogen, enabling the phytoalexins to defend the plant host effectively.

(iv). Production of PR proteins

PR proteins are synthesised *de novo* and constitute one of the main defence mechanisms employed by plants against fungal pathogens (Stintzi *et al*., 1993; Golshani *et al*., 2015). They were first identified in tobacco infected with the Tobacco Mosaic Virus (TMV) and have since been identified in over 20 plant species including grapevine (**Table 3**). Some are involved in preformed defences whilst others are synthesised in

numerous plant tissues after induction by wounding, infection and the presence of elicitors. They accumulate in infected plant organs and also in non-inoculated organs, thus also playing a role in acquired resistance (Golshani *et al*., 2015). A number of PR protein families have been identified in grapevine and these include chitinases, glucanases, osmotin and thaumatin-like proteins. Their antimicrobial properties against fungal pathogens such as *B. cinerea* have been highlighted in numerous studies (Jongedijk *et al*., 1995; Zhu *et al*., 1995; Giannakis *et al*., 1998; Derckel *et al*., 1999; Robert *et al*., 2002; Monteiro *et al*., 2003).

Plant chitinases, also known as PR-3, 4, 8 and 11 proteins, are enzymes with the ability to hydrolyse chitin (Fritig *et al*., 1998). Chitin is a major structural element of fungal cell walls and its hydrolysis renders the fungal pathogens osmotically sensitive. Chitinases can be divided into six different classes according to structural similarities though some researchers report seven classes based on substrate specificity, protein structure and mechanisms of catalysis (Collinge *et al*., 1993; Punja and Zhang, 1993; Kasprzewska, 2003). In white wine, chitinase is notorious for causing haze formation but in plants it has a crucial role in defence against fungal infections (Collinge *et al*., 1993; Waters *et al*., 1996; Pocock *et al*., 2000; Ferreira *et al*., 2004; Marangon *et al*., 2011; Rubio *et al*., 2015; Tohidfar & Khosravi, 2015). In *V. vinifera*, some class IV isoforms of chitinase are constitutively expressed in healthy grape berries from véraison to full maturation, thus forming part of the ontogenic preformed defences (Robinson *et al*., 1997; Derckel *et al*., 1998; Robert *et al*., 2002; Colas *et al*., 2012).

Table 3. List of recognised PR protein families (Table adopted from http://www.bio.uu.nl/~fytopath/PR-families.htm).

Family	Original source plant	Properties
PR-1	Tobacco	Antifungal
PR-2	Tobacco	β-1,3-glucanase
PR-3	Tobacco	Chitinase (class I, II, IV, V, VI, VI)
PR-4	Tobacco	Chitinase I, II
PR-5	Tobacco	Thaumatin-like
PR-6	Tomato	Proteinase-inhibitor
PR-7	Tomato	Endoproteinase
PR-8	Cucumber	Chitinase class III
PR-9	Tobacco	Peroxidase
PR-10	Parsley	Ribonuclease-like
PR-11	Tobacco	Chitinase class I
PR-12	Radish	Defensin
PR-13	*Arabidopsis*	Thionin
PR-14	Barley	Lipid transfer protein
PR-15	Barley	Oxalate oxidase
PR-16	Barley	Oxalate oxidase-like
PR-17	Tobacco	Unknown

Chitinases are also induced by wounding, fungal infections and the presence of elicitors such as oligogalacturonides, laminarin, UV-C radiation and the β-1,4-linked glucosamine oligomer, chitosan (Derckel *et al*., 1998, Aziz *et al*., 2003; 2004; Kortekamp, 2006; Trotel-Aziz *et al*., 2006; Colas *et al*., 2012; Dadakova *et al*., 2015; Katiyar *et al*., 2015). Numerous studies have elucidated the direct role of chitinases in plant defence. Overexpression of a chitinase gene, with known antifungal activity, in tobacco increased host resistance to *B. cinerea* infections (Carstens *et al*., 2003a; 2003b). The duration for accumulation of chitinase in *B. cinerea* infected tissues differs according to the invading fungal strain (Derckel *et al*., 1999). This was evident in studies where induction of chitinase genes, *Vvichit1a* and *Vvichit1b*, upon grapevine infection by *B. cinerea* was shown to be strain specific (Robert *et al*., 2002). Rate of gene induction also differs based on type of elicitor, grapevine cultivar and tissue. An acidic chitinase gene was detected in infected grapevine leaves, but not in berries, indicating tissue specificity in defence responses against *B. cinerea* infections (Bezier *et al*., 2002; Colas *et al*., 2012).

Another group of PR proteins which has been well studied in grapevine defence are the glucanases also known as PR-2 proteins (Golshani *et al*., 2015). During plant infection, β-1,3-glucanase is responsible for the hydrolytic cleavage of β-1,3-glucan, a vital structural component of fungal cell walls. Similar to chitinases, this renders the fungal pathogen osmotically sensitive and vulnerable to cell lysis and death (Mauch *et al*., 1988). It is speculated that the fragments of the fungal cell wall which arise due to chitinase and glucanase hydrolysis could also serve as signalling compounds or elicitors for other plant defence mechanisms (Kortekamp, 2006). The antifungal activity of β-1,3-glucanase has been shown in a number of studies to be synergistic with that of chitinase (Mauch *et al*., 1988; Stintzi *et al*., 1993; Zhu *et al*., 1994; Jongedijk *et al*., 1995; Giannakis *et al*., 1998; Magnin-Robert *et al*., 2007). This is supported by co-expression studies where expression of chitinase and glucanase genes within the same host significantly improved fungal resistance compared to host plants expressing only one of the PR proteins (Zhu *et al*., 1994; Jach *et al*., 1995; Jongedijk *et al*., 1995).

Several researchers have not found any evidence of β-1,3-glucanase expression in healthy plants, leading to the speculation that the PR protein does not play a significant role in preformed defence against fungal infections (Robinson *et al*., 1997; Renault *et al*., 2000). Contrary to these findings, a more recent study detected the expression of β-1,3-glucanase in healthy, uninfected ripening berries. The levels detected increased as the degree of berry ripeness increased, suggesting a possible role in developmental regulation, defence or both (Guillaumie *et al*., 2011). On the other hand, numerous studies have successfully shown that β-1,3-glucanase is induced by fungal infections, wounding, UV light, hormonal signals and the presence of elicitors such as oligogalacturonides and the β-1,3-glucan, laminarin (Derckel *et al*., 1998: 1999; Giannakis *et al*., 1998; Renault *et al*., 2000; Aziz *et al*., 2003:2004; Kortekamp, 2006; Dadakova *et al*., 2015). The rate of accumulation of β-1,3-glucanase in infected tissue is influenced by the type and strain of the invading fungal pathogen (Derckel *et al*., 1999; Ahn *et al*., 2014). Infection of *V. vinifera* L. cv. Chardonnay leaves with *B. cinerea* resulted in the accumulation of β-1,3-glucanase proteins within three days post infection. This was almost simultaneous with the appearance of necrotic lesion formation, suggesting a possible role of the PR protein in triggering or enhancing PCD (Renault *et al*., 2000).

Osmotin and thaumatin-like proteins are PR-1 and 5 proteins which are also referred to as permatins due to their transmembrane permeabilising ability. They cause holes to form on fungal cell membranes, enabling water influx which ultimately causes the hyphal membrane to rupture (Vigers *et al*., 1992; Monteiro *et al*., 2003). They are found in ripening grape berries in high amounts, especially in the pulp (Kretschmer *et al*., 2007; Guillaumie *et al*., 2011). Like chitinases, osmotin and thaumatin-like proteins are associated with haze formation in white wine (Waters *et al*., 1996; Tattersall *et al*., 1997; Pocock *et al*., 2000; Ferreira *et al*., 2004). In *V. vinifera*, VviTL1 (*V. vinifera* thaumatin-like protein 1) was identified and characterised from the cultivar Muscat and was found to accumulate at the same time as berry sugars increase during ripening. It was also shown to be highly soluble in grape juice extracts, thus supporting the work reported by Waters *et al*. (1996) which attributed haze formation in white wine to the high levels of thaumatin-like proteins (Tattersall *et al*., 1997). The gene encoding for osmotin in *V. vinifera*, pVviOSM1, was isolated and

characterised by Loulakakis in 1997. Its basal expression levels were found to be highest in healthy roots, stems and leaves with intermediate levels detected in berries. Thus the expression of both osmotin and thaumatin-like proteins in grapevine is developmentally regulated, with both proteins accumulating differentially in healthy uninfected plants (Kretschmer *et al*., 2007; Colas *et al*., 2012).

Wounding, infection by fungal pathogens and the presence of elicitors such as salicylic acid, methyl jasmonate and UV radiation induces even higher accumulation of osmotin and thaumatin-like proteins in grapevine tissues (Zhu *et al*., 1995; Monteiro *et al*., 2003; Colas *et al*., 2012; Jiang *et al*., 2015; Wang *et al*., 2015). Thaumatin-like proteins accumulate in the exocarp and around the peripheral vascular elements in stressed grape berries, a possible strategy to inhibit fungal penetration through these regions. Both osmotin and thaumatin-like proteins work synergistically in grapevine leaves and berries against *B. cinerea* by inhibiting mycelial growth, spore germination and germ tube growth (Monteiro *et al*., 2003; Colas *et al*., 2012; McFeeters and McFeeters, 2012).

Other PR proteins which have been detected in plants include PR-6 (proteinase inhibitor), PR-7 (endoproteinase), PR-9 (peroxidase), PR-10 (ribonuclease-like protein), PR-12 (defensin), PR-13 (thionin), PR-14 (lipid-transfer protein), PR-15 (germin/oxalate oxidase), PR-16 (germin/oxalate oxidase-like proteins) and PR-17 (unknown activity) (Fritig *et al*., 1998; van Loon & van Strien, 1999; Edreva, 2005; Sels *et al*., 2008). Their different localisations, physiological and defence roles are gradually being elucidated in numerous plant backgrounds such as *Arabidopsis* (Sels *et al*., 2008). Despite their direct role in defence against fungal pathogens such as *B. cinerea*, breeding for grapevine cultivars with enhanced levels of PR proteins raises concerns about the likelihood of high turbidity wines produced from such grapes. Alternatively, the use of tissue specific promoters in genetic engineering platforms could limit over-expression in berries. In addition to PR proteins, there are other proteins/peptides involved in grapevine defence against *B. cinerea* discussed below (McFeeters and McFeeters, 2012).

(v). Defensins

Grapevine also defends itself against fungal pathogens through the action of small, cysteine-rich antimicrobial peptides called defensins (Thomma *et al*., 2002; Van der Weerden and Anderson, 2013; Vriens *et al*., 2014). Their role in defence has been elucidated through overexpression studies in numerous plant backgrounds. Presence of defensins conferred the host plants with partial resistance against an array of invading pathogens (Montesinos, 2007; Hegedus and Marx, 2013). Defensins inhibit fungal activity by binding to specific fungal membrane receptors called sphingolipids. This results in the permeabilization of the fungal membranes ultimately killing the pathogen (Thevissen *et al*., 1999; De Coninck *et al*., 2013). A total of 79 defensin-like sequences have been identified in the *V. vinifera* genome, corresponding to 46 genes or allelic variants (Giacomelli *et al*., 2012). The first defensin peptide from *V. vinifera* was isolated from Pinotage, fully characterised and named VviAMP1 (*Vitis vinifera* antimicrobial peptide 1) (De Beer and Vivier, 2008). The expression of *Vviamp1* was shown to be highly tissue specific and developmentally regulated, only being expressed in berries from the onset of ripening. This expression pattern may however,

be cultivar specific since in Pinot Noir, *Vviamp1* was detected in the mesocarp, leaves, roots and flowers (Giacomelli *et al*., 2012).

In vitro assays showed that the VviAMP1 peptide could significantly inhibit the growth of a wide range of grapevine pathogens including *B. cinerea*. However, the peptide was not induced by external stimuli such as wounding and infection (De Beer and Vivier, 2008). Other defensin peptides that have since been identified in *V. vinifera* include VviAMP2, VviAMP3 and VviAMP4, all isolated from Pinot Noir (Giacomelli *et al*., 2012). VviAMP2 and VviAMP4 are mainly expressed in grapevine inflorescences at anthesis whilst VviAMP3 expression was only detected in seeds two weeks prior to véraison (Giacomelli *et al*., 2012; Nanni *et al*., 2014). All four VviAMPs has been shown to inhibit *B. cinerea* conidia germination and growth significantly with VviAMP2 exhibiting the strongest inhibitory action at lower concentrations (Giacomelli *et al*., 2012).

Although the developmental regulation of grapevine defensins is in line with their role as one of the first lines of preformed defence against invading pathogens for vulnerable tissues involved mainly in reproduction, there is evidence that they are also upregulated by wounding, cold stress, jasmonic acid and *B. cinerea* infection (Penninckx *et al*., 1998; Thomma and Broekaert, 1998; Koike *et al*., 2002; Pervieux *et al*., 2004). Infection of *V. vinifera* cv. Pinot noir with *B. cinerea* caused an upregulation of a number of defensins, suggesting a role of the antimicrobial peptides in induced grapevine defence responses. VviAMP4 accumulated to significantly high levels in *B. cinerea* infected inflorescences compared to uninfected control plants at 4 days post inoculation (Giacomelli *et al*., 2012). Thus the authors suggest that it plays a role in both preformed and induced defence of grapevine inflorescences against *B. cinerea*.

(vi). Polygalacturonase Inhibiting Proteins (PGIPs)

PGIPs are soluble cell wall-associated glycoproteins belonging to the leucine-rich repeat (LRR) protein family (most recently reviewed by Kalunke *et al*., 2015). To date, the only plant PGIP whose structure has been solved by X-ray crystallography is PvPGIP2 from *Phaseolus vulgaris* (Di Matteo *et al*., 2003). Structures of other plant PGIPs have since been deduced *in silico* using advanced structural bioinformatics tools (Lim *et al*., 2009; Matsaunyane *et al*., 2015). This is achieved by threading the amino acid sequences of the PGIPs of interest to the crystal structure of PvPGIP2. The deduced structures are then utilised for *in silico* docking studies. In this way, the availability of the PvPGIP2 crystal structure has made a very important contribution towards the current understanding of the structural requirements for PGIP function (Di Matteo *et al*., 2006).

PGIPs generally exhibit a curved and elongated shape with a twisted scaffold. The highly conserved LRR motif which is characteristic of all proteins in this family is found mainly on the concave surface of the structure (Di Matteo *et al*., 2003). It is involved primarily in protein-protein recognition and interaction (Kobe and Kajava, 2001; Xu *et al*., 2009). Furthermore, PGIPs possess a wide negatively charged cleft in the middle of the inner concave surface. The cleft has a high affinity for fungal-derived molecules such as ePGs resulting in an inhibition interaction (Di Matteo *et al*., 2003).

Research on the structural basis of effective inhibition of ePGs by PGIPs has advanced largely due to the availability of numerous three-dimensional ePG structures (Pickersgill *et al*., 1998; van Santen *et al*., 1999; Federici *et al*., 1999; 2001; Cho *et al*., 2001; van Pouderoyen *et al*., 2003; Bonivento *et al*., 2008). Residues which are crucial for complex stability at the interfaces have been identified from numerous PGIP-ePG interaction studies. These studies were conducted experimentally and using *in silico* docking models and the results complement each other (Sicilia *et al*., 2005; Lim *et al*., 2009; Maulik *et al*., 2009; Prabhu *et al*., 2014, Matsaunyane *et al*., 2015). The deduced inhibition interactions that have been observed can be divided into three classes; competitive, non-competitive and a mixed mode (Federici *et al*., 2001; Sharrock *et al*., 1994; Di Matteo *et al*., 2006).

Competitive inhibition involves PGIP binding to the active site of an ePG, masking and preventing it from binding to any other substrate (**Fig. 5A**). Non-competitive inhibition involves PGIP binding to an allosteric site causing structural changes to the ePG, including the active site (**Fig. 5B**). This in turn renders the active site of the ePG incompatible with any of its normal substrates such as pectin (Protsenko *et al*., 2008). Mixed mode of inhibition involves PGIP binding partially to the ePG active site and also to an area close to the active site (**Fig. 5C**). This not only partially blocks the active site reducing substrate affinity, but also results in conformational changes to other parts of the ePGs (Manfredini *et al*., 2005; Sicilia *et al*., 2005).

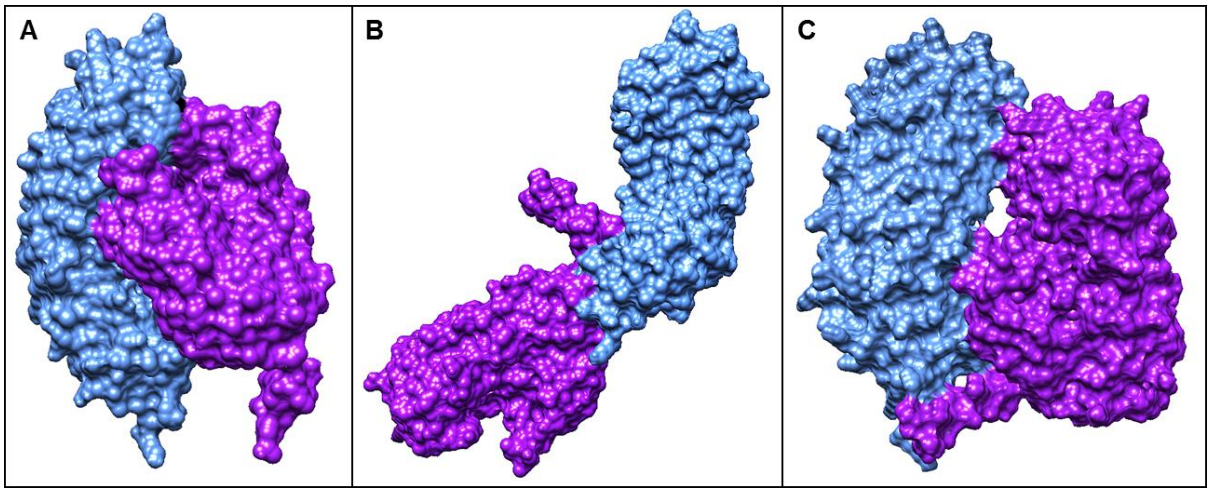

Figure 5. Docking geometry of PGIP-PG interaction complexes showing **(A)** Competitive inhibition of BcPG2 from *B. cinerea* grape strain (purple) by VviPGIP1 (blue). The active site cleft is almost completely buried in the interaction and is not accessible to substrate. **(B)** Non-competitive inhibition of BcPG1 from B05.10 strain (purple) by VviPGIP1. Active site is not covered and thus is left accessible to substrate (**C**) Mixed-mode inhibition of BcPG1 from SAS56 strain (purple) by VviPGIP1 showing a partially covered active site (unpublished data).

The inhibition of BcPGs by plant derived PGIPs has a two-fold function. Firstly, it directly suppresses the activity of BcPGs, thus limiting the amount of damage caused during *B. cinerea* infection. Secondly, it prolongs the existence of longer chain cell wall fragments called oligogalacturonides which are produced when BcPGs macerate the host. These oligogalacturonides are believed to then act as elicitors for numerous defence responses (Cervone *et al*., 1987: 1989; Aziz *et al*., 2004). The role of PGIPs in plant defence has

been elucidated in numerous plant backgrounds including tomato (Powell *et al*., 2000), tobacco (Joubert *et al*., 2006; 2007; Oelofse *et al*., 2006; Venter, 2010), wheat (Janni *et al*., 2006), *Arabidopsis* (Manfredini *et al*., 2005), pear (Sharrock *et al*., 1994; Faize *et al*., 2003) and grapevine (Aguero *et al*., 2005) where the presence of PGIP resulted in reduced fungal susceptibility of the host plant species (De Lorenzo and Ferrari, 2002; D'Ovidio *et al*., 2004; Gomathi and Gnanamanickam, 2004; Juge 2006; Kalunke *et al*., 2015).

Overexpressing *Vvipgip1* from *V. vinifera* cv. Pinotage in tobacco conferred the transgenic population with reduced susceptibility to a hyper-virulent *B. cinerea* grape strain (Joubert *et al*., 2006). The defence phenotype was attributed to VviPGIP1 being an effective inhibitor of BcPG1 and BcPG2, important *B. cinerea* virulence factors. Furthermore, constitutive expression of *Vvipgip1* primed the host plants, prior to infection, by modifying cell wall and hormone profiles (Joubert *et al*., 2007; Alexandersson *et al*., 2011; Nguema-Ona *et al*., 2013). On the other hand, studies have also shown that PGIPs are involved in important plant growth and developmental processes, not directly related to disease response. These include regulating radicle protrusion during seed germination (Kanai *et al*., 2010), cell elongation in hypocotyls (Irshad *et al*., 2008) and competence to form green callus in cell cultures grown in auxin-rich medium (Che *et al*., 2007). These findings suggest that overexpressing *pgip*s in plant hosts possibly also affects numerous endogenous processes unrelated to defence.

2.5 CONCLUSIONS AND PERSPECTIVES

Despite producing high quality grapes, the susceptibility of *V. vinifera* to major fungal pathogens is of great concern worldwide with none of the commercial grapevine cultivars displaying resistance to downy mildew, powdery mildew or grey mould rot. Advances in molecular biology have identified promising avenues such as marker assisted breeding and the use of genetic engineering platforms, such as cisgenics, which could see the future development of resistant commercial grapevine cultivars. However, for the implementation of such technologies on a global scale to be a viable option, a solid understanding of the grapevine-fungi relationships is required.

To date, detailed studies of the infection strategies employed by fungal pathogens during grapevine infections have been documented. Fungal strain-specific traits and different pathogenicity factors on numerous plant hosts have been identified through mutant analysis. On the other hand, inherent defence pathways and the respective genes induced in grapevine during fungal infection have also been identified and elucidated through gene silencing and overexpression studies. This association of specific fungal pathogenicity traits with grapevine defence strategies has not only shed light on plant-fungi interactions but also highlighted the complexity of this dynamic system.

The recent shift towards whole transcriptomic studies of these plant-fungi interactions has improved our understanding of the different pathways and genes involved in the process. It provides a broad picture of the interconnectivity of both fungal and plant systems. Only when the intricate details are clearly understood can research shift towards a systems approach where, for example, plant defence strategies against infection

by a consortium of fungal pathogens can be deciphered. The answer to developing fungal resistant grapevine cultivars could lie in the interaction of these pathogens within the same host.

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Chapter 3

Research results

Interaction of grapevine polygalacturonase inhibiting proteins (PGIPs) and *Botrytis cinerea* endopolygalacturonases (BcPGs): An *in silico* study

Interaction of grapevine polygalacturonase inhibiting proteins (PGIPs) and *Botrytis cinerea* endopolygalacturonases (BcPGs): An *in silico* study

3.1 ABSTRACT

Grey mould rot is a devastating disease in commercial vineyards globally, caused by the fungus *Botrytis cinerea*. During infection, *B. cinerea* utilises endopolygalacturonases called BcPGs to break down pectin in the hosts' cell wall, thus enabling it to enter and access plant nutrients. Six BcPG isoforms have been identified in the *Botrytis* genome and they display different substrate specificities and pH optima with BcPG1 and BcPG2 being major virulence factors on numerous plant hosts. As part of its defence strategy, studies have elucidated that grapevine, like most plant species, utilises polygalacturonase inhibiting proteins (PGIPs) which can directly inhibit the activity of BcPGs. In this study, PGIP amino acid sequences from *Vitis vinifera* (VviPGIP1), *V. caribaea* (PGIP1038) and the hybrid *V.* x *doaniana* Munson (PGIP1012) were utilised to deduce their putative protein structures using domain-based comparative modelling tools against the crystal structure of PvPGIP2 from *Phaseolus vulgaris*. The predicted protein models displayed high structural homology with conserved regions similar to those reported for PvPGIP2. Subtle differences, corresponding to amino acid sequence differences, were identified in structure-based alignments. Structural protein models were also predicted for BcPG isoforms from three *B. cinerea* grey mould strains (B05.10, SAS56 and a hyper-virulent grape strain). Amino acid sequence analysis confirmed that BcPG1 and 2 were highly polymorphic whilst BcPG 3 to 6 were conserved in all three species. Threading the sequences against the crystal structures of a combination of five endopolygalacturonases predicted BcPG models that displayed conservation of the right-handed PG fold and the negatively charged active site cleft. Grapevine PGIP and BcPG structural predictions with the highest quality validation scores were then subjected to molecular docking analyses. *B. cinerea* strain and BcPG isoform were the main factors which influenced docking orientations, not the source of PGIP. In approximately 80% of the docked complexes, grapevine PGIPs were predicted to utilise their concave surfaces, where the conserved leucine-rich repeat (LRR) motif is located, for ligand binding. The remaining 20% utilised the convex surfaces, outside the conserved region. The predicted inhibition interactions of grapevine PGIPs against BcPG1 and BcPG2 displayed *B. cinerea* strain-specificity whilst BcPG3-6 models were all predicted to be non-competitively inhibited, with slight differences in docking orientations specific mainly to each BcPG isoform. Differences were noted in the nature and number of bonds at protein-protein interfaces for all docked complexes. Using alanine scanning mutagenesis, the most significant PGIP and BcPG binding sites for all docked complexes were predicted. Some complexes shared the same PGIP or BcPG hotspots with slight differences is stability, evidenced by the predicted energy released when the residues were computationally replaced with alanine. Forty one percent of the predicted docked complexes shared a PGIP hotspot with at least one other complex, whilst only 24% of the complexes shared at least one BcPG hotspot. The most common PGIP hotspots, identified in

three complexes, were predicted to be O101 and S104 whilst J11 was predicted to be a BcPG hotspot in three other docked complexes. None of the docked complexes were predicted to share the same hotspots for both PGIP and BcPG.

3.2 INTRODUCTION

Grey mould rot of grapevine is caused by *Botrytis cinerea*, a necrotroph which displays high genetic diversity amongst its many strains. These strains are characterised by notable differences in virulence on particular plant hosts (van Kan, 2006; Kretschmer and Hahn, 2008; Atwell *et al*., 2015). The genomic sequences of three strains (B05.10, T4 and BcDW1), differing significantly in virulence, have been assembled and are publicly available, facilitating studying the pathogen further (Amselem *et al*., 2011; Staats and van Kan, 2012; Blanco-Ulate *et al*., 2013). The genetic basis of the pathogenicity of *B. cinerea* has been a focal point of numerous studies with pathogenesis-associated loci, such as those encoding for endopolygalacturonases (BcPGs), having been sequenced from more than 30 *Botrytis* strains including a hyper-virulent South African grape strain and a monoascospore strain, SAS56 (Rowe and Kliebenstein, 2007; Wubben *et al*., 1999).

BcPGs are cell wall degrading enzymes (CWDE) which are secreted by *B. cinerea* during infection. The *B. cinerea* genome possesses at least six BcPG isoforms (BcPG1-6) with differential regulation and substrate specificities (Wubben *et al.*, 1999; Kars *et al*., 2005). BcPG1 and 2 are highly polymorphic and have been identified as major virulence factors during infection (Rowe and Kliebenstein, 2007; Nakajima and Akutsu, 2014). They cause the most *in planta* damage on numerous hosts and silencing or removal of either of the respective encoding genes could reduce fungal virulence (Kars *et al*., 2005). Knockout mutants of BcPG1 exhibited reduced virulence on tomato and apple plants whilst BcPG2 mutants displayed reduced virulence on tomato and broad bean (ten Have *et al*., 1998; Kars *et al*., 2005).

Plants possess polygalacturonase inhibiting proteins (PGIPs) to counter the activity of BcPGs (reviewed by Kalunke *et al*., 2015). VviPGIP1 was isolated from *V. vinifera* cv. Pinotage and forms part of the leucine-rich repeat (LRR) protein family which is characterised by a highly conserved LRR motif, shown to play a pivotal role in recognition of pathogen-derived molecules such as BcPGs (De Ascensao, 2001; Kobe and Kajava, 2001). VviPGIP1 differentially inhibited BcPGs *in vitro*, with inhibition only being detected against BcPG1 and 6 (Joubert *et al*., 2006). Using an *in planta* approach, transient expression of BcPG2 was shown to cause the most tissue damage compared to other BcPG isoforms. Interestingly, the *in planta* environment rendered VviPGIP1 an effective inhibitor of BcPG2 activity, suggesting that the cellular environment was crucial for the BcPG2-VviPGIP1 interaction to occur (Joubert *et al*., 2007).

Thus far, the only PGIP protein whose structure has been solved by X-ray crystallography is PvPGIP2 from *Phaseolus vulgaris* (Di Matteo *et al*., 2003). It is curved and elongated with a right-handed super-helix in the LRR domain. The concave region consists of parallel B1 β-sheets whilst B2 β-sheets are found in the convex exterior parts of the structure. Conserved regions include a negatively charged active site cleft located in the inner concave surface and a β-structure asparagine ladder (Di Matteo *et al*., 2003). In

contrast, numerous three-dimensional structures of ePGs have been solved to date. These include PGs from *Colletotricum lupini* (Bonivento *et al*., 2008), *Fusarium moniliforme* (Federici *et al*., 1999; 2001), *Erwinia carotovora* ssp. *carotovora* (Pickersgill *et al*., 1998), *Aspergillus aculeatus* (Cho *et al*., 2001) and *Aspergillus niger* (van Santen *et al*., 1999; van Pouderoyen *et al*., 2003). They are all characterised by a right-handed parallel β-helix fold with a deep negatively charged substrate-binding cleft on one side of the structure.

The structural basis of the interaction of PvPGIP2 with some of these ePGs has been studied. PvPGIP2 competitively inhibits FmPG from *F. moniliforme* by masking the active site and preventing any substrate from binding. It however, non-competitively inhibits AnPGII from *A. niger* by binding to an allosteric site (Federici *et al*., 2006). This is believed to cause structural changes within the ePG which affects the shape of the active site thus rendering it unable to bind to any substrate (Protsenko *et al*., 2008; King *et al*., 2002). PvPGIP2 exhibits a mixed-type mode of inhibition against BcPG1 from *B. cinerea* by partially blocking the active site, thus reducing substrate affinity (Manfredini *et al*., 2005; Sicilia *et al*., 2005). Residues that are crucial in different interactions have been identified using numerous techniques such as site-directed mutagenesis and shown to differ from one complex to the next (Maulik and Basu, 2013; Benedetti *et al*., 2013; Prabhu *et al*., 2014; Matsaunyane *et al*., 2015).

In grapevine, knowledge of the structural basis of VviPGIP1 inhibition interactions against BcPGs is currently lacking. To gain insight on these structure-function relationships, we performed and analysed molecular docking simulations of VviPGIP1 and two non-*vinifera* grapevine PGIPs (PGIP1012 from *V. x doaniana* Munson (a natural hybrid of *V. mustangensis* and *V. acerifolia*) and PGIP1038 from *V. caribaea*) against BcPG isoforms from three *B. cinerea* strains (B05.10, SAS56 and grape strain). PGIP1012 has an LRR motif identical to that of VviPGIP1 whilst PGIP1038 has four amino acid changes in this conserved region. PGIP1012 however, possesses three non-synonymous nucleotide changes outside the LRR region whilst PGIP1038 possesses seven (Wentzel, 2005). The choice of PGIPs from different grapevine accessions displaying sequence differences could provide insights into potential host-specific inhibition interactions and the influence of amino acid changes on ligand binding. Utilising BcPG isoforms from three *B. cinerea* strains in turn could provide valuable clues into strain-specific inhibition interaction trends. Using *in silico* site-directed mutagenesis, we predicted PGIP and BcPG residues crucial for the stability of the different docked complexes. This study therefore provides a structural basis for understanding the inhibition interaction between grapevine PGIPs and BcPGs.

3.3 MATERIALS AND METHODS

3.3.1 Comparative structural modelling of grapevine PGIPs and BcPGs

Domain-level based comparative structural modelling was utilised to predict putative protein structures of PGIPs and BcPGs using Robetta server (Kim *et al*., 2004). An overview of the workflow utilised in this study is presented in **Figure 1**.

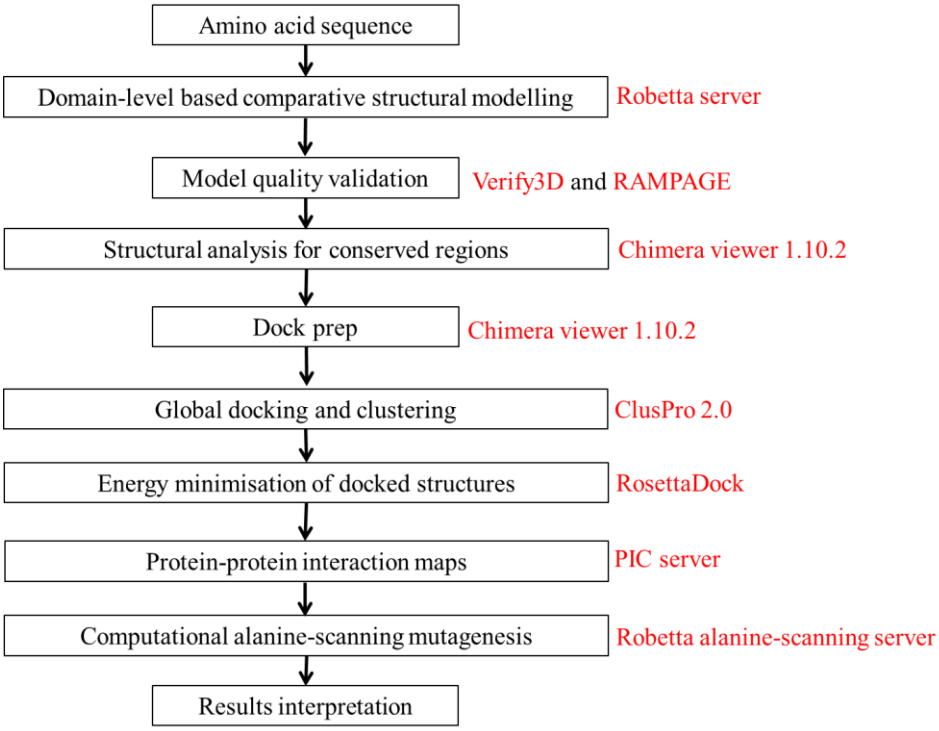

Figure 1. Summary of the different analyses conducted in this study to predict putative protein structures and docking simulations. The *in silico* tools utilised at each step are highlighted in red.

Amino acid sequences of VviPGIP1 (GenBank: AAM74142.1), PGIP1012 and PGIP1038 (Wentzel, 2005) were aligned in MUSCLE (Multiple Sequence Comparison by Log-Expectation) software (Edgar, 2004) to determine percentage sequence similarities. They were then comparatively modelled against the crystal structure of *P. vulgaris* PvPGIP2 [Protein Data Bank identity (PDB ID): 1OGQ]. The process involved cutting the amino acid query sequences into domain-sized fragments which were then threaded to the respective regions on the template sequence. Unaligned regions were treated as loops and modelled last based on template structure. The separate domain models were then combined to form a full length model and the amino acid side chains repacked using a backbone-dependant side-chain rotamer library. The top 5 low energy conformations were generated as the most likely *in silico* structural predictions for each query sequence (Kim *et al*., 2004).

The GenBank ID's of the amino acid sequences for the BcPGs utilised in the study are listed in **Table 1**. These included BcPG1-6 from B05.10 strain (Kars et al., 2005), BcPG1-6 from SAS56 strain (Ten Have *et al*., 1998; Wubben *et al*., 1999) and BcPG1-3 from a hyper-virulent South African grape strain

(Joubert *et al*., 2006; Rowe and Kliebenstein, 2007). Similar to PGIPs, the amino acid sequences were also aligned in MUSCLE (Edgar, 2004) to evaluate sequence similarities. Due to the availability of multiple endopolygalacturonase crystal structures to use as templates, two different approaches were initially tested for predicting BcPG models.

BcPG1 and BcPG2 from B05.10 strain were each comparatively modelled against five individual templates; *C. lupini* polygalacturonase (PDB ID: 2IQ7), *F. moniliforme* endopolygalacturonase (PDB ID: 1HG8), *A. aculeatus* polygalacturonase (PDB ID: 1IA5) and *A. niger* endopolygalacturonases I and II (PDB IDs: 1NHC and 1CZF respectively). The two BcPGs were also modelled against a combination of all five templates. The predicted models generated using a single template and multiple templates were then compared using model validation tools (described in the next section). Utilising multiple templates produced structural predictions with higher quality scores and thus all BcPG models reported in this study were threaded on the five templates mentioned above. Similar to the PGIP structural predictions, the top five low energy conformations for each BcPG were generated in Robetta for further analyses.

Table 1. List of BcPG protein sequences utilised for structural predictions and subsequent docking simulations against VviPGIP1, PGIP1012 and PGIP1038

***B. cinerea* strain**	**BcPG**	**GenBank ID**	**Reference**
B05.10	1	AAV84613.1	Kars *et al*., 2005
	2	AAV84614.1	Kars *et al*., 2005
	3	AAV84615.1	Kars *et al*., 2005
	4	AAV84616.1	Kars *et al*., 2005
	5	AAV84617.1	Kars *et al*., 2005
	6	AAV84618.1	Kars *et al*., 2005
SAS56	1	AAC64374.1	Ten Have *et al*., 1998
	2	AAC24950.1	Wubben *et al*., 1999
	3	AAC24952.2	Wubben *et al*., 1999
	4	AAC24953.1	Wubben *et al*., 1999
	5	AAC24955.1	Wubben *et al*., 1999
	6	AAC24956.2	Wubben *et al*., 1999
Grape strain	1	ABR20946.1	Rowe and Kliebenstein, 2007
	2	ABP88771.1	Rowe and Kliebenstein, 2007
	3	ABR20979.1	Rowe and Kliebenstein, 2007

3.3.2 Quality validation of predicted protein structures

From each amino acid query sequence, a total of five putative structural predictions were generated in Robetta server. Verify3D server (http://services.mbi.ucla.edu/Verify_3D/) was then used to determine the compatibility of the predicted 3D models to the original PGIP or BcPG amino acid sequences. Overall acceptable models exhibited an averaged 3D-1D score ≥ 0.2 for at least 80% of the residues. They were then subjected to further quality evaluations using Ramachandran plots constructed in RAMPAGE Server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). The plots determined the stereochemical quality of

predicted structures using torsion angles of amino acid residues. Regions within the predicted structures where residue conformations were not possible due to high levels of steric hindrance between main and side chain atoms were identified. The highest scoring structures with at least 90% of the residues clustering in the low energy, allowed and generously allowed regions of the Ramachandran plots were selected for each PGIP and BcPG. The models were further validated in Chimera viewer 1.10.2 (http://www.cgl.ucsf.edu/chimera/) where positions of known conserved regions were identified. The final predicted structures were selected based on the outputs of the model validations and they all passed the criteria set for the quality evaluations.

3.3.3 Docking and energy minimisation

Prior to docking, all structural predictions were prepared using the Dock Prep function in Chimera. This deleted water molecules, repaired truncated side chains, added hydrogens and assigned partial charges. The resulting PDB files were then subjected to global docking using ClusPro 2.0 (http://cluspro.bu.edu/home.php) to predict grapevine PGIP-BcPG complexes. The software filtered the different conformations, selecting those with the lowest desolvation and electrostatic energies (Comeau *et al*., 2004). The docked complexes were then clustered on the basis of pairwise RMSD (root mean square deviation) of atomic positions. The cluster with the largest number of members (cluster 00) was then selected as the closest representative of the interaction that is most likely to occur between the two proteins. In the event that the difference in number of members in the top clusters was less than 10, the different conformations were taken to represent the possible interactions between the respective PGIP and BcPG. The docked structures were then refined using RosettaDock (http://rosie.rosettacommons.org/) Docking2 option (Lyskov and Gray, 2008). It utilised the ClusPro output as a starting point, optimising the side chain conformations and rigid-body orientations. The analysis resulted in the 10 best-scoring predicted structures with lowest-interface energy. The lowest energy structure was then selected for further analysis.

3.3.4 Intra-protein and protein-protein interaction analyses

Electrostatic surface potentials of all predicted proteins were calculated in Chimera according to Coulomb's law:

$$\varphi = \Sigma [q_i / (\varepsilon d_i)]$$

where φ-potential, q-atomic partial charges, d-distances from the atoms, and ε-dielectric representing screening by the medium or solvent. A dielectric constant of 4.0 and a distance from surface of 1.4Å was utilised for all models. The different types of interactions at the protein-protein interfaces were analysed using the Protein Interaction Calculator (PIC) server (http://pic.mbu.iisc.ernet.in/) (Tina *et al*., 2007). These included different types of hydrogen bonds, hydrophobic and ionic interactions.

3.3.5 Computational alanine-scanning mutagenesis

The Robetta alanine-scanning server (http://robetta.bakerlab.org/alascansubmit.jsp) was utilised to identify energetically favourable amino acids at each of the PGIP-BcPG interfaces, which were important for the stability of the complexes. The server scanned the interface, replacing each residue with alanine in turn, and evaluated the change in binding free energy of the complex. The method relies on the assumption that at the interface, alanine is a neutral residue due to its small, non-polar methyl group side chain. It thus does not make a significant contribution to the binding, nor cause any substantial change to the protein backbone. The relative free energy was calculated as follows:

$$\Delta\Delta G_{binding} = G_{binding\_wildtype} - G_{binding\_mutant}$$

Residues that were identified as "hotspots" at the interface released binding free energy greater than 1 kcal/mol when replaced with alanine. This was regarded as a good indication that the mutation would destabilise the complex (Kortemme *et al*., 2004). Thus, the more energy was released; the more crucial the residue was for the complex to remain stable.

3.4 RESULTS

3.4.1 Predicted PGIP and BcPG structural models display high quality scores

The final selection of putative protein structural models for each PGIP and BcPG displayed quality scores >80% for compatibility to the original amino acid sequences using Verify3D and stereochemical quality scores >90% using RAMPAGE (**Table 2**). All scores were above the recommended thresholds for acceptable structural predictions.

Table 2. Quality scores of predicted protein structures utilised for further analyses in this study.

	Predicted structure	Verify3D % score	RAMPAGE % score
PGIP	VviPGIP1	96.1	98.0
	PGIP1012	95.8	98.7
	PGIP1038	90.2	99.0
BcPG1	B05.10	99.2	98.9
	SAS56	92.7	98.4
	Grape strain	89.1	98.9
BcPG2	B05.10	98.3	99.4
	SAS56	93.1	98.6
	Grape strain	94.4	98.7
BcPG3	B05.10, SAS56, Grape strain	85.6	98.6
BcPG4	B05.10, SAS56	90.4	98.5
BcPG5	B05.10, SAS56	94.2	98.9
BcPG6	B05.10, SAS56	93.2	98.9

3.4.2 PGIP1012 and PGIP1038 display high amino acid sequence and structural homology with VviPGIP1

The multiple amino acid sequence alignments of the grapevine PGIPs used showed overall homology of at least 97% (**Table 3**) with PGIP1012, isolated from the hybrid exhibiting higher similarity to VviPGIP1 compared to PGIP1038, isolated from the *V. caribaea* species.

Table 3. Percentage identity matrix of grapevine PGIPs utilised in this study. A value of 100 in each column was assigned to the PGIP to which the rest were compared.

	% Amino acid sequence similarity		
VviPGIP1	100	99.1	97.9
PGIP1012	99.1	100	98.8
PGIP1038	97.9	98.8	100

The predicted structural models for VviPGIP1, PGIP1012 and PGIP1038 were viewed and analysed for conserved regions in Chimera viewer (**Figure 2**). All three proteins displayed a right-handed, curved and elongated shape with a twisted scaffold in the LRR domain, possessing both B1 and B2 β-sheets. They also possessed a super-coiled alpha-helix in the N-terminal region and numerous 3_{10} helices. A short loop characterised the C-terminal region. The B1 β-sheets were located on the concave inner side of the structures whilst B2 β-sheets were on the convex exterior region. The β-sheets were of variable lengths, similar to what was reported for PvPGIP2 (Di Matteo *et al*., 2003). Analysis of the electrostatic surface potentials of the proteins revealed the presence of a negatively charged pocket located in the inner concave region of the LRR domain believed to be involved in PG recognition, also similar to PvPGIP2 (Di Matteo *et al*., 2003).

The leucine residues were mainly located on the concave surfaces where the LRR motifs responsible for recognition and inhibition of fungal ePGs are found. Asparagine residues formed the asparagine ladder, believed to play a crucial role in structural stability through the formation of hydrogen bonds with amide and main-chain carbonyl groups. It also plays a role in the bending of the protein. The location of the glycine residues on the β-B2 sheets was similar to that reported for PvPGIP2 (Di Matteo *et al*., 2003) and is believed to play a crucial role in the ability of this part of the structure to bend since glycine does not possess any side chain and thus does not contribute to steric clashes within the structure. Superimposition of VviPGIP1, PGIP1012 and PGIP1038 models highlighted subtle structural differences between the three predictions brought about by amino acid sequence differences. There were six structural positions where the three PGIP models differed; positions 35, 45, 172, 207, 214 and 219. Positions 35 was in the N-terminal region where PGIP1012 and PGIP1038 differ from VviPGIP1 whilst position 45 is just outside this region. Positions 172, 207, 214 and 219 were in the LRR region where PGIP1012 shares 100% homology to VviPGIP1.

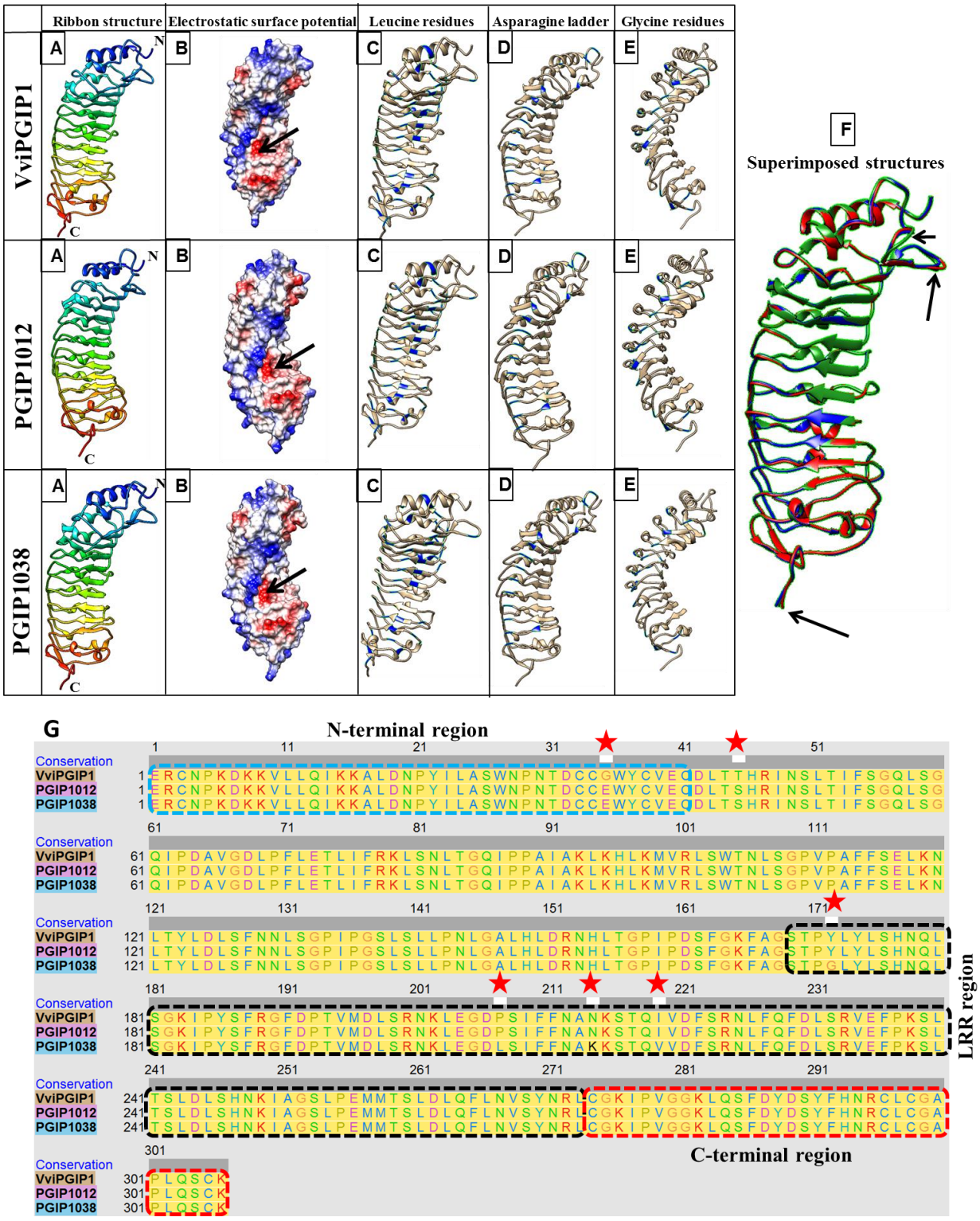

Figure 2. Highest ranking structural predictions for VviPGIP1, PGIP1012 and PGIP1038 showing (for each structure) (**A**) the ribbon structures with N-terminal and C-terminal regions highlighted. (**B**) An arrow on the electrostatic surface plots indicates the position of the conserved negatively charged pockets. The positions of the (**C**) leucine, (**D**) asparagine and (**E**) glycine residues are indicated in blue separately on the ribbon structural predictions of each grapevine PGIP. (**F**) Superimposition of the three PGIP models highlights the structural similarities with the most notable differences (on this plane) indicated by the arrows. (**G**) The structure based multiple sequence alignment shows all the regions which differ amongst the grapevine PGIP structural models (differences are indicated with a red star). The broken lines highlight the N-terminal, LRR and C-terminal regions (in different colours) in the PGIP structural predictions. All images were generated in Chimera viewer.

3.4.3 BcPG1 and 2 from different *B. cinerea* strains display high sequence similarities, whereas the other BcPGs were conserved as a separate group

Amino acid sequence alignment of BcPGs using MUSCLE software (Edgar, 2004) shows that subtle strain-specific amino acid differences are present in BcPG1 and 2 amongst all three strains. The BcPG1s' share 94-96% homology whilst BcPG2s' share 98-99% homology. BcPG3-6 sequences are conserved for all *B. cinerea* strains, but they generally display lower levels of sequence similarity to the virulence factors BcPG1 and 2 (**Table 4** and **Figure 3**).

Table 4. Percentage identity matrix of BcPGs from the three *B. cinerea* strains utilised in this study. A value of 100 highlighted in red in each column represents the BcPG to which the rest of the BcPGs were compared. The highlighted boxes show sequence similarity of BcPG1 and 2 across the different strains. BcPG1 sequences share ≥94% homology whilst BcPG2 sequences share ≥98% homology. BcPG3 and 6 amino acid sequences display the least percentage similarities to the rest of the BcPGs.

BcPG	% Amino acid sequence similarity									
BcPG1 B05.10	47	47	56	65	64	64	66	94	**100**	96
BcPG1 SAS56	48	47	56	65	65	65	66	96	96	**100**
BcPG1 Grape	47	46	57	65	64	64	65	**100**	94	96
BcPG2 B05.10	45	44	51	54	**100**	99	98	64	64	65
BcPG2 SAS56	45	44	51	54	98	98	**100**	65	66	66
BcPG2 Grape	45	44	51	54	99	**100**	98	64	64	65
BcPG3	60	**100**	40	43	44	44	44	46	47	47
BcPG4	40	40	**100**	59	51	51	51	57	56	56
BcPG5	45	43	59	**100**	54	54	54	65	65	65
BcPG6	**100**	60	40	45	45	45	45	47	47	48

*BcPG3-6 amino acid sequences are conserved and thus identical for all three strains

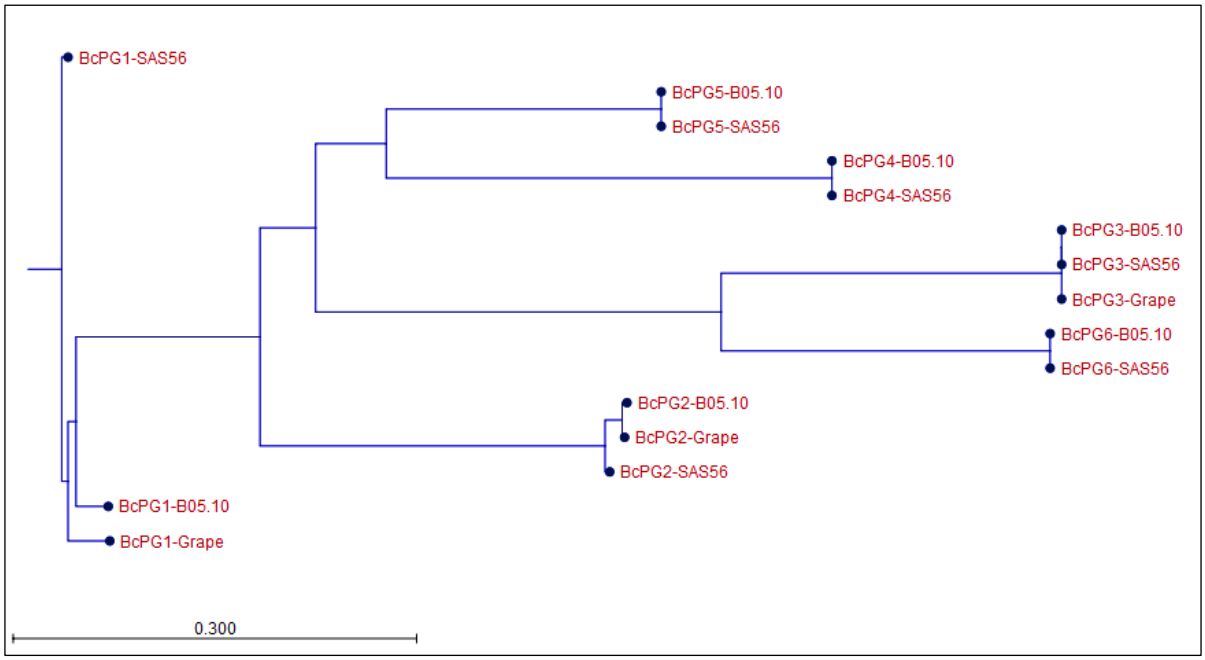

Figure 3. Phylogenetic tree of the full-length amino acid sequences of BcPGs from B05.10, SAS56 and the grape strain contructed in CLS Sequence Viewer 7.6.1 (QIAGEN Bioinformatics).

3.4.4 BcPG structural predictions display conservation of the negatively charged active site cleft

The ribbon representations of the homology modelled BcPGs from the three *B. cinerea* strains all exhibited a parallel right-ended β-helix. They were also all characterised by the presence of a deep negatively charged cleft on one side of the β-helix. BcPG1 structural predictions from the three strains (**Figure 4**) were highly similar as shown by the superimposition of the three ribbon representations. This was not surprising considering that they share >94% amino acid sequence homology. Their main difference was in the α-helix region. The β-helix secondary structures elements appeared to be completely conserved. There was very little variability in the length and conformation of the interstrand loops, none of which appeared to be close to the active site cleft. There were numerous structural differences between the three models, as shown by the structure-based multiple sequence alignment. Furthermore, there were subtle differences in overall electrostatic charge distribution over the entire BcPG1 surfaces for the different strains.

The BcPG2 structural predictions for the three *B. cinerea* strains also mainly differed in the α-helix region as shown by the ribbon representations in **Figure 5**. Similar to what was observed for BcPG1, the electrostatic potential surfaces of the models showed a negatively charged active site cleft on one side of the β-helix with subtle differences in overall charge distribution. Furthermore, the β-helix secondary structure elements were also conserved. Interestingly, the length and conformation of the interstrand loops was highly variable especially in the area around the active site cleft. These structural differences were highlighted by the structure based multiple sequence alignment.

BcPG3-6 structural predictions also possessed a deep cleft on one side of the β strand with variations mainly in the α-helix region (**Figure 6**). The charge distributions on the electrostatic potential surfaces of these four models were relatively different from those of BcPG1 and BcPG2 where the negative charge was mainly in the active site cleft. For BcPG3, 4 and 5 most of the surfaces on the structures were predicted to be negatively charged together with the active site crevice. The structure of BcPG3 was strikingly different from the other BcPGs isoforms, possessing an enlarged N-terminal extension. In terms of charge distribution, BcPG3 displayed high negative charge on the same side of the β strand as the active cleft, whilst BcPG4 had stronger negative charges on the surfaces opposite the active site. BcPG5 had a more even distribution of the negative charges whilst BcPG6 had an electrostatic potential surface profile similar to that of BcPG1 and BcPG2.

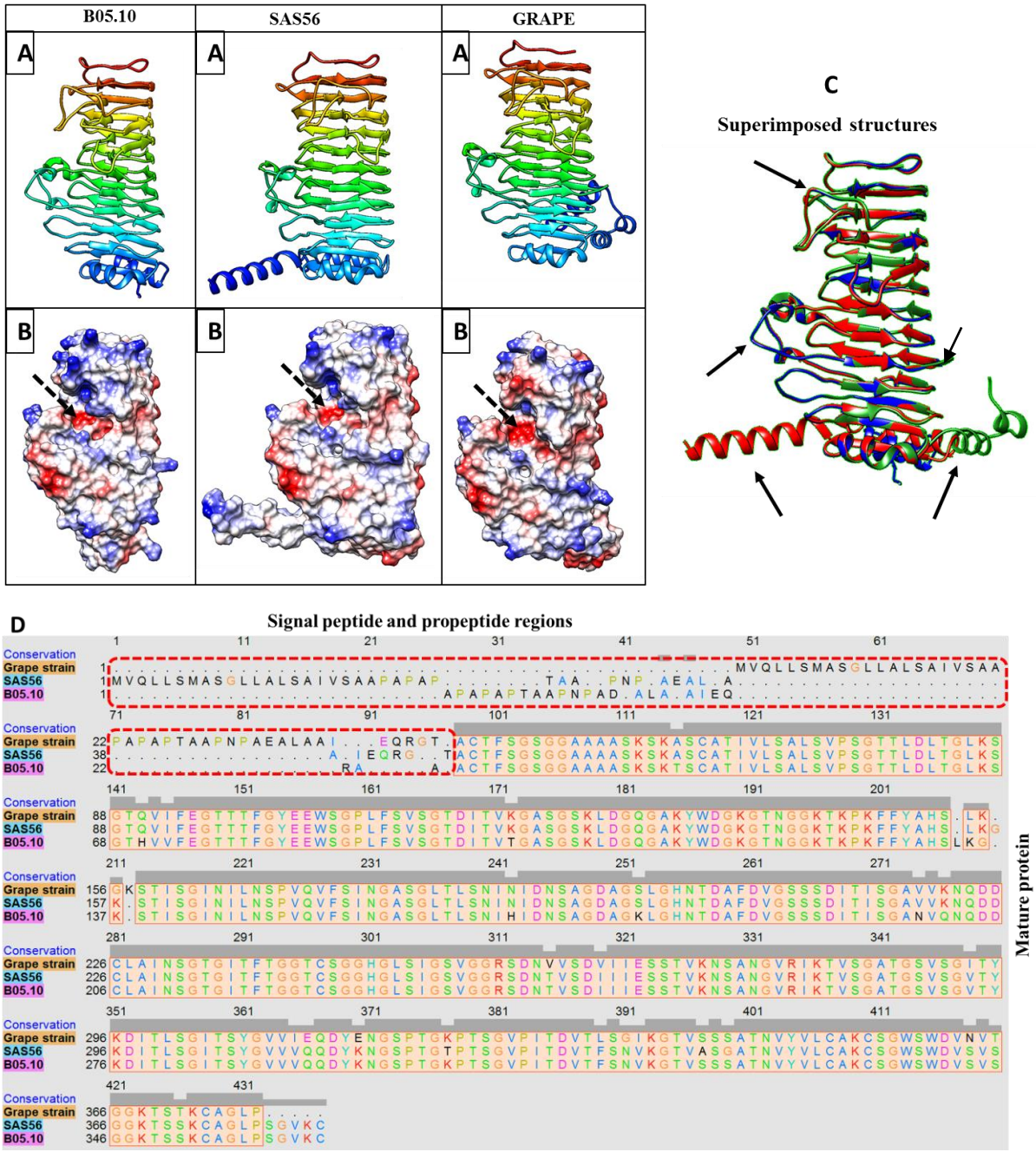

Figure 4. (A) The ribbon representations of the predicted BcPG1 proteins from the three *B. cinerea* strains showing the deep cleft on one side of the β-helix. (**B**) The electrostatic potential surfaces showed positive charges in blue and negative charges in red. For all three strains, the cleft was negatively charged (indicated by broken arrow), though there were slight differences that could be noted in charge distribution. (**C**) Structural supposition of BcPG1 from the three strains showed high conservation of the PG fold with very subtle differences in interstrand loop conformations (indicated by an arrows in this plane). (**D**) A structure-based multiple sequence alignment showed all the regions that differed amongst the BcPG1 structures from the different strains. The signal peptide and propeptide regions were not conserved in the structural predictions. However, higher levels of structural conservation were evident in the mature protein region.

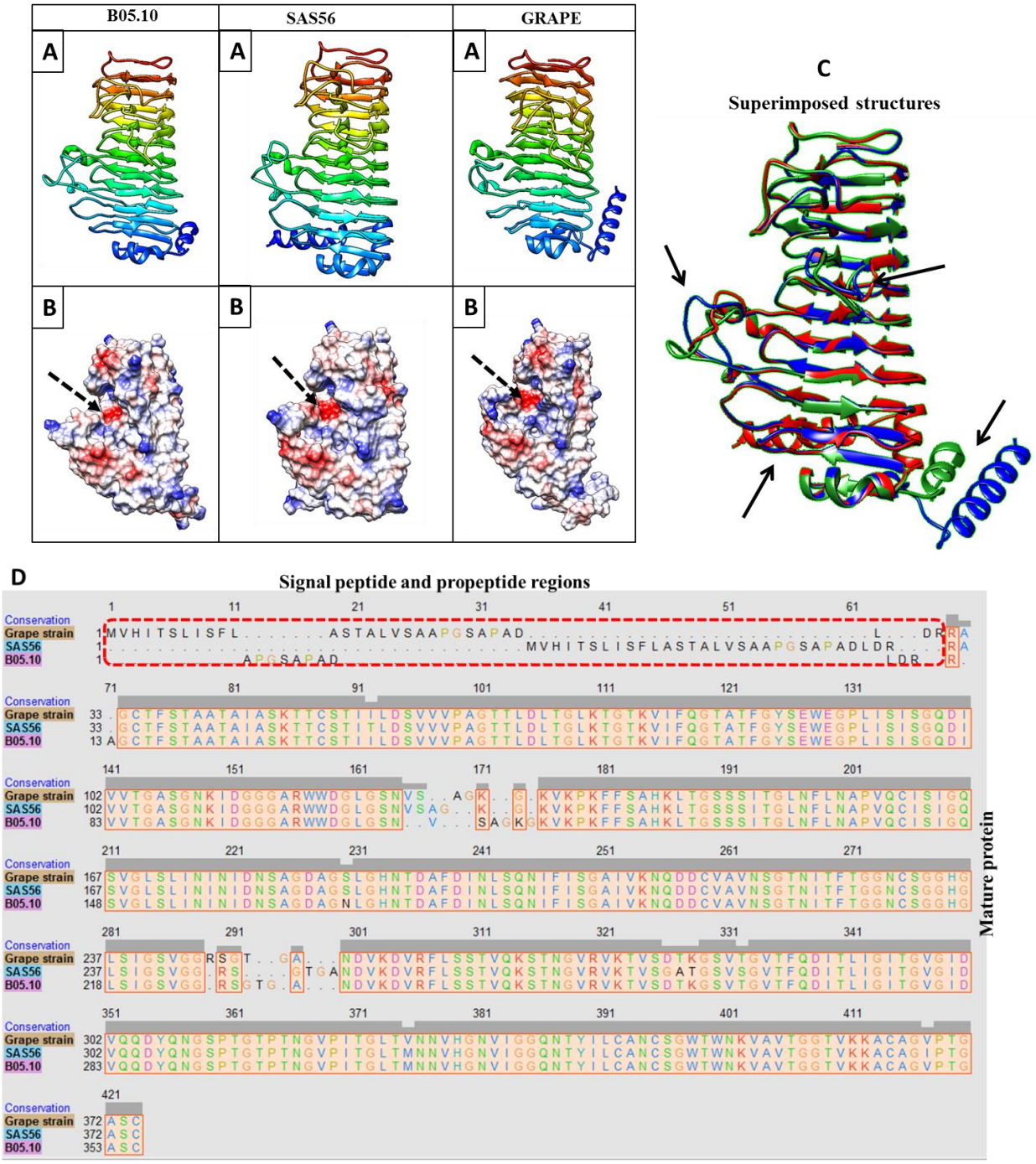

Figure 5. (A) The ribbon representations of the predicted BcPG2 proteins from the three *B. cinerea* strains showing the deep cleft on one side of the β-helix. (**B**) The electrostatic potential surfaces show positive charges in blue and negative charges in red. For all three strains, the cleft was negatively charged (indicated with a broken arrow), though there are slight differences that could be noted in charge distribution. (**C**) Structural supposition of BcPG2 from the three strains showed high conservation of the PG fold with differences in interstrand loop conformations around the active site (indicated by arrows). (**D**) A structure-based multiple sequence alignment highlighting all the regions that differ amongst the BcPG2 structures from the different strains. There was no structural conservation in the signal peptide and propeptide regions with the mature protein region showing high levels of conservation.

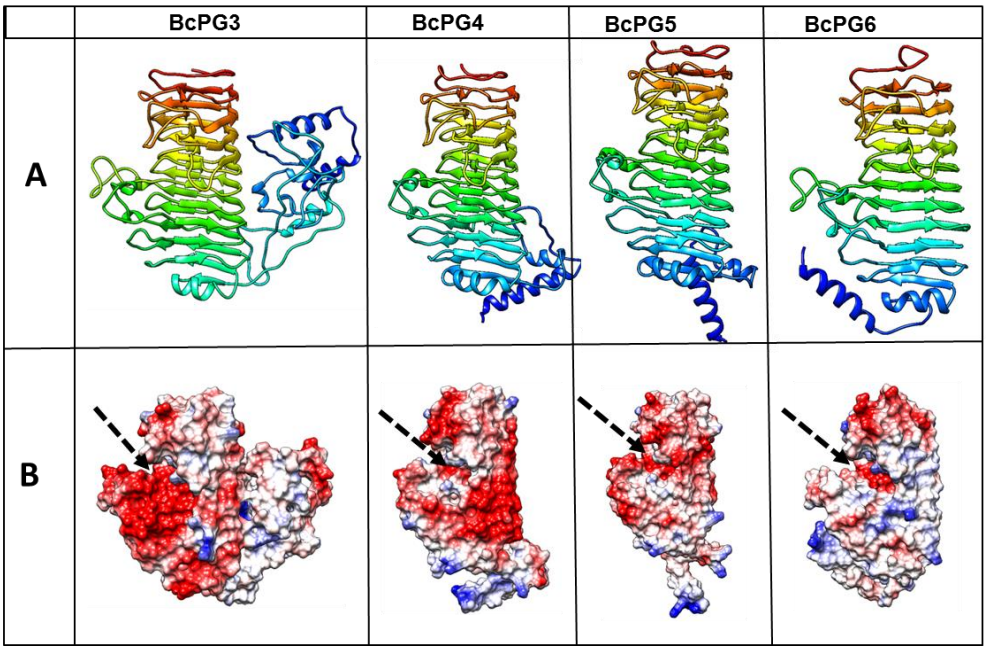

Figure 6. (A) Ribbon representations of BcPG3, 4, 5 and 6 structural models angled to highlight the active site clefts. (**B**) The electrostatic potential surfaces show positive charges in blue and negative charges in red highlighting the differences in charge distribution. The position of the negatively charged active site cleft is indicated with an arrow on each structural prediction.

3.4.5 Interaction of grapevine PGIPs with BcPG1 and 2 seems to be strain specific

Molecular docking simulations predicted the mode of interaction of VviPGIP1, PGIP1012 and PGIP1038 against the different BcPG1's to be highly *B. cinerea* strain dependent (**Figure 7**). The grapevine PGIPs were predicted to non-competitively inhibit BcPG1 from B05.10 and the grape strain; however, they did not utilise the same surfaces to do so. The N-terminal region of BcPG1 from B05.10 was predicted to interact with the different PGIPs whilst for the grape strain it was the C-terminal region. In addition, the PGIPs were predicted to utilise their concave surfaces to interact with BcPG1 from B05.10 and their convex region for the grape strain BcPG1. The predicted inhibition profiles against BcPG1 from SAS56 differed between VviPGIP1 and the two non-*vinifera* PGIPs. VviPGIP1 exhibited competitive, non-competitive and mixed-type mode of inhibition against SAS56-BcPG1 whilst the two non-*vinifera* PGIPs only displayed competitive and mixed-type mode of inhibition. The different docking simulations between the PGIPs and SAS56-BcPG1 involved mainly the concave region of the PGIPs and both N and C-terminals for BcPG1.

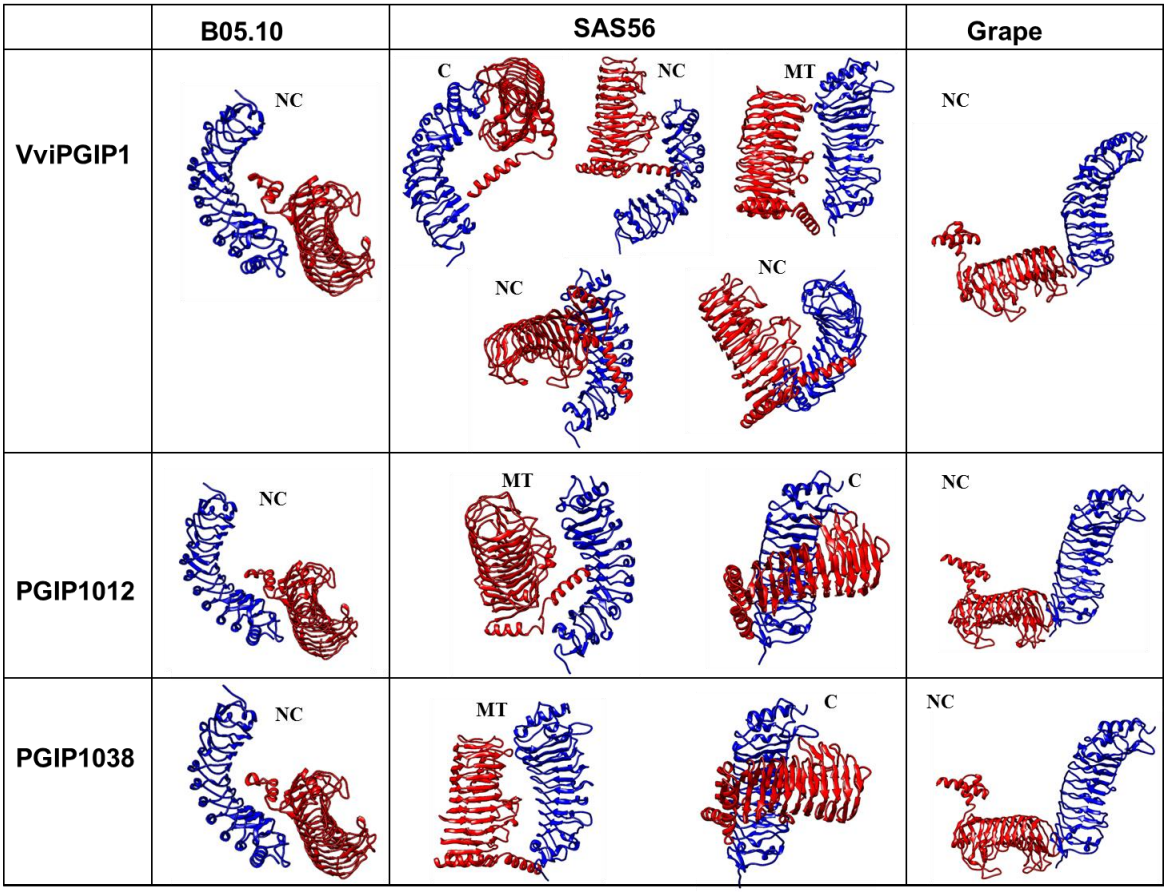

Figure 7. Ribbon representation of the predicted interactions between the grapevine PGIPs and BcPG1 from different *B. cinerea* strains obtained from molecular docking simulations. The PGIPs are shown in blue and BcPG1 is shown in red. ClusPro clustering of docking simulations was utilised to select the complexes that represented each interaction prior to refinement in RosettaDock. The highest ranking complex with the most members in a cluster was always chosen. Subsequent clusters were also chosen to represent the interaction if the difference in cluster size from the highest ranking cluster was less than 10 and they are presented in ranking order from top left per row. **C**-competitive inhibition, **NC**- non-competitive inhibition and **MT**-mixed-type mode of inhibition.

The mode of interaction of the grapevine PGIPs with BcPG2 was also predicted to be *B. cinerea* strain dependent (**Figure 8**). The grapevine PGIPs were all predicted to competitively inhibit BcPG2 from B05.10 with slight variations in docking orientations. Non-competitive inhibition was exhibited against SAS56-BcPG2 whereby the PGIPs were predicted to utilise their concave surfaces for the interaction and BcPG2 utilised its N-terminal region. Interaction between the PGIPs and BcPG2 from *B. cinerea* grape strain was predicted to either be competitive or non-competitive. Only VviPGIP1 and PGIP1012 displayed competitive inhibition in the top ranking docking simulations whilst PGIP1038 only displayed non-competitive inhibition. All the non-competitive docking simulations involved the N-terminal region of grape strain-BcPG2. The non-competitive docking orientation that resembled that of the predicted complex between PGIP1038 and grape strain-BcPG2 was also represented amongst the highest ranking complexes for the VviPGIP1 and PGIP1012 complexes.

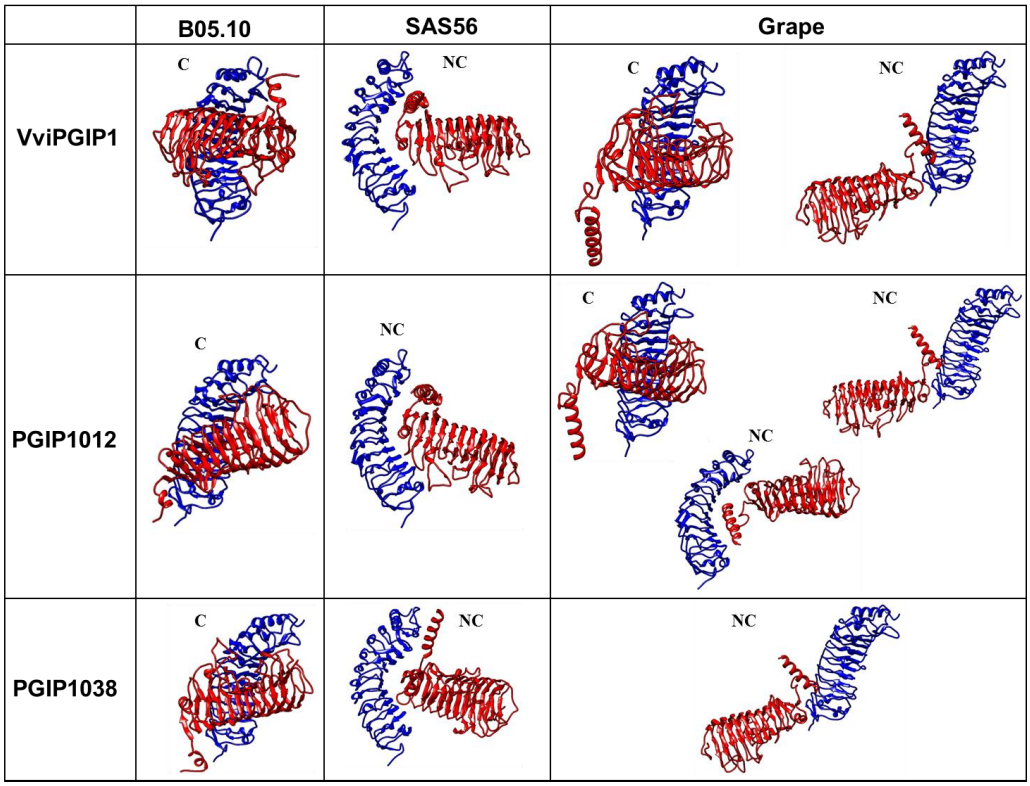

Figure 8. Ribbon representation of the predicted interaction between grapevine PGIPs and BcPG2 from different *B. cinerea* strains obtained from molecular docking simulations. The PGIPs are shown in blue and BcPG1 is shown in red. ClusPro clustering of docking simulations was utilised to select the complexes that represent each interaction prior to refinement in RosettaDock. The highest ranking complex with the most members in a cluster was always chosen. Subsequent clusters were also chosen to represent the interaction if the difference in cluster size from the highest ranking cluster was less than 10. Complexes are presented in ranking order from top left. **C**-competitive inhibition, **NC**-non-competitive inhibition and **MT**-mixed-type mode of inhibition.

3.4.6 Grapevine PGIPs are predicted to non-competitively interact with BcPG3-6

BcPG3-6 amino acid sequences from the different *B. cinerea* strains displayed 100% conservation. Thus docking simulations for each BcPG was carried out against the three grapevine PGIPs utilised in this study. VviPGIP1 and the two non-*vinifera* PGIPs were predicted to utilise their concave surfaces to interact with BcPG3-6 non-competitively (**Figure 9**). However, there were differences in the binding orientations during the interactions. For example, the C-terminal region of BcPG4 was predicted to interact with VviPGIP1 and PGIP1038 whilst it was the N-terminal region which interacted with PGIP1012. BcPG3, BcPG5 and BcPG6 were all predicted to interact with the grapevine PGIPs using the N-terminal regions, with slight variations in docking orientations.

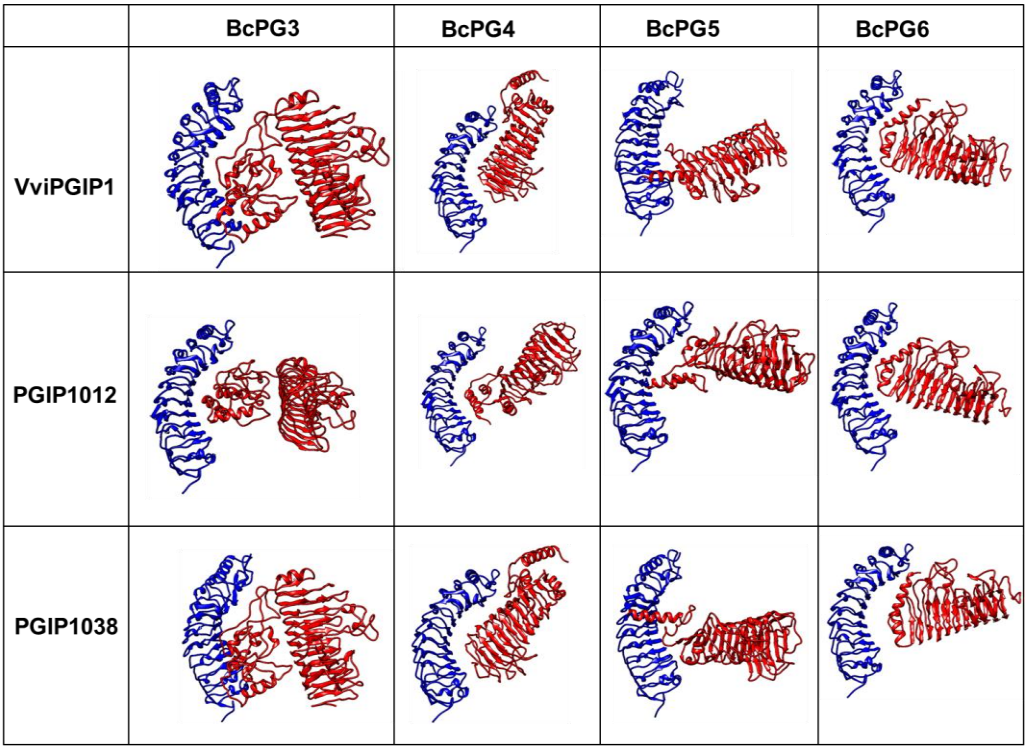

Figure 9. Ribbon representations of the predicted non-competitive interactions between grapevine PGIPs and BcPG3-6 obtained from molecular docking simulations. The PGIPs are shown in blue and the respective BcPGs are shown in red.

3.4.7 Nature and number of protein-protein interactions that are predicted to stabilise the docked complexes differ

The different types of interactions were analysed using the PIC server to determine possible differences in contact surfaces. Interesting results were obtained for complexes which were visually similar such as the non-competitive inhibition of B05.10-BcPG1 by the different PGIPs. The nature and number of bonds keeping each docked complex stable varied (**Table 5**). The same trend was observed for docked complexes between the grapevine PGIPs and BcPG1 grape strain. No hydrophobic interactions were predicted between the PGIPs and BcPG1 grape strain in any of the docked complexes. The grapevines PGIPs were all predicted to competitively inhibit BcPG2 from B05.10 and the docking simulations showed slight variations in the orientation of the complexes. The differences in nature and number of bonds stabilising the different complexes together were evident in the results shown. This trend was observed for other docked complexes as well.

Table 5. Summary of protein-protein interactions highlighting the vast differences in nature and number of bonds predicted to stabilise the top scoring docked complexes involving virulence factors BcPG1 and 2. The grapevine PGIPs were docked against (**A**) BcPG1 from B05.10, (**B**) BcPG1 from SAS56, (**C**) BcPG1 from grape strain, (**D**) BcPG2 from B05.10, (**E**) BcPG2 from SAS56 and (**F**) BcPG2 from grape strain.

A
B05.10 BcPG1

Type of interaction	VviPGIP1-BcPG1	PGIP1012-BcPG1	PGIP1038-BcPG1
Hydrophobic interaction (within 5 Å)	10	8	11
Disulphide bridges	0	0	0
Main chain – Main chain H-bonds	0	0	0
Main chain – Side chain H-bonds	3	0	2
Side chain – Side chain H-bonds	23	9	11
Ionic interactions (within 6 Å)	6	3	5
Cation-Pi interactions (within 6 Å)	0	0	0

B
SAS56 BcPG1

Type of interaction	VviPGIP1-BcPG1	PGIP1012-BcPG1	PGIP1038-BcPG1
Hydrophobic interaction (within 5 Å)	4	2	2
Disulphide bridges	0	0	0
Main chain – Main chain H-bonds	0	0	0
Main chain – Side chain H-bonds	3	6	10
Side chain – Side chain H-bonds	3	0	0
Ionic interactions (within 6 Å)	4	0	0
Aromatic-Aromatic (within 4.5 and 7 Å)	1	1	0
Cation-Pi interactions (within 6 Å)	1	3	1

C
Grape strain BcPG1

Type of interaction	VviPGIP1-BcPG1	PGIP1012-BcPG1	PGIP1038-BcPG1
Hydrophobic interaction (within 5 Å)	0	0	0
Disulphide bridges	1	0	0
Main chain – Main chain H-bonds	0	2	3
Main chain – Side chain H-bonds	7	3	2
Side chain – Side chain H-bonds	5	3	0
Ionic interactions (within 6 Å)	2	0	1
Aromatic-Aromatic (within 4.5 and 7 Å)	0	0	0
Cation-Pi interactions (within 6 Å)	1	0	0

D
B05.10 BcPG2

Type of interaction	VviPGIP1-BcPG2	PGIP1012-BcPG2	PGIP1038-BcPG2
Hydrophobic interaction (within 5 Å)	2	5	0
Disulphide bridges	0	0	0
Main chain – Main chain H-bonds	1	0	0
Main chain – Side chain H-bonds	1	7	9
Side chain – Side chain H-bonds	9	3	22
Ionic interactions (within 6 Å)	6	3	5
Aromatic-Aromatic (within 4.5 and 7 Å)	0	1	0
Cation-Pi interactions (within 6 Å)	1	1	1

E
SAS56 BcPG2

Type of interaction	VviPGIP1-BcPG2	PGIP1012-BcPG2	PGIP1038-BcPG2
Hydrophobic interaction (within 5 Å)	10	7	5
Main chain – Main chain H-bonds	1	0	0
Main chain – Side chain H-bonds	3	3	0
Side chain – Side chain H-bonds	12	3	2
Ionic interactions (within 6 Å)	5	4	5
Aromatic-Aromatic (within 4.5 and 7 Å)	1	2	2
Aromatic-Sulphur (within 5.3Å)	1	0	0
Cation-Pi interactions (within 6 Å)	0	0	1

F
Grape strain BcPG2

Type of interaction	VviPGIP1-BcPG1	PGIP1012-BcPG1	PGIP1038-BcPG1
Hydrophobic interaction (within 5 Å)	5	4	2
Disulphide bridges	0	0	0
Main chain – Main chain H-bonds	0	0	1
Main chain – Side chain H-bonds	2	9	7
Side chain – Side chain H-bonds	11	7	3
Ionic interactions (within 6 Å)	1	0	2
Cation-Pi interactions (within 6 Å)	0	0	1

3.4.8 Significant PGIP and BcPG binding sites differ per complex despite similar docking orientations

The most significant binding sites for each docked complex, represented by the residues which released the highest levels of relative free energy when computationally replaced with alanine, differed from one complex to the next with a few overlaps (**Table 6**). **Figure 10** and **11** show the positions of the hotspots on some of the predicted models. B05.10-BcPG1 was predicted to utilise Q99 as a hotspot when docked against VviPGIP1 whilst C14 was the hotspot when docked against both PGIP1012 and PGIP1038. J11 was the SAS56-BcPG1 hotspot when docked against PGIP1012 and PGIP1038 whilst O256 was predicted for docking simulations against VviPGIP1. The J11 hotspot was also shared by BcPG4 in its predicted docking simulation against PGIP1012. O36 was predicted to be the BcPG5 hotspot when docked against VviPGIP1 and PGIP1012 whilst P5 was predicted for the PGIP1038 complex. Predictions of the most significant PGIP binding sites showed that O101 was a hotspot on PGIP1012-BcPG3, PGIP1038-BcPG4 and PGIP1038-B05.10 BcPG1 complexes. L106 was predicted as a hotspot only on PGIP1038 docked complexes against B05.10-BcBG2 and BcPG5. Similarly, T123 was predicted as a hotspot on PGIP1012 docked complexes against SAS56-BcPG2 and BcPG6. VviPGIP1 shared the S104 hotspot with PGIP1038 in VviPGIP1-BcPG6 and PGIP1038 against BcPG3 and SAS56-BcPG2 docked complexes. T172 was a predicted hotspot for VviPGIP1 and PGIP1012 docked complexes against BcPG5 and BcPG4 respectively. All shared hotspots however, released different levels of relative free energy when computationally replaced with alanine.

Table 6. Summary of the predicted hotspots for the highest ranging docked PGIP-BcPG complexes. The relative free energy (kcal/mol) predicted to be released when each residue is computationally replaced by alanine in the docking simulations is presented in brackets next to the hotspot. Coloured cells in each column represent hotspots which were common in at least two complexes with unique colours for each hotspot. No hotspots were predicted for the PGIP1012-BcPG1 grape strain complex.

Complex	Most significant PGIP binding site	Most significant BcPG binding site
VviPGIP1-BcPG1 (B05.10)	I79 (4.92)	Q99 (6.08)
PGIP1012-BcPG1 (B05.10)	O78 (9.32)	C14 (6.12)
PGIP1038-BcPG1 (B05.10)	O101 (4.84)	C14 (2.15)
VviPGIP1-BcPG1 (SAS56)	Q31 (3.08)	O256 (3.58)
PGIP1012-BcPG1 (SAS56)	N179 (1.92)	J11 (1.93)
PGIP1038-BcPG1 (SAS56)	O244 (2.9)	J11 (1.58)
VviPGIP1-BcPG1 (Grape strain)	L294 (1.18)	Q369 (1.87)
PGIP1012-BcPG1 (Grape strain)	-	-
PGIP1038-BcPG1 (Grape strain)	O272 (1.02)	Q365 (2.35)
VviPGIP1-BcPG2 (B05.10)	T270 (1.54)	H315 (1.53)
PGIP1012-BcPG2 (B05.10)	P246 (3.31)	O256 (1.27)
PGIP1038-BcPG2 (B05.10)	L106 (5.12)	L167 (7.08)
VviPGIP1-BcPG2 (SAS56)	C197 (3.59)	Q38 (2.7)
PGIP1012-BcPG2 (SAS56)	T123 (1.84)	C66 (1.9)
PGIP1038-BcPG2 (SAS56)	S104 (2.84)	C55 (2.36)
VviPGIP1-BcPG2 (Grape strain)	L267 (3.56)	G293 (2.29)
PGIP1012-BcPG2 (Grape strain)	N264 (2.29)	L332 (2.29)
PGIP1038-BcPG2 (Grape strain)	N303 (2.05)	Q48 (1.33)
VviPGIP1-BcPG3	S152 (2.98)	J15 (1.01)
PGIP1012-BcPG3	O101 (2.53)	P99 (1.76)
PGIP1038-BcPG3	S104 (4.31)	R131 (1.84)
VviPGIP1-BcPG4	E54 (2.62)	T249 (4.36)
PGIP1012-BcPG4	T172 (3.02)	J11 (1.05)
PGIP1038-BcPG4	O101 (2.25)	P309 (2.82)
VviPGIP1-BcPG5	T172 (2.19)	O36 (1.27)
PGIP1012-BcPG5	G149 (2.12)	O36 (1.99)
PGIP1038-BcPG5	L106 (3.66)	P5 (2.43)
VviPGIP1-BcPG6	S104 (2.5)	J10 (1.29)
PGIP1012-BcPG6	T123 (2.75)	N32 (3.5)
PGIP1038-BcPG6	T37 (2.28)	H7 (2.18)

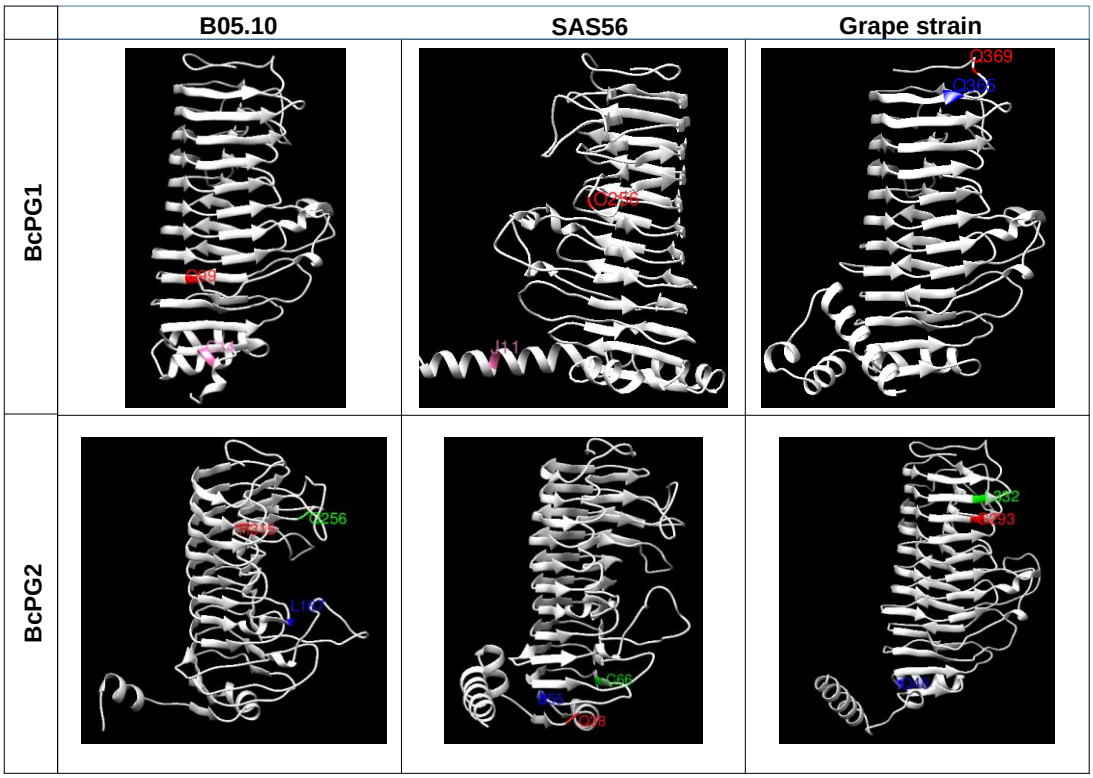

Figure 10. Ribbon representations of BcPG1 and 2 from the three *B. cinerea* strains angled to best show the positions of the predicted hotspots in **Table 6**. The hotspots are coloured according to docking partner. Thus all BcPG1 and 2 hotspots in VviPGIP1-BcPG interactions are coloured in red, PGIP1012-BcPG in green, PGIP1038-BcPG in blue and shared hotspots in pink.

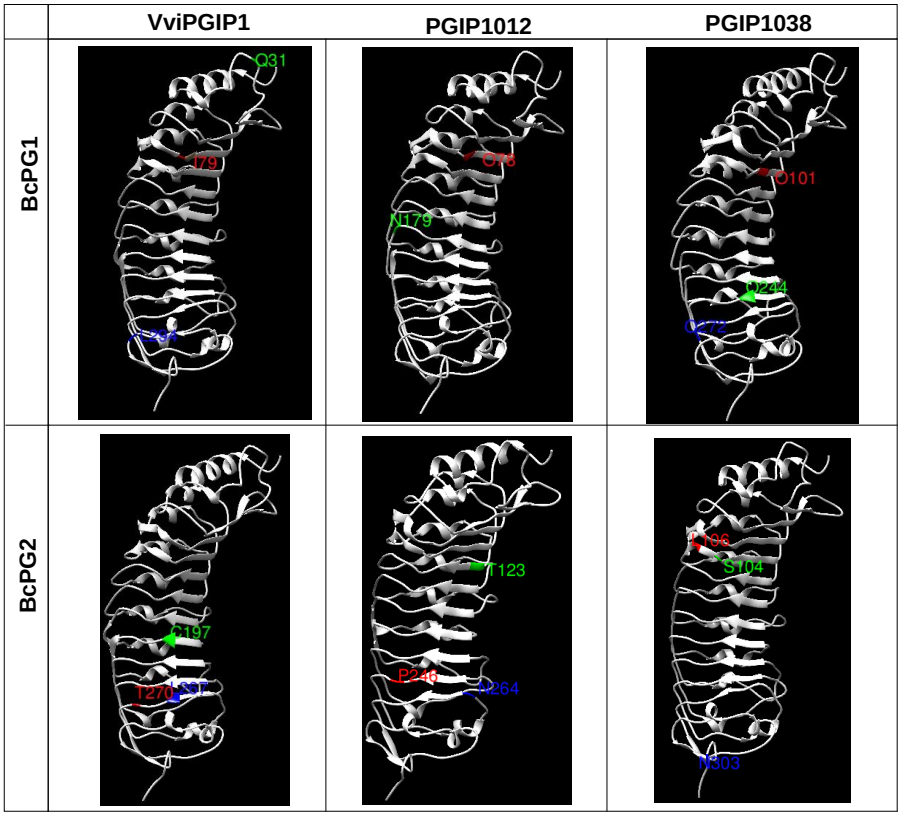

Figure 11. Ribbon representations of grapevine PGIPs angled to best show the positions of the predicted hotspots in BcPG1 and 2 interactions. The hotspots are coloured according to docking partner. Thus all PGIP hotspots in BcPG-B05.10 interactions are coloured in red, BcPG-SAS56 in green, BcPG-Grape strain in blue.

3.5 DISCUSSION

The bioinformatics pipeline utilised in this study yielded high quality structural predictions

The comparative models of the grapevine PGIPs predicted in this study were generated based on the crystal structure of PvPGIP2 (Di Matteo *et al*., 2003). Despite the fact that the grapevine PGIPs share less than 50% amino acid sequence homology with the template structure, it was possible to generate structural predictions which highlighted the subtle differences in the LRR motif and N-terminal region between VviPGIP1 and the two non-*vinifera* PGIPs. Further analysis of the predicted structures revealed the characteristic conservation of the positions of leucine, glycine residues, asparagine ladder as well as the negatively charged electrostatic pocket in the inner concave LRR region (**Figure 2**), similar to what was reported for the PvPGIP2 crystal structure (Di Matteo *et al*., 2006). On the other hand, the comparative models of the six BcPG isoforms from all three *B. cinerea* strains showed conservation of the PG fold and the negatively charged active site cleft (**Figure 4, 5** and **6**), similar to other previously reported PG structures (Federici *et al*., 1999: 2001; van Pouderoyen *et al*., 2003; Sicilia *et al*., 2005; Bonivento *et al*., 2008). All PGIP and BcPG protein models predicted in this study and subsequently utilised for docking simulations passed stringent structural validation tests with high quality scores (**Table 2**). Taken together, these attributes provided confidence in the predicted models being reliable representations of the different proteins and thus their *in silico* functional capabilities.

Subtle differences in the structural PGIP predictions could affect inhibition interactions

The amino acid sequences of both PGIP1012 and PGIP1038 share very high overall homology, >95% to VviPGIP1 (Wentzel, 2005), a trait which was also evident in the resulting *in silico* structural predictions. Despite the observed high levels of sequence and structural homology, marked differences in inhibition potentials are a possibility as evidenced by a comparative study which was conducted on the inhibitory activity of PGIPs from *P. vulgaris* with 99% sequence homology on *F. moniliforme* PGs (Maulik *et al*., 2009). The study convincingly showed that the function and inhibitory potential of the PGIPs was not directly dependent on the degree of sequence similarity. Furthermore, numerous studies have shown that subtle changes in the sequences of plant PGIP encoding genes and amino acids sequences significantly alters the specificity for inhibiting pathogenic PGs (Misas-Villamil *et al*., 2008; Casasoli *et al*., 2009; Maulik *et al*., 2009; Maulik and Basu., 2013; Benedetti *et al*., 2013).

In this study, six structural positions were identified which differed amongst the three PGIP models (**Figure 2**). Four of these (positions 172, 207, 214 and 219) were within the highly conserved LRR region and predicted to be identical between VviPGIP1 and PGIP1012, but differed when compared to PGIP1038. The motif is believed to be crucial for recognition of fungal molecules and functional sites for ligand binding located on the concave surface have been reported (Kobe and Kajava, 2001; Heft *et al*., 2011). In PvPGIP2, the LRR region was further shown to act as a releasing signal which enables the protein to leave the Golgi and move to the cell wall (De Caroli *et al*., 2015). This trait is crucial for PGIPs to be in a favourable position to defend plant cells against CWDE such as BcPGs during *B. cinerea* infection. Despite the fact that

none of the four structural positions were predicted as hotspots on any of the PGIP-BcPG docked complexes (**Table 6**), it cannot be ruled out that they could have influenced the overall strength and/or dynamics of the interactions without necessarily being the most significant binding sites. Due to the 100% structural homology of VviPGIP1 and PGIP1012 models in the LRR region, the two proteins are likely to display similar profiles in terms of recognition of fungal molecules and transport from the Golgi to the cell wall. However, the structural differences outside this region possibly have an impact on inhibition interactions.

Some of the predicted PGIP structural differences were however, not located in the conserved LRR region. Predicted structural positions 35 and 45, where PGIP1012 and PGIP1038 differed from VviPGIP1, were situated in and just outside the N-terminal region respectively (**Figure 2**). In PvPGIP2, the N-terminal region has been shown to act as a holding signal during transit through Golgi stacks (De Caroli *et al*., 2015). Also, it has been hypothesised that PGIP utilises other parts outside the LRR region for binding to fungal PGs and/or masking substrates such as pectin (Spadoni *et al*., 2006). The formation of three-component complexes comprising of ePG, PGIP and pectin was shown to have an effect on inhibition interactions when compared to the classical two-component PGIP-PG interactions. The type and strength of the effect was dependent on the PGIP-PG pairing (Gutierrez-Sanchez *et al*., 2012). This means that not only changes in the LRR motif but also in other regions, could affect the dynamics of inhibition interactions. The two structural positions 35 and 45 on PGIP1012 and PGIP1038 could be crucial for masking pectin in the three-component complexes and the dynamics of the interactions possibly differ from that of VviPGIP1. The strength of the interactions could also be host specific, being influenced by characteristics of the pectin substrate such as degree of methylation.

Structural differences of BcPG isoforms could influence inhibition interactions

All BcPG models generated in this *in silico* study displayed similar structural characteristics described in previous reports of three-dimensional ePG structures solved by X-ray crystallography (Federici *et al*., 1999: 2001; van Pouderoyen *et al*., 2003; Sicilia *et al*., 2005; Bonivento *et al*., 2008). They possessed a negatively charged active site cleft with conservation of the right-handed PG fold. However, the amino acid sequence differences highlighted in the percentage identity matrix (**Table 4** and **Figure 3**), were evident in the overall predicted models of the BcPG isoforms. The BcPG3 amino acid sequence had the lowest percentage similarities to the other isoforms and the predicted structure was also strikingly different with an enlarged N-terminal extension. These structural differences could explain the differential substrate specificities of BcPG isoforms (Wubben *et al*., 1999; Kars *et al*., 2005).

The structural predictions for BcPG1, 2 and 6 possessed highly similar charge distributions on their electrostatic surface plots compared to BcPG3, 4 and 5 (**Figures 4**, **5** and **6**). This attribute is known to influence the ability of proteins to bind to other molecules in a highly specific manner, thus determining the compatibility of the inhibition interaction (Jones and Thornton, 1996; Sheinerman *et al*., 2000). Interestingly, experimental findings have previously shown that VviPGIP1 displays differential inhibition profiles against purified B05.10 BcPGs. It effectively inhibited individual BcPG1, 2 and 6 using a combination of *in vitro*

and *in planta* platforms (Joubert *et al*., 2006; 2007). No inhibition was detected against the other BcPG isoforms. The amino acid sequence differences amongst the BcPG isoforms possibly had a strong influence on the dynamics of protein threading in our predicted models, leading to differences in surface charge distributions. The similar surface charge distributions on BcPG1, 2 and 6 most likely complements those of grapevine PGIPs, allowing for stable interactions to occur. This aspect could have influenced the PGIP-BcPG inhibition interaction trends reported by Joubert *et al*., (2006; 2007).

Subtle differences in BcPG1 and 2 models possibly led to strain specific PGIP-PG interactions

The predicted inhibition profiles of grapevine PGIPs against BcPG1 and 2 were *B. cinerea* strain specific. This was despite the fact that the amino acid sequences of BcPG1 and 2 from the three strains shared ≥94 and ≥98 percentage similarities respectively (**Table 4**). BcPG1 and 2 are *B. cinerea* virulence factors on numerous plant hosts (ten Have *et al*., 1998; Kars *et al*., 2005; Choquer *et al*., 2007; Nakajima and Akutsu, 2014). Several other studies, with known virulence factors such as botrydial and *Bcpme1*, have experimentally reported strain specific trends in B05.10, SAS56 and T4 deletion mutants on several crop species (Colmenares *et al*., 2002; Valette-Collet *et al*., 2003; Kars *et al*., 2005; Siewers *et al*., 2005).

BcPG1 from B05.10 and the grape strain were both predicted to be non-competitively inhibited by all three PGIPs, however, the docking orientations differed (**Figure 7**). On the other hand, the grapevine PGIPs displayed a combination of competitive, non-competitive and mixed-type modes of inhibition in their predicted docked complexes against SAS56-BcPG1. Interestingly, computational mutagenesis predicted that B05.10-BcPG1 formed stronger complexes with all three grapevine PGIPs compared to the other strains, as evidenced by higher levels of relative free energy predicted to be released when the PGIP hotspots were replaced with alanine as well as nature and number of bonds at the interface (**Table 5** and **6**). Grape strain BcPG1 formed the weakest complexes with all three grapevine PGIPs, with VviPGIP1 complexes being slightly stronger with more bonds at the protein-protein interface (**Table 5**). We suggest that the strength of the bonds at the different interfaces could be an indication of inhibition potential. Thus, grapevine PGIPs appear to be poor inhibitors of BcPG1 from the grape strain. The predicted bonds on the VviPGIP1-BcPG1 grape strain complex, compared to the non-*vinifera* PGIPs, is suggestive of VviPGIP1 being a slightly stronger inhibitor.

On the other hand, BcPG2 from B05.10 was predicted to be competitively inhibited by all three PGIPs, whilst SAS56-BcPG2 complexes displayed non-competitive inhibition profiles. The top scoring B05.10-BcPG2 complexes displayed both competitive and non-competitive inhibition profiles (**Figure 8**). Based on the docking orientations for all three PGIPs for both BcPG1 and 2 per strain, our results suggest that the subtle amino acid differences in BcPG sequences, evident in the predicted structures, were the main contributors towards the strain specificity trend. This could be an indication of evolutionary pressure on the proteins, suggestive of their crucial role to *B. cinerea* virulence during host infection.

The pairing of PGIP and BcPG influenced strength and stability of docked complexes

The stability of the docked complexes reported in this study differed, as evidenced by differences in nature and number of bonds at the protein-protein interfaces (**Table 5**) coupled with the amount of energy predicted to be released when hotspots were computationally replaced with alanine (**Table 6**). Several complexes were predicted to share a common PGIP or BcPG hotspot, but not both. However, even the shared hotspots differed in predicted free energy released during computational mutagenesis. The uniqueness of PGIP hotspots was not surprising since PvPGIP2 has also been shown to display differential inhibition profiles against numerous ePGs by utilising different structural surfaces at the protein-protein interfaces (Sicilia *et al*., 2005; Federici *et al*., 2006; Benedetti *et al*., 2013). On the other hand, B05.10-BcPG1 hotspots during interaction with PvPGIP2 have previously been identified *in silico* as D161, D182, D183 and H204 (Sicilia *et al*., 2005) whilst our study predicted C14 and Q99 as B05.10-BcPG1 hotspots during interaction with grapevine PGIPs. This suggested that the source of PGIP strongly influenced these clear differences, further highlighting the versatility of PGIP-PG interactions.

In summary, the work presented in this study provides valuable insights into the structure-function relationships between grapevine PGIPs and *B. cinerea* BcPGs and yielded several testable hypotheses that could direct further site-directed mutagenesis and protein-protein interaction studies to complement the *in silico* predictions achieved here. Our results are in line with previous studies which have shown that subtle sequence and/or structural differences can have a large impact on PGIP-PG interaction dynamics. Additionally, we have shown that the strength of the inhibitory interaction is unique to each PGIP-BcPG pairing. To our knowledge, this study provides the first report of *in silico* interaction of grapevine PGIPs against all six BcPG isoforms. One of the major outcomes of this study is an appreciation of the importance of including more than one pathogen strain in resistance/susceptibility phenotyping on a specific plant host. In context of the objectives, to clarify a surprising hyper-susceptibility phenotype observed when non-*vinifera* grapevine PGIP encoding genes were overexpressed in *V. vinifera*, the docking results raise the possibility that the specific phenotype was an outcome based on the specific *Botrytis* strain on the specific engineered genetic background. This aspect will be further investigated in the study.

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Chapter 4

Research results

Non-*vinifera pgip*s as defence genes against *Botrytis cinerea* B05.10 and *Erysiphe necator* in tobacco and grapevine

Non-*vinifera pgip*s as defence genes against *Botrytis cinerea* B05.10 and *Erysiphe necator* in tobacco and grapevine

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4.1 ABSTRACT

The roles of grapevine polygalacturonase inhibiting proteins (PGIPs) in plant defence have been elucidated through overexpression studies in native and heterologous plant backgrounds. In tobacco, the constitutive expression of *Vvipgip1* from *Vitis vinifera* reduced host susceptibility to a hyper-virulent *B. cinerea* strain isolated from grapes. Additionally, tobacco plants overexpressing non-*vinifera pgip*s (from American wild vines and *Vitis* hybrids) displayed even more pronounced reduced susceptibility compared to *Vvipgip1*-expressing plants. However, a transgenic *V. vinifera* grapevine population overexpressing two of the non-*vinifera* PGIP encoding genes displayed striking hyper-susceptibility to the same *B. cinerea* grape strain. *In silico* analyses of the structural features of the different grapevine PGIP encoding genes, *B. cinerea* endopolygalacturonases (from three different strains), as well as docking simulation analyses of the PGIP-BcPGs strongly pointed to potentially important strain-specificity in these interactions. This prompted an analysis of the grapevine PGIP overexpressing tobacco and grapevine transgenic populations with another *B. cinerea* strain, as well as knock-out pectin degradation and galacturonic acid catabolism mutants of this strain (using whole plant infection assays), as well as the grapevine powdery mildew pathogen (using a detached leaf assay). The transgenic grapevine population that was found to be hyper-susceptible to the grape strain of *B. cinerea* did not exhibit this phenotype when infected with the B05.10 strain, but interestingly, displayed increased penetration resistance against infection with the biotrophic powdery mildew pathogen, showing shorter *E. necator* hyphal development and multiple failed penetration attempts on the transgenic leaves. Host-specific differences were further confirmed when the B05.10 strain infection on the transgenic tobacco carrying the same grapevine PGIP genes, also showed reduced susceptibility, similar to when this population was infected with the hyper-virulent grape strain. Moreover, mutant infections revealed host-specificity of virulence and potential recognition factors, in line with findings using docking simulation analyses of PGIP-BcPG interactions.

4.2 INTRODUCTION

Polygalacturonase-inhibiting proteins (PGIPs) have been identified and characterised from both monocots and dicots and their defence roles during fungal attack elucidated in numerous plant backgrounds (reviewed by Kalunke *et al.*, 2015). They inhibit the activity of *B. cinerea* cell wall degrading BcPGs, secreted during

host infection. In grapevine, a single copy PGIP encoding gene, *Vvipgip1*, was isolated and characterised from *V. vinifera* L. cv. Pinotage (De Ascensao, 2001). Using *in vitro* and *in planta* platforms, VviPGIP1 was shown to be an effective inhibitor of the virulence factors BcPG1 and 2 (Joubert *et al*., 2006; 2007; Nakajima and Akutsu, 2014). Its defence role was further elucidated through a tobacco overexpression study where it conferred the host with reduced susceptibility to a *B. cinerea* grape strain in whole plant antifungal assays (Joubert *et al*., 2006). Furthermore, overexpression of PGIP encoding genes from non-*vinifera* and American grapevine species (known for their low susceptibility to fungal infections compared to *V. vinifera*) in tobacco also reduced host susceptibility to *B. cinerea* grape strain to a greater extent than that observed in *Vvipgip1*-expressing lines (Venter, 2010).

Two of the aforementioned non-*vinifera* PGIPs (*pgip*1012 from *V.* x *doaniana* Munson and *pgip*1038 from *V. caribaea*) were constitutively expressed in *V. vinifera* cv. Redglobe and the resulting transgenic population challenged with the *B. cinerea* grape strain. Contrary to the enhanced defence phenotype in tobacco, transgenic grapevine displayed hyper-susceptibility to infection (Moyo, 2011). These contrasting defence phenotypes to the same *B. cinerea* grape strain on different hosts (carrying similar PGIPs) suggested host context as an important factor to consider. Moreover, through *in silico* analysis (Chapter 3 of this thesis), the importance of pathogen-strain was shown, prompting the current study that has as aim to evaluate comparative defence phenotypes of both tobacco and grapevine PGIP transgenics when infected with *Botrytis* strains and mutants. The grapevine population was also challenged with a biotrophic pathogen (in comparison with the necrotrophic *Botrytis*).

B. cinerea mutant analysis has been utilised in numerous other studies and the results have provided valuable insights on strain and plant host specificity of pathogenicity factors (Aziz *et al*., 2004; Nakajima and Akutsu, 2014; Blanco-Ulate *et al*., 2015). *Bcpme1* (pectin methylesterase) knockout mutants in *B. cinerea* Bd90 strain displayed reduced pathogenicity on *Arabidopsis*, apple fruits and grapevine leaves (Valette-Collet *et al*., 2003). Yet, deletion of the same gene did not affect pathogenicity of B05.10 on tomato and grapevine leaves (Kars *et al*., 2005). In this study, *Δbcpg1*, *Δbcpg2* and *Δbcpg6* were tested on both transgenic tobacco and grapevine plants. BcPG1 and 2 have previously been identified as virulence factors on numerous plant species. Targeted mutagenesis of *Bcpg1* gene from B05.10 strain significantly reduced secondary lesion formation on infected apples, tomato leaves and fruits (ten Have *et al*., 1998), whilst BcPG2 mutants showed reduced virulence on tomato leaves and broad bean (Kars *et al*., 2005).

The knock-out mutants in genes involved in galacturonic acid catabolism in our study were *Δbcgar1* and *Δbcgar2*. Galacturonic acid is the most abundant pectin component in the plant cell wall and is believed to be a major carbon source for *B. cinerea*. Mutation of genes involved in the catabolic pathway did not affect pathogenicity of B05.10 strain on apple fruit, bell peppers and tomato leaves. However, reduction in virulence was detected when the same mutants were tested on *Nicotiana benthamiana* and *Arabidopsis*, highlighting host specific trends (Zhang *et al*., 2011; Zhang and van Kan, 2013). To our knowledge, no work has been reported on the pathogenicity of any of the mutants utilised in this study on tobacco (*N. tabacum*) and grapevine leaves.

Therefore the main objective of this study was to determine if the hypersusceptible phenotype displayed by transgenic grapevine against *B. cinerea* grape strain infection (Moyo, 2011), was linked to either the choice of *B. cinerea* strain used or host background. Additionally, challenging the transgenic grapevine population with another one of its natural pathogens, *E. necator*, provided us with a platform to compare defence phenotypes of the grapevine population against two pathogens with contrasting feeding lifestyles and infection strategies.

4.3 MATERIALS AND METHODS

4.3.1 *B. cinerea* cultures and growth conditions

Wild type B05.10 and mutant strains (**Table 1**) utilised in this study were a gift from JAL van Kan (Wageningen University, Netherlands). Fungal cultures were routinely grown from glycerol stocks on 5% Malt Extract Agar and 1% Yeast Extract (Merck (Pty) Ltd, Modderfontein, South Africa). They were maintained in darkness at 20-21°C for 4-5 days in an upside-down position. Unlike the *B. cinerea* grape strain which sporulates in the dark, B05.10 cultures were placed under constant artificial light conditions for 4 consecutive days to induce sporulation. Conidia were then harvested in 5 ml sterile water with 0.001% Tween 20, and the suspension filtered through sterile glass wool to remove mycelium fragments. The viability and germination potential of spores was evaluated by plating the spore suspension on 1.4% (w/v) water agar and incubating it overnight under a 16/8 hour light/dark cycle at 23°C. The spores were then hydrated in water overnight at 4˚C before being counted with a haemocytometer and diluted to $1X10^5$ spores/ml in 50% sterile grape juice prior to infection.

Table 1. *B. cinerea* B05.10 knock-out mutants utilised for whole plant infection assays

Function	Enzyme	Mutant	Virulence phenotype	Reference
Pectin degradation	ePG	*Δbcpg1*	Reduced secondary lesions in tomato and apple	Ten Have *et al*., 1998
		Δbcpg2	Delayed lesion formation and expansion in tomato and broad bean	Kars *et al*., 2005
		Δbcpg6	No published data	-
Galacturonic acid catabolism	GAR	*Δbcgar1, Δbcgar2*	Reduced lesion size in tobacco and *Arabidopsis* but no change in apple, pepper and tomato	Zhang *et al*., 2011; Zhang and van Kan, 2013

4.3.2 Tobacco growth conditions and *B. cinerea* B05.10 and mutant lines whole plant infection assays

N. tabacum SR1 (Petit Havana) seeds (control) as well as seeds from F1 transgenic plants overexpressing *Vvipgip1* (line 37 as described in Joubert *et al*., 2006) and *pgip*1012 (line 14, as described in Venter 2010), were sterilised using 0.5% chlorine gas according to Clough and Bent (1998). Transgenic seeds were germinated on Murashige and Skoog (MS) medium supplemented with 15g/litre sucrose and 100 mg/mL kanamycin as selection agent, whereas Control SR1 seeds were germinated on MS medium without kanamycin. All seedlings were transferred to moss peat plugs (Jiffy Products International AS, Norway), and hardened off under 16/8 hour light/dark conditions at 23°C. Peat plugs were maintained in an upright

position in small pots filled with vermiculite and plants were allowed to grow until six mature leaves formed before infection. Five biological replicates per plant line were generated for these infections. All these lines displayed a strong resistance phenotype when infected with the *B. cinerea* grape strain (Joubert *et al*., 2006; Venter, 2010).

The infections were performed essentially as described in Joubert *et al*. (2006). Prior to infection, plants were acclimatised in perspex high humidity chambers for 24 hours at room temperature. Counting from the top, leaf 3, 4 and 5 were infected with six infection spots per leaf and 3000 spores per spot on the adaxial side. WT B05.10 and mutant strains listed in **Table 1** were utilised. Infected plants were maintained in the high humidity chambers and a 16/8 hour light/dark cycle was maintained throughout the course of the infection assay. Disease progression was monitored by measuring lesion diameter using a digital calliper (EuroTool ©) at 24 hour intervals from 48 hours post infection when primary lesions started forming. Infection was allowed to progress till 6 dpi (days post infection), the last day when all lesions were measurable. The technical repeats (nine infection spots) were averaged per biological repeat (plant line). MS Excel 2010 was used to calculate averages and standard deviations. Two-factor ANOVA was utilised to compare significant differences between infections across all plant genotypes. Paired t-tests were then used to determine significant differences between the B05.10 wildtype infections and mutant infections on each plant genotype. Statistically significant data was determined at $p<0.05$.

4.3.3 Grapevine growth conditions and *B. cinerea* B05.10 and mutant lines whole plant infection assay

Untransformed *V. vinifera* cv. Redglobe plant lines and seven randomly chosen transgenic grapevine lines from a previously characterised transgenic population (Moyo, 2011), namely: *pgip*1012-1, *pgip*1012-9, *pgip*1038-15, *pgip*1038-51, *pgip*1038-52, *pgip*1038-62 and *pgip*1038-69 were utilised for the whole plant infection assays. The full genetic and phenotypic characterisation of the transgenic lines was previously performed and reported by Moyo (2011). All these lines displayed a hyper-susceptible phenotype when infected with the *B. cinerea* grape strain (Moyo, 2011). At least two biological replicates per plant line were utilised for the assay. *In vitro* plantlets were maintained on MS medium supplemented with 15 g/ litre sucrose under sterile conditions in a 16/8 hour light/dark photoperiod. Explants were excised and transferred to Grodan plugs (Grodan A/S, Denmark) where they were maintained until healthy roots formed. The plugs were then potted in a soil mixture (1 part sterile soil: 1 part perlite: 1 part vermiculite: 1 part palm peat) and kept in a greenhouse at 23˚C under natural light conditions.

Plants were grown until the primary shoot was approximately one metre long and at several mature (fully expanded) healthy leaves were present. Four fully expanded mature leaves per plant were infected on the adaxial side with three infection spots per leaf and 2000 spores per spot. The handling of spores and plants for the antifungal assay, data collection and analysis were as described for tobacco. The technical repeats (four infection spots) were averaged per biological repeat (plant line). MS Excel 2010 was used to calculate averages and standard deviations. Two-factor ANOVA was utilised to compare significant

differences between infections across all plant genotypes. Paired t-tests were then used to determine significant differences between the B05.10 wildtype infections and mutant infections on each plant genotype. Statistically significant data was determined at $p<0.05$.

4.3.4 Detached leaf infection of transgenic grapevine with the biotroph *E. necator*

A heavily infected grapevine source plant was kept at 100% humidity in a room maintained at 25°C to promote growth of *E. necator*. Five healthy plant lines, with no disease symptoms, were utilised for the infection assay, namely, *pgip*1012-16, *pgip*1012-28, *pgip*1038-2, *pgip*1038-69 and the untransformed control. A total of six young and glossy leaves of same sizes were selected per plant line. The leaves were detached from the main plant using a sterile blade and leaf surfaces were sterilised by spraying the leaves with 70% ethanol followed by three subsequent washes in sterile dH_20 for 5 min each, with gentle shaking. The leaf surfaces were then patted dry using sterile paper towels and the remaining moisture was air-dried in a laminar flow cabinet. The dry leaves were then placed on 1% (w/v) water agar plates (120 X 120 mm), with the adaxial side up and the petiole cut to about 1cm and inserted in the medium.

Heavily infected leaves from the source plant were then lightly brushed on the adaxial side of each leaf and the conidia tapped off on to the leaves in the culture dishes according to the method described in Feechan *et al*., 2011. The infected leaves were kept under optimum conditions for *E. necator* growth. Relative humidity was maintained at 100% with culture dish lids closed at a constant temperature of 25°C in the dark for the first 24 hours. Thereafter the infected leaves were subjected to 16/8 hour light/dark conditions for the duration of the infection. From 48 hpi, the lids were opened once a day to reduce the humidity inside the culture dishes.

At 48 hpi, 6 random 5 X 5 mm samples were cut from two infected leaves per plant line and infections were visualised using the LEO® 1450VP scanning electron microscope (SEM) (Central Analytical Facility, Stellenbosch University) at different magnifications. Two more leaves per plant line were stained with trypan blue solution for one hour in a boiling water bath and subsequently decolourised in 2.5 g/ml chloral hydrate solution for 30 min. Fungal structures were then viewed and photographed under the light microscope at 40X magnification.

Fungal growth was allowed to progress on the remaining two leaves till 7 dpi and symptoms visualised under a stereomicroscope. One of the leaves was then stained with trypan blue to visualise fungal structures under the light microscope as described above. The defence phenotypes were then classified according to the guidelines outlined in Feechan *et al*., (2011) for defining defence mechanisms in grapevine species against *E. necator* infections (**Figure 1)**. From the other infected leaf, five discs of 1cm in diameter were cut out with a cork borer. The discs from each plant line were combined and rinsed in 10 ml sterile water with 0.1% (w/v) Tween 20 with gentle shaking for 5 min. Five aliquots were then taken from each conidial suspension and spores counted using a haemocytometer before being averaged to determine spore load for each leaf/plant line (also according to Feechan *et al*., 2011).

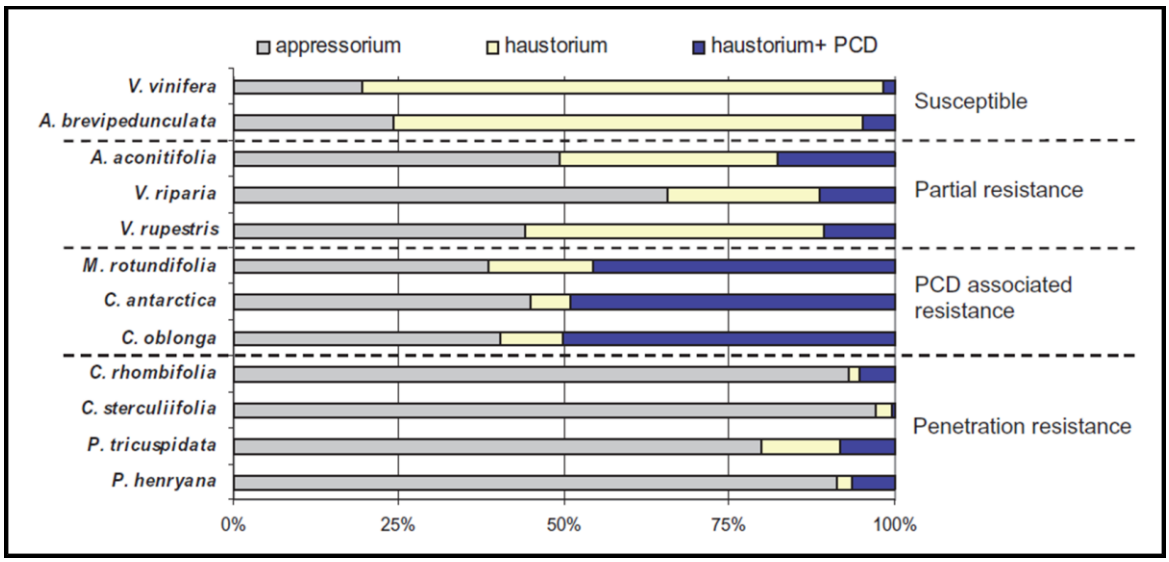


Figure 1. Resistance mechanisms employed by numerous grapevine species against *E. necator* at 48 hours post infection. The frequency of appressorium and haustorium formation together with programmed cell death (PCD) characteristic of each defence phenotype is shown in percentages (Adopted from Feechan *et al*., 2011).

4.4 RESULTS

4.4.1 Constitutive expression of non-*vinifera pgip*s reduced susceptibility of transgenic tobacco to *B. cinerea* WT B05.10 similar to grape strain infections, but neither reduced nor enhanced susceptibility of transgenic grapevine, unlike the hyper-susceptibility induced by the grape strain

Infection conditions were optimal for *Botrytis* infections on both plant hosts as evidenced by approximately 97% of the infection spots developing into primary lesions and ultimately secondary spreading lesions. Primary lesions formed within the first 24 hpi on grapevine and after 48 hours on tobacco. On both plant hosts, primary lesions then progressed to expanding secondary lesions during the course of the antifungal assay. There were no distinctive differences in the visual appearance of lesions formed on control compared to transgenic plants.

In tobacco, lesions on leaf 3, 4 and 5 were averaged per plant line. Secondary lesion growth on the untransformed WT and *Vvipgip1*-37 transgenic lines was significantly faster than on *pgip*1012 plants from as early as 2 dpi (**Figure 2A**). *Vvipgip1*-37 plant lines had smaller lesions than the control lines at 5 dpi whilst *pgip*1012 transgenic lines had the smallest lesions throughout the infection assay, indicative of a strong reduction in susceptibility to the WT B05.10 strain. The *pgip*1012 expressing lines developed lesions which were at least 20% smaller than both the WT and *Vvipgip1* lines by day 5. All differences were statistically significant at $p<0.05$. This trend was similar to *B. cinerea* grape strain infections (Venter, 2010), where *Vvipgip1*-expressing lines also displayed reduced susceptibility to infection compared to control plants whilst *pgip*1012-expressing lines had even smaller secondary lesion expansion over time compared to both *Vvipgip1* lines and the controls (**Insert A** to **Figure 2A –** the inserted Figure contains a broader range of non-*vinifera* PGIP expressing lines tested in Venter, 2010).

In grapevine, no notable visual differences in the onset of lesion formation, rate of lesion growth or appearance of lesions on transgenic leaves infected with WT B05.10 compared to the untransformed controls were observed (**Figure 2B**). Analysis of lesion diameters during the course of infection did not reveal any statistically significant differences at $p<0.05$ on transgenic compared to control plants. This trend was very different in comparison to the hyper-susceptible phenotype reported during *B. cinerea* grape strain infections (Moyo, 2011) (**Insert B** to **Figure 2B -** the inserted Figure contains a broader range of non-vinifera PGIP expressing lines tested in Moyo, 2011).

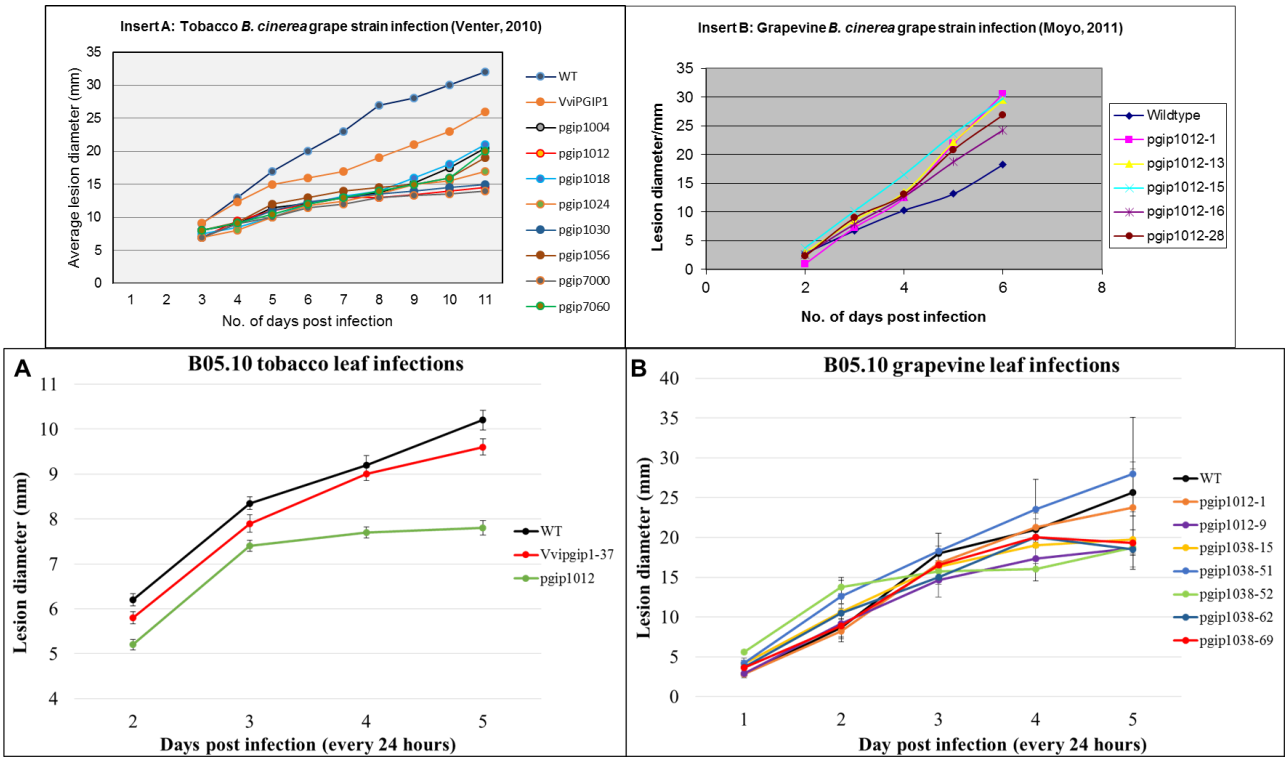


Figure 2. **Insert A** and **B** from previous studies are included for comparative purposes to show the different trends on transgenic tobacco and grapevine infected with two different *B. cinerea* strains. Lesion development on (**A**) transgenic tobacco (n=5) and (**B**) grapevine plants overexpressing non-*vinifera pgip*s infected with *B. cinerea* WT B05.10 strain (n≥2). Untransformed WT plants were included as controls. Similar results were obtained in tobacco but the two *B. cinerea* strains displayed different defence phenotypes on grapevine. Error bars represent the standard deviation of the technical repeats for each plant line.

4.4.2 B05.10 mutant infections on tobacco and grapevine plants reveal potential virulence and recognition factors, displaying host specific trends

Similar to WT B05.10 infections, all mutant infection spots on both tobacco and grapevine leaves developed into primary lesions which later progressed into secondary spreading lesions. Lesion measurements from the last day when all lesions could still be measured accurately (5 dpi), were grouped together per plant population (control plants, *Vvipgip1*-37, *pgip*1012 and *pgip*1038) and averaged accordingly. Two-factor ANOVA tests (**Table 2**) showed statistically significant differences between *Botrytis* infections in grapevine but not in tobacco.

Table 2. Summary of two-factor ANOVA tests conducted at 5 dpi on (**A**) tobacco and (**B**) grapevine. At $p<0.05$, there were no statistically significant differences in *Botrytis* infections on all plant genotypes in tobacco. However, the differences were statistically significant in grapevine (p value highlighted in red) for the different infections.

A

Source of Variation	SS	df	MS	F	P-value	F crit
B05.10/mutant	9.515989	5	1.903198	1.332897	0.32601	3.325835
Plant genotype	0.939833	2	0.469917	0.329104	0.727075	4.102821
Error	14.27866	10	1.427866			
Total	24.73448	17				

B

Source of Variation	SS	df	MS	F	P-value	F crit
B05.10/mutant	428.8668	5	85.77336	16.84225	**0.000138**	3.325835
Plant genotype	11.29263	2	5.646317	1.108697	0.367373	4.102821
Error	50.9275	10	5.09275			
Total	491.0869	17				

In tobacco, lesions on leaf 3, 4 and 5 were averaged per plant line, per mutant. At 5 dpi, none of the knockout mutants showed reduced virulence compared to WT B05.10 infections on all tobacco plant lines tested. On control tobacco plants, Δ*bcpg2* infections resulted in significant increase in lesion size whilst lesions from the rest of the mutants were similar to those of WT B05.10, whilst on *Vvipgip1* transgenic plants Δ*bcgar1* displayed the same trend. Infections with Δ*bcpg1* and Δ*bcgar2* had significantly larger lesions compared to infections with WT B05.10 on *pgip*1012 expressing plants (**Figure 3**).

In grapevine, only Δ*bcpg1* infections showed a significant reduction in rate of lesion expansion at 5 dpi on both transgenic and control plants compared to WT B05.10 infections, however, the lesion sizes were the same on all plant genotypes (**Figure 4**). Apart from Δ*bcpg1*, none of the other mutant infections displayed any statistically significant differences in lesion size compared to B05.10 infections on control plants. On *pgip*1012 transgenic plants, Δ*bcpg2*, Δ*bcpg6* and Δ*bcgar1* infections resulted in significantly larger lesions at 5 dpi compared to WT B05.10 infections, whilst on *pgip*1038 only Δ*bcpg2* and Δ*bcpg6* displayed this trend. No significant differences in lesion expansion between B05.10 and Δ*bcgar2* infections were observed on all three plant genotypes.

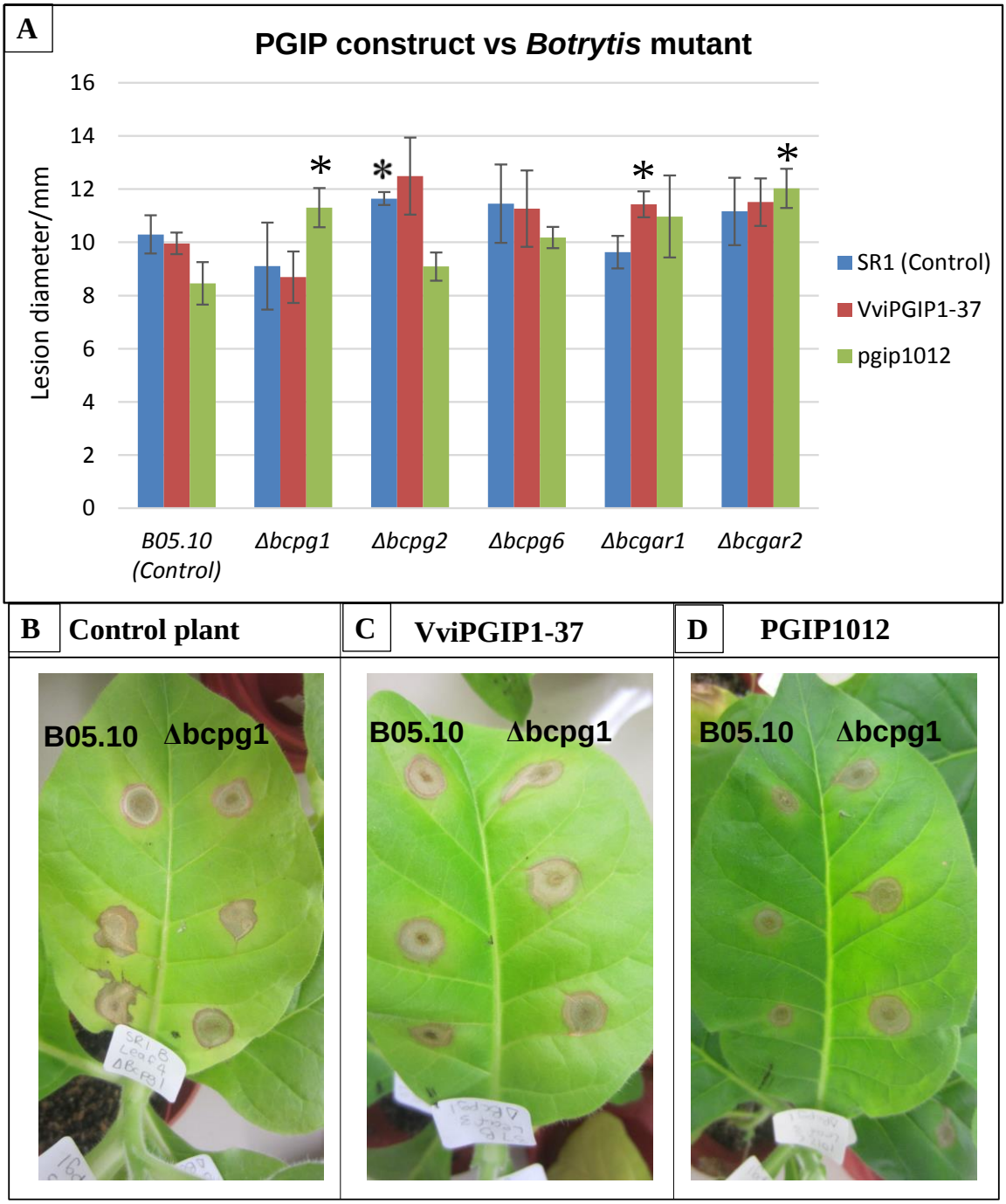


Figure 3. (A) Bar chart showing averaged lesion diameter at 5 dpi on tobacco plants (transgenic population and controls) infected with WT B05.10 and its knock-out mutants in genes involved in pectin degradation and galacturonic acid catabolism. Statistically significant differences between B05.10 infections and the respective mutant on a specific plant genotype at $p<0.05$ are indicated with an asterisk. Error bars indicate standard deviation (n=5). (**B**) Representative pictures showing secondary lesions on tobacco leaves infected with WT B05.10 and Δ*bcpg1* at 5 dpi. Each leaf was infected with six spots, three spots on one side of the midrib for WT B05.10 and three spots on the other side for the mutant strain. There were no significant differences in WT B05.10 and Δ*bcpg1* infections in terms of lesion sizes on the control and (**C**) VviPGIP1-37 plants. However, Δ*bcpg1* lesions on *pgip*1012 plants (**D**) were significantly larger than WT B05.10 infections. No distinctive differences in visual appearance of lesions were observed for all infections.

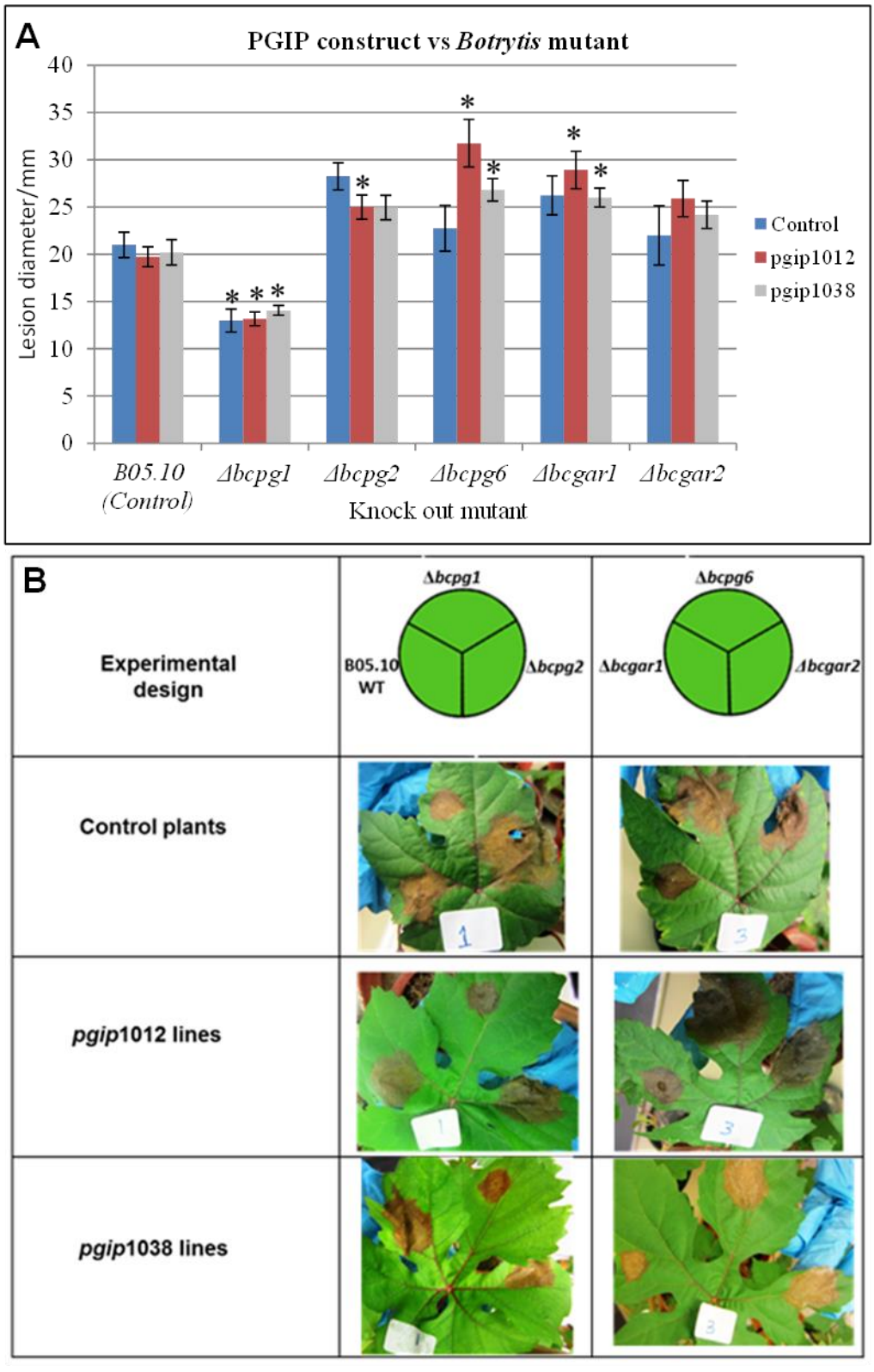


Figure 4. (A) Bar chart showing averaged lesion diameter at 5 dpi on grapevine plants (transgenic population and controls) infected with WT B05.10 and its knock-out mutants in genes involved in pectin degradation and galacturonic acid catabolism. Statistically significant differences between B05.10 infections and the respective mutant on a specific plant genotype at $p<0.05$ are indicated with an asterisk. Error bars indicate standard deviation ($n\geq2$). (**B**) Representative pictures showing visual symptom development on grapevine leaves infected with WT B05.10 and the knock out mutants. The experimental design pie charts show positions of the different lesions on the grapevine leaves. Only Δ*bcpg1* infections had significantly smaller lesions on all plant lines compared to WT B05.10 infections.

4.4.3 Transgenic grapevine leaves exhibited a combination of partial and penetration resistance to the biotroph *E. necator*

During the first 48 hpi, there were no noticeable visual differences in infection trends (symptoms) on the transgenic grapevine leaves versus the control leaves. However, when the infected leaves were studied under the SEM and light microscope, there were clear differences in fungal growth patterns. On control leaves, >95% of the fungal spores formed appresoria and thin, elongated secondary hyphae, indicative of a successful infection and susceptible phenotype according to the Feechan *et al*., (2011) guidelines for classification of defence phenotypes (**Figure 1**). Also, no PCD at infection sites was detected. On transgenic leaves, <70% of the spores formed an appresorium with only 50% of the total spores developing secondary hyphae, typical of partial resistance mechanism, but without any signs of PCD. The hyphae were much shorter with thick globular ends compared to those on control leaves, indicative of multiple failed penetration attempts (**Figure 5**). None of the infected leaves stained with trypan blue at 48 hpi showed any signs of cell death around infection sites. At 7 dpi, similar dense networks of sporulating hyphae representing typical *E. necator* infections were visible on surfaces of both transgenic and control leaves under the stereomicroscope. There were no significant differences in spore load on transgenic compared to control leaves at this advanced stage of infection (**Figure 6**).

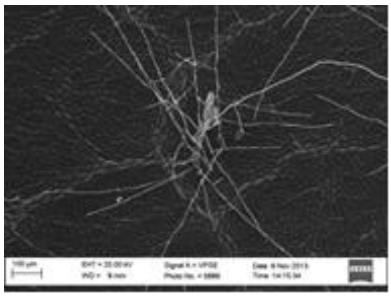
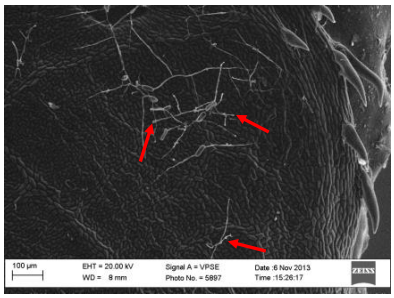
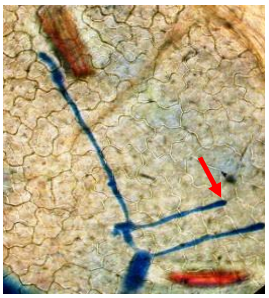
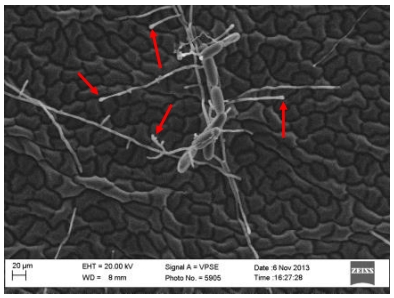
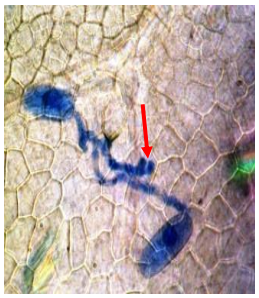
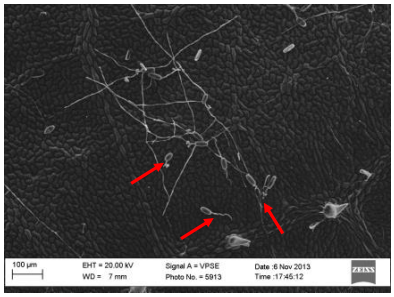
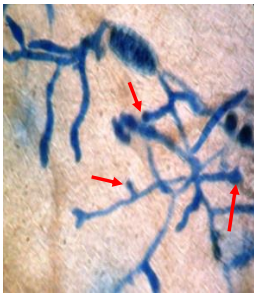
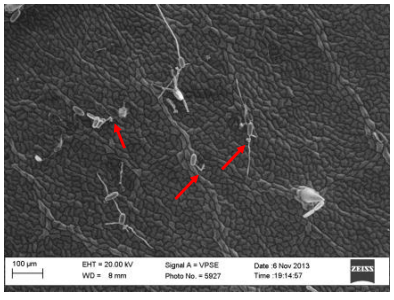
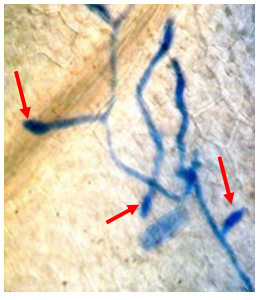
Plant line	Representative SEM view: 48 hpi	40X magnification: Light microscope 48 hpi	Stereomicroscope micrographs: 7 dpi
Wildtype			
***pgip*1012-16**			
***pgip*1012-28**			
***pgip*1038-2**			
***pgip*1038-69**			

Figure 5. Comparison of *E. necator* spores on transgenic grapevine leaves overexpressing non-*vinifera pgip*s and control leaves at 48 hpi and 7 dpi. The SEM pictures were taken at optimal magnifications to capture the differences in secondary hyphae morphology and possible resistance mechanisms. All light microscope pictures were taken at 40X magnification with different optical zooms. Stereomicroscope pictures were taken at different optical zooms for optimal observation of infection structures. The red arrows point out the features which were unique to the infections on transgenic leaves, namely, short lobed hyphae and appresoria formation without any further evidence of secondary hyphae formation.

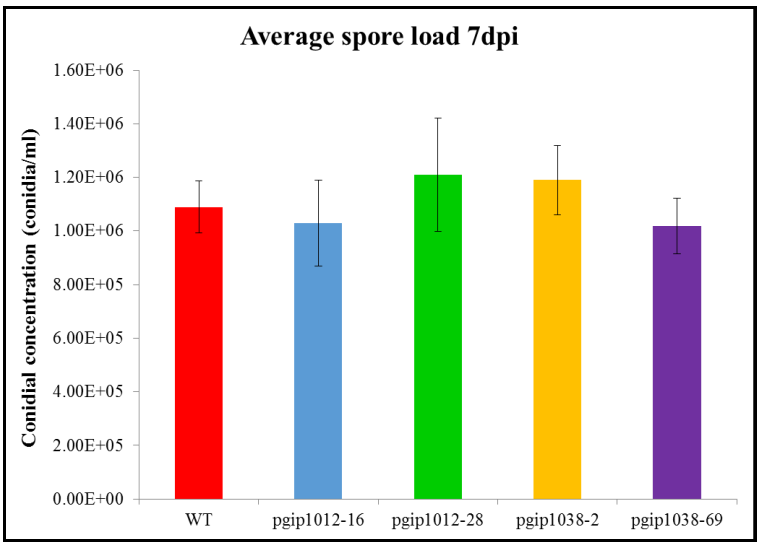


Figure 6. Bar chart showing average *E. necator* spore concentrations on infected transgenic and control grapevine leaves at 7 dpi. Error bars represent standard deviation of the five technical replicates.

4.5 DISCUSSION

The hyper-susceptible phenotype of transgenic grapevine to *B. cinerea* grape strain infection is host and strain specific whilst expressing grapevine PGIPs in tobacco consistently reduced host susceptibility to infection irrespective of strain.

The reduced susceptibility of transgenic tobacco plants overexpressing *Vvipgip1* to WT B05.10 was consistent with findings reported by Joubert *et al*., (2006), where the transgenic population displayed reduced susceptibility to a hyper-virulent *B. cinerea* grape strain. The same transgenic tobacco plants were also previously shown to exhibit even stronger resistance phenotypes to different *B. cinerea* grape strain compared to both *Vvipgip1*-expressing plant lines and the untransformed controls (**Figure 2A** and **Insert A**). Results from this study showed that the reduced susceptibility was a consistent defence phenotype regardless of the *B. cinerea* strain used. The only notable difference was in the degree of resistance displayed by the *pgip*1012 transgenic tobacco population; 23% in the present study against B05.10 strain compared to 33-60% reported by Venter (2010) against the grape strain. This could be attributed to differences in virulence of the strains on tobacco with average lesion diameters from *B. cinerea* grape strain infections reaching more than 20 mm by 5 dpi whilst in the B05.10 strain lead to smaller lesions (averaged at 10 mm) at the same time point.

B05.10 is a laboratory strain which is a haploid derivative of a virulent mono-ascospore SAS56 strain from Europe whilst the grape strain is a hyper-virulent strain isolated from heavily infected berries from a South African vineyard (Joubert *et al*., 2006; Amselem *et al*., 2011). Therefore, the two strains have undergone different evolutionary pressures on different substrates/hosts. Having co-existed with plants in a natural field environment, the grape strain has most likely continuously adapted mechanisms to evade host recognition and defence responses as the plant also evolves its defence strategies. This ‘arms race’ ensures the continued survival of *B. cinerea* even with competition from other pathogens and would render it more

aggressive in comparison to a laboratory strain which has most likely been maintained with minimal or no competition over several generations. The adapted aggressiveness of the grape strain could be specialised to overcome general plant defence responses since *Botrytis* is known to infect a very wide host range (Williamson *et al*., 2007). This would explain the high virulence of the same grape strain to *A. thaliana* (Denby *et al*., 2004).

In grapevine, infection of the same transgenic population with two *B. cinerea* strains yielded different responses. Unlike the hyper-susceptibility to *B. cinerea* grape strain (Moyo, 2011), the transgenic population displayed an unaltered defence phenotype to B05.10 infections, similar to that of the control plants. Thus, the presence of non-*vinifera* PGIPs in a *V. vinifera* background did not confer the host with enhanced resistance against either of the two strains. The native VviPGIP1 has been shown to display strong developmental regulation and tissue specificity, only being expressed in leaves after infection (Joubert *et al*., 2013). Constitutively expressing the non-*vinifera* PGIPs in grapevine leaves could have affected the inherent defence mechanisms in a way that impacted the two *B. cinerea* strains differently, favouring one strain over the other. It is also possible that changes to the metabolic processes of the plants, which are unrelated to the classical PGIP-PG inhibition interactions, such as priming, could have occurred and also favoured the grape strain during infection (Alexandersson *et al*., 2011; Nguema-Ona *et al*., 2013). These aspects need to be studied further to conclude on the likely mechanisms.

As seen for tobacco, disease symptoms on (untransformed) grapevine leaves infected with B05.10, over time, were less severe compared to those reported in Moyo (2011) with the *B. cinerea* grape strain infections, highlighting differences in virulence of the two strains. This was also expected since the evolution of the two strains differs, as explained earlier. The grape strain could have also evolved to overcome grapevine-specific defence responses and maybe even manipulate the host to induce defence responses which would benefit the necrotrophic feeding lifestyle of *B. cinerea*. Possible mechanisms could include: Through the action of small regulatory RNAs (sRNAs), *B. cinerea* grape strain could either block the ability of the host to recognise the pathogen on the surface or suppress signalling pathways upon entering the host cells (Weiberg *et al*., 2013; 2014; Knip *et al*., 2014; Wang *et al*., 2015; Weiberg and Jin, 2015). Additionally, BcPG1 from the grape strain could have elicited the oxidative burst, triggering PCD which ultimately benefits the necrotrophic lifestyle of the invading pathogen (Govrin *et al*., 2006). The strength of the elicitation is probably linked to structural differences in the signal peptide and propeptide regions of BcPG1 from the two strains (**Chapter 3** of thesis). Docking simulations clearly showed that these differences, together with other subtle differences in the mature protein region, can influence the interaction kinetics at the protein-protein interfaces. Furthermore, computational mutagenesis predicted stronger complexes between B05.10-BcPG1 and the grapevine PGIPs compared to grape strain BcPG1 complexes. The weaker complex formation by the grape strain BcPG1 could be a strategy that the pathogen evolved to enable it to utilise BcPG1 to elicit PCD instead of the classical PGIP-BcPG inhibition interaction.

Comparison of the transgenic tobacco and grapevine defence phenotypes, against both B05.10 and the grape strain, shows clear host-specific phenotypes. Since *B. cinerea* utilises cell wall degrading enzymes

to penetrate the host, the profiles of the tobacco and grapevine cell walls could, in part, explain the different phenotypes between the two hosts (Underwood, 2012; Pogorelko *et al*., 2013; Bellincampi *et al*., 2014). Furthermore, Blanco-Ulate *et al*. (2014) showed that conditions in the host cell wall matrix influence *B. cinerea*'s infection strategy. Previous studies have shown that tobacco cell wall fractions possess higher levels of methylated homogalacturonan compared to grapevine (Nguema-Ona *et al*., 2012; Moore *et al*., 2014). An increase in the degree of pectin methylation has been associated with an increase in resistance of plant hosts to fungal infections through limiting the activity of ePGs (Lionetti *et al*., 2012). It is possible that this attribute could have contributed towards grapevine being a natural host to *B. cinerea* whilst tobacco is not. Profiling the cell walls of transgenic tobacco and grapevine leaves against their respective controls, could provide valuable insights on this trait.

BcPG1 from B05.10 is a potential virulence factor on grapevine leaves but not on tobacco whilst BcPG2 and 6 are not on either host

The significant reduction in pathogenicity of *Δbcpg1* compared to the WT B05.10 on both transgenic and control grapevine plants was indicative of BcPG1 being a B05.10 virulence factor on grapevine leaves. The trend was host specific since it was not identified as a virulence factor on either control or transgenic tobacco leaves. The same mutant previously displayed reduced virulence on tomato leaves, fruits, and apples (ten Have *et al*., 1998). Additionally, *bcpg1* expression was detected in grape berries infected with B05.10 (Kelloniemi *et al*., 2015) and with a noble rot strain, BcDW1 (Blanco-Ulate *et al*., 2014). Since there were no significant differences in the level of reduction in *Δbcpg1* virulence on control compared to transgenic grapevines (**Figure 4**), the host specific trend was probably not linked to the presence or absence of non-*vinifera* PGIPs in the host. Instead, it could be linked to the inherent differences in cell wall profiles between the two hosts (Nguema-Ona *et al*., 2012; Moore *et al*., 2014), an aspect that merits further study.

BcPG2 and 6, on the other hand, were not identified as virulence factors on either transgenic or control tobacco and grapevine plants in our study. A transcriptomic study showed that *bcpg2* was not expressed during BcDW1 berry infection (Blanco-Ulate *et al*., 2014). However, it was detected on tomato fruit and lettuce leaves infected with B05.10 whilst Δ*bcpg2* displayed reduced virulence on tomato and broad bean (ten Have *et al*., 1998; Kars *et al*., 2005). To our knowledge, no other study has reported on BcPG6 mutant analysis thus it was difficult to interpret our results. There is evidence that it could be host and plant organ specific since *bcpg6* expression was detected on BcDW1 infected berries and on tomato fruit and lettuce leaves infected with B05.10 (Blanco-Ulate *et al*., 2014).

Silencing *Bcgar1* and *Bcgar2* did not reduce B05.10 virulence on tobacco and grapevine leaves

BcGAR1 and BcGAR2 encode for two non-homologous galacturonate reductase genes which catalyse the conversion of D-galacturonic acid to L-galactonate in the first step of the galacturonic acid catabolism pathway. Galacturonic acid is believed to be a major carbon source for *B. cinerea* during infection (Zhang *et al*., 2011). A previous study showed that the pathogenicity of B05.10 *Δbcgar1* and *Δbcgar2* on *N.*

benthamiana and *A. thaliana* leaves was significantly reduced whilst it was not affected on bell peppers, apple fruit and tomato leaves (Zhang and van Kan, 2013). Since the tobacco plants in our study were *N. tabacum* and BcGAR1 and 2 were not identified as virulence factors on either control or transgenic tobacco and grapevine, the roles of BcGAR1 and BcGAR2 in pathogenicity of *B. cinerea* are most likely to be host specific and influenced by host specific metabolic processes or plant cell wall dynamics.

BcPG1, BcPG2, BcPG6, BcGAR1 and BcGAR2 as potential recognition factors or decoys

Transgenic tobacco overexpressing *pgip*1012 infected with *Δbcpg1* and *Δbcgar2* had significantly larger lesions at 5 dpi compared to leaves infected with the WT B05.10 strain. Similarly, *pgip*1012 transgenic grapevine infected with *Δbcpg2*, *Δbcpg6* and *Δbcgar1* displayed the same trend together with *pgip*1038 plants infected with *Δbcpg6* and *Δbcgar1* (**Figure 4A**). Since the elicitor activity of BcPG1 was previously shown to be due to recognition of specific motifs within the protein by grapevine cells (Poinssot *et al*., 2003), it is possible that the same concept of recognition could apply to other pathogenicity factors. The products of these genes could play a role as 'recognition' factors during infections on the respective hosts. Their absence therefore could lead to a situation where the hosts would take longer to identify the presence of an invading pathogen leading to more damage, as evidenced by larger lesion sizes at 5 dpi.

Additionally, they could act as 'decoys', not directly contributing to fungal virulence but rather being perceived by host defence proteins during infection, in turn guarding effector genes from recognition. Thus, silencing the decoy could reduce perception of the invading pathogen by the host plant leading to more damage in the early stages of infection (van der Hoorn and Kamoun, 2008; Dodds and Rathjen, 2010). Interestingly, there were more potential recognition factors or decoys identified during infection of transgenic tobacco and grapevine plant lines compared to controls. This could be linked to the presence of VviPGIP1 and non-*vinifera* PGIPs in the cell walls of the transgenic tobacco and grapevine plants. It has been shown that constitutive expression of *Vvipgip1* in tobacco caused hormonal and cell wall changes which primed the host prior to infection (Alexandersson *et al*., 2011; Nguema-Ona *et al*., 2013). Expressing non-*vinifera* PGIPs in tobacco and grapevine could have a similar priming effect. One of the consequences of the primed state could be the quicker perception of fungal recognition factors and/or decoys. Our mutant analyses raise some interesting new concepts regarding the roles of these factors and should be followed by further experimentation to test the recognition/decoy concept.

Constitutive expression of non-*vinifera* PGIPs in grapevine could have indirectly primed the plants against *E. necator*

The genetic backgrounds of the plants utilised for the powdery mildew infections did not affect spore load as shown in **Figure 6**. Based on the classification of resistance mechanisms described in Feechan *et al*., (2011), the transgenic grapevine plant lines displayed partial and penetration resistance to *E. necator*. However, neither the controls nor the transgenic plants employed PCD as a defence strategy against infection. The lack of PCD as a defence strategy could be specific to *V. vinifera* cv. Redglobe since Cabernet Sauvignon was

utilised to define the *V. vinifera* susceptible phenotype in Feechan *et al*., (2011). Alternatively, it could be attributed to unique properties of the *E. necator* strains utilised in the different studies.

The detached leaf infection conditions were optimal for *E. necator* growth with a huge spore load which favoured successful host infection. Additionally, the detached leaf environment could have triggered a wound response in the leaves, influenced by the plant genetic background, which involved changes in metabolic processes which indirectly affected the ability of *E. necator* to penetrate the host. Several PGIP functions in plant hosts have been elucidated to date, with the main ones being direct inhibition of fungal ePGs, prolonging existence of elicitor compounds in the cell wall and priming the host prior to infection (Kalunke *et al*., 2015). Priming enhances the ability of the host to mobilise pathogen induced defence responses. Constitutive expression of *Vvipgip1* in tobacco primed the host, as evidenced by changes in hormone profiles and cell wall composition prior to infection (Alexandersson *et al*., 2011; Nguema-Ona *et al*., 2013). The presence of non-*vinifera* PGIPs in transgenic grapevine plants could possibly have also primed the grapevine plants, leading to an enhanced pre-formed defence against all pathogens, including *E. necator*. Our interpretation is that the increased penetration resistance seen was probably not directly linked to the typical functions of the PGIPs, but rather indirect associated impacts such as a priming mechanism.

Summary and Conclusions

On a general level, the far-reaching impact that plant host pathogen strain selection has on characterising defence genes in heterologous systems was highlighted in this study. Parallel antifungal assays utilising an array of strains on the same host could provide a more comprehensive way of defining defence phenotypes. This study compellingly showed that the hyper-susceptible phenotype displayed by transgenic grapevine during *B. cinerea* grape strain infections is specific to the host-pathogen pairing. The molecular basis of the hyper-susceptible pairing will be investigated further in this study. On the other hand, the partial resistance of the transgenic grapevine population to a biotroph raises the possibility that non-*vinifera* PGIPs primed the plants prior to infection - further work will be conducted to explore this possibility.

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Chapter 5

Research results

Evaluating the hyper-susceptible phenotype of transgenic grapevine overexpressing non-*vinifera* *pgip*s to *Botrytis cinerea* grape strain infection further

Evaluating the hyper-susceptible phenotype of transgenic grapevine overexpressing non-*vinifera pgip*s to *Botrytis cinerea* grape strain infection further

5.1 ABSTRACT

A hyper-susceptible phenotype against a hyper-virulent strain of *Botrytis* in a transgenic grapevine population overexpressing known polygalacturonase-inhibiting protein (PGIP) defense genes from grapevine was shown to be strain specific. In this study, analyses were used to rule out certain uncertainties regarding the impact of the overexpression of the PGIPs to this phenotype. We tested whether the transgenes and perhaps the native grapevine PGIP encoding gene might have been silenced in the population; as well as whether or not the different grapevine PGIPs were indeed effective inhibitors of *Botrytis* endopolygalacturonases (ePGs). Our results ruled out that the PGIP encoding genes were silenced and *in vitro* and *in planta* assays confirmed the grapevine PGIPs to be able to interact and inhibit BcPGs in the transgenic environment. We further hypothesised that the presence of non-*vinifera* PGIPs in *V. vinifera* could have influenced crucial plant defence mechanisms to the detriment of the host, while providing strain specific-benefit to the *B. cinerea* grape strain. Analysis of uninfected transgenic grapevine leaves revealed a reduction in abundance of cell wall components associated with cell wall strengthening, pointing to potential weakened cell walls in the transgenics. The cell wall profile and specifically AGPs provide an interesting perspective to the phenotype as well as the fact that the transgenics also emitted significantly lower levels of defence-related sesquiterpenes compared to the controls when infected by the grape strain.

5.2 INTRODUCTION

Polygalacturonase-inhibiting proteins (PGIPs) are mostly present in multi-gene families in plant species (as reviewed by Kalunke *et al*., 2015). These PGIP families typically consist of members that have different substrate specificities for their fungal-derived targets (Gomathi *et al*., 2004), the endopolygalacturonases (ePGs), that are secreted to attack plant host cell walls. PGIPs form 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 (Kalunke *et al*., 2015).

Several grapevine PGIP genes have been functionally characterised in both transgenic tobacco and grapevine populations (Venter, 2010; Moyo, 2011) and host-specific responses, as well as *Botrytis* strain-specific phenotypes have been observed (**Chapter 4** of thesis). One of the most striking results obtained was that a *V. vinifera* grapevine population that overexpresses PGIP encoding genes from non-*vinifera* species showed two very distinct infection phenotypes when two different *Botrytis* strains were used for the infection. Infections with a hyper-virulent *Botrytis* strain isolated from grapes caused a hyper-susceptible phenotype in the transgenic populations characterised by explosive fungal growth and lesion formation, whereas a laboratory strain (B05.10) caused similar infection symptoms on both the transgenic and control

systems. In tobacco overexpressing the same *pgip* genes though, both strains struggled to maintain active infections with a strong defense response in the transgenics versus the control (Venter, 2010; **Chapter 4** of thesis).

Therefore, in this study, our main aim was to investigate the *B. cinerea* grape strain-specific hyper-susceptible phenotype displayed on the transgenic grapevine population. The study tested the following assumptions: (1) the non-*vinifera* PGIPs are able to effectively inhibit *Botrytis* BcPGs; (2) the hyper-susceptible phenotype is not linked to gene-silencing of the native *Vvipgip1*, or the non-*vinifera* PGIP encoding genes; and (3) overexpression of the *pgip* genes could influence cell wall and other priming-related changes, as was seen in the transgenic PGIP tobacco population (Nguema-Ona *et al*., 2013).

Since knowledge on the *in vitro* and *in planta* interaction of the non-*vinifera* PGIPs against individual BcPGs is currently lacking, we utilised these two analytical approaches to fill the knowledge gap. We then compared inhibition profiles of PGIP1012 and PGIP1038 against individual BcPGs to those previously reported for VviPGIP1 in Joubert *et al*. (2006; 2007). We also analysed *pgip* gene expression profiles during the early stages of infection (first 48 hpi) on grapevine plants infected with *B. cinerea* grape strain. In order to determine if there were any priming-related cell wall changes in the transgenic population that could impact defence responses, we profiled cell wall compositions of healthy, uninfected leaves. Also, we analysed the emission profiles of defence-related sesquiterpenes from infected grapevine leaf material for potential differences that could benefit *B. cinerea* during infection. The *B. cinerea* grape strain specific hyper-susceptible phenotype in grapevine is discussed as well as possible hypotheses and future work to understand this surprising phenotype.

5.3 MATERIALS AND METHODS

5.3.1 *In vitro* interaction of BcPGs and non-*vinifera* PGIPs

Two healthy, mature leaves were harvested from four previously characterised (Moyo, 2011) greenhouse-grown transgenic grapevine lines (*pgip*1012-16, *pgip*1012-28, *pgip*1038-2 and *pgip*1038-69) and the untransformed control. The material was immediately flash frozen in liquid nitrogen and later ground to a fine powder before crude total protein was extracted. The leaves from the same plant line were kept separate and extracted as biological repeats. The extraction protocol entailed homogenising 500 milligrams 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. The supernatants were collected and 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.

All plant protein extracts were then diluted to the same concentration and utilised in an agarose diffusion assay (Taylor and Secor, 1988) against purified B05.10 BcPGs (kindly provided by Dr Jan van Kan, Wageningen University, Netherlands). The purified BcPGs were suspended in sodium acetate buffer, pH 5. They were all diluted to the same concentration before being utilised for the agarose diffusion assay. The assay utilises polygalacturonic acid (PGA) as substrate for BcPGs. 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 BcPG activity is inhibited by active PGIP being co-inoculated in the same well. All assays were carried out in triplicate (technical repeats) for each biological repeat.

5.3.2 *In planta* interaction of BcPGs with non-*vinifera* PGIPs

5.3.2.1 *Transient expression of BcPGs in Nicotiana benthamiana*

N. benthamiana seeds were sown in MS (Murashige and Skoog, 1962) plates supplemented with 15 g/l sucrose before being transferred to peat plugs. Three separate batches of plants were utilised for the transient expression of individual BcPGs, co-infiltration studies and protein activity assays during infiltration respectively. The plants were then transferred to potting soil (Double Grow, Durbanville, South Africa) and maintained in the greenhouse at 26˚C, watered every third day and supplemented with Nutrisol natural organic plant food (Envirogreen (PTY) Limited, Fleuron, Braamfontein, South Africa) once every two weeks until they were eight weeks old. *Agrobacterium* strain EHA105 (Hood *et al*., 1993) transformed with individual BcPGs, or the empty vector pMOG800 control (Honee *et al*., 1998) were utilised for the infiltration studies in *N. benthamiana* using a protocol described in Joubert *et al*., 2007. The transformed *A. tumefaciens* cells were grown to a final OD_{600}=1.2 and all cultures were adjusted to OD_{600}=0.6 for the transient expression of individual BcPGs in eight week old *N. benthamiana* plants. Infiltrations were done using 1 ml disposable syringes (without the needle) and 100 µl of cell suspension was infiltrated per spot. Chlorophyll fluorescence was measured using a Plant Efficiency Analyser (Hansatech Instruments LTD, Kings Lynn, England) prior to infiltration and 24 hours post infiltration (hpi) as described in Joubert *et al*., 2007. Changes in chlorophyll fluorescence at 24 hpi and visual symptom development at 48 hpi were used to monitor the effect of transiently expressing individual BcPGs in *N. benthamiana*. All infiltrations were done in triplicate on separate leaves.

5.3.2.2 *Co-infiltration of BcPG2 with non-vinifera PGIPs*

A. tumefaciens transformed with *pgip*1012 and *pgip*1038 were used for the co-infiltration study against BcPG2. Eight week old *N. benthamiana* plants were also utilised for this study. Different ratios of PGIPs to BcPG2 (**Table 1**) were compared. Changes in chlorophyll fluorescence at 24 hpi and visual symptom development at 48 hpi were used to monitor the effect of co-infiltrating BcPG2 with the individual and combined PGIPs in *N. benthamiana*. All infiltrations were done in triplicate on separate leaves. The previously characterised VviPGIP1 (Joubert *et al*., 2007) was included in the study as a control.

Table 1. Cell densities of *A. tumefaciens* used for the co-infiltration of BcPG2 with PGIP1012 and PGIP1038. A combination of the non-*vinifera* PGIPs with VviPGIP1 was included to ascertain the possible effect on inhibition potential of combining the numerous PGIPs in an *in planta* environment

Non-*vinifera* PGIP:BcPG2, pART:BcPG2, VviPGIP1 + non-*vinifera* PGIP:BcPG2	**OD_{600}[a]**
1:10	0.06:0.6
1:5	0.12:0.6
1:1	0.6:0.6
5:1	0.6:0.12
10:1	0.6:0.06

[a] Optical density at 600 nm

Eight week old *N. benthamiana* plants infiltrated with individual BcPGs and also co-infiltrated with PGIP:BcPG2 combinations at 1:1 ratio were utilised to check for protein activity during the course of the infiltration study. Infiltrated leaves were harvested at different time points (T0 to 96 hours post infiltration) and frozen in liquid nitrogen before being ground to a fine powder using a pestle and mortar. Crude protein extracts were isolated from the frozen tissue (as described in Joubert *et al*., 2007) and protein activity tested using the agarose diffusion assay.

5.3.3 Whole plant infection assay with *Botrytis cinerea*

A *B. cinerea* grape 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 by plating the spore suspension on 0.8% (w/v) water agar. The highly viable spores were then hydrated in water overnight at 4˚C before being counted on a haemocytometer and diluted to the desired concentration in 50% grape juice prior to infection.

Untransformed Redglobe plant lines and four randomly chosen transgenic grapevine lines (*pgip*1012-16, *pgip*1012-28, *pgip*1038-2 and *pgip*1038-69) were used for the whole plant infection assays against *B. cinerea* grape strain. At least two biological repeats per plant line were used. Whole plants were pre-incubated in perspex high humidity chambers for 24 hours prior to infection. The perspex chambers were maintained at room temperature under a 16/8 hour light/dark cycle prior to and throughout the course of the infection. Fully expanded mature leaves from 8 to 12 week old greenhouse grown plants were selected for the assays. Four leaves of relatively similar size per plant were infected on the adaxial side with three infection spots per leaf and 1000 spores per spot. Fungal spores with a germination potential >90% were utilised. These infections were used as source materials to follow the grapevine *pgip* gene expressions during the infections.

5.3.4 Following *pgip* expression profile during *Botrytis* infection on grapevine leaves

Leaf material was harvested with a cork borer at; T0 – harvested immediately after infection at the infection spot; 24 hpi – harvested at the infection spot; 48 hpi – harvested on the same leaf, adjacent to the spreading lesion. For each plant line, a different plant was utilised for each harvesting time-point to ensure that an activated wound response did not play a role in the results obtained. The leaf material was immediately frozen in liquid nitrogen at harvesting and ground to a fine powder before being utilised for total RNA extraction using a method developed for *Eucalyptus* RNA extraction (Suzuki *et al*., 2003). The RNA was DNase treated according to the manufacturer's instructions using DNase 1 recombinant (Roche Diagnostics GmbH, Germany) to remove genomic DNA, prior to the synthesis of cDNA using SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen Cooperation, Carlifornia), also according to the manufacturer's instructions. Transgene specific primers (Foward: 5'-CTCCACTCCAGAGCTGCAAGT-3' and reverse: 5'–GCGATCATAGGCGTCTCGCATA-3') and *Vvipgip1* primers (Foward: 5'–GCTGCAAGTGACGGATTTGA-3' and reverse: 5'–CCTTCTAGATCGATGGAACTCGTA-3') were designed using Primer Express Software Version 3.0 (Applied Biosystems) and they produced single amplicons of 66 and 124 bp respectively with cDNA being used as template.

The cDNA was then utilised for qRT-PCR experiments using the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Cape Town, South Africa). The qPCR mix was prepared in a 20 µl final volume as follows: 10 µl of KAPA SYBR FAST qRT-PCR 2X Universal Master Mix, 0.4 µl of forward primer (200 nM), 0.4 µl of reverse primer (200 nM), 0.4 µl of Rox Low, 20 ng of cDNA template and nuclease-free PCR-grade water to adjust the final volume. An ABI 7500 (Applied Biosystems) instrument was used for all qRT-PCR runs and it was programmed as follows: one cycle for enzyme activation/initial denaturation at 95°C for 1 min, 40 cycles comprising of denaturation at 95°C for 3 sec and annealing at 55°C for 45 sec. A final dissociation step was included to identify the specificity of the reaction through a melting curve analysis. Data analysis was conducted using Linear Regression of Expression (LRE) Analyzer version 0.9.9 (Rutledge, 2011). LRE calculates target quantity directly from fluorescence readings via linear regression analysis. Amplification efficiency within individual reactions is determined and bacteriophage lambda gDNA (Roche Diagnostics, Germany) is used as a universal quantitative standard for optical calibration. Each sample was tested in triplicate.

5.3.5 Cell wall analysis of uninfected transgenic grapevine leaves

Fully mature greenhouse grown grapevine plants expressing *pgip*1012 and *pgip*1038 were utilised for the cell wall analysis. Five transgenic lines (*pgip*1012-16, *pgip*1012-28, *pgip*1038-2, *pgip*1038-61 and *pgip*1038-69) were randomly selected for the analysis and the untransformed wildtype was included as a control. Mature leaves were harvested and immediately frozen in liquid nitrogen. The samples comprised of four biological repeats per plant line (four separate extractions per plant line). The leaves were milled to a fine powder in liquid nitrogen. The ground tissue was then boiled in 80% ethanol for 20 minutes and the insoluble material was subjected to a series of washes; 2 hours in methanol, 2 hours in 1:1 v/v

methanol:chloroform, 2 hours in absolute chloroform, 2 hours in 2:3 v/v chloroform:acetone and 2 hours in absolute acetone. The samples were then air dried and resuspended in purified water before being frozen in liquid nitrogen and freeze dried.

The freeze dried plant material, referred to as alcohol insoluble residue (AIR), was utilised for comprehensive microarray polymer profiling (CoMPP) essentially as described in Moller *et al*., (2007). Approximately 10 mg per sample was used for the CoMPP analysis in triplicate. The AIR samples were subjected to a sequential extraction of cell wall glycans using CDTA, to solubilise pectins, followed by NaOH, to solubilise non-cellulosic polysaccharides. A heatmap showing mean spot signals was generated, with the highest signal in the data set being assigned a value of 100 and the rest of the data adjusted accordingly. The CoMPP data was statistically analysed using Statistica software package (Statsoft Inc., Tulsa, USA). One-way Analysis of Variance was performed at 95% confidence levels.

5.3.6 Profiling the sesquiterpene emission profiles of grapevine leaves during *B. cinerea* infection

Two transgenic plant lines were randomly selected for this analysis, *pgip*1012-16 and *pgip*1038-69. Their sesquiterpene emission profiles during the first 48 hours post infection were compared to the untransformed control. The samples utilised were from infected leaves sampled at three time points namely T0, 24 and 48 hpi. From each sample, 200 mg of frozen leaf tissue was weighed into a 20 mL solid phase micro extraction (SPME) vial and 2 mL of tartaric acid buffer (5 g/L, pH 3.2) containing preservatives (2 g/L ascorbic acid and 0.8 mg/L sodium azide) was added. Twenty microliters of the internal standard (3-Octanol, 0.5 mg/L) was then added to each vial and the mixtures incubated at 100°C for 1 hr (to extract the total volatiles) and subsequently cooled to room temperature. Head space solid-phase micro-extraction (HS-SPME) GC single quadrupole MS was then utilised to profile sesquiterpene emission during *B. cinerea* infection according to the method outlined in Young *et al*. (2016).

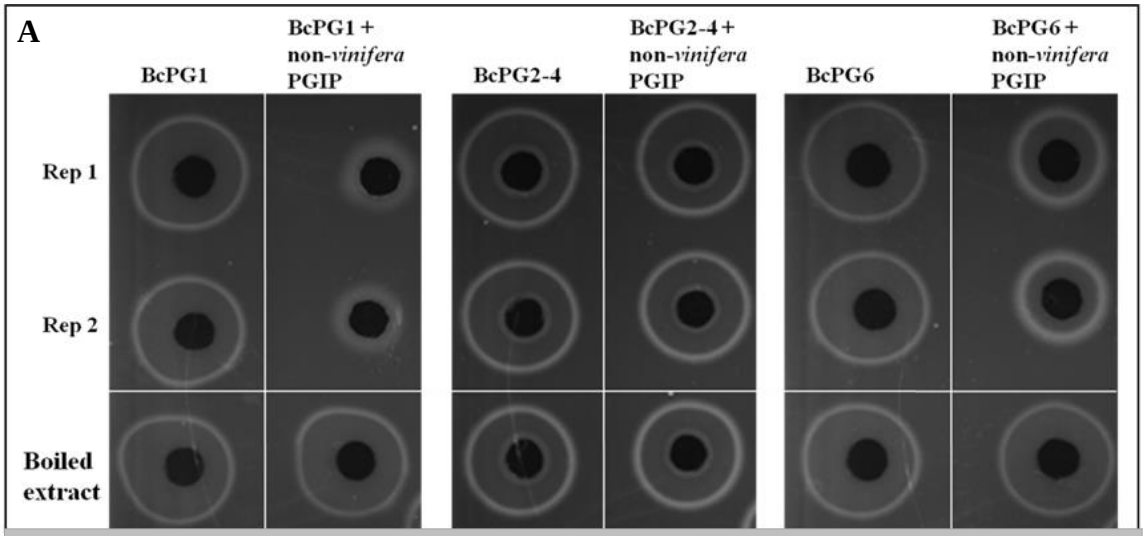
The percentage recovery was >80% for samples which were spiked with a known concentration of standard, suggesting that the method was an effective tool for analysing volatile compounds in the grapevine matrix. Peaks representing different sesquiterpenes were identified using the Wiley library at >90% identity. The peak areas of the identified peaks in the GC-MS chromatograms were calculated and relatively quantified against the α-ionone standard curve. The data generated was statistically analysed with SIMCA 14 (MKS Data Analytics Solutions, Sweden), using univariate scaling.

5.4 RESULTS

5.4.1 Non-*vinifera* PGIPs display a similar inhibition profile to VviPGIP1, inhibiting BcPG1 and 6 *in vitro*

The crude plant protein extracts from the transgenic grapevine plants effectively inhibited purified BcPG1 and BcPG6 in an *in vitro* agarose diffusion plate assay. No inhibition was detected against BcPG2, BcPG3 and BcPG4 (**Figure 1A**). Purified BcPG5 was not available and thus could not be included in the analysis. The results obtained were similar to those previously reported for VviPGIP1 (Joubert *et al*., 2006; 2007) (**Figure 1B)**.

A



B

	VviPGIP1 (Joubert *et al*., 2006; 2007)	PGIP1012	PGIP1038
BcPG1	✓	✓	✓
BcPG2	×	×	×
BcPG3	×	×	×
BcPG4	×	×	×
BcPG6	✓	✓	✓

Figure 1. (**A**) Representative results of inhibition activity assays of 1 mg/ml plant protein extracts against 1U/ml purified BcPGs. One enzyme unit is defined as the quantity of BcPG required to increase the zone size on the agarose gel by 1mm^2 over a period of 16 hrs. Results show reduction in zone size when BcPG1 and 6 are co-inoculated with the plant extracts from non-*vinifera* PGIP expressing plants. A boiled plant extract control was included in the analyses to ascertain that reduction in zone size was not a result of an artefact of the method used (**B**) Summary of results compared to VviPGIP1 published data. ✓ - Inhibition detected: × - no inhibition detected.

5.4.2 Non-*vinifera* PGIPs inhibit BcPG2 *in planta*, but not *in vitro*, similar to VviPGIP1

The transient expression of individual BcPG constructs in *N. benthamiana* caused differences in both chlorophyll fluorescence measurements and visual symptom development. Transient expression of BcPG2 caused the most tissue damage which was visualised as necrotic lesions and total tissue collapse at 48 hours post infiltration. BcPG1 only caused some slight chlorosis, but no necrosis, whilst no visual symptoms were

observed for the rest of the BcPGs (**Figure 2A-C**). As expected, transient expression of the empty vector control, pMOG800, did not result in any symptom development. These results are in line with those published in Joubert *et al*., 2007 where only BcPG2 expression caused necrotic lesion formation.

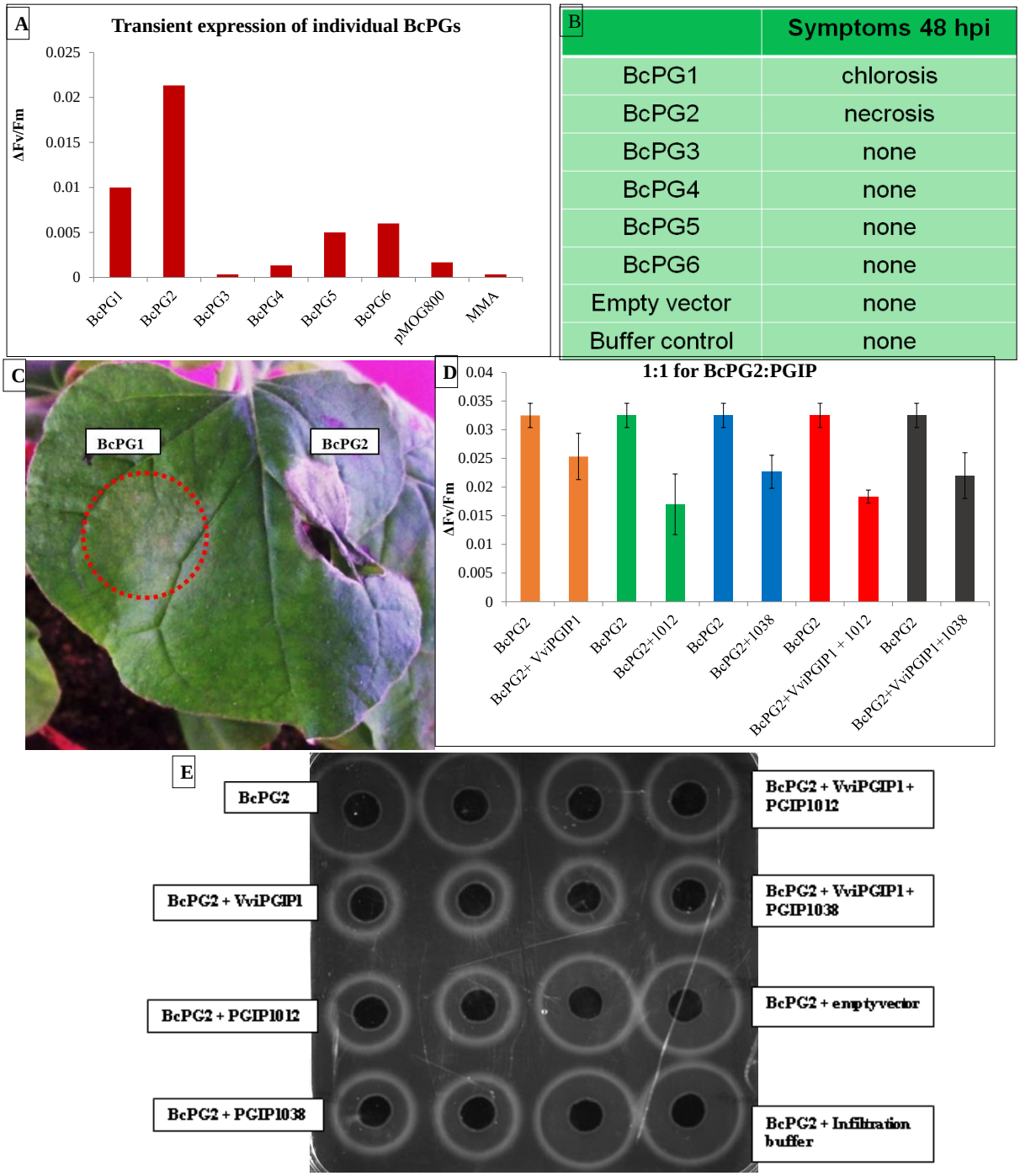


Figure 2. (**A**) Individual transient expression of BcPG2 caused the most tissue damage which was picked up at 24 hours post infiltration by measuring change in chlorophyll fluorescence. (**B** and **C**) This result was later confirmed at 48 hours post infiltration by visual symptom development. (**D**) Co-infiltration of BcPG2 with the non-*vinifera* PGIPs resulted in significant reduction in tissue damage. A combination of the non-*vinifera* PGIPs with VviPGIP1 in the same plant did not have a negative impact on the inhibition potential of the non-*vinifera* PGIPs against BcPG2 as shown by change in chlorophyll fluorescence. (**E**) Agarose diffusion plate assay results for co-infiltration of BcPG2 with the numerous PGIPs at 48 hours post infiltration at a ratio of 1:1. There was a reduction in zone size for the co-infiltrated leaf material, an indication of *in planta* inhibition of BcPG2 by the different PGIPs and their combinations.

Co-infiltration of BcPG2 with either PGIP1012 or PGIP1038 resulted in a reduction in change of chlorophyll fluorescence, an indication of reduced tissue damage (**Figure 2D**). The change in chlorophyll fluorescence of BcPG2 infiltration was dose dependant. As expected, co-infiltration of BcPG2 with the empty vector control did not result in any reduction in symptom development. A combination of VviPGIP1 with either PGIP1012 or PGIP1038 in an *in planta* environment did not negatively affect the inhibition potentials of the individual PGIPs or display any additive effects. These results were similar to those previously reported for VviPGIP1 coinfiltrations where BcPG2 was effectively inhibited *in planta* without any evidence of *in vitro* interaction (Joubert *et al*., 2007). Furthermore, protein activity assays using the agarose diffusion plate assay (Taylor and Secor, 1988) on infiltrated plant material showed that BcPG2 activity could still be detected from 48 hours post infiltration. The co-infiltration of BcPG2 with the numerous PGIPs therefore showed that both the individual PGIPs and the combinations of VviPGIP1 with a non-*vinifera* PGIP, produced active protein which effectively inhibited BcPG2 *in planta* (**Figure 2E**).

5.4.3 Both native and transgenic *pgip*s are expressed during the course of *B. cinerea* infections

At least 90% of all infection spots developed into primary lesions at 48 hpi, an indication that the incubation conditions were favourable for *Botrytis* germination and proliferation. LRE data analysis of RT-PCR results detected expression of native *Vvipgip1* in both transgenic and control plants within the first 48 hpi with *pgip*1012-16 and *pgip*1038-2 displaying expression levels almost half those of the WT and *pgip*1012-28, which were at the same level. *PGIP1038-69* displayed the highest level of *Vvipgip1* expression, a three-fold change compared to the WT (**Figure 3**). The non-*vinifera pgips* in the transgenic lines were constitutively expressed at high levels throughout the infection assay with a five-fold change between *pgip*1012-16 and *pgip*1012-28 expression patterns compared to *pgip*1038-2 and *pgip*1038-69. There was a 600 fold-change in expression levels for the non-*vinifera* pgips compared to the native *Vvipgip1* expression. *PGIP* expression profiling of leaf samples taken at 9 days post infection (results not shown) showed that this trend persisted for the entire duration of the antifungal assay. This data confirmed that the *pgip* genes were expressed in the transgenic population without obvious signs of gene silencing occurring.

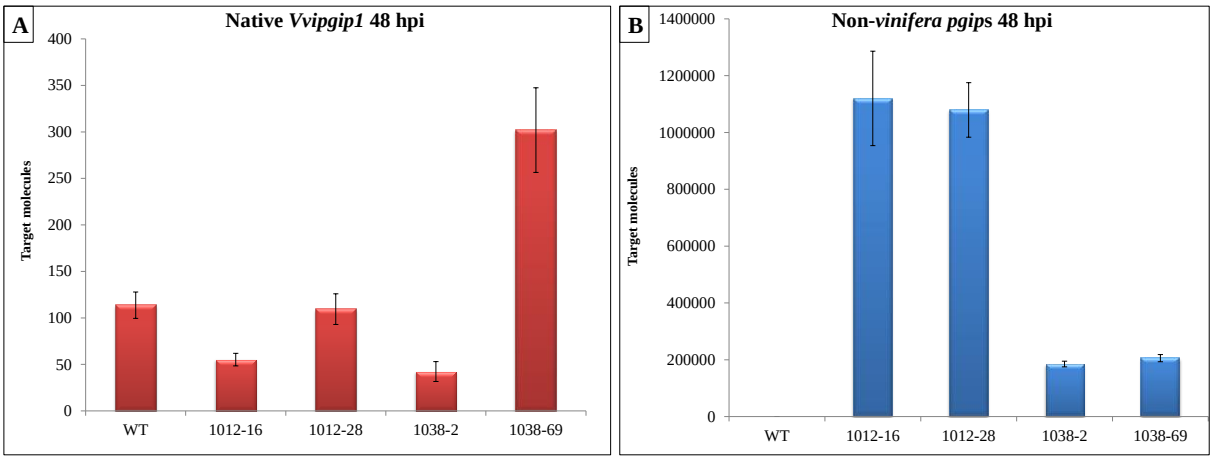


Figure 3. (**A**) Native and (**B**) transgenic *pgip* expression profiles using LRE data analysis on leaf material from the whole plant time-course infection assay at 48 hpi. Both the native and transgenic *pgip*s were expressed during the course of the assay. Error bars represent the standard deviation from the three technical repeats.

5.4.4 Transgenic grapevine leaves exhibit cell wall changes prior to infection

Since previous work has shown that overexpressing *Vvipgip1* in tobacco primed the host by changing the cell wall profiles prior to infection (Nguema-Ona *et al*., 2013), in this study, cell wall profiles of uninfected transgenic and control grapevine leaves were profiled for potential compositional differences using CoMPP analysis (Moller *et al*., 2007). The method involves the sequential extraction of cell wall polymers from homogenised ground leaf material using CDTA and NaOH to extract pectins and non-cellulosic polysaccharides respectively. The supernatants are used to generate microarrays which are probed with monoclonal antibodies (mAbs) that bind to cell wall components resulting in spot signals which are then relatively quantified (Moller *et al*., 2007). The CoMPP analysis of leaves from transgenic grapevine plants showed significant differences in cell wall epitope abundance compared to the controls prior to infection. Although there were slight differences amongst cell wall profiles of the different transgenic lines, they all displayed the same trends which differed from the controls. These differences were observed in both the CDTA and NaOH sequential extracts. In **Figure 4**, the heatmap generated from the CoMPP data shows differences in mean spot signals between the untransformed wildtype and the transgenic lines. Binding of the mAbs JIM5 (binds to homogalacturonan (HG) with a low degree of esterification (DE)), JIM7 (bind to HG with a high DE), LM18, LM19 and LM20 (bind to HG partially methyl esterified) in the CDTA extract indicated the predominant extraction of a major pectin polysaccharide homogalacturonan (HG) from all the AIR samples. CDTA is a calcium chelating agent and it is utilised for the extraction of chelator-soluble fractions. There were no significant differences in HG epitope abundance between the wildtype and the transgenic plant lines in the CDTA extract suggesting that the degree of calcium-crosslinking between the two plant populations were similar. This was further supported by the mean spot signals obtained for mAB 2F4, which binds to $Ca2^{+}$ cross-linked HG, where no significant differences in epitope abundance were detected.

The CDTA extract however, showed significantly higher levels of the highly soluble arabinogalactan proteins (AGPs) epitopes in the transgenic plant lines compared to the untransformed control. These

differences were consistent and significant across four different mAbs that bind specifically to AGPs namely JIM8, JIM13, LM2 and LM14. The NaOH buffer, which mainly solubilises the non-cellulosic cell wall polysaccharides, predominantly extracted xyloglucan, mannan, arabinan, extensins and residual levels of AGPs. The untransformed wildtype had higher abundance of β-1,4-D-galactan, β-1,3-D-glucan and de-esterified HG compared to the transgenic plant lines. All differences in epitope abundance were shown to be statistically significant at $p<0.05$ using one-way ANOVA.

A

		HG with a low DE (mAb JIM5)	HG with a high DE (mAb JIM7)	HG Partially methylesterified (mAb LM18)	HG Partially methylesterified (mAb LM19)	HG Partially methylesterified (mAb LM20)	HG Blockwise de-esterified (mAb PAM1)	HG Ca2+ crosslinked (mAb 2F4)	Xylogalacturonan (mAb LM8)	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	(1→4)-β-D-galactan (mAb LM5)	(1→5)-α-L-arabinan (mAb LM6)	Linearised (1→5)-α-L-arabinan (mAb LM13)	(1→4)-β-D-(galacto)(gluco)mannan (mAb LM21)	(1→4)-β-D-(gluco)mannan (mAb LM22)	(1→3)-β-D-glucan (mAb BS-400-2)	Xyloglucan (XXXG motif) (mAb LM15)	Xyloglucan / unsibstituted β-D-glucan (mAb LM25)	(1→4)-β-D-xylan (mAb LM10)	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	Cellulose (crystalline) (CBM3a)	Extensin (mAb LM1)	Extensin (mAb JIM11)	Extensin (mAb JIM20)	AGP (mAb JIM8)	AGP (mAb JIM13)	AGP (mAb LM14)	AGP, β-linked GlcA (mAb LM2)
CDTA	Wildtype	59	85	67	69	25	0	14	0	26	18	7	8	0	0	0	0	0	16	0	0	0	0	0	11	34	51	20	22
	pgip 1012-16	54	78	63	64	21	0	12	0	25	17	1	7	0	0	0	0	0	18	0	1	0	0	0	11	41	57	25	28
	pgip 1012-28	49	76	60	59	22	0	11	0	21	15	4	7	0	0	0	0	0	19	0	0	0	0	0	7	38	56	24	25
	pgip 1038-2	60	86	69	67	26	0	12	0	27	18	6	8	0	0	0	0	0	15	0	0	0	0	0	10	43	60	26	28
	pgip 1038-61	64	85	71	73	19	0	15	0	20	12	8	10	0	0	0	0	4	19	0	1	0	0	0	9	44	63	28	32
	pgip 1038-69	67	92	74	76	27	0	17	0	34	26	7	9	0	0	1	0	0	17	0	0	1	0	4	15	49	69	30	31
NaOH	Wildtype	0	0	4	13	0	18	0	0	36	9	22	21	0	46	0	44	0	47	0	0	34	0	13	31	18	57	7	8
	pgip 1012-16	0	0	3	11	0	14	0	0	36	8	14	18	0	47	0	35	0	49	0	2	33	0	16	32	16	52	7	8
	pgip 1012-28	0	0	5	14	0	15	0	0	37	8	18	21	0	62	3	38	5	65	2	3	36	0	15	30	17	53	8	9
	pgip 1038-2	0	0	0	9	0	8	0	0	30	7	16	16	0	50	0	33	0	50	0	0	37	0	8	20	12	45	6	7
	pgip 1038-61	0	0	0	0	0	7	0	0	24	3	15	12	0	57	3	19	1	57	0	1	37	0	8	21	11	45	8	9
	pgip 1038-69	0	0	0	11	2	14	0	0	35	8	19	20	0	43	0	31	0	48	0	0	41	0	13	31	16	54	7	8

B

AGP monoclonal antibody	Plant line	Mean spot density	Group 1	Group 2	Group 3	Group 4	Group 5
JIM 8	Wildtype	34					XXXX
	1012-16	41			XXXX		
	1012-28	38				XXXX	
	1038-2	43		XXXX	XXXX		
	1038-61	44		XXXX			
	1038-69	49	XXXX				
JIM 13	Wildtype	51					XXXX
	1012-16	57			XXXX	XXXX	
	1012-28	56				XXXX	
	1038-2	60			XXXX		
	1038- 61	63		XXXX			
	1038-69	69	XXXX				
LM2	Wildtype	22				XXXX	
	1012-16	28		XXXX			
	1012-28	25			XXXX		
	1038-2	28		XXXX			
	1038-61	32	XXXX				
	1038-69	31	XXXX				
LM14	Wildtype	20					XXXX
	1012-16	25			XXXX	XXXX	
	1012-28	24				XXXX	
	1038-2	26			XXXX		
	1038-61	28		XXXX			
	1038-69	30	XXXX				

C

Monoclonal antibody	Plant line	Mean spot density	Group 1	Group 2	Group 3	Group 4	Group 5
BS-400-2	Wildtype	44	XXXX				
	1012-16	35		XXXX	XXXX		
	1012-28	38		XXXX			
	1038-2	33			XXXX	XXXX	
	1038-61	19					XXXX
	1038-69	31				XXXX	
PAM 1	Wildtype	18	XXXX				
	1012-16	14		XXXX			
	1012-28	15		XXXX			
	1038-2	8			XXXX		
	1038- 61	7			XXXX		
	1038-69	14		XXXX			
LM5	Wildtype	22	XXXX				
	1012-16	14			XXXX		
	1012-28	18		XXXX			
	1038-2	16			XXXX		
	1038-61	15			XXXX		
	1038-69	19		XXXX			

Figure 4. CoMPP analysis of wildtype and transgenic grapevine leaves prior to infection. (**A**)The heatmap illustrates the average relative abundance of the full set of cell wall glycan-associated epitopes detected in grapevine leaf cell wall material. Colour intensity on the heatmap correlates to mean spot signals. The highest signal in the data set was assigned a value of 100 and all other signals were adjusted accordingly. (**B**) A subset of the results showing statistical groupings in the CDTA extract with relatively lower levels of AGP epitope abundance in the wildtype compared to all the transgenic plant lines using the mAbs JIM8, JIM13, LM14 and LM2. One-way ANOVA showed that these differences were statistically significant (groups represented by different colours) at $p<0.05$. (**C**) A subset of the results showing statistical groupings in the NaOH extract. There was a clear separation between the wildtype and the transgenic plants into statistically significant groups at $p<0.05$ for β-1,3-D-glucan using mAb BS-400-2, β-1,4-D-galactan using mAb LM5 and HG-blockwise de-esterified using mAb PAM1.

5.4.5 Transgenic grapevine plants exhibit an altered sesquiterpene emission profile during *B. cinerea* infection

Previous work in our environment has shown that infecting tobacco leaves with *B. cinerea* results in an upregulation of genes involved in sesquiterpene biosynthesis (unpublished data). We thus studied the sesquiterpene emission profiles of transgenic and control plants during *B. cinerea* infection and identified significant differences between the two populations. The most abundant sesquiterpene emitted by the infected leaves was α-copaene. Low levels were detected at T0 and 24 hpi in all samples. However, the levels increased significantly at 48 hpi with higher levels being emitted by control samples compared to the transgenics (**Figure 5A**). Two other sesquiterpenes, α-humulene and β-caryophyllene, were detected in all samples at 48 hpi but not at any of the earlier time points (**Figure 5B** & **5C**). Again, higher levels were detected in control samples compared to the transgenics. Unsupervised Principal Component Analysis (PCA) of the data set showed that at T 0 and 24 hpi, there was no clear separation between the sesquiterpene emission profiles of the wildtype and the transgenic leaves. However, at 48 hpi there was a clear separation (**Figure 5D**). The loadings plot shows the sesquiterpenes which contributed towards the clear differences in emission profiles (**Figure 5E**).

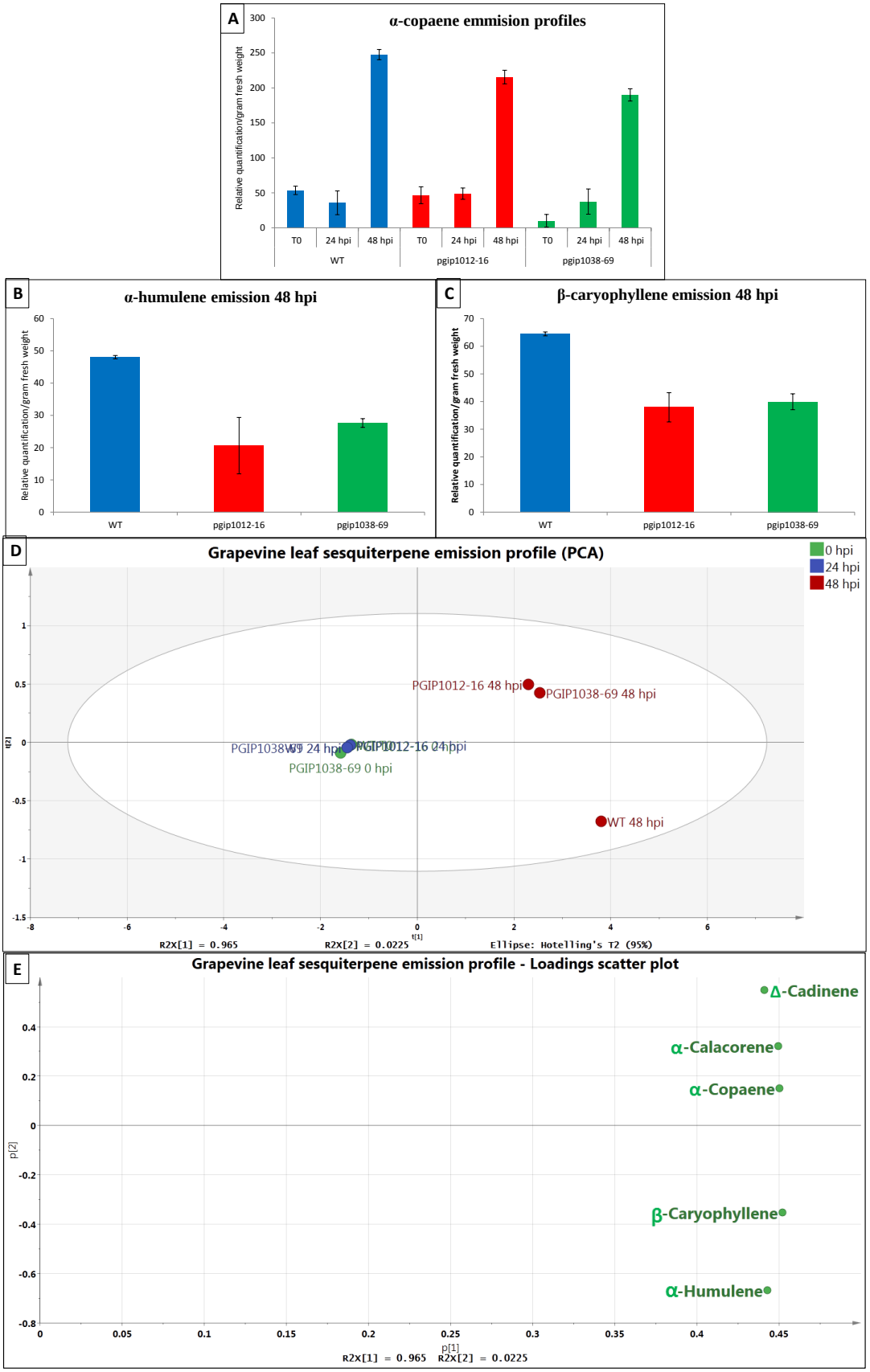


Figure 5. Defence-related sesquiterpene emission profiles of grapevine leaves infected with *B. cinerea* during the first 48 hpi. The untransformed wildtype emitted significantly higher levels of (**A**) α-copaene, (**B**) α-humulene and (**C**) β-caryophyllene at 48 hpi. All compounds were quantified against the α-ionone standard curve. Error bars represent standard deviation from three technical repeats (**D**) Using univariate scaling, the sesquiterpene emission profiles of the transgenic leaves clearly seperated from the wildtype at 48 hpi on a PCA plot using SIMCA 13.0.2. (**E**) The loadings plot showed that the sesquiterpenes pulled the data to the right seperating 48 hpi from the earlier time points.

5.5 DISCUSSION

The hyper-susceptibility of the transgenic grapevine population when challenged with the *B. cinerea* grape strain (Moyo, 2011) was an interesting outcome, more-so when further studies showed that it was a strain specific defence phenotype (**Chapter 4** of thesis). This study aimed to provide insight on potential factors that could have contributed to this and the results are discussed below.

Post transcriptional *pgip* gene silencing did not contribute towards the hyper-susceptible phenotype of transgenic grapevine

In this study, we followed *pgip* expression during the first 48 hpi to determine if gene silencing could have occurred thus contributing to the hyper-susceptible phenotype. Our results showed that *Vvipgip1* was upregulated whilst the non-*vinifera pgip*s were constitutively expressed in all transgenic plants during the course of the infection assay. Numerous studies have reported the upregulation of PGIP encoding genes in plant hosts following stress stimuli such as mechanical wounding, exposure to elicitors and *B. cinerea* infection (Review by Kalunke *et al*., 2015). For example, in *Arabidopsis*, *AtPGIP1* and *AtPGIP2* were strongly upregulated in response to *B. cinerea* infection (Ferrari *et al*., 2003).

In grapevine, expression patterns for *Vvipgip1* have been studied and it has been shown to be tissue specific and developmentally regulated, only being expressed in roots and berries during and soon after véraison (Joubert *et al*., 2013). This restriction of *pgip* expression patterns could be suggestive of functions of grapevine PGIPs in other biological processes which do not necessarily involve the classical PGIP-PG inhibition interactions. Moreso, since transcriptional analysis has shown that véraison is associated with a strong oxidative burst in berries which is characterised by a rapid accumulation of H_2O_2 (Pilati *et al*., 2007). Promoter and gene expression analysis have shown a strong induction of *Vvipgip1* in all tissues due to wounding, infection, presence of elicitors, but also oxidative stress (such as the oxidative burst linked to the metabolic changes linked to véraison) (Joubert *et al*., 2013).

Several other studies have also reported the upregulation of grapevine *pgip* during infection, elicitation or stress. Treatment of grapevine cell cultures with oligogalacturonides elicited plant defence responses which included an upregulation of *pgip* (Aziz *et al*., 2004) whilst analysis of gene expression profiles of grapevine leaves and berries infected with *B. cinerea* revealed an upregulation of *pgip* within the first 6 hpi on leaves, reaching the highest levels at 48 hpi. It was also upregulated on partially infected mature *V. vinifera* clusters (Bezier *et al*., 2002). Additionally, veraison berries infected with *B. cinerea* displayed an upregulation of *pgip* (Kelloniemi *et al*., 2015).

Non-*vinifera* PGIPs are effective inhibitors of BcPGs

In vitro and *in planta* platforms previously reported in the profiling of VviPGIP1-BcPG interactions were utilised and thus VviPGIP1 was included as a control in our study. As previously reported (Joubert *et al*., 2006; 2007), VviPGIP1 effectively inhibited BcPG1 and 6 *in vitro* whilst BcPG2 was only inhibited *in planta*. The inhibition profiles of PGIP1012 and PGIP1038 against the same BcPGs displayed a similar trend

without deviation. BcPG1 and 2 have been classified as major *B. cinerea* virulence factors on numerous plant hosts and knock-out mutants of these genes display a significant reduction in pathogenicity (ten Have *et al*., 1998; Kars *et al*., 2005; Nakajima and Akutsu, 2014). The strong activity of these two virulence factors was confirmed in our infiltration studies where transient expression of BcPG2 caused the most tissue damage, causing severe necrosis within the first 48 hpi, whilst BcPG1 only caused chlorosis (**Figure 2**). Co-infiltration studies revealed that *in planta*, the non-*vinifera* PGIPs were able to effectively inhibit BcPG2 despite not being able to do so *in vitro*. Since the same trend was previously reported for VviPGIP1 (Joubert *et al*., 2007), it could be that grapevine PGIPs only inhibit BcPG2 in three-component complexes which includes a substrate not available in the *in vitro* setup, thus the need for an *in planta* environment (Gutierrez-Sanchez *et al*., 2012).

BcPG1 on the other hand, was inhibited *in vitro* by the non-*vinifera* PGIPs. It appears that the PGIP-BcPG inhibition is more stable compared to that involving BcPG2, hence it occurs *in vitro*. This is supported by docking simulations where predicted PGIP complexes with B05.10-BcPG1 released higher levels of energy during computational mutagenesis of residues at the protein-protein interfaces compared to B05.10-BcPG2 complexes (**Chapter 3** Table 6 of thesis). Additionally, BcPG1 from B05.10 was shown to be a virulence factor on grapevine leaves with *Δbcpg1* infections displaying a significant reduction in pathogenicity compared to WT B05.10 infections (**Chapter 4** Figure 4 of thesis), further confirming its role in the classical PGIP-PG interaction. To simulate multiple PGIPs in the same host (since the transgenic grapevine plants possess both native VviPGIP1 and a non-v*inifera* PGIP) we co-infiltrated a combination of VviPGIP1 with either PGIP1012 or PGIP1038 and this did not negatively affect the inhibition profiles or protein activities of the PGIPs.

Cell wall changes in transgenic leaves prior to infection could benefit *Botrytis* during infection

The plant cell wall is one of the first physical barriers that *B. cinerea* encounters during infection, and thus differences in cell wall composition between plant hosts plays a potential role in determining the extent of damage that the fungus can inflict (Maganu & Paolocci, 2013). A model for the cell wall of tobacco plants overexpressing *Vvipgip1* was previously proposed in our environment (Nguema-Ona *et al*., 2013). In the model, *Vvipgip1* overexpression resulted in the upregulation of a cinnamyl alcohol dehydrogenase (CAD) gene involved in lignification whilst also downregulating XTH/EXT. These changes strengthened the cell wall prior to any infection in a priming phenotype that was also linked to altered hormone levels (Alexandersson et al., 2011). In our study, comparison of the cell wall compositions of uninfected transgenic and control grapevine leaves also yielded some interesting results. Firstly, the transgenic grapevine plants showed significantly lower levels of β-1,3-D-glucan epitope, a linear polymer for callose formation in plants. Callose plays a major role in plant defence against invading fungal pathogens where it forms plugs at the site of infection which seal tissue wounds and forms a protective interface between the pathogen and the plasma membrane (Conrath *et al*., 2002; Hulten *et al*., 2006; Luna *et al*., 2011). Furthermore, callose deposition has been shown to be one of the priming defence mechanisms that plants employ prior to any infection

(Reviewed by Pastor *et al*., 2013). Thus a plant with higher levels of callose possesses a reinforced cell wall at the point of potential attack and this leads to improved defence against the invading fungal pathogen.

Secondly, the transgenic grapevine leaves showed significantly lower levels of β-1,4-D-galactan epitope binding to mAb LM5 (Jones *et al*., 1997) compared to the wildtype. β-1,4-D-galactan is a neutral sugar side chain for RG I in the plant cell wall. The galactan-rich pectin side chains from sugar beet (*Beta vulgaris*) and potato (*Solanum tuberosum*) have the ability to bind to cellulose *in vitro* (Zykwinska *et al*., 2005; 2008). The researchers proposed that this binding potentially functions as a cell wall 'coat' which provides a continuum between the cellulose and pectin networks. The abundance of the side chains thus influences the extent of binding which ultimately determines cell wall assembly and strength (Zykwinska *et al*., 2005; 2008). The reduced levels of β-1,4-D-galactan in the transgenic plants could have resulted in a weaker cell wall 'coat' thus making it much easier for *B. cinerea* to access and ultimately macerate the pectin.

Thirdly, the transgenic grapevine population had significantly lower levels of blockwise de-esterified HG compared to the wildtype. Higher levels of pectin de-esterification are associated with an increase in susceptibility of HG to endopolygalacturonase degradation (Lionetti *et al*., 2007). When comparing relative epitope abundance, de-esterified HG levels were generally lower than both β-1,4-D-galactan and β-1,3-D-glucan in all samples. It seems as if the non-*vinifera pgip*s in the transgenic grapevines resulted in cell wall compositional changes which contributed to both strengthening and weakening of the defence barrier. However, based on epitope abundance, the dominating components were linked to cell wall weakening in transgenic plants.

Lastly, transgenic grapevine leaves had higher abundance of AGP epitopes compared to the wildtype. AGPs have been identified in numerous plants species including *Arabidopsis*, rice, poplar and tomato (Ma and Zhao, 2010; Showalter *et al*., 2010; Fragkostefanakis *et al*., 2012; Showalter *et al*., 2016). They have highly diverse functions ranging from cell division to regulation of cell wall biosynthesis (Reviewed by Tan *et al*., 2012). In *Arabidopsis*, 85 AGP encoding genes with diverse localisations and expression patterns were identified (Showalter *et al*., 2010). Functional analysis on some of the *At*AGPs has revealed a diverse range of functions (Zhang *et al*., 2011; Hijazi *et al*., 2014; Seifert *et al*., 2014). Whether or not grapevines AGPs exhibit the same diverse trends requires further investigation. To our knowledge, the grapevine AGP family has not been characterised from the available sequenced genome. As a first step towards interpreting our cell wall AGP trends, we identified 57 putative grapevine AGPs (VviAGPs), classified them into six-subfamilies based on sequence similarities and characterised the expression patterns which showed high levels of developmental regulation with some genes displaying organ-specific expression patterns. VviAPG12 and 16 were strongly expressed in leaves and could potentially be involved in cell wall strengthening through formation of self-aggregates and also cross-links with pectin (Tan *et al*., 2013; Hijazi *et al*., 2014) (Please refer to **Addendum A** to **Chapter 5** for this analysis).

An altered sesquiterpene emission profile possibly contributes to a hyper-susceptible grapevine-*Botrytis* phenotype

By profiling the sesquiterpene emission during *B. cinerea* infection, it was confirmed that the transgenic grapevine leaves emitted significantly lower levels of α-copaene, α-humulene and β-caryophyllene at 48 hpi compared to the wildtype. These results complimented previous work where an upregulation of genes involved in terpene biosynthesis was observed in tobacco leaves infected with *B. cinerea* (unpublished data). This response could be specific to leaves since grape berries infected with *B. cinerea* displayed downregulation of genes involved in triterpenoid and sesquiterpene biosynthesis (Agudelo-Romero *et al*., 2015).

The sesquiterpene α-copaene was the most prominent volatile compound in our study. This was interesting since it was also the most prominent volatile induced in tomato leaves infected with *B. cinerea* (Thelen *et al*., 2005). The authors suggested the use of the compound as a marker for the detection of *B. cinerea* infection but did not make any inference to its role in defence. On the other hand, *β*-caryophyllene has been directly implicated in the inhibition of *B. cinerea* spore germination and hyphal growth (Zhang *et al*., 2008) whilst α-humulene has antimicrobial activity (Dorman & Deans, 2000). Thus, the lower levels of sesquiterpenes detected in infected transgenic leaves could be taken as an indicator of a compromised defence strategy which benefited the invading pathogen.

Summary and Conclusions

In this study, the transgenic grapevine population displaying strain-specific hyper-susceptibility to *B. cinerea* grape strain was analysed to try and understand the phenotype from this host-pathogen pairing. The phenotype could not be attributed to lack/compromised PGIP inhibition profiles or post transcriptional gene silencing of either native or transgenic *pgip*s. However, changes in the cell wall prior to infection and altered sesquiterpene emission profiles during the early stages of infection could have compromised plant defence. These results could be a sign of changes in several metabolic processes which are probably also linked to structural features which then contributed to the hyper-susceptible phenotype. The co-evolution of the *B. cinerea* grape strain with the host could also be a major contributing factor to the observed phenotype, since it could have adapted ways to overcome or even hijack host defence responses for its own benefit. Thus, studying the behaviour of both host and pathogen during infection would be valuable in understanding the unique phenotype specific to this host-pathogen pairing. A grapevine-*Botrytis* grape strain interactome study was thus conducted to investigate other potential defence mechanisms in the transgenic grapevine population and gain insight on the infection strategies employed by the fungus.

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Addendum A

ADDENDUM A to CHAPTER 5:

Genome-wide identification, classification and expression analysis of arabinogalactan proteins in *Vitis vinifera*

ADDENDUM A: Genome-wide identification, classification and expression analysis of arabinogalactan proteins in *Vitis vinifera*

S1.1 ABSTRACT

Arabinogalactan proteins (AGPs) are members of the hydroxyproline-rich glycoprotein (HRGP) family in plants, well known for their role in growth and development. They have been identified and characterised in rice, tomato and *Arabidopsis* but no work has been published for grapevine. In this study, an *in silico* data mining approach was utilised to search for putative AGP encoding genes from the annotated grapevine genome. A total of 57 putative *Vitis vinifera* AGP encoding genes (termed *Vviagp*s henceforth) were identified and classified into six sub-families based on well-documented characteristics unique to this gene family. Subjecting all genes to ortholog and paralog detection to determine their evolutionary relatedness showed that the high levels of amino acid sequence similarities utilised to classify the VviAGPs into subfamilies were not a positive indicator for gene relatedness. The grapevine genome-wide expression atlas data (based on 54 *V. vinifera* cultivar Corvina samples at different developmental stages) was utilised to study the global gene expression patterns of VviAGPs across all organs. Hierarchical clustering analyses showed high levels of developmental regulation with moderate tissue specific expression patterns. The majority of VviAGPs expressed in berries exhibited developmental regulation with very few being expressed in all stages of development. Interestingly, the strong developmental expression patterns identified in Corvina applied to 10 other Italian cultivars which encompassed both red and white grape varieties at four grape developmental stages; fruit set, pre-veraison, post-veraison and ripe stages. The main driver of separation on an OPLS-DA plot was developmental stage and not cultivar. The data generated in this study can be used to select VviAGPs with characteristics of interest for functional and cellular localisation studies.

S1.2 INTRODUCTION

The HRGP super-family comprises of minimally glycosylated proline-rich proteins (PRPs), moderately glycosylated extensins (EXTs) and highly glycosylated AGPs. Studies have shown that a continuum exists within the super-family from the minimally glycosylated PRPs to the extensively glycosylated AGPs, resulting in hybrid HRGPs with properties from at least two families e.g. EXT-AGP hybrids (Seifert and Roberts, 2007; Showalter *et al*., 2010). However there still exist a large number of members of the super-family belonging exclusively to a particular sub-family (Showalter, 1993; Ma and Zhao, 2010).

The heavily glycosylated AGPs are proteoglycans in nature composed of >90% (w/w) carbohydrate and <10% protein. The core protein backbone varies in length and is predominantly rich

in proline/hydroxyproline, alanine, serine and threonine (PAST). Furthermore, it is *O*-glycosylated by complex carbohydrates consisting primarily of galactose and arabinose-rich polysaccharide units (Seifert and Roberts, 2007). AGPs are mainly found in the cell wall, plasma membrane, apoplastic space, intracellular multi-vesicular bodies and secretions of terrestrial and aquatic plants (Herman & Lamb, 1992; Showalter, 2001). They have been detected in numerous plant species including *Arabidopsis*, rice, carrot, pear, tobacco, poplar and grapevine (Saulnier *et al*., 1992; Pellerin *et al*., 1993; Smallwood *et al*., 1996; Youl *et al*., 1998; Ma and Zhao, 2010; Showalter *et al*., 2010; Ma *et al*., 2015; Showalter *et al*., 2016).

AGPs have been directly and indirectly implicated in diverse plant processes including cell division and expansion, pollen germination, tip elongation in pollen tubes, xylem differentiation, somatic embryogenesis, cellulose deposition, cell wall biosynthesis and strengthening, signal transduction, root growth, cell aggregation and programmed cell death (PCD) in plant cell cultures (Clarke *et al*., 1979; Gao & Showalter, 1999; Majewska-Sawka and Nothnagel, 2000; Deepak *et al*., 2010; Zhang *et al* 2011; Costa *et al*., 2013; Lamport & Varnai, 2013; Tan *et al*., 2012:2013; Hijazi *et al*., 2014; Seifert *et al*., 2014). Their broad functional range could explain the diversity of organs where AGPs have been detected. These include both above and below-ground plant tissues such as roots, seeds, leaves, stems, buds and reproductive organs (Recent review by Pereira *et al*., 2015).

The identification and classification of AGPs was previously based on factors such as the ability of the proteins to bind to a synthetic phenylazo dye, *β*-glucosyl Yariv reagent or the presence of a GPI (glycosylphosphatidylinositol) anchor signal sequence at the C-terminus. *β*-glucosyl Yariv reagent binds and selectively precipitates AGPs, thus making it possible to purify the proteoglycans. It is also used for localisation studies where it stains AGPs, thus enabling determination of cellular distribution (Yariv *et al*., 1967). The GPI-anchor is associated with the plasma membrane and it is believed to play a pivotal role in facilitating the exchange of information between the intracellular and the extracellular space (Schultz *et al*., 1998). A number of studies have since shown that some well characterised AGPs neither react with the Yariv reagent, nor possess a GPI-anchor signal sequence. Thus the current trend in identification and classification of AGPs into distinct sub-families is based on their polypeptide backbones and the presence/absence of highly conserved domains (Showalter, 2001). This is done in combination with the use of glycan-specific monoclonal antibodies (mAbs) such as JIM8, JIM13 (John Innes Monoclonal), LM2 and LM14 (Leeds Monoclonal) that specifically bind to AGP epitopes in different plant cells, tissues and organs (Knox, 1997; Seifert and Roberts, 2007; Moller *et al*., 2007; 2008).

Based on the current classification system, AGP sub-families include classical, lys-rich, AG peptides, eNod-like and FLAs (Showalter, 2001). Classical AGPs have a higher % PAST compared to the other classes. Lys-rich AGPs belong to the classical sub-family. They however additionally possess short stretches of lysine rich residues between the hydrophobic C-terminus and the proline-rich domain. The FLAs possess at least one or more fasciclin conserved domain believed to be

involved in cell adhesion and protein-protein interactions. The eNod-like AGPs possess a conserved early nodulin domain related to the copper-containing phytocyanins.

AGPs with short protein backbones are classified as AG peptides. The majority of AGPs, though not all, that have been identified from different plant species possess a secretory signal peptide at the N-terminus (Schultz *et al*., 2002; Seifert and Roberts, 2007; Ma and Zhao, 2010; Showalter *et al*., 2010: 2016; Fragkostefanakis *et al*., 2012). There are some discrepancies in the criteria used to classify AGPs into different sub-families from one research group to another. For example, classical AGPs are generally characterised by the presence of an N-signal sequence with a C-terminus GPI anchor (Pereira *et al*., 2015). However, such generalisations seem to serve as a guideline and not necessarily a rule. This is evident in previous studies where from the 22 classical AGPs that were identified in *Arabidopsis*, only 14 possessed the GPI anchor sequence and 19 had the signal peptide (Showalter *et al*., 2010). Furthermore, in *Arabidopsis*, poplar and tomato, AGPs with 42% PAST were classified under the classical sub-family whilst in rice a cut-off of 50% was utilised (Ma & Zhao, 2010; Showalter *et al*., 2010; 2016; Fragkostefanakis *et al*., 2012). This makes cross comparison of AGPs from different plant species based on non-standard class groupings difficult.

In grapevine, AGP abundance increased in Cabernet Sauvignon and Crimson seedless berries as they ripened (Moore *et al*., 2014). This corresponds with the accumulation of other cell wall proteins during ripening (Huang *et al*., 2005). In other preliminary data, there were no clear differences in AGP abundance between ripe and overripe Shiraz berries at 23 to 26°Brix. However, at 28°Brix, a slight decrease in AGPs associated with the pectin rich fraction was observed (unpublished data from Moore, 2014). Despite the large contribution that AGP detection methods such as the use of *β*-glucosyl Yariv reagent and mABs have made to the current knowledge on these complex proteins, their major drawback is their inability to determine and identify single AGPs.

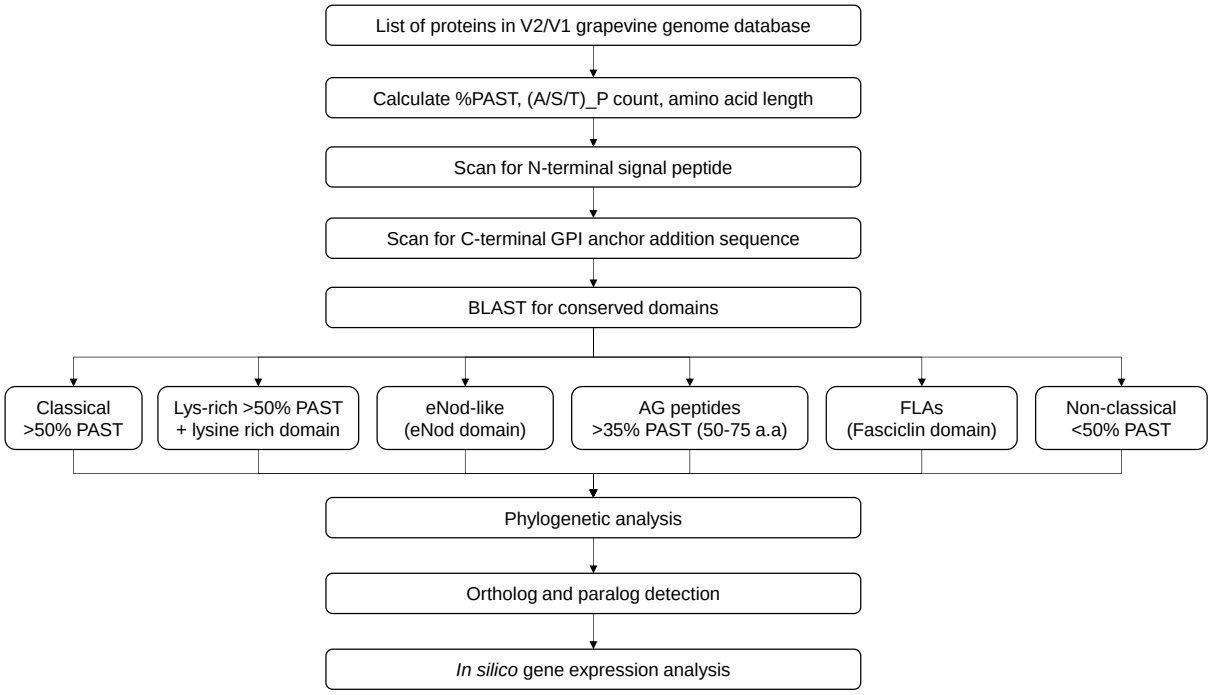
In this study, the annotated grapevine genome and publicly available expression datasets made it possible to utilise genetic approaches to identify and characterise putative AGPs *in silico*. Due to the discrepancies in classification of AGPs utilised by different researchers, we decided to follow the guidelines from the work published on rice AGPs outlined in Ma & Zhao, 2010. The only difference which we incorporated was the criteria used for the classification of non-classical AGPs. Ma & Zhao (2010) classified proteins as non-classical AGPs if they possessed atypical regions such as an asparagine (Asn) rich region whilst the same AGPs in tomato were classified as Asn-rich AGPs (Fragkostefanakis *et al*., 2012). In our study non-classical AGPs possess the general attributes of AGPs but do not meet the requirements to be classified in other sub-families due to their % PAST, amino acid length and lack of conserved domains. Furthermore, all putative VviAGPs were screened for repeats associated with either extensins or PRPs thus eliminating HRGP hybrids from our final list. The publicly available grapevine atlas dataset (Fasoli *et al*., 2012) was a valuable resource to investigate VviAGP expression patterns in different organs. In order to assess if the observed patterns were cultivar specific, we compared the Corvina berry expression patterns to those of 10 other Italian

cultivars. Our findings suggested that the trends applied to all grapevine cultivars. The work presented in this chapter aims to shed light on the characteristics of the complex grapevine AGP family.

S1.3 MATERIALS AND METHODS

S1.3.1 Identification of putative AGP encoding genes from the grapevine genome

The identification of grapevine AGPs was based on the method outlined by Ma and Zhao, 2010. All grapevine proteins were downloaded from V2 of the annotated grapevine genome and used to identify putative AGPs. Only those proteins which were also represented in V1 annotation were utilised for further analysis. A custom Perl script was utilised to categorise the proteins based on their percentage PAST resulting in a list of candidate AGPs. The proteins were then subjected to amino acid analysis and their lengths, (A/S/T)_P count and percentage prolines were determined. The presence of the N-terminal signal sequence was determined using Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The presence of the GPI anchor sequence on the C-terminus was determined using the big-PI Plant Predictor tool (http://mendel.imp.ac.at/gpi/plant_server.html). Conserved domains on all candidate AGPs were identified using the Conserved Domain tool on NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). To identify FLAs, several prediction algorithms were utilised (http://www.ebi.ac.uk/interpro/, IPR000782, http://pfam.sanger.ac.uk/, PF02469, http://smart.embl-heidelberg.de/ and SM00554. A summary of the screening steps including the successive analyses is presented in **Supplementary Figure 1.**



Supplementary Figure 1. Bioinformatics workflow summarising identification, classification and *in-silico* data mining of grapevine AGP encoding genes.

S1.3.2 Classification of grapevine AGPs into sub-families

The putative grapevine AGPs were classified based on the criteria outlined in Ma and Zhao, 2010. The proteins were predominantly made up of Ala-Pro, Ser-Pro and Thr-Pro with not more than 11 amino acid residues between consecutive proline residues. They had an (A/S/T)_P count ≥2 and did not possess repeats associated with the closely related extensins or PRPs (such as multiple Ser-Pro3 and Ser-Pro4 separated by Tyr, Lys and Val residues). All AGPs which possessed the fasciclin conserved domain were classified as FLAs. Classical AGPs were selected on the basis of possessing >50% PAST, longer than 75 amino acids in length and did not possess any conserved domain other than the GPI anchor.

The AG peptides were classified on the basis of having >35% PAST and amino acid length of between 50 and 75. The lysine-rich AGPs were identified from the classical AGPs since their PAST had to be >50%. These were proteins with at least 5 lysine residues per stretch of 16 residues as determined by the Largest_K_count_for_16_mers program. The eNod AGPs were grouped based on the presence of the conserved domains characteristic to this group. AGPs which did not possess any conserved domain and did not fit the criteria for being classified under either classical or AG peptides yet possessed enough attributes to be identified as AGP encoding genes were classified as non-classical AGPs.

S1.3.3 Phylogenetic, ortholog and paralog relationships within the grapevine AGP family

A phylogenetic tree of the grapevine AGPs was constructed in CLS Sequence Viewer 7.6.1 to get a general overview of gene relatedness. An ortholog and paralog detection code was then utilised to determine evolutionary relatedness within the grapevine AGP family at an *e*-value threshold of 0.00001. This resulted in gene clusters of VviAGPs sharing possible common ancestry.

S1.3.4 Global expression analysis of VviAGPs in all organs

The grapevine expression atlas (GSE36128) (Fasoli *et al*., 2012) was utilised to study the expression patterns of the grapevine AGP family in different organs. It is based on 54 Corvina samples from floral, green and woody tissues at different developmental stages. SIMCA 13.0.2 (Umetrics, Sweden) was used to statistically analyse and visualise any groupings within the dataset using univariate scaling. The raw expression values were log2 transformed and mean centred before being subjected to hierarchical clustering using Gene Cluster 3.0. The generated heatmaps were viewed and analysed for developmental and tissue specific patterns in Java TreeView.

S1.3.5 Berry specific VviAGP expression analysis

The expression of VviAGPs in berries was also studied in 10 Italian grape cultivars harvested at fruit set (FS), pre-veraison, post-veraison and ripe stages. The expression data was kindly provided by

Prof. Mario Pezzotti through our collaboration with the Department of Biotechnology, University of Verona, Italy. A summary of the cultivars is given in **Supplementary Table 1**. SIMCA 13.0.2 (Umetrics, Sweden) was used to statistically analyse and visualise any groupings within the dataset. The raw expression values were log2 transformed and mean centred before being subjected to hierarchical clustering using Gene Cluster 3.0. The generated heatmaps were viewed and analysed in Java TreeView.

Supplementary Table 1. Summary of Italian grapevine cultivars whose AGP berry expression patterns were analysed.

Cultivar	Colour of grapes	Developmental stages
Sangiovese	Red	Fruit set, pre-veraison, post-veraison, ripe
Barbera	Red	Fruit set, pre-veraison, post-veraison, ripe
Negroamaro	Red	Fruit set, pre-veraison, post-veraison, ripe
Refosco	Red	Fruit set, pre-veraison, post-veraison, ripe
Primitivo	Red	Fruit set, pre-veraison, post-veraison, ripe
Vermentino	White	Fruit set, pre-veraison, post-veraison, ripe
Garganega	White	Fruit set, pre-veraison, post-veraison, ripe
Glera	White	Fruit set, pre-veraison, post-veraison, ripe
Moscatobianco	White	Fruit set, pre-veraison, post-veraison, ripe
Passerina	White	Fruit set, pre-veraison, post-veraison, ripe

S1.4 RESULTS

S1.4.1 A total of 57 AGP encoding genes identified from grapevine genome

Based on all of the selection criteria mentioned earlier, a total of 57 putative AGPs were identified from the grapevine genome and classified into six subfamilies (**Supplementary Table 2 and 3**). They all possess an N-terminal signal peptide and 26 possess a C-terminal GPI addition sequence.

Supplementary Table 2. Summary of results obtained in the identification and classification of grapevine AGPs.

Subfamily	Total AGP genes	Signal peptide	GPI anchor	Length (a.a)	% PAST
Classical	11	11	3	>75	>50
Lys-rich	5	5	1	>75	>50
eNod-like	3	3	2	200-350	>35
AG peptides	20	20	11	50-75	>35
Non-classical	1	1	0	80-90	<50
FLAs	17	17	9	200-470	>35

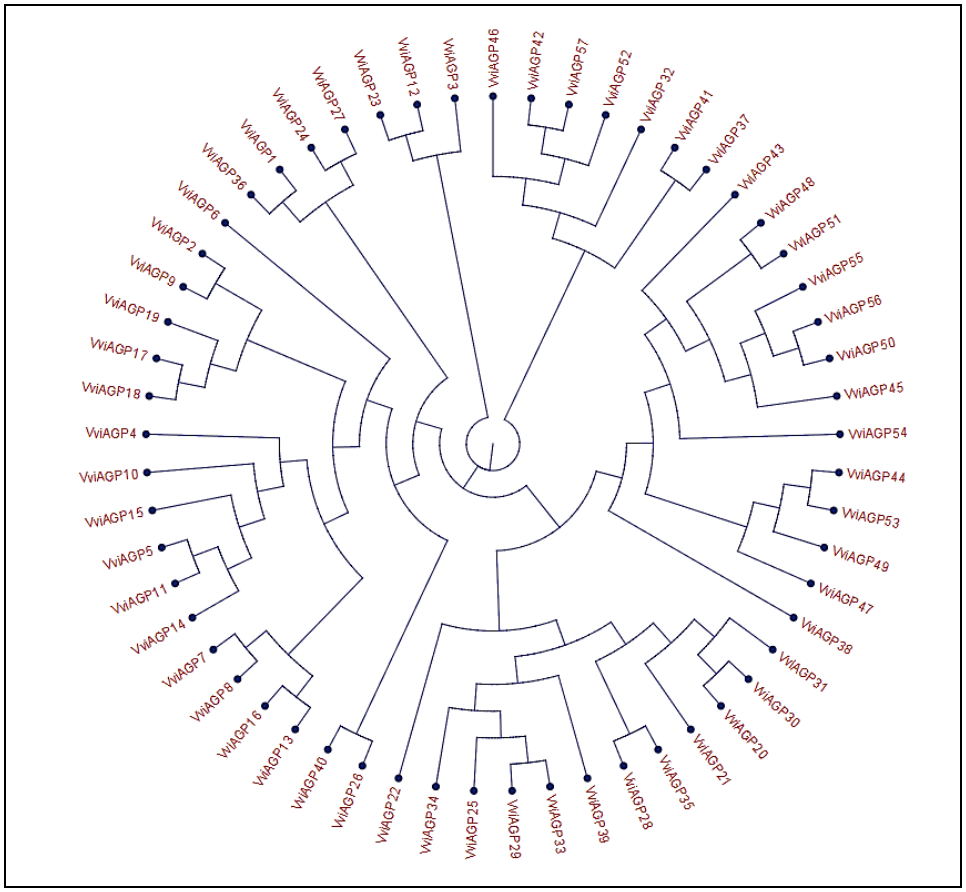
Supplementary Table 3. Identification and classification of AGP encoding genes from the grapevine genome

Gene ID (V1)	Code	Subfamily	Amino acid Length	Signal sequence	GPI anchor	(A/S/T)_P count	Largest_K_count_ for_16mers
VIT_10s0003g02720	VviAGP1	Classical	292	Yes	Yes	22	1
VIT_00s0407g00050	VviAGP2	Classical	134	Yes	No	5	1
VIT_08s0040g02000	VviAGP3	Classical	78	Yes	No	3	1
VIT_09s0054g00510	VviAGP4	Classical	222	Yes	No	17	2
VIT_06s0004g01990	VviAGP5	Classical	211	Yes	No	23	2
VIT_05s0077g01460	VviAGP6	Classical	167	Yes	No	8	2
VIT_00s0533g00050	VviAGP7	Classical	177	Yes	Yes	28	0
VIT_18s0001g06560	VviAGP8	Classical	138	Yes	Yes	24	1
VIT_00s0199g00010	VviAGP9	Classical	137	Yes	No	6	1
VIT_09s0002g09100	VviAGP10	Classical	452	Yes	No	37	3
VIT_06s0004g01980	VviAGP11	Classical	214	Yes	No	32	2
VIT_00s0174g00330	VviAGP12	Lys-rich	433	Yes	No	26	5
VIT_01s0011g02500	VviAGP13	Lys-rich	275	Yes	No	43	5
VIT_18s0166g00250	VviAGP14	Lys-rich	272	Yes	No	22	6
VIT_05s0049g00520	VviAGP15	Lys-rich	217	Yes	No	15	5
VIT_07s0129g00560	VviAGP16	Lys-rich	235	Yes	Yes	39	6
VIT_09s0002g06900	VviAGP17	eNod-like	320	Yes	No	40	1
VIT_12s0059g02640	VviAGP18	eNod-like	232	Yes	Yes	22	1
VIT_19s0014g02460	VviAGP19	eNod-like	341	Yes	Yes	47	3
VIT_16s0050g02310	VviAGP20	AG peptides	67	Yes	Yes	3	0
VIT_04s0008g00700	VviAGP21	AG peptides	66	Yes	Yes	4	1
VIT_08s0007g07980	VviAGP22	AG peptides	61	Yes	Yes	2	2
VIT_00s0204g00070	VviAGP23	AG peptides	67	Yes	No	3	1
VIT_09s0002g02950	VviAGP24	AG peptides	74	Yes	No	6	0
VIT_08s0007g08010	VviAGP25	AG peptides	67	Yes	Yes	3	1
VIT_17s0000g00680	VviAGP26	AG peptides	72	Yes	No	4	2
VIT_09s0002g02970	VviAGP27	AG peptides	73	Yes	No	6	0
VIT_08s0007g07330	VviAGP28	AG peptides	60	Yes	Yes	3	1
VIT_08s0007g08000	VviAGP29	AG peptides	67	Yes	Yes	4	1
VIT_15s0046g01360	VviAGP30	AG peptides	75	Yes	No	4	0
VIT_11s0016g02760	VviAGP31	AG peptides	65	Yes	Yes	4	0
VIT_14s0036g00320	VviAGP32	AG peptides	71	Yes	No	2	5
VIT_08s0007g07990	VviAGP33	AG peptides	67	Yes	Yes	4	1
VIT_08s0007g08020	VviAGP34	AG peptides	68	Yes	Yes	3	1
VIT_06s0004g00440	VviAGP35	AG peptides	59	Yes	Yes	3	2
VIT_05s0049g02150	VviAGP36	AG peptides	56	Yes	No	2	1
VIT_01s0127g00110	VviAGP37	AG peptides	58	Yes	No	2	1
VIT_03s0063g01600	VviAGP38	AG peptides	57	Yes	No	3	1
VIT_08s0040g01820	VviAGP39	AG peptides	70	Yes	Yes	3	1
VIT_01s0011g02680	VviAGP40	Non-classical	90	Yes	No	3	3

VIT_08s0032g00910	VviAGP41	Fasciclin-like AGPs	466	Yes	No	10	4
VIT_01s0011g06570	VviAGP42	Fasciclin-like AGPs	412	Yes	No	3	3
VIT_12s0028g02990	VviAGP43	Fasciclin-like AGPs	251	Yes	Yes	7	2
VIT_12s0057g01020	VviAGP44	Fasciclin-like AGPs	407	Yes	Yes	8	5
VIT_08s0040g02020	VviAGP45	Fasciclin-like AGPs	259	Yes	No	6	3
VIT_01s0011g01980	VviAGP46	Fasciclin-like AGPs	353	Yes	No	9	2
VIT_09s0018g01330	VviAGP47	Fasciclin-like AGPs	312	Yes	Yes	22	4
VIT_08s0040g01970	VviAGP48	Fasciclin-like AGPs	247	Yes	Yes	10	3
VIT_15s0048g01750	VviAGP49	Fasciclin-like AGPs	416	Yes	No	28	3
VIT_08s0040g02010	VviAGP50	Fasciclin-like AGPs	243	Yes	Yes	8	3
VIT_08s0040g02070	VviAGP51	Fasciclin-like AGPs	239	Yes	Yes	8	4
VIT_01s0011g06650	VviAGP52	Fasciclin-like AGPs	403	Yes	No	9	1
VIT_19s0015g00530	VviAGP53	Fasciclin-like AGPs	417	Yes	No	13	4
VIT_12s0059g00570	VviAGP54	Fasciclin-like AGPs	254	Yes	Yes	15	4
VIT_08s0040g01980	VviAGP55	Fasciclin-like AGPs	274	Yes	Yes	13	4
VIT_08s0040g01990	VviAGP56	Fasciclin-like AGPs	254	Yes	Yes	10	4
VIT_01s0011g06640	VviAGP57	Fasciclin-like AGPs	392	Yes	No	7	2

S1.4.2 Phylogenetic, ortholog and paralog similarities within the grapevine AGP family

Phylogenetic analysis of the grapevine AGPs did not show any groupings based on sub-family classification or chromosome location (**Supplementary Figure 2**).



Supplementary Figure 2. Circular cladogram of grapevine AGPs generated in CLC Sequence Viewer.

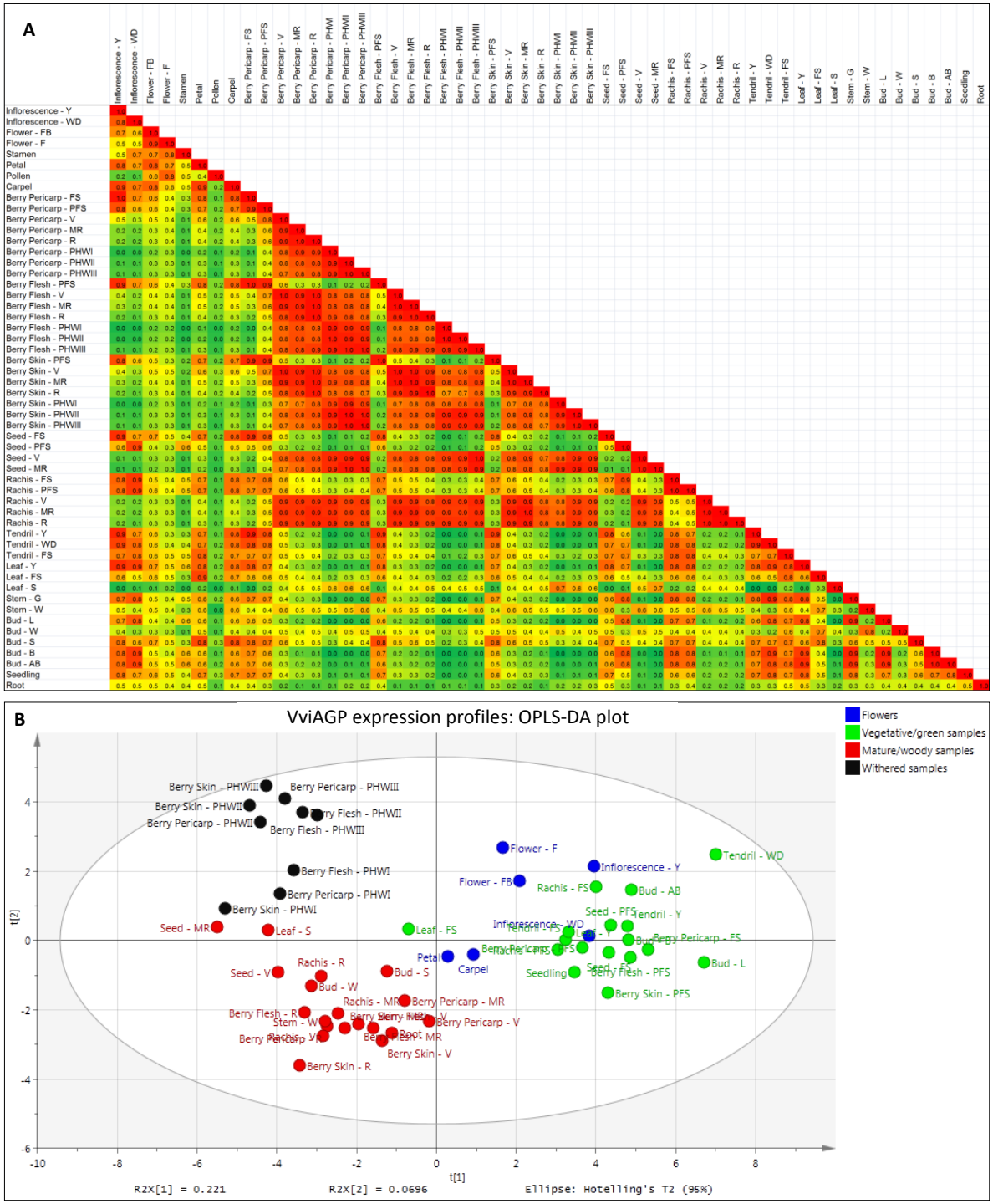
Using the ortholog and paralog detection code, eight clusters of AGPs based on similarity were obtained at an *e*-value threshold of 0.00001. Each of the resulting groupings had between 2 and 14 members, with 35 of the 57 identified AGPs being in a particular cluster (**Supplementary Table 4**). Thus 22 of the AGPs identified in this study did not share any orthologous or paralogous similarity to any other member of the super-family. Apart from cluster 1, all clusters were made up of AGPs from the same subfamily. However not all AGPs from the same subfamily clustered together. None of the lys-rich and non-classical AGPs clustered with any other AGP. This indicated that though some AGPs share high levels of amino acid sequence similarity, they do not necessarily share an evolutionary genetic background.

Supplementary Table 4. Ortholog and paralog clustering of grapevine AGPs. Genes that do not appear on the list did not share any similarity with any other AGPs at an *e*-value of 0.00001.

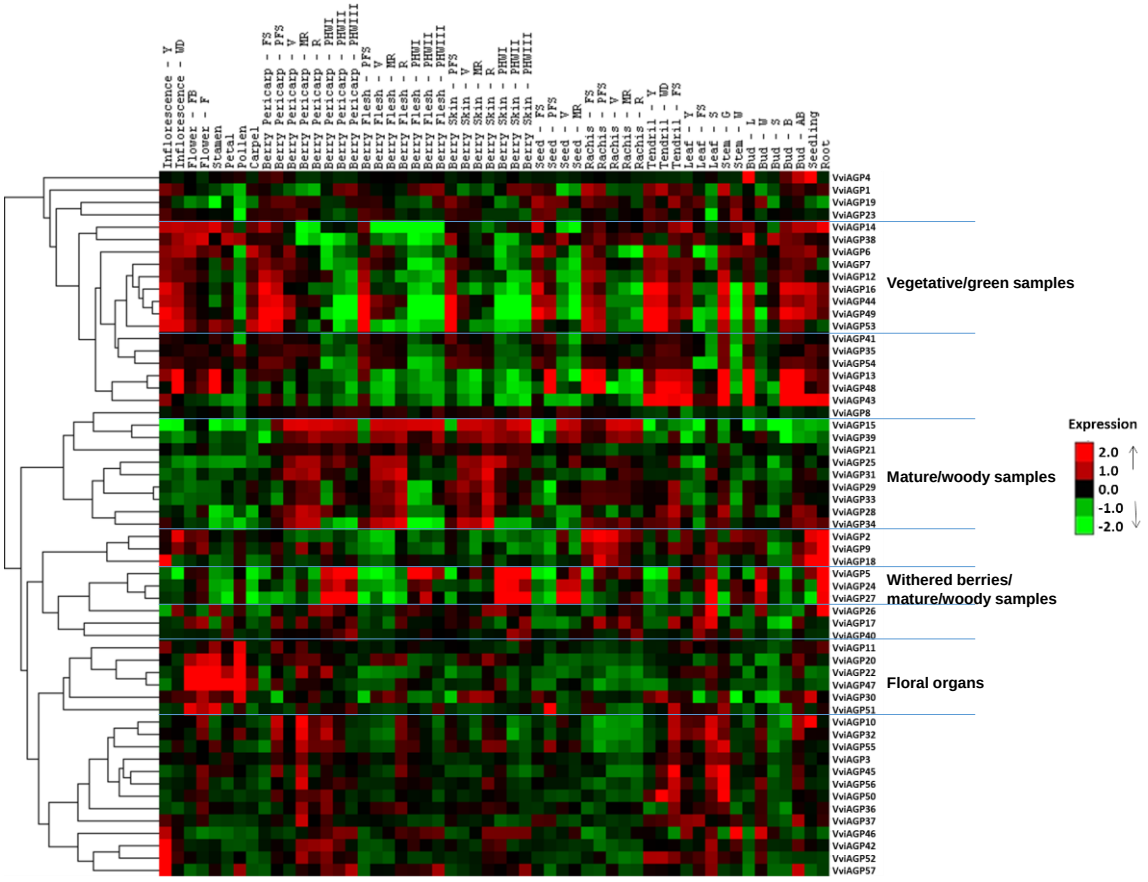
Cluster	Gene Code	Subfamily	Cluster	Gene Code	Subfamily
1	VviAGP3	Classical	**3**	VviAGP42	Fasciclin-like AGPs
1	VviAGP41	Fasciclin-like AGPs	**3**	VviAGP46	Fasciclin-like AGPs
1	VviAGP43	Fasciclin-like AGPs	**3**	VviAGP52	Fasciclin-like AGPs
1	VviAGP44	Fasciclin-like AGPs	**3**	VviAGP57	Fasciclin-like AGPs
1	VviAGP45	Fasciclin-like AGPs	**4**	VviAGP20	AG peptides
1	VviAGP47	Fasciclin-like AGPs	**4**	VviAGP30	AG peptides
1	VviAGP48	Fasciclin-like AGPs	**4**	VviAGP31	AG peptides
1	VviAGP49	Fasciclin-like AGPs	**5**	VviAGP17	eNod-like
1	VviAGP50	Fasciclin-like AGPs	**5**	VviAGP18	eNod-like
1	VviAGP51	Fasciclin-like AGPs	**5**	VviAGP19	eNod-like
1	VviAGP53	Fasciclin-like AGPs	**6**	VviAGP24	AG peptides
1	VviAGP54	Fasciclin-like AGPs	**6**	VviAGP27	AG peptides
1	VviAGP55	Fasciclin-like AGPs	**7**	VviAGP28	AG peptides
1	VviAGP56	Fasciclin-like AGPs	**7**	VviAGP35	AG peptides
2	VviAGP25	AG peptides	**8**	VviAGP2	Classical
2	VviAGP29	AG peptides	**8**	VviAGP9	Classical
2	VviAGP33	AG peptides			
2	VviAGP34	AG peptides			
2	VviAGP39	AG peptides			

S1.4.3 Expression analysis of AGPs in grapevine atlas shows developmental regulation in all organs

The four classes mentioned in Fasoli *et al*., 2012 (floral, vegetative/green, mature/woody and withered samples) were utilised to study possible groupings within the VviAGP expression dataset. Comparisons of the VviAGP transcriptomes from each of the 54 samples were conducted using a Pearson's distance correlation matrix (**Supplementary Figure 3A**). There was a clear difference between vegetative/green and mature/woody samples. The mature/woody samples show a strong correlation whilst the pollen transcriptome showed the least resemblance to other non-floral samples. This trend was confirmed on an OPLS-DA scatter plot (**Supplementary Figure 3B**) where the AGP expression data separated into the four developmental classes with a few overlaps. There were no distinct groupings based on organ identity in the dataset. Hierarchical clustering of the expression data (**Supplementary Figure 4**) further highlighted the different AGPs which are upregulated in different organs and with high developmental regulation. For example, VviAGP16 is upregulated in all vegetative/green tissues whilst VviAGP25 and VviAGP39 are upregulated in mature/woody and withered samples. Interestingly some AGPs such as VviAGP47 are only upregulated in floral organs, thus showing signs of organ specificity.



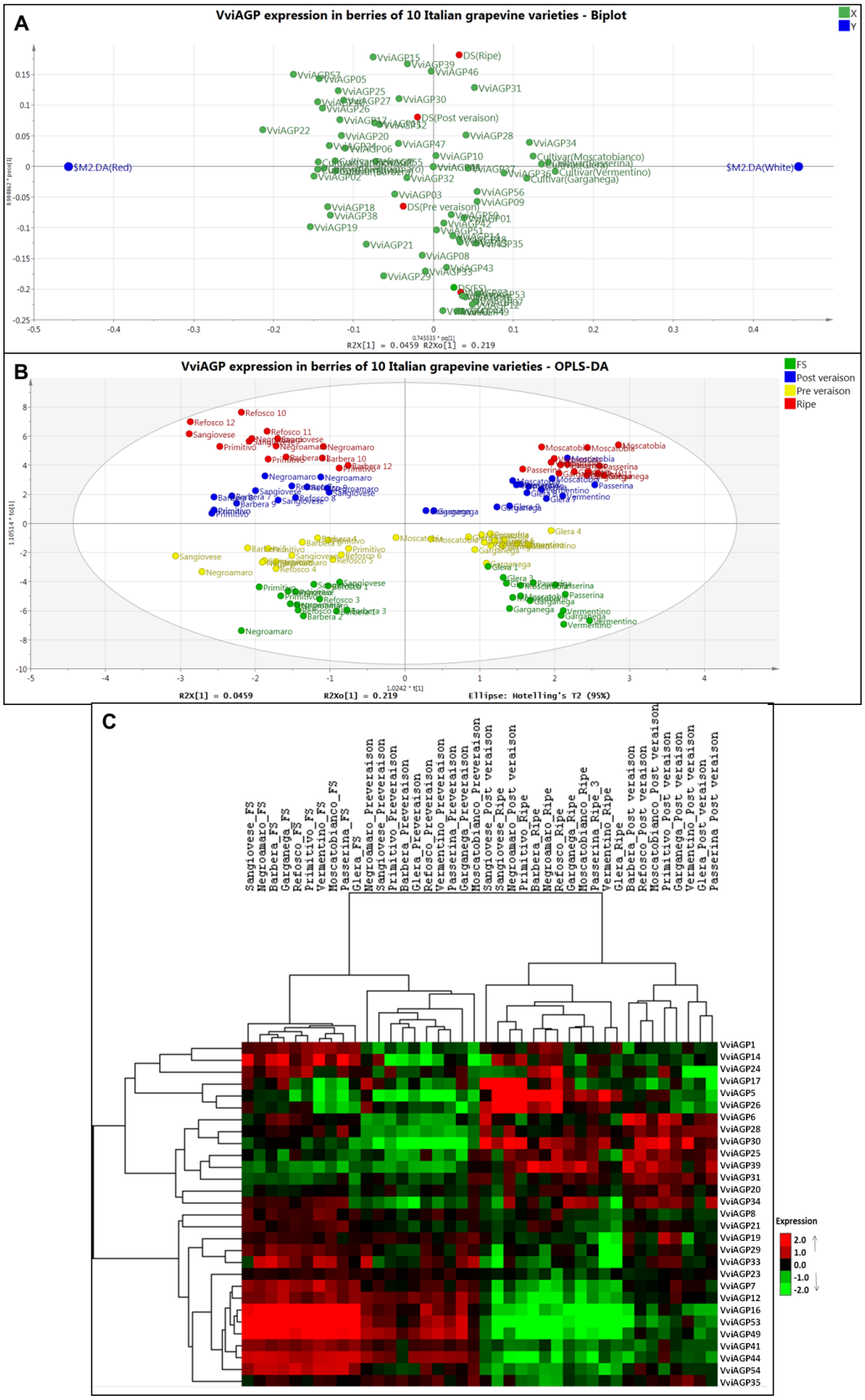
Supplementary Figure 3. (**A**) Correlation matrix of the AGP transcriptome data using the averaged expression values of the biological replicates. Analysis was performed in Excel and Pearson's distance was used as the metric. Red - strong relationship; yellow - low relationship; green - no relationship. (**B**) Scores scatter plot of the OPLS-DA model applied to all 54 samples generated in SIMCA. Outlying observations were excluded from the analysis.



Supplementary Figure 4. Heatmap showing expression patterns of VviAGPs across all 54 samples in the grapevine atlas dataset highlighting organs upregulated in some clusters.

S1.4.4 Developmental regulation of VviAGPs in berries is not cultivar specific

There was a clear separation between green and red grapevine varieties on an OPLS-DA plot of VviAGP expression in berries from 10 Italian cultivars (**Supplementary Figure 5A**). The colour of the grapes pulled the data apart on the x-axis whilst developmental stage caused separation on the y-axis. The loadings plot (**Supplementary Figure 5B**) showed that development stage (y-axis) was the main driver of separation in the whole dataset. Subjecting the expression data to hierarchical clustering revealed that 29 VviAGPs were expressed in berries of all 10 cultivars studied (**Supplementary Figure 5C**). High levels of developmental regulation similar to the grapevine atlas data were observed. For example, VviAGP2 and VviAGP4 were not expressed in either Corvina berries or the other 10 grapevine cultivars. VviAGP16 was upregulated in berries at fruit set in both datasets whilst VviAGP25 and VviAGP39 were upregulated in mature berries in both datasets.



Supplementary Figure 5. (**A**) Scores scatter plot of the OPLS-DA model showing VviAGP expression patterns in five red and five white Italian grapevine cultivars. There is a separation between the red and grape cultivars and developmental stage within each grouping. (**B**) The loadings plot shows that the main driver of separation is developmental stage, not the colour or cultivar of berries. (**C**) The heatmap shows hierarchical clustering based on developmental stage, not cultivar. Data was filtered in Cluster to remove all genes with less than 100% presence across all samples.

S1.5 DISCUSSION

Advances in biotechnology such as the sequencing of whole plant genomes and the availability of bioinformatics tools have made it possible to conduct *in silico* studies of complex gene families such as the HRGPs. In this study, we investigated a sub-family of the HRGPs, the AGPs, in the grapevine genome at the protein composition and gene expression level. Numerous bioinformatics tools were applied to identify features which are specific to this group of proteins. The same tools have been utilised in the identification of AGPs in *Arabidopsis*, poplar, rice and tomato (Shultz *et al*., 2002; Ma & Zhao, 2010; Showalter *et al*., 2010: 2016; Fragkostefanakis *et al*., 2012). We managed to identify a total of 57 putative AGPs and classify them into six different sub-families based on their amino acid composition and conserved domain constitution.

In an effort to understand how the grapevine AGPs are related to each other from an evolutionary perspective, gene orthologs and paralogs were identified and utilised for cluster analysis. This approach has the potential to provide useful information from a functional perspective, though the functions of the grapevine AGPs remain to be tested (Tan *et al*., 2012). AGPs within a particular subfamily did not necessarily all cluster together except for the eNod-like AGPs. This suggested that despite possessing the same conserved domains, e.g. the fasciclin motif, the proteins do not necessarily share the same evolutionary background and thus could potentially also exhibit diverse functions. Similar analysis has not been documented for other plant species where AGP families have been characterised, thus it is currently not clear if this trait is specific to VviAGPs. However, the functional diversity would not be surprising since AGPs from the same subfamily can be found in different localisations within the plant cell and also different tissues within the same plant (Herman & Lamb, 1992; Showalter, 2001). In rose plants, AGPs with different protein composition, size and hydrophobicity were identified from the plasma membrane (Serpe & Nothnagel, 1996). This is an indication that there is co-existence of AGPs from different sub-families within the same cellular space. Whether or not there is an overlap in function as well is still highly elusive. In our current work, localisation studies are required in order to determine if this pattern holds true for the VviAGPs as well.

Organ-specific expression profiles of VviAGPs

AGPs are known to be expressed in numerous plant organs such as leaves, stems, roots, seeds, floral structures and in tissues such as the xylem (Showalter, 2001; Pereira *et al*., 2015). Out of the 34 AGPs identified in tomato, 20 were expressed in fruit (Fragkostefanakis *et al*., 2012) whilst in rice, OsFLA14 (Fasciclin protein 14 from *Oryza sativa*) was only expressed in the later stages of panicle development (Ma and Zhao, 2010). Our data mining results showed that some grapevine AGPs e.g. VviAGP47 were upregulated mainly in Corvina floral organs. Within the organs, there were also differences in gene expression patterns based on organ age. For example, VviAPG14 was expressed in green vegetative tissue whilst VviAGP34 was expressed in mature/woody tissue. In leaves,

VviAGP12 and 16 were expressed in young leaves whilst VviAGP24 and 27 were expressed in senescing leaves. These trends could be indicative of VviAGPs with different roles within the same organ.

Organ and tissue specific AGP expression patterns have been observed in numerous other plant backgrounds. In cotton, *GhAGP31* was detected in ovules, roots and hypocotyls but very little or nothing was detected in cotyledons, petals, anthers and leaves (Gong *et al*., 2012). A tomato AGP was detected in high levels in young leaves, roots and fruit with very low levels in older, wounded or infected organs (Pogson & Davies, 1995). In pine, two AGPs were detected in high abundance in differentiating xylem tissue whilst very little or nothing was detected in embryos, needles and megagametophytes (Loopstra & Sederoff, 1995). In *Arabidopsis*, organ-specific AGP expression patterns were observed with some genes being expressed in roots, others in pollen, stems and stamens separately (Showalter *et al*., 2010). Rice, poplar and tomato AGPs also exhibited organ-specific expression patterns (Ma & Zhao, 2010; Fragkostefanakis *et al*., 2012; Showalter *et al*., 2016). It would thus be interesting to ascertain the functional roles of the organ specific VviAGPs.

Developmentally regulated expression of VviAGPs and their potential roles in plant defence

AGPs are also known for their developmentally regulated expression patterns (Showalter, 2001). The Corvina expression patterns showed high levels of developmental regulation in different organs. The trends were confirmed in berries of 10 Italian cultivars. A list of plant species, organs and the appropriate references where developmentally regulated AGP expression patterns were detected is listed in Showalter, (2001). There have been other recent studies which have also elucidated the developmentally regulated expression patterns of AGPs (Negri *et al*., 2011; Zhang *et al*., 2011; Costa *et al*., 2013; Moore *et al*., 2014).

The developmental regulation of AGPs in berries is very interesting. More-so when taken together with the accumulation of AGP epitopes in berries during ripening (Moore *et al*., 2014). The potential role that the grapevine AGPs could play in the ripening process requires further investigation. It is however possible that the AGPs are not actively involved in the ripening process but rather, they accumulate during veraison when the fruit becomes more susceptible to a wide array of bacterial, viral and fungal infections (Ficke *et al*., 2002; Gadoury *et al*., 2003). For example, young green berries are highly resistant to the necrotrophic fungus *B. cinerea* whilst mature, ripening berries exhibit higher levels of susceptibility characterised by high levels of infections between veraison and berry ripening (Kretschmer *et al*., 2007; Deytieux-Belleau *et al*., 2009).

It cannot be ruled out that one of the functional roles of the AGPs in berries could be potentially similar in effect to that of volatile organic compounds like the sesquiterpene β-carophyllene, which are also known to increase in abundance in grape berries at pre-harvest stage (Lucker *et al*., 2004). β-carophyllene has strong antifungal activity against *Botrytis cinerea* spore germination and hyphal growth (Zhang *et al*., 2008). Therefore, the accumulation of AGPs during

ripening could play a significant role in grapevine pre-formed ontogenic defence response against different types of stress.

Our speculations are supported by numerous studies which have associated AGPs with plant defence responses against both biotic and abiotic stresses (Deepak *et al*., 2010). Grapevine roots infected with the phylloxera parasite (*Daktulosphaira vitifolia* Fitch) exhibited an up-regulation of an AGP encoding gene (Du *et al*., 2014). In cotton, cold stress during early stages of seedling development resulted in up-regulation of a cell wall non-classical AGP gene, *GhAGP31* in roots. Furthermore, overexpression of *GhAGP31* in *Arabidopsis* significantly enhanced the cold tolerance of the transgenic seedlings (Gong *et al*., 2012). In a separate study, tobacco cells exposed to salt stress also displayed an up-regulation of a classical AGP (Lamport *et al*., 2006). Cabbage plants exposed to heat treatment accumulated significant levels of AGPs coupled with an increase in H_2O_2, suggesting a role of this group of proteins in enhancing thermotolerance (Yang et al., 2006). Also, some of the AGPs from tomato were constitutively expressed under hypoxia whilst others were upregulated under anoxia (Fragkostefanakis *et al*., 2012).

These and other studies have elucidated the role of AGPs in plant defence in general, but the exact mechanism is however not completely clear. It could be similar to AGP31 from *A. thaliana*, through interaction with galactans and methylesterified polygalacturonic acid in the cell wall matrix, forming a dense interconnected network which acts as a reinforced physical barrier to pathogen entry (Hijazi *et al*., 2014). Or the AGPs could be interconnected to pectin, masking it from pathogens such as *B. cinerea* which require pectin as a substrate for growth (Tan *et al*., 2013). There is also evidence that AGP31 can also form self-aggregates with a potential role in cell wall assembly and strengthening (Hijazi *et al*., 2014). All these mechanisms have the potential to prolong the time it takes for a pathogen to penetrate the berry surface or for environmental factors such as very low temperatures to cause significant damage to the berry. This would ultimately give the host plant ample time to recognise the invader or dangerous environmental condition and mount defence responses before much damage is done. An in-depth study of the cell walls of different grapevine organs at different developmental stages is however required to determine if these interactions occur during ripening.

Summary and conclusion

The novel identification, classification, phylogenetic and expression analysis of grapevine AGPs in this study provides valuable insights into the characteristics of the family. The developmental regulation and tissue specific expression patterns exhibited by some of the VviAGPs suggests specialised functional roles. Future work could include the functional characterisation of a selection of VviAGPs exhibiting interesting expression patterns.

***Acknowledgements*:** The work presented in this addendum was a collaborative effort. The initial generation of the list of putative grapevine AGP encoding genes from the grapevine genome was conducted by Dr Dan Jacobson (Institute for Wine biotechnology, Stellenbosch University). The grapevine gene atlas data together with the berry expression data from the ten Italian cultivars was kindly provided by Prof. Mario Pezzotti through our collaboration with the Department of Biotechnology, University of Verona, Italy. The classification of VviAGPs and the rest of the data analyses presented in this chapter was conducted by Miss Mukani Moyo under the supervision of Prof. Melane A. Vivier (Institute for Wine Biotechnology, Stellenbosch University).

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Chapter 6

Research results

A first overview analysis of a Grapevine-*Botrytis* leaf interactome study using a transgenic PGIP and control population

A first overview analysis of a Grapevine-*Botrytis* leaf interactome study using a transgenic PGIP and control population

6.1 ABSTRACT

A transcriptomic plant-pathogen interaction study was conducted to decipher the molecular basis of a strain specific hyper-susceptible phenotype caused by a *Botrytis* strain isolated from grapevine on transgenic grapevines constitutively expressing polygalacturonase-inhibiting proteins (PGIPs). This interactome analysis was conducted to study the infection and defence progression from both the fungal and plant perspectives within the first 48 hours post infection on the leaves. Here we present the first overview of the results to provide credence to the characterisation already performed to understand this phenotype and/or expand our understanding of this interesting host-pathogen specific interaction. It was clear that the *B. cinerea* grape strain utilised BcPG1 as part of its infection machinery, but not BcPG2. There was also an induction of genes involved in galacturonic acid catabolism and break-down of different cell wall components; these expression patterns did not differ between the transgenic and control plants. However, expression patterns of genes involved in the synthesis of the phytotoxic botrydial and botcinic acid were higher in the strains that infected the transgenic plants compared to the controls. In grapevine, *Vvipgip1* was expressed in both control and transgenic plant lines during infection, however, it did not display expression profiles characteristic of gene upregulation in response to infection. Further analysis revealed relatively higher levels of other defence-related genes from 24 to 48 hours post infection in infected control plants compared to the transgenic population. These included pathogenesis related proteins, stilbene synthases and phenylalanine ammonia lyase. Collectively the preliminary overview supports the idea that the PGIP overexpression altered the plant metabolism, possibly in a priming mechanism that actually benefit the grape strain, which seems to be able to activate strongly the ability to kill the host tissues through toxin production and perhaps even modulate the host defence mechanism.

6.2 INTRODUCTION

B. cinerea is one of the most extensively studied fungal pathogens due to its wide host range which includes more than 1 400 plants from cultivated and economically important fruit crops, ornamental flowers and vegetables (Williamson *et al*., 2007; Elad *et al*., 2016). Infection of plant tissue such as fruits, stems, leaves and flowers typically results in irreversible damage leading to pre and postharvest losses. A lot of studies have focused on identifying *B. cinerea* pathogenicity factors in an effort to understand the pathogen better (Nakajima and Akutsu, 2014).

Its infection strategy has been shown to start as early as pre-penetration, where conidial germination is accompanied by an upregulation of infection-related genes (Leroch *et al*., 2013). Upon landing on host tissue, *B. cinerea* induces mitogen activated protein kinases (MAPKs), signalling compounds for the

transduction of extracellular signals. The perception of these host-derived signals is believed to trigger conidial germination and appresorium formation (Schamber *et al*., 2010). Several genes involved in the MAPK cascade have been identified and these include *Botrytis* MAP kinase required for pathogenesis (Bmp1) and serine/threonine protein kinases (Ste7 and Ste11). Knockout mutants in these genes display poor germination with low conidiation, delayed vegetative growth, low penetration efficiency and significant loss in pathogenicity (Doehlemann *et al*., 2006; Schamber *et al*., 2010).

After successful germination and appresorium formation, *B. cinerea* releases a combination of metabolites, enzymes and toxins to enable host takeover. These include cutinases to breach the cuticle layer (van Kan *et al*., 1997), endopolygalacturonases (BcPGs) and pectin methylesterases (BcPMEs) to break down the pectin backbone of host cell walls (Kars *et al*., 2005; ten Have *et al*., 1998; Vallette-Collet *et al*., 2003) and phytotoxins such as botrydial and botcinic acid to induce tissue collapse (Colmenaras *et al*., 2002; Siewers *et al*., 2005; Dalmais *et al*., 2011). Mutants in genes involved in the synthesis of these enzymes and toxins display significant reduction or complete loss of pathogenicity on numerous plant hosts with some of the pathogenicity factors exhibiting strain specificity (Vallette-Collet *et al*., 2003; Kars *et al*., 2005). It is the combined action of all the virulence factors which kills host cells. Being a necrotroph, *B. cinerea* utilises the dead plant tissue to acquire nutrients for growth and proliferation (van Kan, 2006).

Plants are able to sense the presence and damaging action of *B. cinerea* leading to induction of a cascade of host-specific defence responses (Jones and Dangl, 2006). These include strengthening the cell wall barrier by increasing lignin deposition (Denness *et al*., 2011); rapid accumulation of reactive oxygen species (ROS) to induce oxidative stress in *B. cinerea* whilst triggering programmed cell death at the site of infection (Aziz *et al*., 2004); production of toxic phytoalexins which inhibit fungal growth (Timperio *et al*., 2012) and production of pathogenesis-related (PR) proteins with antimicrobial activity (Dadakova *et al*., 2015). Marker genes for the various defence mechanisms have been identified and their expression profiles are usually indicative of active defence responses.

Previous studies focused on either the fungal or plant transcriptome separately during infection to study fungal infection strategies or plant defence mechanisms. However, with the advent of advanced molecular tools, whole transcriptomic studies of host-pathogen interactions are becoming more common (Kelloniemi *et al*., 2015; Kong *et al*., 2015). The availability of sequenced genomes for both grapevine and *B. cinerea* enables interaction of the two organisms to be studied in detail (Jaillon *et al*., 2007; Amselem *et al*., 2011). Hence in this study, *Vitis vinifera* plants overexpressing non-*vinifera pgip*s were challenged with the hyper-virulent *B. cinerea* grape strain and a whole transcriptomic study of the interactome on the infected leaves was investigated. The transgenic population was previously shown to exhibit strain specific hyper-susceptibility to the *B. cinerea* grape strain which was also shown to be a host specific phenotype. Furthermore, it displayed evidence of compromised plant defence strategies before and during *B. cinerea* infection, which somehow rendered the transgenic population partially resistant to the biotroph, *Erysiphe necator*. From the fungal side, BcPG1 was shown to be important for *B. cinerea* pathogenicity on grapevine plants, with knock-out mutants displaying significantly reduced virulence on both control and transgenic

plants (Moyo, 2011; Chapter 4 and 5 of thesis). Therefore, the primary objective of the study was to decipher the molecular basis of the observed hyper-susceptible phenotype from both the pathogen and the host's perspectives. These preliminary results build on the foundation set by previous chapters of this thesis on understanding the dynamics of this unique host-pathogen pairing.

6.3 MATERIALS AND METHODS

6.3.1 *B. cinerea* fungal cultures

A *B. cinerea* grape strain isolated from a South African vineyard (as reported in Joubert *et al*., 2006) was grown on sterile apricot halves (Naturlite, Tiger Food Brands Limited, South Africa). The cultures were kept in a dark growth chamber at 23˚C until sporulation occurred. Spores were then harvested in sterile water and the spore suspension was plated on 0.8% (w/v) water agar in order to evaluate their viability and germination potential. The highly viable spores were then hydrated in sterile water overnight at 4˚C before being counted on a haemocytometer under the light microscope and diluted to the desired concentration in 50% grape juice prior to infection.

6.3.2 Grapevine whole plant infection assay

Eight to twelve week old potted, greenhouse-grown *V. vinifera* cv. Redglobe plants expressing *pgip*s from *V. x doaniana* Munson (PGIP1012) and *V. caribaea* (PGIP1038) were utilised for the whole plant antifungal assay. Untransformed Redglobe plants were included as controls. All plants were acclimatised in high humidity perspex chambers 24 hours prior to infection under a 16/8 hr light/dark cycle and maintained under the same conditions for the duration of the assay. A total of 30 plants displaying a synchronised growth pattern were selected. Six of the plants were controls whilst the remaining 24 were transgenic plant lines, 12 from the PGIP1012 population and 12 from the PGIP1038 population (**Table 1**).

Two fully grown leaves of relatively similar size per plant were infected with 8 spots of 1000 spores/spot *B. cinerea* grape strain suspension on the adaxial side. Leaf tissue was harvested at three time points: T0 samples were harvested immediately after infection, T24 samples at 24 hours post infection (hpi) and T48 samples at 48 hpi. Local response samples were collected at the site of infection at a radius of about 17 mm around the infection spot. Uninfected whole leaves adjacent to the infected ones were harvested at the same time as the local response samples to study the systemic response. Samples were collected from two plants per plant line for both local and systemic response and the biological replicates were kept separate. Upon sampling, all samples were immediately frozen in liquid nitrogen before being ground to a fine powder using a Retsch MM400 mixer mill (Retsch™ GmbH, Germany) for further analyses.

Table 1. Summary of plant population and samples collected at each time point. x = biological replicate.

Plant line	Time Point	Local response	Systemic response
Wildtype	T0	xx	xx
	T24	xx	xx
	T48	xx	xx
***pgip*1012-16**	T0	xx	xx
	T24	xx	xx
	T48	xx	xx
***pgip*1012-28**	T0	xx	xx
	T24	xx	xx
	T48	xx	xx
***pgip*1038-2**	T0	xx	xx
	T24	xx	xx
	T48	xx	xx
***pgip*1038-69**	T0	xx	xx
	T24	xx	xx
	T48	xx	xx

6.3.3 RNA extraction and cDNA library construction

Total RNA was extracted from ground leaf samples using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Pty Ltd, Johannesburg, South Africa) as per the manufacturer's instructions. A total of 100 mg of ground tissue per sample was used as input material. RNA integrity was determined on an Agilent 2100 Bioanalyzer using the eukaryote total RNA nano assay (Agilent Technologies, Santa Clara, CA95052, United States). Four micrograms of intact RNA were then utilised to construct 60 double stranded cDNA libraries using the KAPA Stranded mRNA-Seq Kit (Kapa Biosystems, South Africa), following the manufacturers protocol. The TrueSeq Indexed Adapter sequences were obtained online from the Illumina adapter sequence document (http://support.illumina.com/downloads/illumina-customer-sequence-letter.html) and custom synthesized by Integrated DNA Technologies, Inc. The indexed libraries were amplified on a thermal cycler, prior to library amplification clean-up. Each purified library was then analysed on a Bioanalyzer High Sensitivity DNA Assay to determine the fragment size distribution, concentration and overall quality.

6.3.4 RNA sequencing and bioinformatics pipeline

The cDNA libraries were combined in equal amounts for single-read sequencing (100 bp) at the Functional Genomics Center (University of Verona, Italy) on an Illumina HiSeq 2000 sequencing platform. The data generated was analysed using two separate bioinformatics pipelines (**Figure 1**). The first pipeline was executed in Chipster, a user friendly data analysis platform containing a comprehensive collection of NGS data analysis tools (Kallio *et al*., 2011). The quality of the sequenced reads, sequence content and base quality scores were checked using the FastQC tool.

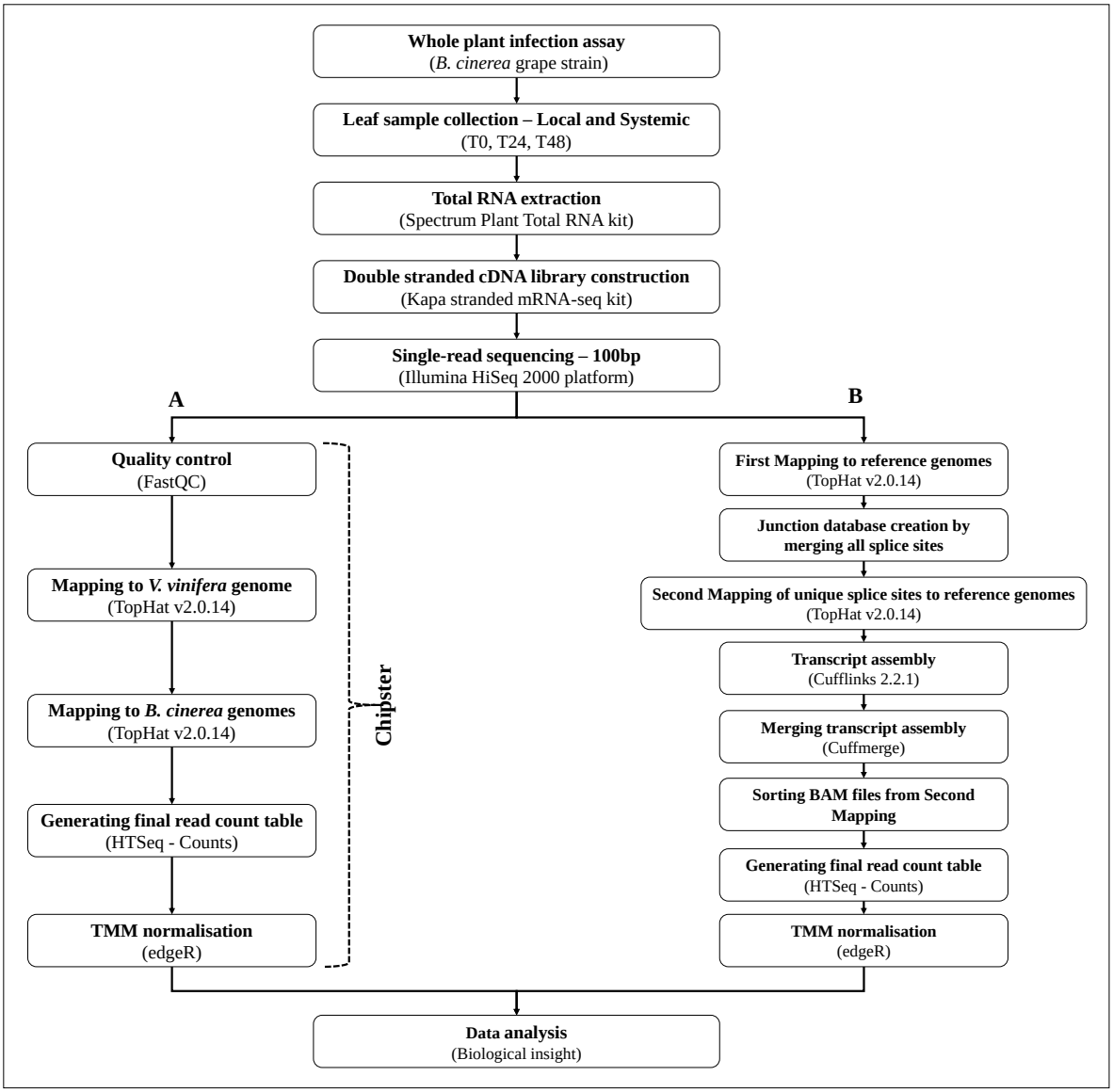


Figure 1. Flow chart of bioinformatics pipelines used to analyse the RNAseq data. The first approach (**A**) was executed in Chipster whilst the second approach (**B**) utilised a pipeline outlined in Trapnell *et al*., 2012.

The resulting high quality reads were aligned separately against the genome sequences of *Vitis vinifera L.* PN40024 (IGGP_12X.28; http://genomes.cribi.unipd.it/DATA/V1) and *Botrytis cinerea* B05.10 (ASM83294v1; http://fungi.ensembl.org/Botrytis_cinerea/Info/Index) using TopHat v2.0.14 (Trapnell *et al*., 2009). The number of reads mapping to each gene was calculated using HTSeq, and a final read count table was generated independently for reads mapping to the two different organisms. The raw count values were converted to counts-per-million (CPM) and normalized by the TMM (Trimmed Mean of M values) method using edgeR package, to eliminate composition biases between libraries.

In the second bioinformatics approach, a data analysis pipeline, as outlined in Trapnell *et al*., (2012), was utilised in collaboration with Dr Edoardo Bertolini (Institute of Life Sciences, Scuola Superiore Sant'anna University, Italy). The pipeline was included to identify gene or splice variants. The splicing site

details for all libraries obtained by first mapping to different genomes were merged independently and the unique splice site information was included in the following step for second mapping using TopHat. This was done to eliminate redundant and inaccurate information. The transcripts were then assembled from the mapped reads using Cufflinks assembly v2.2.1 (Trapnell *et al*., 2010). The assembled files were merged with reference transcriptome annotation in to a unified annotation using the Cuffmerge platform in Cufflinks (Trapnell *et al*., 2010), to produce a final transcriptome assembly. The final reads counts were calculated and the data was normalized as mentioned in the first pipeline. The normalised data was then subjected to principal component analysis (PCA) in Simca ver. 14 (MKS Data Analytics Solutions, Sweden). Differentially expressed (DE) genes were identified at FDR (false discovery rate) of 5% and subjected to Gene Ontology (GO) enrichment analysis in AgriGO v1.2 (Du *et al*., 2010) using the singular enrichment tool with hypergeometric scaling at $p \leq 0.05$. Redundant GO terms were trimmed using REVIGO (Supek *et al*., 2011).

6.4 RESULTS

6.4.1 Infection conditions were optimal for *B. cinerea* growth

The germination potential of the *B. cinerea* grape strain spores utilised for the antifungal assay was at least 90%. Approximately 98% of the infection spots developed into primary lesions after T24 and by T48 they had started developing into secondary spreading lesions (**Figure 2**). There were no notable visual differences at this stage in the onset of lesion formation on transgenic leaves compared to the untransformed controls.

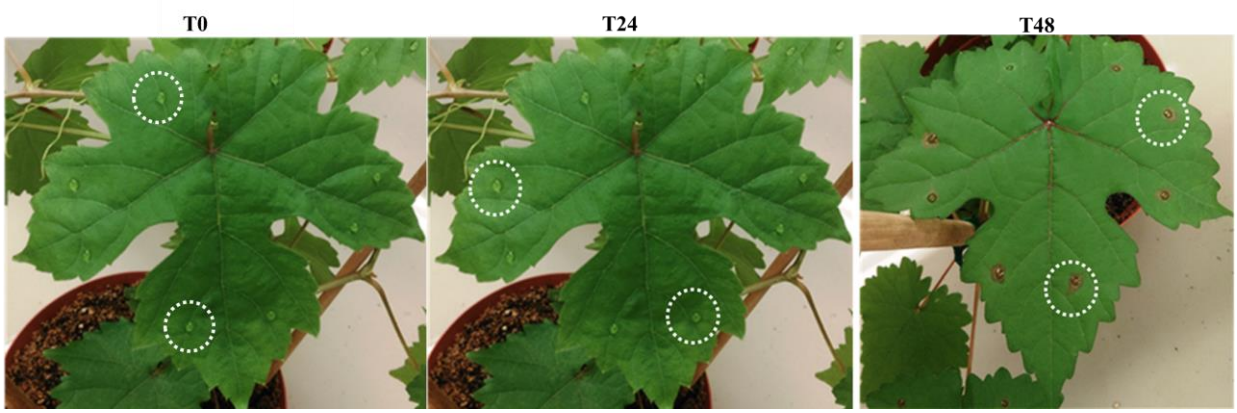


Figure 2. Representative images showing infection progression on grapevine leaves. Secondary lesions were visible at T48. The lesion/infection spot and its immediate surrounding (circled in white) were collected for local response.

6.4.2 RNA sequencing statistics

A total of 1,455,589,498 raw reads were generated by sequencing 60 RNA libraries. After removing the low quality reads, the remaining data was aligned to the *V. vinifera* and *B. cinerea* reference genomes separately and 75.5% of the total reads mapped uniquely to the grapevine genome whilst 0.4% mapped uniquely to the fungal genome (**Table 2**).

Table 2. Summary of mapped reads from grapevine plants infected with *B. cinerea*

Genome	Total input reads	Total number of reads uniquely mapped from 60 libraries	Percentage mapped
V. vinifera	1,455,589,498	1,098,437,729	75.5
B. cinerea	1,455,589,498	5,899,410	0.4

6.4.3 *B. cinerea* grape strain gene expression overview

The two separate analysis methods mentioned earlier (**Figure 1**) yielded the same gene expression trends. Thus the results presented are representative of both analyses. The gene expression data mapped to the *B. cinerea* genome was subjected to a multi-dimensional scaling (MDS) plot to visualise any similarities in expression profiles between local and systemic response samples. The local and systemic profiles clearly separated on the MDS plane, indicative of different gene expression profiles for the two treatments (**Figure 3A**). The systemic samples displayed negligible gene expression levels on the MDS plot (close to zero), which was expected since they came from uninfected grapevine leaves. Thus all gene expression data from the *B. cinerea* perspective presented henceforth is from the local response.

Expression profiles showed a close relationship between T24 and T48 profiles, separate from T0. Out of the 12 004 genes in the *Botrytis* genome, at T0 the total number of transcripts expressed on each plant line was between 600 and 1000. This number increased to between 7000 and 9000 at T24 and remained in the same range at T48 (**Figure 3B**). Unsupervised Principal Component Analysis (PCA) was used to summarise the relationships/associations among samples. The first and second components (P1 and P2), collectively best explained about 60% of the variation (**Figure 3C)**. P1 drove the separation on the vertical plane, with T0 clearly separating from T24 and T48, complementing the trends highlighted on the MDS plot for local response. Thus sampling time point was the main driver of separation and not the plant genotype.

The loadings scatter plot (**Figure 3D**) shows that the bulk of the *B. cinerea* genes contributed to the separation on P1 by pulling the data to the left. Unfortunately, due to the current poor annotation of the *Botrytis* genome, the identities of most of the genes could not be established in this preliminary analysis of the data. Also GO enrichment analysis of the differentially expressed genes was not informative (results not shown). Genes that have been functionally analysed in other studies were however, used to follow the infection strategy of *B. cinerea* grape strain on grapevine leaves.

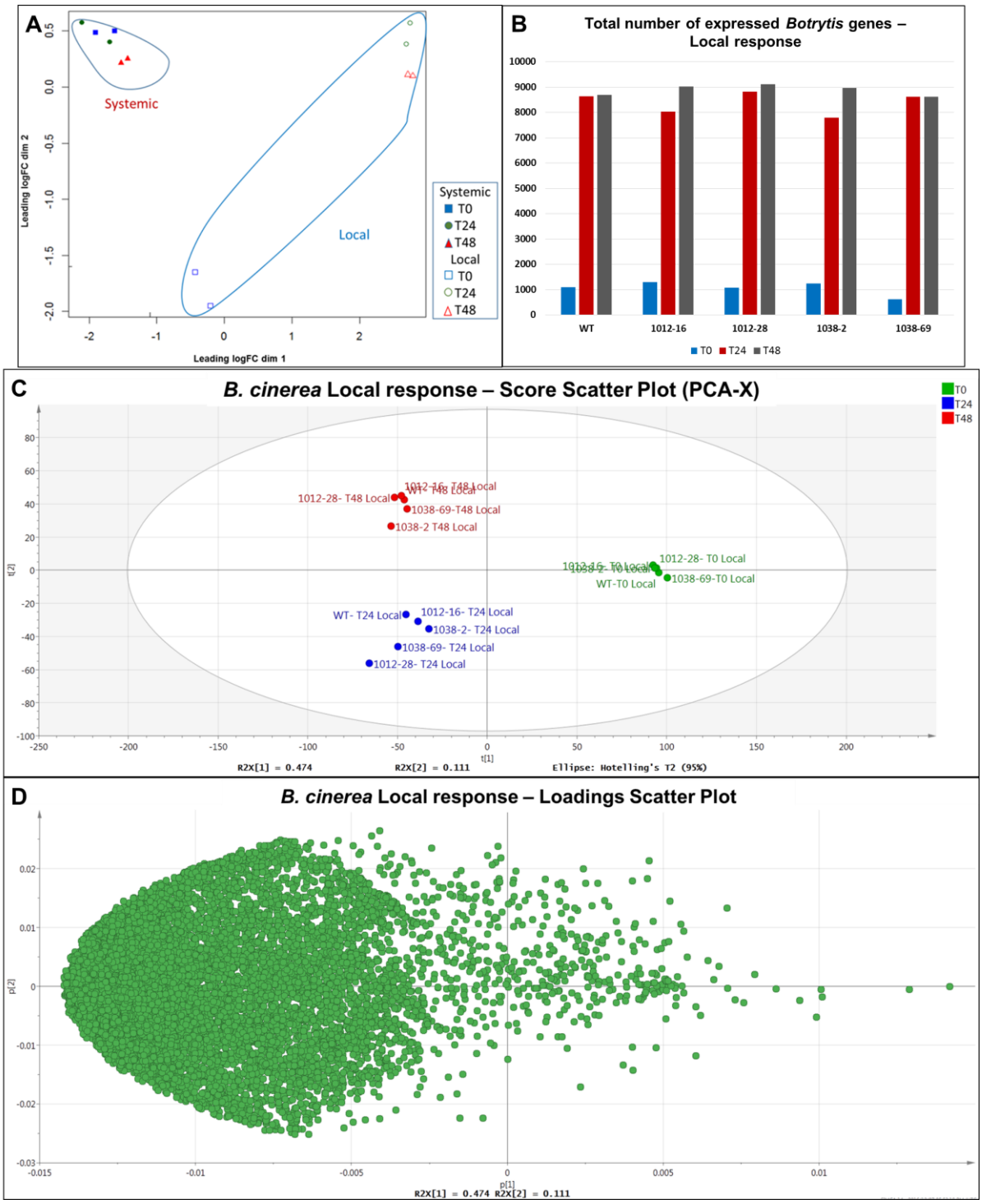


Figure 3. A. MDS plot showing differences in expression profiles within the *B. cinerea* dataset in two dimensions. Distance corresponds to leading logFC between each pair of samples **B**. Bar chart showing changes in total number of expressed transcript during the course of the infection per plant line. **C**. Scores scatter plot of *B. cinerea* local response expression profiles coloured according to infection time point. **D**. Loadings scatter plot showing a strong pull to the left on P1.

6.4.4 *B. cinerea* grape strain infection strategy

Genes involved in germination of *B. cinerea* and normal development of hyphae such as *Bmp1*, *Bcste11* and *Bccrz1* were strongly upregulated at T24 on all plant lines. A cutinase gene, *BccutA* was also highly expressed at T24 with levels reducing at T48 (**Figure 4**). Genes involved in the galacturonic acid catabolism pathway were not expressed at T0. However, there was a strong induction of galacturonate reductase genes (*Bcgar1* and *Bcgar2*) together with galactonate dehydratase (*Bclgd1*) at T24 on all plant lines. Expression levels then reduced at T48. Genes encoding for enzymes catalysing the last two stages of the pathway, 2-keto-3-deoxy-galactonate aldolase (*Bclga1*) and glyceraldehyde reductase (*Bcglr1*), were also induced at T24 but increased reaching highest level of expression at T48.

Genes encoding for cell wall degrading endopolygalacturonases (BcPGs) were not expressed at T0 with low levels of expression detected at T24. This was then followed by an upregulation of *Bcpg1*, *Bcpg3*, *Bcpg4*, *Bcpg5* and *Bcpg6* at T48 with *Bcpg1* displaying the highest levels of expression. Very low levels of expression were detected for *Bcpg2* without any upregulation as infection progressed. *Bcpg6* expression values were also relatively low with expression on some plant lines only being detected at T48. *B. cinerea* phytotoxins, botrydial (*Bcbot*) and botcinic acid (*Bcboa*), were induced at very low levels at T24 with highest levels of expression at T48. Interestingly, the levels were between two and five-fold higher in the local lesions on transgenic plants compared to those on control plants.

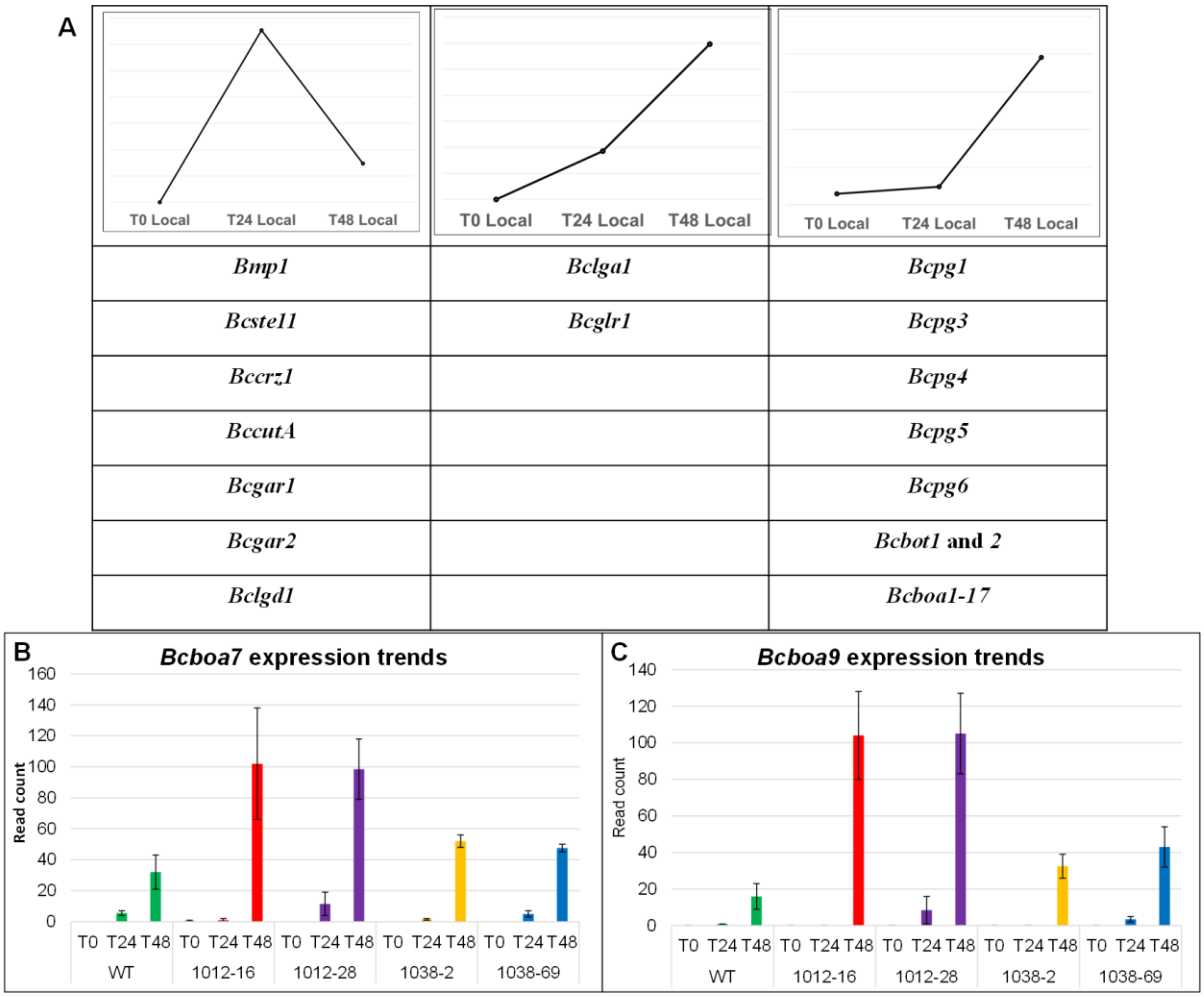
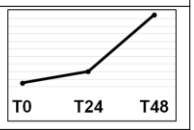
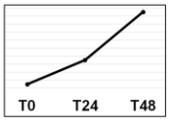
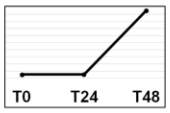
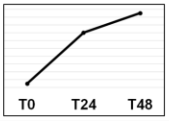
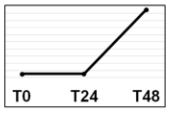
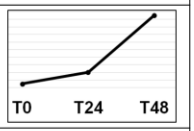
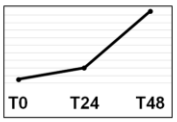
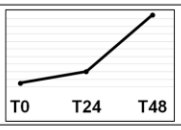
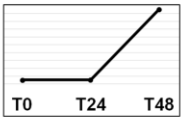
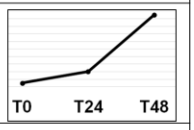
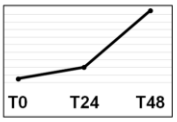


Figure 4. A. Expression trends of a selection of putative *B. cinerea* grape strain virulence factors generated through cluster analysis in STEM (Short Time Series Expression Miner) ver. 1.3.8 ($p<0.05$). **B** and **C**. Expression trends of botcinic acid genes displayed relatively higher expression on transgenic plants compared to the controls. Error bars represent standard error of the transcript abundance of the two biological repeats per plant line at each time point.

Expression patterns of other carbohydrate active enzymes (CAZymes) involved in the disassembly of the host cell wall during *B. cinerea* grapevine infections were analysed. The list was based on enzymes previously identified in grape berries infected with *B. cinerea* (reported in Blanco-Ulate *et al*., 2014). At least one gene involved in disassembling a plant cell wall target identified in infected berries was also identified in our dataset (**Table 3**). Similar to *Bcpg* expression profiles, they were not expressed at T0 on all plant lines. Some were strongly upregulated at T24, such as the RG hydrolases whilst others were only expressed at T48, such as α-L-rhamnosidases. Pectin-methylesterases were slightly upregulated at T24, with a strong induction at T48. All the CAZymes studied reached highest levels of expression at T48.

Table 3. List of CAZymes upregulated during *B. cinerea* grape strain leaf infections. Infection profiles were generated through cluster analysis in STEM (Short Time Series Expression Miner) ver. 1.3.8 ($p<0.05$).

Plant cell wall target	CAZyme subfamily	Functional annotation	Genes displaying upregulation during infection	Expression profile
HG backbone	GH28	Polygalacturonases	Bcpg1, Bcpg3, Bcpg4, Bcpg5, Bcpg6, BC1G_01617	
	PL1	Pectin lyases	BC1G_07527, BC1G_12517	
	CE8	Pectin methylesterases	Bcpme1, Bcpme2, BC1G_1114	
RG-1 backbone	GH28, GH105/GH28	RG hydrolases	BC1G_05961, BC1G_13970	
	GH78	α-L-rhamnosidases	BC1G_06328	
	CE12	RG acetylesterases	BC1G_14009	
XyG backbone	GH16, GH16/CBM18	Glucanases and XyG transglucosylase	BC1G_00409, BC1G_02932, BC1G_04948, BC1G_09991	
Xylan backbone	GH10, GH11, GH10/CBM1, GH11/CBM1	β-xylanases	BcXyn11A, BC1G_01778	
Cellulose	GH5, GH5/CBM1, GH45	1,4-β-glucanases	BcCEL5A, BC1G_03038, BC1G_13862	
	GH6/CBM1, GH6/CBM2, GH7, GH7/CBM1	1,4-β-cellobiosidases	BC1G_10880, BC1G_14702	
Side chains/adducts	GH2, GH35, GH31, GH43, GH93, GH47, GH92, GH51, GH54/CBM42, GH62/CBM13, GH95	β-galactosidases α-xylosidases α-L-1,5-arabinanases α-mannosidases α-arabinofuranosidases α-L-fucosidases	BC1G_02410, BC1G_03567, BC1G_12184 BC1G_12859, BC1G_11115 BcAra1 BC1G_09742, BC1G_12174 BC1G_04994 BC1G_08975	

6.4.5 *V. vinifera* defence against *B. cinerea* grape strain infection: WT Local Response

The differential expression data was utilised to study enriched functional categories in the untransformed WT plants. From T0 to T24, functional categories for genes involved in photosynthesis were upregulated whilst GO terms for defence related genes were downregulated (**Figure 5A**). At T24 to T48, there was a switch in trends with GO terms for genes involved in stress response being upregulated whilst functional categories for genes involved in photosynthesis and growth were downregulated (**Figure 5B**). Genes represented in the top 10 enriched functional categories were analysed further for expression trends as infection progressed and compared to expression profiles in transgenic plants (**Figure 6**). Similar to the WT, GO terms for defence-related biological processes were upregulated from T24 to T48 whilst photosynthesis and growth related GO terms were downregulated.

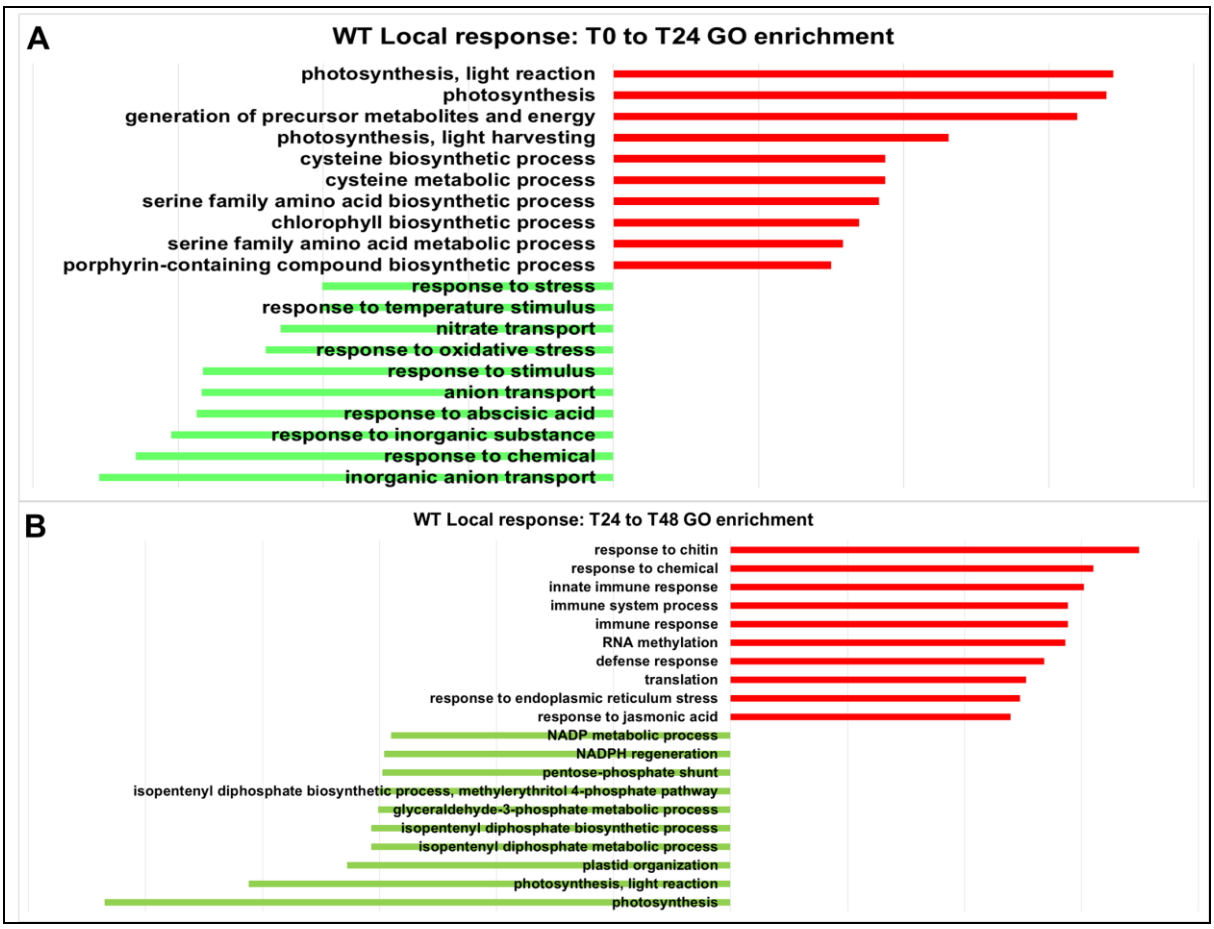


Figure 5. GO enrichment analysis of differentially expressed genes in WT plants from (**A**) T0 to T24 and (**B**) T24 to T48. Green bars represent the top 10 downregulated GO terms whilst red bars represent top 10 upregulated GO terms at each time point. The length of the bar represents the fold enrichment of the GO terms, thus the GO term 'photosynthesis, light reactions' is the most enriched for upregulated WT local response genes whilst 'inorganic anion transport' is the most enriched for downregulated WT local response genes from T0 to T24.

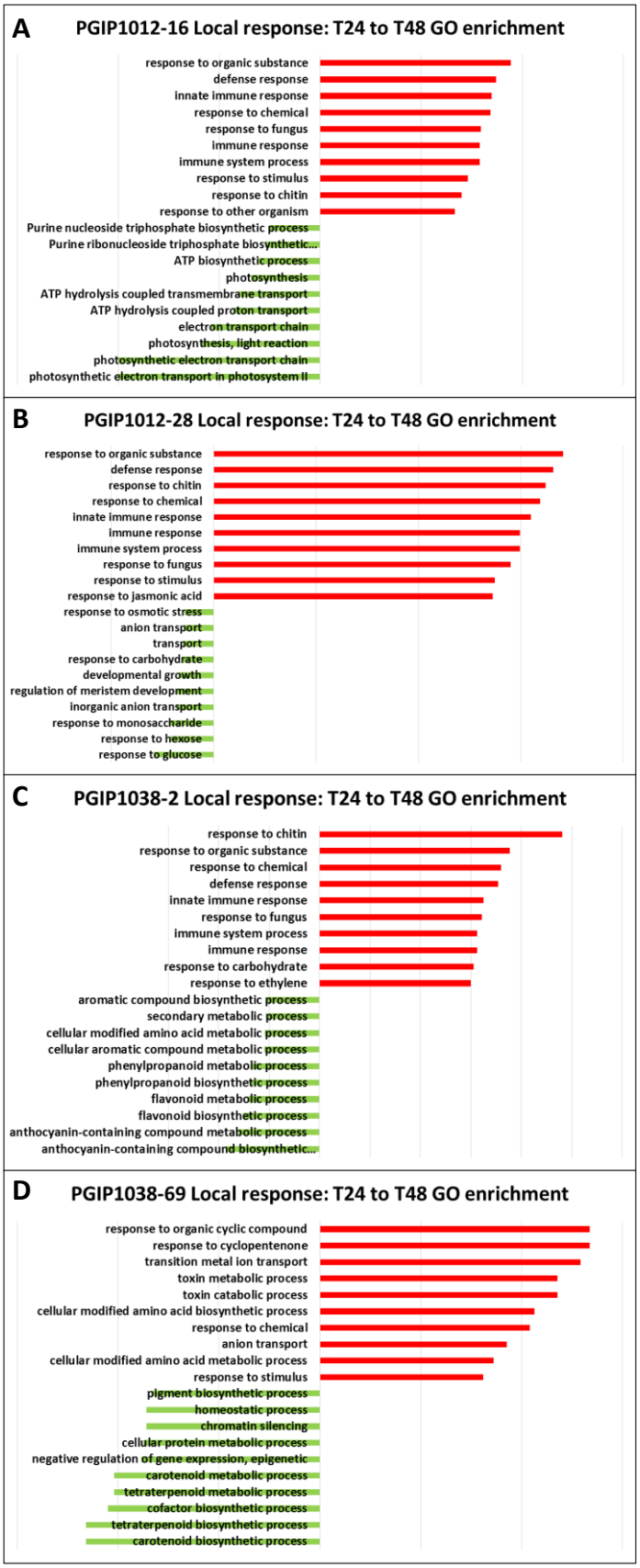


Figure 6. GO enrichment analysis of differentially expressed genes in the transgenic lines (**A**) PGIP1012-16, (**B**) PGIP1012-28, (**C**) PGIP1038-2 and (**D**) PGIP1038-69. Green bars represent the top 10 downregulated GO terms whilst red bars represent top 10 upregulated GO terms from T24 to T48. The length of the bar represents the fold enrichment of the GO terms.

6.4.6 *Vvipgip1* expression profile in locally infected grapevine leaves

The expression profile of *Vvipgip1* during the first 48 hpi was studied on both local and transgenic grapevine plants infected with *B. cinerea* grape strain (**Figure 7**). It was expressed on all plants during the course of infection with the WT displaying relatively lower levels throughout compared to the transgenic plant lines. Also, *Vvipgip1* expression on WT generally decreased as infection progressed whilst the expression profile and levels on transgenic plants was highly variable.

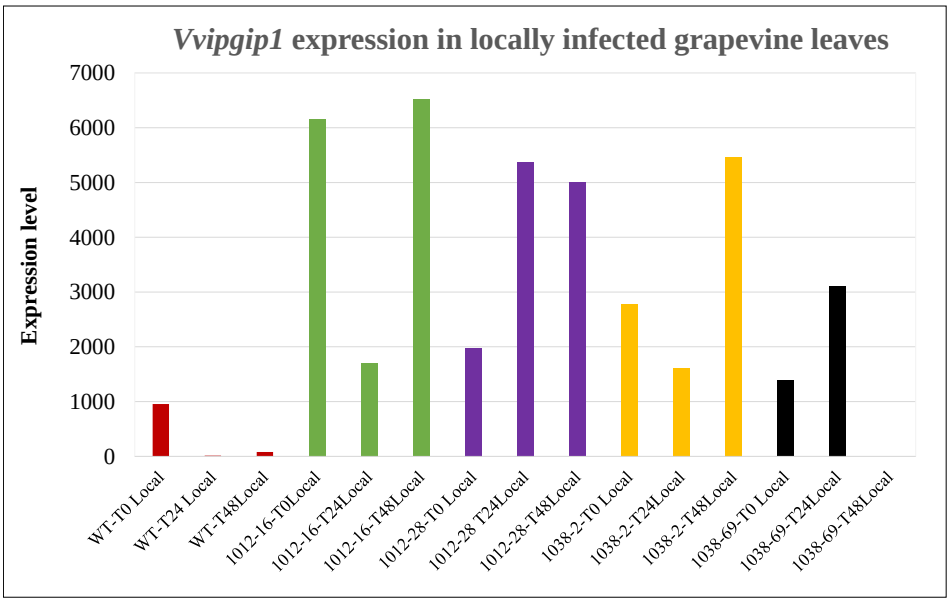


Figure 7. Expression profile of *Vvipgip1* on local and transgenic grapevine leaves at the local infection.

6.4.7 Transgenic plants display lower expression levels for defence-related genes compared to controls

GO terms for genes encoding for markers of active plant defence against pathogen attack such as PR proteins, PAL and stilbene synthases (VviSTS) were represented in the top ten functional categories enriched from T24 to T48 in all plant samples. The genes were expressed at very low levels at T0 and T24. They were then strongly induced, reaching their highest levels of expression in the infection time course at T48. Expression levels at T48 were higher in control plants compared to transgenic population (**Figure 8**). A similar trend was observed for genes involved in both monoterpenes and sesquiterpene biosynthesis (VviTPS) as well as the shikimate pathway.

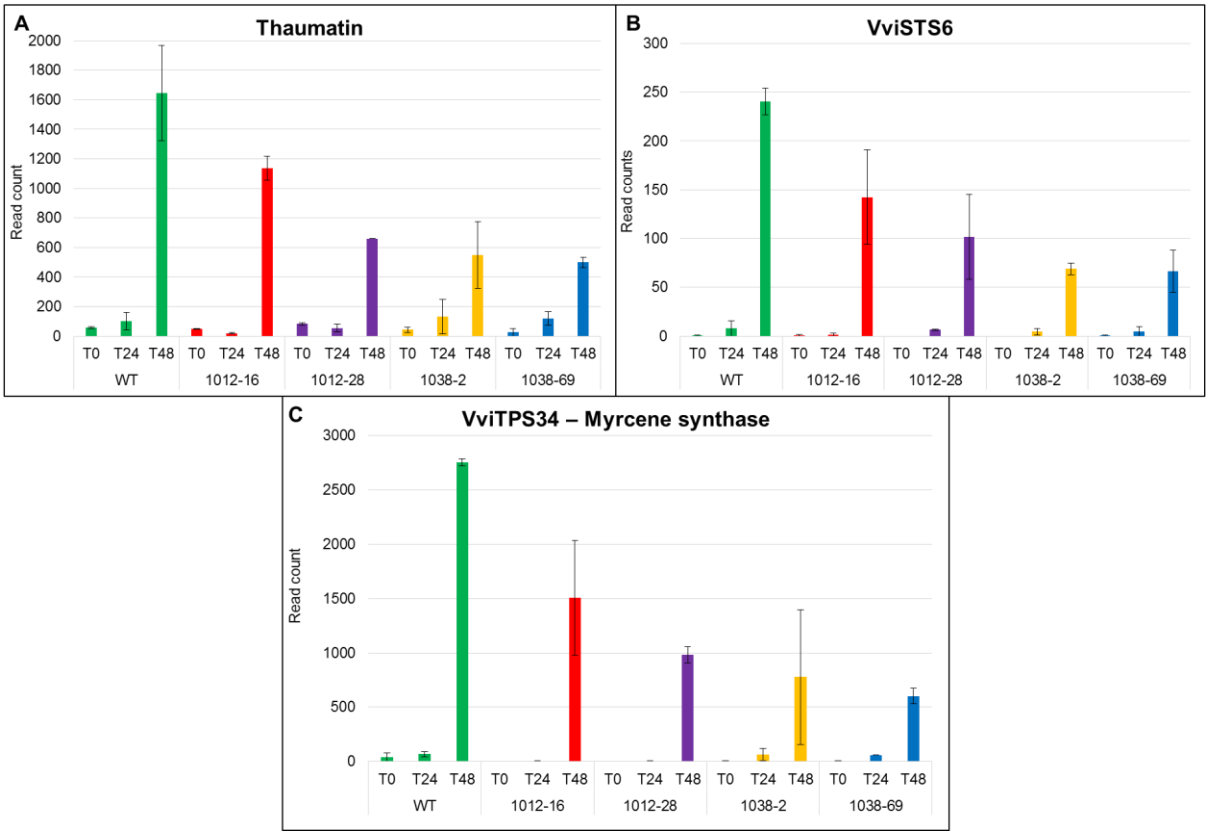


Figure 8. Expression profiles of a representative sample of defence-related genes in grapevine leaves during *B. cinerea* infection showing (**A**) a PR protein, (**B**) stillbene synthase and (**C**) terpene synthase. Error bars represent standard error of the transcript abundance of the two biological repeats per plant line at each time point.

6.5 DISCUSSION

The preliminary results of the grapevine-*Botrytis* interactome study provided valuable insights into the molecular basis of the hyper-susceptible phenotype displayed by *V. vinifera* plants overexpressing non-*vinifera pgip*s. It also provided details of the infection strategy utilised by *B. cinerea* grape strain during grapevine leaf infections.

Putative *B. cinerea* grape strain virulence factors on grapevine leaves

By following gene expression profiles of *B. cinerea* grape strain on both transgenic and control leaves in the first 48 hours post infection we managed to identify putative virulence factors. These include genes which were upregulated on all plant lines at any specific time point. Genes involved in spore germination, conidiation and appresorium formation were upregulated at T24, decreasing at T48. They included *Bmp1*, *Bcste11* and *Bccrz1* (**Figure 4**). *Bmp1* is a virulence factor on carnation flowers and tomato leaves (Zheng *et al*., 2000) whilst *Bcste11* is a virulence factor on tomato and apple (Schamber *et al*., 2010). *Bccrz1* was also shown to be a virulence factor on tomato, bean and apricot (Schumacher *et al*., 2008a: 2008b). These studies utilised different *B. cinerea* strains and convincingly showed that these genes were virulence factors on a diverse host range with knock out mutants displaying reduced pathogenicity. Based on their expression profiles, they could be *B. cinerea* grape strain virulence factors on grapevine leaves as well. Infecting both transgenic and control plants with knockout mutants could shed more light on this issue.

A cutinase gene, *BccutA*, was strongly upregulated on all plant lines at T24, reducing in levels at T48. This gene was functionally characterised through mutant analysis and *B. cinerea* SAS56 knock-out mutants retained their full pathogenicity on gerbera flowers and tomato fruits (van Kan *et al*., 1997). Since several studies have reported strain and host specificity of virulence factors (Nakajima and Akutsu, 2014; Chapter 4 of thesis), it cannot be ruled out that *BccutA* could be a *B. cinerea* grape strain virulence factor on grapevine leaves. On the other hand, genes encoding for cell wall degrading enzymes were upregulated at T24 reaching their highest levels in our time course infection assay at T48. Amongst the six BcPG isoforms, *Bcpg1* displayed the highest expression levels. It has been identified as a virulence factor on apples, tomato leaves and fruits (ten Have *et al*., 1998; Kars *et al*., 2005) and was expressed on grape berries infected with *B. cinerea* noble rot strain, BcDW1 (Blanco-Ulate *et al*., 2014). Also our previous work showed that B05.10 *bcpg1* mutants displayed significant reduction in virulence on both transgenic and control plants (Chapter 4 of thesis).

Bcpg2 expression levels were very low in our transcriptomic dataset. Also the expression profiles on both transgenic and control plants did not display induction as infection progressed. Interestingly, a previous study showed that *Bcpg2* was not expressed on grape berries infected with BcDW1 suggesting that it was not an important virulence factor (Blanco-Ulate *et al*., 2014). Also in our previous work (Chapter 4 dissertation), B05.10 *Bcpg2* mutants did not display reduced virulence on both transgenic and control plants. Thus three different *B. cinerea* strains display similar profiles on different tissues of the same plant species. Since *Bcpg2* was shown to be a virulence factor on tomato leaves and broad bean (Kars *et al*., 2005), it could be a host specific virulence factor, not being required for full virulence on grape berries and leaves.

The expression profiles of *B. cinerea* genes involved in galacturonic acid catabolism showed a clear progression in the pathway with genes encoding for enzymes catalysing the first steps being induced earlier. *Bcgar1*, *Bcgar2* and *Bclgd1* were strongly induced at T24 whilst *Bclga1* and *Bcglr1* reached highest expression levels at T48. Previous work has shown that mutation of *Bcgar1* and *Bcgar2* did not reduce pathogenicity of B05.10 on apple fruit, tomato leaves, bell peppers and grapevine leaves. However, reduction in virulence was detected when the same mutants were tested on tobacco and *Arabidopsis* (Zhang *et al*., 2011; Zhang and van Kan, 2013; Chapter 4 of dissertation). These results display host specificity of *Bcgar1* and 2 as virulence factors. Unfortunately, all the previous studies utilised the same *B. cinerea* strain, B05.10. Therefore, strain specificity of *Bcgar1* and *Bcgar2* as *B. cinerea* grape strain virulence factors cannot be ruled out in our results. Other putative *B. cinerea* grape strain virulence factors during grapevine leaf infections identified in our transcriptomic dataset were genes involved in the synthesis of phytotoxins. They are discussed in detail in the next section.

B. cinerea grape strain expressed higher levels of phytotoxins on transgenic plants compared to the controls

Following the *B. cinerea* grape strain infection strategy showed that it expressed the same virulence-associated genes on all plant lines. The only difference identified thus far is the higher expression levels of

genes involved in synthesis of botrydial (*Bcbot*) and botcinic acid (*Bcboa*) on transgenic compared to the control plants at T48. These phytotoxins induce severe chlorosis, cell and tissue collapse leading to colonisation of plant organs (Colmenares *et al*., 2002). They display functional redundancy with knockout mutants in either botrydial or botcinic acid synthesis genes having no effect on virulence. However, double knock out mutants show significantly reduced virulence (Pinedo *et al*., 2008; Dalmais *et al*., 2011). *Bcboa6* and *Bcboa9* have been shown to be crucial for the synthesis of botcinic acid as mutants in both genes abolishes its production but without affecting pathogenicity of the *B. cinerea* strain. On the other hand, double mutants in *Bcboa6* and *Bcbot2* do not produce either of the toxins and they display severe reduction in pathogenicity (Dalmais *et al*., 2011).

Botrydial has been identified in *Capsicum annum* infected with *B. cinerea* and the highest levels of the phytotoxin were found on tissues with the most severe necrotic lesions (Deighton *et al*., 2001). It was also shown to induce the hypersensitive response in *Arabidopsis* resulting in elevated levels of programmed cell death (PCD), a plant defence strategy which benefits a necrotroph like *B. cinerea* (Rossi *et al*., 2011). Interestingly, the action of botrydial was shown to be controlled by hormone signalling within the plant hosts, mediated by salicylic and jasmonic acid (Rossi *et al*., 2011). A previous study in our environment showed that overexpressing *Vvipgip1* in tobacco plants altered the cell wall and hormone profiles of the transgenic population in comparison to control plants (Alexandersson *et al*., 2011). Since the transgenic grapevine population displays changes in cell wall compositions and emission of defence related sesquiterpenes (Chapter 5 of thesis), indicative of changes in metabolic processes within the population, it is possible that hormone profiles could have also been altered and that these changes modulated *B. cinerea* to implement a different infection strategy which involved stronger activation of phytotoxic genes. Alternatively, the potential metabolic changes in the transgenic population (Chapter 5 of thesis) could have weakened defence signalling, leading to *B. cinerea* grape strain being able to suppress, block or even hijack crucial host defence mechanisms to its own benefit leading to the hyper-susceptible phenotype. These aspects should be studied further to confirm the hormone profiles in the transgenic and control lines as well as investigate further the mechanisms that *Botrytis* could have used to modulated host responses.

***Vvipgip1* is expressed in all plant lines during infection, without evidence of induction due to infection**

The native *V. vinifera Vvipgip1* has previously been linked to defence against infection by different *B. cinerea* strains in overexpression studies where it conferred transgenic tobacco plants with reduced susceptibility to infection (Joubert *et al*., 2006; Chapter 4 of thesis). Analyses of the transgenic tobacco population attributed the defence phenotype to the effective inhibition of BcPGs by VviPGIP1 and priming of the cell wall and hormone profiles (Joubert *et al*., 2006; 2007; Alexandersson *et al*., 2011). In grapevine, several studies have highlighted the upregulation of *pgip* in response to infection and/or stress (Bezier *et al*., 2002; Aziz *et al*., 2004). Furthermore, gene expression and promoter analysis showed that *Vvipgip1* was upregulated in all organs due to *B. cinerea* infection (Joubert *et al*., 2013). Contrary to these finding, in our study, gene expression analysis of infected WT plants showed a different trend (**Figure 7**). There was no

evidence of induction of *Vvipgip1* in leaves in response to infection on either control or transgenic plant lines. In a separate study, Trincadeira berries infected with *B. cinerea* also did not show an upregulation of *Vvipgip1*, instead, expression levels decreased in EL35 grapes (Agudelo-Romero *et al*., 2015). Taken together, it is possible that the role of grapevine PGIPs in defence against *B. cinerea* grape strain could be host specific. Also, grape PGIPs may have evolved for other functions in grapevine not directly related to defence.

Transgenic grapevine plants express lower levels of defence-related genes compared to the controls at the local lesions

The defence strategies of control plants during *B. cinerea* grape strain infection were followed for the first 48 hours post infection at the local lesions. GO enrichment analysis showed that from T24 to T48 the controls upregulated genes with defence-related functional categories such as 'defence response', 'response to fungus' and 'response to stimulus' (**Figure 5**), similar to previous reports on cucumber infected with *B. cinerea* at 96 hpi (Kong *et al*., 2015). This was indicative of successful *B. cinerea* grape strain recognition coupled with mounted defence responses (Windram *et al*., 2012). Analysis of expression patterns of defence-related genes encompassed in the top 10 enriched GO terms and comparing them to the transgenic population (**Figure 6**) revealed interesting trends.

Marker genes for active plant defence responses against pathogen attack were strongly upregulated at T48 in all plant lines, with higher expression levels in control plants compared to the transgenics. These included genes encoding for chitinase, osmotin, thaumatin and PAL (phenylalanine ammonia lyase). Chitinases break down chitin in fungal cell walls whilst thaumatin and osmotin permeabilise fungal membranes. On the other hand, PAL is a marker gene for lignin biosynthesis and is thus indicative of cell wall strengthening defence strategies (Monteiro *et al*., 2003). Several other studies have identified the induction of these genes in response to pathogen attack (Monteiro *et al*., 2003; Aziz *et al*., 2004; Agudelo-Romero *et al*., 2015; Golshani *et al*., 2015). Also, overexpression of chitinase and thaumatin genes in strawberry, tobacco, cucumber and lilies conferred the transgenic plants with enhanced resistance to *B. cinerea* (Carstens *et al*., 2003; Kishimoto *et al*., 2004; Vellicce *et al*., 2006; Nunez *et al*., 2015).

A similar trend was observed for stilbene synthase genes, key enzymes in the biosynthesis of phytoalexins such as resveratrol, pterostilbene, α-viniferin and ε- viniferin (Chong *et al*., 2009). Resveratrol is the best studied, exhibiting fungitoxic activity which inhibits spore germination and disrupts the plasma membrane (Adrian & Jeandet, 2012). Hence, overexpression of a stilbene synthase in grapevine reduced susceptibility of the host to *B. cinerea* (Dabauza *et al*., 2015). Taken together, we suggest that the lower levels of these marker defence genes in transgenic plants rendered the population less able to fight *B. cinerea* grape strain compared to the control.

The transgenic plant lines also exhibited lower expression levels of genes encoding for enzymes which catalyse the different steps in the shikimate pathway. This pathway is the biosynthetic route for tryptophan, tyrosine and phenylalanine (Herrman, 1995). The aromatic amino acids are precursors for

secondary metabolites and plant hormones such as salicylic acid, a signalling molecule involved in both local and systemic defence responses (Durner *et al*., 1997; Chen *et al*., 2009). Hence, the lower expression levels in transgenic plant lines could mean lower production of salicylic acid which would translate to poor signalling for other defence responses.

Lastly, lower expression levels of genes involved in both monoterpene and sesquiterpene biosynthesis were detected in transgenic plant lines compared to the controls at T48. This was an interesting expression trend in view of our previous results where transgenic leaves emitted significantly lower levels of sesquiterpenes during *B. cinerea* infection at T48 compared to the controls (Chapter 5 of thesis). The induction of sesquiterpenes during *B. cinerea* infection has also been reported in tomato (Thelen *et al*., 2006) and they display strong antifungal activity against *B. cinerea* (Tsao and Zhou, 2000).

Conclusion and perspectives

The preliminary results of the grapevine-*Botrytis* interactome presented in this study have enabled us to profile WT grapevine response to *B. cinerea* grape strain infections together with the infection strategy employed by the fungus on leaves during the early stages of infection. Comparing this profile to that of the transgenic plants has provided valuable insights into the molecular basis of the hyper-susceptibility of *V. vinifera* plants overexpressing non-*vinifera pgip*s to *B. cinerea* grape strain. Our results suggest a shift in the infection strategy utilised by *B. cinerea* grape strain on transgenic plants which could be linked to previously reported metabolic changes within the transgenic population. The altered infection strategy could have then manipulated host defence responses to the benefit of the pathogen and detriment of the host. The results presented in this chapter are from the local response and analysis of systemic transcriptomic data could still provide more clues to explain the hyper-susceptible phenotype of the transgenic grapevine population. Future work will include metabolic analysis to confirm some of the results obtained with the transcriptomic data, data mining of the systemic response expression data and verification of the reported expression trends through RT-PCR. A more thorough analysis of this interactome in under way and the results of this current study can guide the analysis to specifically understand how the grape strain modulated the host response to cause the outcomes that were observed in this study.

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Chapter 7

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Cultivated grapevine is of high economic value with harvested grapes being used to make wine, brandy, vinegar, grape fruit or consumed whole as table grapes and raisins. These products make significant contributions to the economies of most grape growing countries. Unfortunately biotic stress factors such as fungal infections are often associated with a decline in crop quality and they threaten the productivity of the grapevine industry.

Numerous methods are currently being utilised to try and limit damage caused by these fungal infections, although the main preventative and curative action remains the application of fungicides throughout the growth season (Bisson *et al*., 2002). Unfortunately, the emergence of fungal strains exhibiting acquired fungicide resistance has pushed fungicide manufacturers to come up with stronger active ingredients. This has been met with a lot of concerns with regards to health hazards. Potential risks associated with some of the new and improved fungicides towards the environment and consumers have been reported, giving conservationists and consumers more reason to oppose their continued use (Mirlean *et al*., 2007; Komarek *et al*., 2010).

In an effort to reduce the dependence on fungicides, there has been a lot of attention on agricultural practices that harness the innate resistance traits of cultivated grapevine. Plant breeders have used conventional methods for centuries to breed and select for disease resistance. These methods are not only time consuming, but the resulting hybrids sometimes exhibit undesirable traits such as poor quality grapes, requiring extensive back-crossing. Genetic engineering is a plant improvement tool which provides a more direct approach and although genetically modified grapes are not currently accepted for commercialisation, most of what is currently known about defence genes and how they interact with fungal molecules is based on utilising genetic engineering platforms for knowledge generation (Punja, 2001; Lusser *et al*., 2012; Rai and Shekhawat, 2014).

The Grapevine Molecular Biology Group at the Institute for Wine Biotechnology is similarly utilising genetic engineering platforms to study plant-pathogen interactions. A number of inherent grapevine defence genes have been identified and their roles in defence elucidated through overexpression studies. One group of genes which has sparked interest in our research group encodes for polygalacturonase inhibiting proteins (PGIPs). Only a single copy of the gene exists in the commercially cultivated *Vitis vinifera* and is named *Vvipgip1* (De Ascensao, 2001). Previous work has shown that VviPGIP1 inhibits *Botrytis cinerea* cell wall degrading endopolygalacturonases (BcPGs) *in vitro* and *in planta* and overexpression of the gene in tobacco reduced susceptibility of the host to infection (Joubert *et al*., 2006; 2007).

Based on the confirmed defence role of *Vvipgip1*, attention then shifted to PGIP encoding genes from American wild vines and *Vitis* hybrids. These non-*vinifera* grapevine species exhibit inherent high levels of fungal resistance compared to the highly susceptible *V. vinifera*. The main interest was to evaluate if the non-*vinifera* PGIPs contributed to the higher degree of innate immunity. Overexpression of non-

vinifera PGIPs in tobacco resulted in enhanced resistance of the host to *B. cinerea*, more than what was observed with the *Vvipgip1* lines (Venter, 2010). It was thus highly unexpected that overexpression of two non-*vinifera* PGIPs (PGIP1012 and PGIP1038 from *V. x doaniana* Munson and *V. caribaea* respectively) in *V. vinifera* resulted in enhanced susceptibility, not resistance (Moyo, 2011). We hypothesised that the phenotype was not linked to the inability of grapevine PGIPs to inhibit BcPGs, but rather to the non-ePG functions of PGIPs that influenced plant host defence mechanisms to the benefit of the invading pathogen. The major findings are highlighted below and placed in context of literature and future work.

Non-*vinifera* PGIPs are effective inhibitors of *B. cinerea* BcPGs and display subtle structural differences which, together with BcPG structural differences, could influence the dynamics of inhibition interactions

Non-*vinifera* PGIP1012 and PGIP1038 were convincingly shown to inhibit BcPGs using *in vitro* and *in planta* approaches (Chapter 5 of thesis). The resulting trends were similar to what was previously reported for VviPGIP1 where *in vitro* inhibition was only detected against BcPG1 and 6, with BcPG2 being inhibited *in planta* (Joubert *et al*., 2006; 2007). Although the two approaches did not detect any differences in inhibition profiles between non-*vinifera* PGIPs and VviPGIP1, *in silico* structural modelling was able to identify subtle differences in the predicted protein structures in regions which could affect inhibition interactions (Chapter 3 of thesis). Similarly, structural predictions of BcPG isoforms from three *B. cinerea* strains revealed subtle differences which could influence inhibition interactions. This was evident in the predicted inhibition profiles of grapevine PGIPs against the *B. cinerea* virulence factors, BcPG1 and 2, which displayed strain specificity. Furthermore, docking simulations revealed the versatility of PGIP-BcPG interactions, with each pairing being unique in terms of nature of bonds at the protein-protein interface, stability of the complex and predicted hotspots. Future work could include studying the interaction of grapevine PGIPs and BcPGs in three-component complexes with pectin, to gain more understanding on possible *in planta* impacts of the subtle protein structural differences.

The hyper-susceptibility of the transgenic grapevine population to *B. cinerea* grape strain is a host and strain specific phenotype

Transgenic tobacco plants expressing non-*vinifera pgip*s displayed the same defence phenotype to infection against two different *B. cinerea* strains. However, when the transgenic grapevine population, which previously displayed hyper-susceptibility to *B. cinerea* grape strain (Moyo, 2011), was challenged with a different *B. cinerea* B05.10 strain, it displayed a different defence phenotype (Chapter 4 of thesis). Additionally, there were host specific trends in *B. cinerea* virulence and potential recognition/decoy factors. These could have been brought about by differences between the cell wall matrices of grapevine and tobacco since it has been shown that conditions in the host cell wall influence *B. cinerea*'s infection strategy (Blanco-Ulate *et al*., 2014). The strain specific trends in this study complemented the *in silico* PGIP-BcPG docking simulations (Chapter 3 of thesis) which showed strain specificity of virulence factors. This study clearly

showed that the hyper-susceptibility of the transgenic grapevine population was a specific response to *B. cinerea* grape strain infection. It also pointed towards the roles of grapevine PGIPs in defence being host specific, conferring transgenic tobacco with resistance against *B. cinerea* infections, but not *V. vinifera*. It is possible that in grapevine, PGIPs have evolved for other roles in growth and development, which are unrelated to defence.

Overexpressing non-*vinifera pgip*s in grapevine could have impacted on these alternative roles in a way that enabled *B. cinerea* grape strain to elevate its infection strategy, to the detriment of the host. The different phenotypes displayed by the transgenic grapevine population when infected with two different *B. cinerea* strains, were probably linked to differences in infection strategies employed by the invading pathogens. These were likely to have been influenced by the evolutionary backgrounds of the strains. Since the grape strain co-evolved with grapevine, it possibly adapted mechanisms to evade or even hijack defence responses to its own benefit. Taken together, the dynamics of this host-pathogen pairing influenced the infection strategy employed by the invading pathogen and most likely, the host defence responses as well. The study revealed the impact that host-pathogen pairings could have on defining defence phenotypes. Future studies could include characterising the transgenic tobacco and grapevine defence phenotypes against another *B. cinerea* strain to confirm strain specificity.

Native and non-*vinifera pgip*s in transgenic grapevine are expressed during *B. cinerea* infection

PGIP gene expression analysis of locally infected leaf tissue was performed on untransformed and non-*vinifera* PGIP expressing lines within the first 48 hours following *B. cinerea* infection (Chapter 5 of thesis). Previous work has shown that *B. cinerea* utilises its main infection arsenal during this period with plants responding by inducing high levels of defence genes (Bezier *et al*., 2002; AburQamar *et al*., 2006; Blanco-Ulate *et al*., 2014; Kelloniemi *et al*., 2015). Our results showed that *Vvipgip1* was expressed in leaves during infection, as previously reported in Joubert *et al*., 2013. The non-*vinifera* PGIPs, which are under a constitutive promoter, were highly expressed throughout the first 48 hrs post infection. These findings, in combination with the protein activity determined using *in vitro* and *in planta* platforms (Chapter 5 of thesis), revealed that the transgenic population was in a position to make use of both the native and transgenic PGIPs for defence during the early stages of *B. cinerea* infection. Thus, our results show that post transcriptional *pgip* gene silencing did not play a role in the hyper-susceptible phenotype displayed by the grapevine transgenic population.

An altered sesquiterpene emission profile is a possible contributor to the hyper-susceptible phenotype

Sesquiterpene emissions of both transgenic and control *V. vinifera* leaves infected with *B. cinerea* were evaluated using GC-MS (Chapter 5 of thesis). Again, the first 48 hpi were targeted for analysis. In our study, we could not measure emission of volatile organic compounds concurrently as whole plant infections occurred. This was mainly due to limited resources and experimental logistics. Instead, locally infected leaves were harvested at three time points, ground to a fine powder and sesquiterpenes extracted. Despite the

aforementioned limitation of our approach, we managed to detect significant differences in emission profiles between the untransformed control and the transgenic plants. At 48 hours post infection, the transgenic leaves emitted significantly lower levels of defence related sesquiterpenes compared to the control lines. This was further supported by transcriptomic data (Chapter 6 of thesis), which showed that the transgenic plants expressed significantly lower levels of terpene synthase genes involved in sesquiterpene biosynthesis. Thus expressing non-*vinifera* PGIPs in *V. vinifera* affected both the metabolism and emission of defence-related sesquiterpenes. Since some of the sesquiterpenes have been implicated in the inhibition of spore germination and hyphal growth (Zhang *et al*., 2008), the profile displayed by the transgenic population could have enabled *B. cinerea* to germinate and proliferate faster, to the detriment of the plant host thus contributing towards the hyper-susceptible phenotype observed.

Grapevine plants expressing non-*vinifera* PGIPs show altered cell wall composition prior to infection

Cell wall analysis of uninfected control and transgenic grapevine leaves showed significant differences in several components (Chapter 5 of thesis). Transgenic leaves had higher levels of AGPs and lower levels of β-1,4-D-galactan, β-1,3-D-glucan and de-esterified HG prior to fungal infection. The reduction in cell wall polysaccharide composition can be linked to weakening of plant cell walls (Zykwinska *et al*., 2005: 2008; Pastor *et al*., 2013). Previous work in our environment has shown that expression of *Vvipgip1* in tobacco causes cell wall changes which are associated with cell wall strengthening prior to any infection, leading to a primed state (Joubert, 2004; Alexandersson *et al*., 2011). From our results, it appears that expressing non-*vinifera pgip*s in *V. vinifera* might have the opposite effect. The two studies show that overexpressing grapevine PGIPs in either tobacco or *V. vinifera* changes the cell wall of the host, however, the changes can either be to the benefit or detriment of the plant host during *B. cinerea* infection. Thus the proposed role of PGIPs contributing towards priming defence in plants could be host specific. On the other hand, the consequence of the increase in AGP epitope abundance towards the *B. cinerea* infection phenotype is still elusive and requires further investigation. The grapevine AGP family was thus identified and characterised (Addendum A to Chapter 5 of thesis) as a first step towards understanding the potential roles that an increase in AGP abundance in transgenic *V. vinifera* cell walls could have towards the hyper-susceptible phenotype.

The grapevine AGP family: What do we currently know about it?

Cell wall analysis of transgenic grapevine leaves showed that they exhibited higher levels of AGP epitope abundance compared to the untransformed controls. Previous studies have associated AGPs with cell wall strengthening through formation of self-aggregates and cross-links with pectin (Tan *et al*., 2013; Hijazi *et al*., 2014). Thus the high AGP epitope abundance was an interesting trend since the same grapevine leaves exhibited lower levels of cell wall polysaccharide components, a trait associated with weakened cell walls. We know from characterisation of AGP gene families in *Arabidopsis*, rice, poplar and tomato (Showalter *et al*., 2010; 2016; Ma & Zhao, 2010; Fragkostefanakis *et al*., 2012) that members of the same family can exhibit highly diverse functions. Thus it is possible that the highly abundant AGPs in transgenic leaves might

not be associated with cell wall strengthening. More work is however, required to ascertain if this is true. To that effect, we identified 57 AGP encoding genes from the grapevine genome and classified them using numerous bioinformatic tools. Expression analysis revealed high levels of developmental regulation, a pattern reported in other studies (Showalter, 2001). The novel characterisation of the grapevine AGP family presented in this dissertation provides a foundation upon which functional analysis of target genes can be built upon (Addendum A to Chapter 5). Future work could include utilising the interactome data (Chapter 6 of thesis) to identify VviAGPs which could have a potential role in defence, based on expression profiles, and then conducting functional and localisation studies on them.

Transgenic grapevine plants show partial resistance to the biotroph *Erysiphe necator*

Infection of untransformed and *V. vinifera* leaves expressing *pgip*1012 and *pgip*1038 with *E. necator* showed that the transgenic lines were partially resistant to infection whilst the controls were highly susceptible (Chapter 4 of thesis). *E. necator* enters host cells by rupturing the hosts' cell wall which in turn triggers a cascade of plant defence responses (Feechan *et al*., 2011). Using scanning electron microscopy and light microscopy we convincingly showed that programmed cell death was not amongst the defence strategies employed by transgenic grapevine leaves. Instead, penetration resistance played a role towards the observed phenotype, suggestive of a physical barrier to entry. The cell wall changes displayed by the transgenic population (Chapter 5 of thesis) could have indirectly prevented *E. necator* spores from penetrating the leaf surfaces through self-aggregates which are known to be formed by AGPs (Hijazi *et al*., 2014). These could have been more abundant in transgenic leaves due to the higher VviAGP composition, creating a cell wall matrix that *E. necator* struggled to penetrate.

The grapevine-*Botrytis* interactome: Lessons learnt

The hyper-susceptible phenotype displayed by transgenic grapevine was shown to be specific to the host-pathogen pairing (Chapter 4 of thesis). This prompted us to study the strain specific infection strategy together with the host specific defence strategy as a dynamic interaction (Chapter 6 of thesis). From the host perspective, native *Vvipgip1* not being upregulated in response to infection within the first 48 hours suggested that in grapevine, PGIPs could have evolved for other functions not primarily linked to defence. However, they seem to be activated to take on defence roles when overexpressed in heterologous backgrounds like tobacco (Chapter 4 of thesis). The presence of non-*vinifera* PGIPs in *V. vinifera* clearly changed the cell wall profiles of transgenic plants (Chapter 5 of thesis) and probably hormone profiles as well. This could have then impacted the *B. cinerea* grape strain to adapt its infection strategy in a manner which heightened the ability of the pathogen to respond to the host resulting in upregulation of genes involved in synthesis of phytotoxins with strong necrotic activity (Colmenares *et al*., 2002; Siewers *et al*., 2005). Additionally, overexpressing non-*vinifera pgip*s in grapevine could have changed metabolic processes within the host which enabled *B. cinerea* grape strain to hijack plant defence responses and utilise them to its

advantage. An improved annotation of the *Botrytis* genome could shed more light on the observed trends at the local response.

Summary and future perspectives

The hyper-susceptibility of *V. vinifera* plants expressing non-*vinifera* PGIPs to *B. cinerea* infection was a highly unexpected outcome (Moyo, 2011). It challenged our current understanding of the roles of PGIPs in plant defence during *B. cinerea* infection. In this dissertation, we have provided evidence that supports our hypothesis that the phenotype was not linked to the inhibitory activity of grapevine PGIPs against BcPGs, but rather to the non-ePG functions of PGIPs which influenced grapevine defence mechanisms in a way that benefitted *B. cinerea*. To our knowledge, this is the first study where overexpressing PGIPs in a plant host has been convincingly shown to negatively influence plant defence-related systems.

The findings presented in this thesis take strides in advancing the existing knowledge on grapevine defence against *Botrytis* infections. It has highlighted that despite grapevine PGIPs displaying defence roles against *B. cinerea* in heterologous hosts, in their native environment they have probably evolved for different roles. They accumulate in berries at véraison and are induced by fructose and sucrose, sugars that characterise berry ripening and softening (Joubert *et al.*, 2013). It could perhaps be that grapevine PGIPs are involved in changing the berry cell wall composition by interfering/disrupting the pectin-ePG networks, a role which might extend to other organs leading to cell wall weakening in leaves of transgenic plants. Additionally, unlike the priming effect they have in heterologous hosts, overexpressing grapevine *pgip*s in their native background changes plant metabolic processes which potentially benefit or favour the invading pathogen. However, the benefits to the invading pathogen do not appear to be universal, probably being linked to fungal strain properties such as adaptation to the host brought about by co-evolution dynamics, infection strategy and maybe its feeding lifestyle as well. Lastly, the uniqueness of grapevine PGIP-BcPG pairings and their influence on inhibition interactions was elucidated.

On a broad overview, this study has generated numerous resources that could be valuable in future studies. The docked models generated from the *in silico* PGIP-BcPG interactions, provide a foundation on which to build and elucidate current and future grapevine PGIP-BcPG studies. The genome-wide identification and characterisation of the complex family of VviAGPs serves as a basis towards the functional characterisation of grapevine AGP encoding genes with potential impacts on plant defence against *B. cinerea*. The transcriptomic data from the grapevine-*Botrytis* interactome study is an information-rich dataset which can be used in other grapevine-related studies.

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