Analysis of antifungal resistance phenotypes in transgenic grapevines

by

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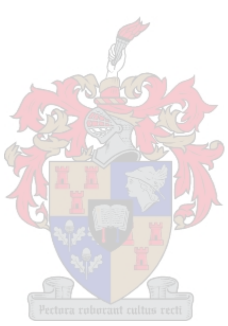

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science

at
Stellenbosch University
Institute for Wine Biotechnology, Faculty of AgriSciences

Supervisor: Prof MA Vivier

December 2012

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Declaration

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Summary

The latest strategies in the protection of crops against microbial pathogens are rooted in harnessing the natural, highly complex defense mechanisms of plants through genetic engineering to ultimately reduce the application of chemical pesticides. This approach relies on an in-depth understanding of plant-pathogen interactions to develop reasonable strategies for plant improvement. Among the highly specialized defense mechanisms in the plant's arsenal against pathogen attack, is the *de novo* production of proteinaceous antimicrobial peptides (AMPs) as part of the plant's innate immunity. These AMPs are small, cysteine-rich peptides such as plant defensins that are known for their broad-spectrum of antifungal activity. These plant defensin peptides have been found to be present in most, if not all plant species and the defensin encoding genes are over-represented in plant genomes. Most of these defensins are generally the products of single genes, allowing the plant to deliver these molecules relatively rapidly and with minimal energetic expense to the plant. These factors contribute to establishing AMPs as excellent candidates for genetic engineering strategies in the pursuit of alternative crop protection mechanisms.

The first antimicrobial peptide identified and isolated from grapevine, Vv-AMP1, was found to be developmentally regulated and exclusively expressed in berries from the onset of ripening. Recombinantly produced Vv-AMP1 showed strong antifungal activity against a wide range of plant pathogenic fungi at remarkably low peptide concentrations *in vitro,* however, no *in planta* defense phenotype could thus far be linked to this peptide. In this study, the antifungal activity of Vv-AMP1 constitutively overexpressed in its native host (*Vitis vinifera)* was evaluated against grapevine-specific necrotrophic and biotrophic fungi. Firstly, a hardened-off genetically characterised transgenic *V. vinifera* (cv. Sultana) population overexpressing Vv-AMP1 was generated and morphologically characterized. In order to evaluate the *in planta* functionality of Vv-AMP1 overexpressed in grapevine, this confirmed transgenic population was subjected to antifungal assays with the necrotrophic fungus, *B. cinerea* and the biotrophic powdery mildew fungus, *Erysiphe necator*. For the purpose of infection assays with a biotrophic fungus, a method for the cultivation and infection with *E. necator* was optimized to generate a reproducible pathosystem for this fungus on grapevine. Detached leaf assays according to the optimized method with *E. necator* revealed programmed cell death (PCD) associated

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resistance linked to overexpression of Vv-AMP1 that can be compared to that of the highly resistant grapevine species, *Muscadinia rotundifolia.* Contrastingly, whole-plant infection assays with *B. cinerea* revealed that Vv-AMP1 overexpression does not confer *V. vinifera* with elevated resistance against this necrotrophic fungus.

An *in silico* analysis of the transcription of defensin-like (DEFL) genes previously identified in grapevine was included in this study. This analysis revealed putative co-expression of these DEFL genes and other genes in the grapevine genome driven by either tissue- or cultivar specific regulation or the plant's response to biotic and abiotic stress stimuli.

In conclusion, this study contributed to our knowledge regarding Vv-AMP1 and revealed an *in planta* defense phenotype for this defensin in grapevine. *In silico* analysis of the DEFL genes in grapevine further revealed conditions driving expression of these genes allowing for inferences to be made regarding the possible biological functions of DEFL peptides in grapevine.

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Opsomming

Die nuutste strategieë wat deel vorm van die beskerming van plant gewasse teen mikrobiese patogene het hul oorsprong in die inspanning van die natuurlike, hoogs gekompliseerde verdedigingsmeganismes van die plant deur middel van genetiese enginieurswese ten einde die gebruik van chemiese plaagdoders te verlaag. Hierdie benadering maak staat op ‘n in-diepte begrip van plant-patogeen interaksies om verstandige strategieë vir plantverbetering te kan ontwikkel. Van hierdie hoogs-gespesialiseerde verdedigingsmeganismses in die plant se arsenaal teen patogeen aanvalle sluit die *de novo* produksie van proteinagtige antimikrobiese peptiede (AMPs) in as deel van die plant se ingebore immuunstelsel. Hierdie AMPs is klein, sisteïen-ryke peptiede soos die plant “defensins” en is bekend vir hul breë-spektrum antifungiese aktiwiteit. Hierdie plant defensinpeptiede word aangetref in meeste, indien nie alle plant spesies nie en die defensin koderende gene word oor-verteenwoordig in plant genome. Meeste van hierdie defensins is gewoonlik die produkte van enkele gene wat die plant in staat stel om hierdie molekules relatief spoedig en met minimale energie verbruik in die plant te vorm. Hierdie faktore dra by tot die vestiging van AMPs as uitstekende kandidate vir genetiese ingenieursstrategieë as deel van die strewe na alternatiewe gewasbeskermingsmeganismes.

Die eerste antimikrobiese peptied wat geïdentifiseer en geïsoleer is uit wingerd, Vv-AMP1, word beheer deur die ontwikkelingsstadium en word eksklusief uitgedruk in korrels vanaf die aanvang van rypwording. Rekombinant-geproduseerde Vv-AMP1 het sterk antifungiese aktiwiteit getoon teen ‘n wye reeks plantpatogeniese swamme teen merkwaardige lae peptied konsentrasies *in vitro*, alhoewel geen *in planta* verdedigingsfenotipe tot dusver gekoppel kon word aan hierdie peptied nie. In hierdie studie was die antifungiese aktiwiteit van Vv-AMP1 wat ooruitgedruk is in sy natuurlike gasheerplant (*Vitis vinifera*) ge-evalueer teen wingerd-spesifieke nekrotrofiese- en biotrofiese swamme. Eerstens is ‘n afgeharde geneties-gekarakteriseerde transgeniese *V. vinifera* (cv. Sultana) populasie wat Vv-AMP1 ooruitdruk gegenereer en morfologies gekarakteriseer. Om die *in planta* funksionaliteit van Vv-AMP1 ooruitgedruk in wingerd te evalueer is hierdie bevestigde transgeniese populasie blootgestel aan antifungiese toetse met die nekrotrofiese swam, *B. cinerea* en die biotrofiese swam, *Erysiphe necator*. Vir die doel om infeksiestudies uit te voer met ‘n biotrofiese swam is ‘n metode geoptimiseer vir die

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kweek en infeksies met *E. necator* wat gelei het tot 'n herhaalbare patosisteem vir hierdie swam op wingerd. Blaarstudies, volgens die pas-verbeterde metode vir *E. necator* infeksies het 'n geprogrammeerde seldood-geassosieërde weerstand, gekoppel aan die ooruitdrukking van Vv-AMP1 onthul, wat vergelyk kan word met dié van die hoogs-weerstandige wingerdspesie, *Muscadinia rotundifolia*. Hierteenoor het heel-plant infeksie studies met *B. cinerea* onthul dat Vv-AMP1 ooruitdrukking geen verhoogde weerstand teen dié nekrotrofiese swam aan *V. vinifera* bied nie.

'n *In silico* analise van die transkripsie van defensin-agtige (DEFL) gene wat vroeër in wingerd geïdentifiseer is, is by hierdie studie ingesluit. Hierdie analise het vermeende gesamentlike uitdrukking van hierdie DEFL gene en ander gene in die wingerd genoom onthul wat aangedryf word deur weefsel- of kultivar-spesifieke regulering of die plant se reaksie tot biotiese en abiotiese stress stimuli.

Ten slotte, hierdie resultate het bygedra tot ons kennis in verband met Vv-AMP1 en het 'n *in planta* verdedigingsfenotipe vir hierdie defensin in wingerd onthul. *In silico* analiese van die DEFL gene in wingerd het verder toestande onthul wat die uitdrukking van hierdie gene aandryf wat ons toelaat om aannames te maak ten opsigte van die moontlike biologiese funksies van DEFL peptiede in wingerd en ondersteun die opstel en toets van hipoteses vir die rol en megansimes van aksie van die wingerd defensin familie.

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This thesis is dedicated to

Leo and my parents

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Biographical sketch

Kari du Plessis was born on 26 December 1985 and raised in Strand. She matriculated from Strand High School in 2003 and commenced her studies at the University of Stellenbosch in 2007 where she enrolled for a BSc-degree in Biodiversity and Ecology which she obtained in 2009. She received a BScHons-degree in Wine Biotechnology in 2010 after which she enrolled for an MSc-degree in Wine Biotechnology at the Institute for Wine Biotechnology at Stellenbosch University.

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Acknowledgements

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

- **Prof MA Vivier,** Institute for Wine Biotechnology at Stellenbosch University, who acted as my supervisor during my MSc studies, for accepting me as a student, for her continued insight, encouragement and support throughout this project;

- **Mr D Jacobson,** Institute for Wine Biotechnology at Stellenbosch University, who provided support with bioinformatics and computational biology concepts and execution and continued support and encouragement throughout this project;

- **Mr P Jones,** my fellow MSc student, Institute for Wine Biotechnology at Stellenbosch University, who performed bioinformatical and mathematical analyses and contributed to my in silico work;

- **My colleagues, friends and fellow students** in the Plant Molecular Biology Laboratory, for their support, insight, advice and encouragement throughout this project;

- **The National Research Foundation (NRF), THRIP, Stellenbosch University and Winetech** for financial assistance;

- **My parents**, for their unconditional love and support throughout my studies without whom my pursuit of science as a career would be impossible;

- **Leo**, my husband, for everything.

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Preface

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal Plant Physiology. Chapters three and four comprise contributions from other students, postdoctoral fellows and researchers as outlined below and acknowledged in the various chapters. All other research results, the compilation of the research results and their interpretation and the written format of the thesis were prepared by the candidate, in interaction with her supervisor.

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Chapter 2 **Literature review**
Plant defensins: A review

Chapter 3 **Research results**
Evaluation of the defense phenotype of transgenic *Vitis vinifera* overexpressing the defensin, Vv-AMP1

Chapter 4 **Research results**
In silico analysis of gene expression patterns of defensin-like peptides in *Vitis vinifera* L.

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Chapter 1

General introduction and project aims

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General Introduction and Project Aims

1.1 Introduction

Plants are continuously exposed to a plethora of potentially harmful pathogens, but despite their sessile nature, the prevalence of successful pathogen infection remains relatively infrequent. However, plant diseases caused by pathogens contribute to crop losses of an estimated 10% worldwide (Strange and Scott, 2005). Therefore, one of the greatest challenges since the onset of modern agriculture is the successful disease management of these crops.

Currently, the primary means implemented to eradicate crop disease is the repeated application of chemical pesticides (Shah, 1997; Pezet et al., 2004). The safety and health risks associated with the application of these pesticides have become well known, leading to increasingly more stringent legislature with regards to the allowed concentration of these pesticide applications worldwide (Phung et al., 2012; Hillcocks, 2012). Furthermore, as a result of the evolutionary arms race between plants and their pathogens, the emergence of pathogens with resistance to these pesticides has become increasingly more prevalent (Staub, 1991; Hayashi et al., 2002; Gressel, 2011; Jansen et al., 2011). The costs involved with this crop protection mechanism as well as the potentially detrimental impact that it may have on natural ecosystems leads to the aggressive pursuit of alternative means to limit the spread of crop diseases (Holland et al., 2012).

In an effort to reduce and eventually eliminate the use of chemical pesticides as the primary means of crop protection against pathogens, several alternative strategies have been attempted. These strategies include production of biological control agents, breeding programs for the production of new resistant cultivars and even crop rotation strategies as reviewed in Compant et al. (2012). Although some of these strategies proved to be successful in combating some pathogenic insects, success with regards to antimicrobial strategies remained limited. Therefore, the latest strategies in the protection of crops against microbial pathogens are rooted in harnessing the natural, highly complex defense mechanisms evolved by plants themselves through genetic engineering. This approach relies on an in-depth

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understanding of plant-pathogen interactions to develop reasonable strategies for plant improvement.

The natural plant defense mechanisms have been honed and fine-tuned over millennia through the ongoing evolutionary arms race between plants and their microbial pathogens. These highly complex defense strategies of plants involve structural and biochemical defense mechanisms that can either be induced upon pathogen attack or constitutively maintained (Bowles, 1990; Broekaert et al., 1997). This structural defense includes the reorganization and subsequent strengthening of the cell wall through the accumulation of a multitude of structural proteins upon pathogen attack as well as the passive protection of the cell wall through for example the cuticle (Heil and Bostock, 2002; Ferreira et al., 2007). These strategies provide the plant with a physical barrier to reduce successful penetration and infection of pathogenic microorganisms.

However, among the highly specialized defense mechanisms in the plant's arsenal against pathogen attack, the *de novo* production of proteinaceous antimicrobial compounds as part of the plant's biochemical defense mechanism remains at the forefront of plant innate immunity (Ahn et al., 2002; van Loon et al., 2006; Ferreira et al., 2007). The inducible nature of these endogenous proteins relies on the plant's recognition of pathogen signal molecules thereby causing a rapid cascade of signaling events leading to the production of these defense-related proteins.

Among the defense-related proteins that form part of the chemical defense response of plants are several enzyme inhibitors as well as a group of low molecular weight antimicrobial peptides that has been extensively studied in recent years. Antimicrobial peptides (AMPs) are small, highly basic, cysteine rich peptides of no more than 90 amino acid residues or <10kDa that are known to possess some form of antibiotic activity (Broekaert et al., 1997). These activities have proven to confer various levels of resistance to numerous plant species against a wide range of fungal, bacterial, insect and even parasitic plant pathogens. Members of this AMP family have been found to be present in most, if not all plant species hereby underscoring the importance of these peptides in plant defense. Furthermore, these peptides are generally the products of single genes, allowing the plant to deliver these molecules relatively rapidly and with minimal energetic expense to the plant

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(Thomma et al., 2002). Most antimicrobial peptides have also been found to be non-toxic when ingested by eukaryotic organisms despite their antibiotic nature. These factors contribute to establishing AMPs as excellent candidates for genetic engineering strategies in the pursuit of alternative crop protection mechanisms.

The main focus of genetic engineering strategies is the transfer of the disease resistance characteristics of a resistant plant donor to a susceptible host in an attempt to endow the susceptible host with similar resistance to the specific pathogen. This is achieved through inserting genes targeted for plant defense into inherently susceptible host plants. Due to the rapid advancement of plant transformation technology in recent years, these aims are now successfully achieved in various plant systems under laboratory conditions (Lay and Anderson, 2005). Even though evidence exists for the adverse effects that overexpression of AMP encoding genes may have on growth and reproduction of transgenic plants (Elfstrand et al., 2001; Anderson et al., 2009; Stotz et al., 2009), several success stories in transgenic research have gained public support around a normally highly controversial industry. The use of small antifungal peptides in the engineering of disease resistant crops proved to be highly successful in field trials (Gao et al., 2000; Portieles et al., 2010). Both of these studies implemented plant defensin peptides as a means of antifungal resistance.

These plant defensin peptides form part of the antimicrobial peptide superfamily of peptides and are known to play an imperative role in the protection of the reproductive structures of almost all known plant species (Broekaert et al., 1995; Thomma et al., 2002; Lay and Anderson, 2005; Ferreira et al., 2007). Upon further bioinformatical investigation it was established that plant defensin-encoding genes are over-represented in various plants species, contributing a monumental 3% of all genetic material in *Arabidopsis* (Silverstein et al., 2005; Silverstein et al., 2007). These findings possibly underscore the importance of these plant peptides in not only plant defense but general plant biology as well.

Similar challenges are being addressed in the disease control of grapevine, the most important and widely cultivated fruit crop in the world (Vivier and Pretorius, 2002). Grapes are commercially cultivated in more than 60 countries over a combined estimated area of 8 million hectares (http://faostat.fao.org). The most commercially

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important grapes are derived from the European grapevine, *Vitis vinifera* L. and are prone to infection by various fungal pathogens since this species has very limited innate immunity against a multitude of necrotrophic and biotrophic pathogenic fungi. The severe pathogen susceptibility characteristic of *V. vinifera* has been recently attributed to the domestication history and reliance on a small group of well-known cultivars that are very closely related (Myles et al., 2011). Vegetative propagation is considered to have stagnated the grapevine gene pool. The lack of continuous breeding of unique cultivars through crosses, and their subsequent adoption by the industries may have allowed for more successful adaptation to pathogens. The *Vitis* species retained high levels of genetic diversity and is known to have adapted to pathogens; this genetic diversity has not been used fully yet, but would be important in future grapevine improvement strategies (Myles et al., 2011).

The completion of the *V. vinifera* genome sequence and the increasing number of molecular profiling tools and datasets becoming available for grapevine as a consequence, has made it possible to evaluate the presence and importance of defensins in this species. The first grapevine defensin encoding gene was isolated and characterized from grape berries and shown to have strong activity against fungal pathogens *in vitro* (De Beer and Vivier, 2008; De Beer, 2008; Tredoux, 2011). The current study builds on the previous work by analyzing the potential defense phenotypes of a transgenic grapevine population, constitutively overexpressing a grapevine defensin. Moreover, knowledge of defensins in grapevine will potentially be extended by an *in silico* approach to mine available transcriptomic grapevine data for defensin expression as well as co-expressing genes.

1.2 Project background and specific aims

Since the identification of the first antimicrobial peptide from grapevine known as Vv-AMP1 (*Vitis vinifera* antimicrobial peptide 1), this peptide has been isolated and characterized (De Beer and Vivier, 2008). Expression of the Vv-AMP1 encoding gene was found to be developmentally regulated, limited to berry tissue from the onset of ripening onwards. Expression of the *Vv-AMP1* gene was not inducible through external hormone stimulus, wounding or pathogenic infection. Upon further evaluation it was found that recombinant production of Vv-AMP1 yielded a highly

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heat-stable protein with a molecular mass of 5.495 kDa that accumulated primarily in the apoplastic region of the plant cell. Furthermore, the recombinantly produced Vv-AMP1 peptide proved to inhibit growth of several fungal pathogens *in vitro*. The peptide was active at low concentrations and acted upon the cell membrane of the pathogens, without changing their morphology (i.e. no hyperbranching or other abnormalities occurred) (De Beer and Vivier, 2008). Subsequent *in vitro* antifungal assays confirmed the antifungal activity of Vv-AMP1 against a wide range of grapevine specific pathogens at exceptionally low peptide concentrations (Tredoux, 2011). These promising results provided clear evidence for the antifungal activity of Vv-AMP1 *in vitro* and therefore prompted further investigations of the antifungal activities of this peptide *in planta*.

Subsequent attempts were made to overexpress this peptide in two plant systems. Vv-AMP1 was overexpressed in tobacco, however, these transgenic lines showed no significant difference with regards to resistance to *Botrytis cinerea* in detached leaf infection assays, perhaps due to peptide instability or non-functionality in the heterologous environment (De Beer, 2008). Furthermore, Vv-AMP1 was also constitutively overexpressed in its native host, hereby generating a genetically characterized transgenic population consisting of nine independently transformed transgenic *V. vinifera* (cv. Sultana) lines (Tredoux, 2011). Preliminary results proved Vv-AMP1 to provide only marginal resistance to its native host in detached leaf antifungal assays against the necrotrophic *B. cinerea* (Tredoux, 2011). The *in planta* antifungal activity of Vv-AMP1 overexpressed in its native grapevine host therefore remained relatively unexplored and formed an important part of the proposed study (see below).

Several putative antimicrobial peptide encoding genes have been identified in the grapevine genome (personal communication with Abré de Beer, formerly of the Institute for Wine Biotechnology; Tredoux et al., 2011; Giacomelli et al., 2012), providing scope for further evaluation of the roles that these genes play in grapevine defense and possible alternative biological functions that remains currently unknown.

This project was therefore established in order to broaden our knowledge regarding AMPs in grapevine. The initial focus was on the complete characterization of the defense phenotypes of the transgenic *V. vinifera* (cv. Sultana) population

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overexpressing the Vv-AMP1 defensin in order to establish whether this peptide can provide its native host with elevated resistance to grapevine pathogens. This required *in planta* infection assays of the hardened off transgenic grapevine population overexpressing the Vv-AMP1 peptide with grapevine-specific necrotrophic and biotrophic fungal pathogens.

An additional element of this study was the evaluation of other defensin-like genes (DEFL genes) and their possible biological roles in grapevine. This focus will require the *in silico* mining and analysis of the publically available transcriptomic expression data for grapevine. This would be achieved by collecting expression data for the DEFL genes of interest and performing a combination of pair-wise correlations and Markov clustering methods to establish putative co-expression of these genes in response to specified experimental conditions. Similar methods would be implemented to identify genes in the grapevine genome that shows putative co-expression with the DEFL genes that would allow inferences to be made about the possible biological functions that these DEFL genes are involved in. Hereby, DEFL gene expression in response to biotic and abiotic stresses could be evaluated as well as the possible involvement of these peptides in plant growth and development. Furthermore, these analyses would elucidate whether DEFL genes are expressed in a tissue or cultivar specific manner. These investigations all aim to contribute to our knowledge of plant defensins and their possible future role in the plant protection as part of genetic engineering approaches.

The specific aims of this study were as follows:

1. The morphological characterization of a transgenic *V. vinifera* (cv. Sultana) population overexpressing the Vv-AMP1 defensin peptide.
    a. Establishment, clonal multiplication and maintenance of an *in vitro* collection of the transgenic Vv-AMP1 lines and untransformed *V. vinifera* (cv. Sultana) wild type lines.
    b. Establishment and maintenance of a hardened-off working population of the transgenic Vv-AMP1 lines and untransformed *V. vinifera* (cv. Sultana) wild type lines under greenhouse conditions. Recording of morphological characteristics of these plants with regards to leaf

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morphology, internode lengths and general growth during development to evaluate possible morphological phenotypes caused by the constitutive expression of the Vv-AMP1 peptide.

c. *In planta* infection assays of the transgenic Vv-AMP1 grapevine population in order to determine whether the overexpression of Vv-AMP1 provides these plants with elevated resistance against grapevine pathogens compared to the untransformed wild type.
    i. The establishment of a reproducible pathosystem for the cultivation and infection assays with grapevine powdery mildew fungus, *Erysiphe necator*, by optimizing for infected leaf age and method of conidia inoculation.
    ii. Infection assays with the biotrophic fungus, *E. necator* on the transgenic and control lines and monitoring the outcome of the infection from both the plant and fungal perspective.
    iii. A whole-plant infection assay with a spore suspension of the necrotrophic fungus, *Botrytis cinerea* on the transgenic and control lines and monitoring the outcome of the infection by comparing the development of lesion sizes.

2. The *in silico* analysis of antimicrobial peptide encoding genes in *V. vinifera*
    a. Collection of all publically available transcriptomic microarray data sets
    b. Collection and preparation of all known *V. vinifera* DEFL gene sequences from previous analyses
    c. Clustering analyses in order to determine which DEFL genes form expression clusters driven by predetermined experimental conditions
    d. Evaluation of the experimental conditions driving expression clusters of DEFL genes in grapevine
    e. Identification and hypothesis generation with regards to the putative functions of DEFL genes in grapevine

The research results obtained from this study are presented in Chapters 3 and 4 of this thesis after a literature review that serves as a concise overview of the biological relevance of plant defensins in Chapter 2. The main findings, their relevance and future prospects are discussed in Chapter 5.

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Chapter 2

Literature Review

Plant defensins – A Review

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Literature Review

2.1 Introduction

All living organisms are exposed to numerous pathogens that can potentially threaten their growth and survival. Plants are however particularly vulnerable to pathogen attack due to their immobility, constituting a significant challenge to the cultivation of economically important crops since the onset of modern agriculture. Despite the implementation of various disease control mechanisms, plant diseases caused by fungal pathogens lead to estimated crop losses of 10% annually (Strange and Scott, 2005). Furthermore, fungal pathogens may produce mycotoxic compounds hereby further threatening food security related to the relevant crops, specifically in grain crops that are harvested and stored.

The current mechanisms of crop protection include the implementation of chemical pesticides to reduce the catastrophic destruction caused by fungal pathogens (Shah, 1997; Pezet et al., 2004). These chemical pesticides are however well known to pose safety risks to farmers, consumers and ecological environments (Hillcocks, 2012). Furthermore, successful pathogens evolve to become highly resistant to these chemical fungicides despite the continuous production of more resistant cultivars through breeding programs (Staub, 1991; Hayashi et al., 2002; Gressel, 2011; Jansen et al., 2011).

Surprisingly, despite the devastating effects that fungal pathogens have on crop cultivation, successful pathogenic fungal infection remains the exception not the rule. Plants have evolved highly specialized mechanisms to deter and restrict the growth of pathogenic microorganisms and it is in harnessing these natural mechanisms that alternative approaches to minimizing crop disease can be actively pursued. In-depth knowledge of plant-pathogen interactions, supported by the availability of genome sequences of both host plants and pathogens are greatly facilitating research in this field. Some of the approaches rely on the generation of transgenic crops through the enhancement and optimization of the plant’s inherent defense responses.

Unlike higher vertebrates that can implement specific or acquired immunity, plants implement mechanisms of innate immunity as their first line of defense against pathogens (Lamb et al., 1989). This innate immunity of plants includes various defense strategies that include physical and chemical defense responses that can either be constitutively

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maintained or induced upon pathogen attack (Bowles, 1990; Bloch and Richardson, 1991; Broekaert et al., 1995; van Loon et al., 2006; Ferreira et al., 2007). Physical defense includes the reorganization and subsequent strengthening of the cell wall through the accumulation of a multitude of structural proteins as reviewed in Showalter et al. (1993). These proteins provide the plant with a physical barrier to reduce successful penetration and infection of pathogenic microorganisms.

However, among the highly specialized defense mechanisms in the plant's arsenal against pathogen attack, the *de novo* production of proteinaceous antimicrobial compounds remains at the forefront of plant innate immunity as an ancient defense system not only employed by plants, but all known multicellular organisms (Bowles, 1990; Broekaert et al., 1997). These pathogenesis-related proteins (PR-proteins) can either be constitutively expressed or their production can be induced in response to pathogen attack. This inducible production of these endogenous proteins relies on the plant's recognition of pathogen signal molecules known as elicitors. Upon elicitor recognition, a rapid cascade of events leads to the production of these defense-related proteins that are generally transcribed and translated from a single gene. This process of single gene transcription allows the plant to deliver these so called effector molecules relatively rapidly and with minimal energetic expense to the plant upon pathogen attack (Thomma et al., 2002). Furthermore, these PR proteins can accumulate in plant tissues that has not been directly infected by the pathogen as part of systemic response known as induced systemic resistance (IRS).

Among the defense-related proteins that form part of the chemical defense response of plants are several enzyme inhibitors that include α-amylase and proteinase inhibitors, as well as hydrolytic enzymes such as 1, 3-β-glucanases and chitinases. Furthermore, within this group of PR proteins, the production of a group of low molecular weight antimicrobial peptides (AMPs) has been extensively studied in recent years (Bowles, 1990; Bloch and Richardson, 1991; Broekaert et al., 1995; van Loon et al., 2006; Ferreira et al., 2007).

AMPs generally share a range of physico-chemical properties. They are small, highly basic, cysteine-rich peptides of no more than 90 amino acid residues, forming peptides smaller than 10 kDa. These peptides generally contain an even number of cysteine residues that participate in intramolecular disulphide bond formation. These bonds provide the peptide with thermostability and structure that allows the necessary interaction with the cellular membranes of target microorganisms (Broekaert et al., 1997). However, there is

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great variation in the mechanisms of defense against specific pathogens exerted by the various AMPs. Therefore, based upon the variation of primary amino acid sequences, the number and arrangement of cysteine residues and their three dimensional structure, several distinct plant antimicrobial peptide families have been identified. These protein families include the plant defensins (Broekaert et al., 1995; Terras et al., 1995; Thomma et al., 2002), thionins (Bohlmann and Apel, 1991; Broekaert et al., 1995), lipid transfer proteins (Garcia-Olmedo et al., 1995; Garcia-Olmedo et al., 1998), hevein- and knottin-type proteins (Broekaert et al., 1990; Garcia-Olmedo et al., 1998; Choon Koo et al., 2002) and the plant cyclotides (Craik et al., 1999; Trabi and Craik, 2004) to name a few.

In the continued pursuit for the generation of commercially viable plant crops, plant defensins as targets for genetic engineering has gained particular interest and has been the most widely studied peptide family within the AMP group. This review will be focused on plant defensins, their biological functions and their involvement in the complex host-pathogen interaction mechanism in plants in general and specifically in grapevine.

2.2 Plant defensins

Plant defensin peptides are not only produced by most, if not all plant species, but this class of peptides also is known to be conserved between vertebrates and invertebrates (Broekaert et al., 1995; Javaux et al., 2001; Thomma et al., 2002; Gao et al., 2009). Since the initial discovery of defensins in the macrophages and granulocytes of rabbits (Petterson-Delafield et al., 1980), similar peptides were subsequently discovered in a multitude of species across several genera. The first plant defensins were however first identified and isolated from the endosperm of barley and wheat grains in 1990 (Colilla et al., 1990; Mendez et al., 1990). Originally these peptides were called γ-thionins due to the high level of similarity in cysteine content and secondary structure that these molecules showed to the previously identified and described thionins (Carrasco et al., 1981). Eventually, after identification of several more of these γ-thionin-like proteins, analyses confirmed these peptides to be structurally more similar to mammalian and insect defensins than to plant thionins. In 1995 Terras and colleagues identified and analyzed two antifungal peptides from radish seeds (*Raphanus sativus*) (RsAFP1 and RsAFP2) and renamed the γ-thionins to plant defensins (Terras et al., 1995).

The plant defensins are highly basic, small peptides of ~5 kDa or 45-54 amino acid residues, characterized by typically eight cysteine residues linked through four disulfide

bridges. Plant defensins also typically have an aromatic amino acid residue located at position 11, a glutamate residue located at position 29 and two aromatic residues located in positions 13 and 14, respectively (numbering relative to RsAFP2) (Terras et al., 1995). The plant defensin family is furthermore recognized based on the highly conserved three-dimensional structure that is shared between its members. This structure comprises of a single α-helix and three anti-parallel β-strands. At the core of this conserved structure is a cysteine-stabilized alpha-beta motif (CSαβ) that connects two cysteine residues located on the α-helix to two cysteine residues located one amino acid apart on the second β-strand through two disulfide bridges (Bloch et al., 1998; Fant et al., 1998; Almeida et al., 2002; Janssen et al., 2003; Lay et al., 2003a, b).

Despite the highly conserved three dimensional structures within the plant defensin family, these peptides show very low sequence homology in their amino acid sequences. However, peptides isolated from species within the same family share higher sequence identity compared to those from other plant families (Odintsova et al., 2007). Furthermore, linking amino acid sequences of plant defensins to functional and biological activities proved to be problematic. This great diversity in primary structure may account for the great functional diversity found within this group of AMPs, where one amino acid variation can cause a dramatic structure-function variation between closely related defensin peptides.

2.3 Biological role of plant defensins

Plant defensins are well known to fulfill an integral role in the innate immunity of plants, but they have also been linked to a range of alternate biological functions. The following section touches on the role of plant defensins in plant growth and development, abiotic stress resistance, in addition to their role in defense against pathogens.

2.3.1 The role of plant defensins in plant physiology and development

The ability of defensins as ion channel blockers has been identified when maize kernel defensins, γ1-zeathionin and γ2-zeothionin, were shown to inhibit voltage-gated sodium channels of mammalian GH3 cells (Kushmerick et al., 1998). These inhibitory activities were further substantiated in a study using the whole-cell voltage patch clamp technique with the *Medicago sativa* defensin, MsDEF1 in tsA-201 cells expressing the calcium L-type

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channel. This plant defensin proved to block nearly 90% of the calcium current through this channel, even though the *M. truncatula* defensin, MtDEF2 and the previously characterized Rs-AFP2 did not display similar calcium blocking activities (Spelbrink et al., 2004). These activity variations were proposed to be due to the structural similarity of MsDEF1 peptide to the fungal toxin, KP4 from *Ustilago maydis* well known as a voltage gated calcium channel blocker (Spelbrink et al., 2004).

These results confirmed that some plant defensins have the ability to block ion channels, a fact that was important since both fungal hyphae and the plant root tip are both known to be dependent on a sustained Ca^{2+} gradient for growth and development. The effect of plant defensins on root tip growth were subsequently evaluated by exposing germinated *Arabidopsis* seedlings to MsDEF1, MtDEF2 and Rs-AFP2 (Allen et al., 2008). These plant defensins inhibited root tip growth relative to the peptide concentration applied (Allen et al., 2008). Similarly, Vijayan et al. (2008) reported a 50% reduction in *A. thaliana* root length in response to 10 µg/ml TvD1 (*Tephrosia villosa defensin 1)* exposure. The inhibition of root tip growth is not considered a general defensin characteristic, since exceptions have been identified. For example, defensins MsDEF1, MtDEF2 and Rs-AFP2 do not inhibit *M. truncatula* root tip growth, hereby indicating that all plant species may not possess the potential receptor required for this type of defensin activity.

A plant defensin was further found to be associated with flower inflorescences and defense when Tregear et al. (2002) studied the molecular events associated with the occurrence of a mantled phenotype of oil palm plantlets *in vitro.* The mantled flower phenotype of the oil palm (*Elaeis guineensis*) is caused by the feminization of both female and male flowers that causes subsequent infertility and hampers the ability of these plantlets to be multiplied by micropropagation of somatic embryogenesis. A putative plant defensin gene, *EGAD1*, was found to be expressed in both normal and mantled plant tissues at the callus stage. However, this putative defensin was found to be specifically expressed in the plant inflorescence in the normal, intact plantlets with no expression displayed in roots or leaves. Further analysis of the promotor region of the *EGAD1* gene provided evidence of two *cis* elements related to stress and defense responses hereby underscoring the potential role of plant defensins in flower development (Tregear et al., 2002).

The relevance of plant defensins in flower development have been further accentuated by the consideration of the mechanisms involved in plant self-incompatibility (SI). Plant SI is a

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range of systems employed by plants to limit the occurrence of self-pollination in an effort to maintain genetic diversity (Nasrallah, 2002). The SI mechanisms for *Brassica* species have been well-studied and proved to be controlled by a single multigene SI locus described as the S locus. In the activation of the SI response, two genes were initially identified to be involved in the recognition step of the SI stimulus. These genes encoded for an S locus-specific glycoprotein (SLG) and an S receptor kinase (SRK) (Nasrallah et al., 1991). In an attempt to identify more elements involved in the SI of *Brassica* spp., a subsequent evaluation of the pollen coat offered evidence of a peptide that could interact with the SLG (Doughty et al., 1993). Upon further analysis, this peptide was found to belong to the plant defensin family and was renamed PCP-A1 (pollen coat protein class A, 1). These analyses further proved that PCP-A1 is not encoded by the S-locus and the authors proposed that it serves as a cofactor in the activation of the S receptor (Doughty et al., 1993).

The potential developmental role of plant defensins was further revealed in a study of the defensin DEF2 that was identified in the pistil of tomato plants (*Solanum lycopersicon)* during flower development (Stotz, 2009). This evaluation included several expression studies that confirmed the necessity of DEF2 in tomato flowers during early flower development as well as the necessity of the inactivation of DEF2 expression during pollen development to ensure a normal process (Stotz et al., 2009). Taken together, these studies and their results implicate plant defensins in new biological activities that involves serving as a signal for plant development and growth, although the mechanisms are still largely unknown.

2.3.2 Plant defensins and their role in abiotic stress responses

Plant defensins have been reported to form part of the plant's ability to respond to external environmental stimuli. These abiotic stimuli are summarized in Table 2.1. The expression of a pepper defensin gene CADF1 (*Capsicum annuum* defensin 1) in leaves that otherwise show no expression of this gene had been reported in response to drought and salinity stress (Do et al., 2004). Furthermore, plants grown in soils with a water deficit have shown a predisposition to disease development as reviewed by Boyer (1995). These factors may indicate the combinatorial effect that plant defensins have on plant protection against water deficit stress and subsequent pathogen-related diseases.

Table 2.1 Abiotic stimuli identified as inducers of plant defensin activity.

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Defensin name	Stimulus	Origin plant	Reference
PgD1	Wounding	*Picea glauca*	Pervieux et al., 2004
AhPDF1.1	Zn+	*Arabidopsis halleri*	Mirouze et al., 2006
PDF1.2	Jasmonic acid	*Arabidopsis thaliana*	Thomma et al., 1998
PDF1.2	Methyl Jasmonate	*Arabidopsis thaliana*	Manners et al., 1998
CADEF1	Salicylic acid	*Capsicum annum*	Do et al., 2004
CADEF1	Drought	*Capsicum annum*	Do et al., 2004
Tgas118	Abscissic acid	*Lycopersicon esculentum*	Van den Heuvel et al., 2001
Tad1	Cold stress	*Triticum aestivum*	Koike et al., 2002

Another environmental condition that has been identified as an inducer of plant defensin activity is exposure to cold temperatures. The first plant defensin implicated in cold-temperature resistance was identified among a range of cold-induced genes in winter wheat (*Triticum aestivum*) (Koike et al., 2002). This defensin, TAD1 (*T. aestivum* defensin 1), did not show induction upon exposure to plant hormones that are known to drive defensin expression such as abscissic acid, salicylic acid and methyl jasmonate, however it showed strong and rapid expression following exposure to low temperatures. *TAD1* transcription could be detected as early as 24 hrs after cold exposure and was maintained for 14 days thereafter. However, when evaluating recombinantly produced TAD1 through ice crystal morphology analysis, no antifreeze activity was observed, but the peptide had antibacterial activity against *Pseudomonas cichorii* (Koike et al., 2002). In the light of these data, the authors postulated that this plant defensin confers pathogen resistance to winter wheat during periods of low temperature exposure. To further substantiate these findings Gaudet et al. (2003) found two plant defensin encoding genes in winter wheat that were not expressed in plants grown at a constant temperature of 20°C. The expression of these plant defensin encoding genes was however induced after exposure to 2°C and remained expressed for 14 days after the treatment.

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Interestingly, an *Arabidopsis* mutant known as hos10-1 (high expression of osmotically responsive genes) provides a possible link between water deficit stress, cold temperature stress and ABA metabolism in some plant species (Zhu et al., 2005). This hos10-1 mutant is highly sensitive to cold temperatures and further shows hypersensitivity to water deficit and salinity related stresses. In the absence of the *hos10* transcription factor encoding gene, several genes could not express. One of these genes is responsible for ABA biosynthesis, while another codes for a plant defensin. The highly sensitive phenotype of this *Arabidopsis* mutant could be linked to a deficiency of ABA, the defensin and the absence of the transcription factor itself (Zhu et al., 2005). These findings indicated a link between dehydration stress and ABA metabolism in a wide range of plant species and also provided evidence that plant defensins are not only involved in plant protection against pathogens, but abiotic stresses as well.

Heavy metals and zinc in particular, have detrimental effects on the growth of numerous plant species. Therefore, with the constant increasing levels of heavy metal contamination, mechanisms to increase zinc tolerance in plants are actively studied. In 2006, the possibility that defensins may be involved in heavy metal tolerance in plants was explored by Mirouze et al. (2006). The molecular mechanism of zinc tolerance in the zinc hyper-accumulating plant *A. halleri*, was evaluated. A cDNA library of this plant was expressed in *Saccharomyces cerevisiae* and zinc tolerant strains were selected by incubation on medium containing toxic concentrations of zinc. Of the nine cDNAs selected, four were found to encode for similar peptides with remarkable sequence similarity to plant defensins (Mirouze et al., 2006). Subsequent functional evaluation of these genes provided evidence to the effect that defensins are involved in the zinc tolerance of plants. These analyses proved that three out of the four genes are induced upon Zn exposure and that the AhPDF1.1 defensin shows the strongest induction 6 to 72 hours after Zn exposure. Furthermore, the constitutive accumulation of the defensin pool in *A. halleri* is approximately 200 fold higher than in *A. thaliana* before Zn exposure and increases to a 500 fold higher concentration upon Zn exposure (Mirouze et al., 2006). Recombinantly produced AhPDF1.1 further provided evidence of antifungal activity against *Fusarium oxysporum* and *Alternaria brassicola* in a follow-up study (Marquès et al., 2009). Although the exact mechanism of the possible zinc tolerance conferred by plant defensins are not yet known, these findings substantiates the possible role that defensins have in heavy metal tolerance in plants.

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2.3.3 Plant defensins and their role in biotic stress responses

Antimicrobial peptides have been studied extensively with approximately 1900 AMPs registered in the online AMP database (http://aps.unmc.edu/AP/main.php). 79% of these AMPs were reported to have antibacterial activity whereas only 34% were assigned antifungal activity. Plant AMPs and defensins in particular are however predominantly known for their antifungal activity even though many reports of the bactericidal activities of plant defensins exists (Osborn et al., 1995; Segura et al., 1998; Koike et al., 2002). However, the antibacterial activities of plant defensins are greatly dependent on not only the bacterial strain involved, but the plant species as well. Therefore, it can be assumed that specific plant defensins target inhibition of specific bacterial strains and complete bacterial protection would require activity from a range of specific plant defensins.

Although plant defensins are well known for their antimicrobial activities, these peptides have been implicated in several other defense-related roles as well. Plant defensins have been reported to be involved in insecticidal plant activities by inhibiting the activity of insect digestive enzymes such as α-amylases and proteases, hereby limiting the prevalence of insect herbivory (Bowles, 1990; Colilla et al., 1990; Mendez et al., 1990; Broekaert et al., 1997). In 2002, Chen et al. identified a small cysteine-rich peptide from *Vigna radiata* known as VrCRP (*V. radiate* cysteine-rich protein 1). Recombinantly produced VrCRP caused the death of larvae from the pathogenic bruchid, *Callosobruchus chinensis*, when ingested in artificial seed assays. The same researchers identified a *V. radiate* defensin, VrD1, that showed the same anti-insect activities in artificial seed assays with *C. chinensis* (Chen et al., 2005). VrD1 was further proven to inhibit α-amylase activity in *Tenebrio molitor* (Liu et al., 2006). These findings should be considered in the light of plant protection against viral diseases as well. Even though plant defensins have not yet been directly implicated in the inhibition of viral replication *in vivo* (Carvalho and Gomes, 2009), it is important to note that several plant viruses depend on intermediate insect hosts as a transmission vector for plant infection. For example, the grapevine leaf-roll associated virus GLRaV3 is known to be transmitted through grafting and infection with mealy bugs (Martelli, 1993; Ling et al., 2004). Therefore, by inhibiting the source of transmission through the insecticidal activities of plant defensins, the spread and proliferation of the specific virus could potentially be limited as well.

The protecting activities of plant defensins are however not limited to insect pathogens, but have been further implicated in plant resistance against other pathogenic plants. In 2007,

Letousey et al. performed expression profiling of two *Helianthus annuus* cultivars. One of these sunflower cultivars were known for its high levels of resistance against an obligate root plant parasite that causes broomrape (*Orobanche cumana*), whereas the other was highly susceptible to the same parasitic plant. The analysis screened for expression levels of 11 known defense-related genes and revealed elevated transcript levels of a defensin, HaDef1 in the roots of the resistant cultivar. Upon infection by the broomrape parasitic plant, transcript levels of this defensin further increased in comparison to the susceptible cultivar (Letousey et al., 2007). A follow-up study provided further evidence for this anomaly when *O. cumana* seedlings showed great sensitivity to purified HaDef1 and even though the germination of *O. cumana* and *O. ramose* seeds were not affected by the presence of the same defensin, HaDef1 caused extensive damage to the root tissues of seedlings from broomrape species (Zèlicourt et al., 2007). These findings provided evidence of the ability of plant defensins to inhibit growth and activity of other plant cells, thereby contributing to the innate immunity of the plant host.

Despite these fascinating reports of the multiple defense responses that plant defensins are involved with, the antifungal activity of these peptides remain at the forefront of plant defensin research. Since the identification of the first plant defensins in the early 1990s, research has revealed the presence and antifungal activity of plant defensins in a wide range of plant species. Similar to the antibacterial activity of plant defensins, the antifungal activity of these defensins depends not only on the tested fungus and the specific plant defensin peptide in question, but on the peptide concentration as well. This pathogen-specificity of plant defensins may be an indication of the specificity of the mode of antifungal activities of these peptides; a quality that may reduce the prevalence of pathogens that develop resistance to antifungal peptides with a wide range of antimicrobial activity (Nicolas et al., 2003).

2.4 Plant defensins and their mode of antifungal action

Despite the wide range of antimicrobial activities identified within the plant defensin family, the antifungal activities of these peptides are the most frequently studied and will therefore be further discussed. Although the complete mechanism by which plant defensins inhibit fungal growth remains to be established, significant progress has been made in understanding the role of plant defensins in plants' complex and multilayered defense strategy.

Thevissen et al. (1999) were the first group to explore the possibility that the fungal plasma membrane could be the target of plant defensin activity. They identified a rapid influx of Ca^{2+} and efflux of K^{+} and the subsequent changes in membrane potential caused by the plant defensins RsAFP2 and Dm-AMP1 (*Dahlia merckii* antimicrobial protein 1) upon interaction with the pathogenic fungus, *Neurospora crassa*. Subsequent evaluations of this possible membrane association of plant defensins revealed that Dm-AMP1 causes the fungal plasma membrane to become permeable (Thevissen et al., 1999). These results were obtained by using Sytox green dye that is known to only penetrate cells with compromised cell membranes, thus allowing the visual evaluation of fungal membranes being compromised in the presence of Dm-AMP1 (Thevissen et al., 1999). Although the results of these studies revealed that plant defensins bind fungal membranes and cause possible permeabilization, the exact targets of these peptides in the fungal membrane were still elusive. By performing competition assays with Dm-AMP1 labeled with radioactivity and two unrelated plant defensins, Rs-AFP2 and Hs-AMP1, Thevissen et al. (2000) provided evidence that specific binding to fungal membranes can only be achieved by similar defensins and that different classes of defensins bound distinct membrane sites (Thevissen et al., 2000).

For the purpose of identifying the specific membrane binding sites of plant defensins, *S. cerevisiae* mutants were evaluated that showed resistance to Dm-AMP1 activity, Thevissen et al. (2003) implicated the inositol phoshotransferase enzyme (*IPTI*) gene as being responsible for the susceptible phenotype of the wild type strain. The *IPTI* gene is involved in the synthesis of a membrane complex rich in sphingolipids (van der Rest et al., 1995), suggesting that sphingolipids are the possible membrane receptors for the Dm-AMP1. Several studies evaluating the Dm-AMP1 and Rs-AFP2 plant defensins further substantiated the discovery that sphingolipids act as plant defensin binding receptors (Im et al., 2003; Thevissen et al., 2003; Aerts et al., 2006).

New evidence regarding fungal membrane binding and pore formation of plant defensins is driving the question whether plant defensins cause a direct permeablization of the fungal membrane, or rather indirectly through the induction of an intracellular signal cascade mechanism.

It has been shown that programmed cell death (PCD) is involved in the inhibitory activity of plant defensins (Aerts et al., 2006; Aerts et al., 2007; van Weerden et al., 2008; Aerts et al., 2009; Mello et al., 2011). Aerts et al. (2006) initially identified an association between

the levels of sphingolipids to the level of susceptibility to oxidative stress, the latter leading to apoptosis. Furthermore, Aerts et al. (2007) provided evidence for a link between the production of reactive oxygen species (ROS) and the antifungal effects of a peptide by evaluation of the ROS production by *Candida albicans* exposed to the defensin, Rs-AFP2. Upon further evaluation it was revealed that inhibition of *C. albicans* by this plant defensin is achieved through PCD (Aerts et al., 2009). Although PCD is known to be a consequence of the induction of an intracellular signal cascading event, it has not yet been established whether Rs-AFP2 induces this signal cascade in response to interaction with the fungal membrane, or if it has an intracellular target that is accessed by entering the fungal cytoplasm. A separate investigation of the *Pisum sativum* defensin, PsD1 (*Pisum sativum* defensin 1) proved that this defensin interacts with cyclin F from the *N. crassa* fungus as its intracellular target (Lobo et al., 2007). Similarly, NaD1 (*Nicotania alata* defensin 1) was found to enter the fungal cytoplasm through the membrane of *F. oxysporum* to bind a currently unidentified intracellular target (van der Weerden et al., 2008). Both PsD1 and NaD1 were therefore implicated in antifungal activities related to entry through the fungal cell membrane, however, NaD1 was also found to instigate the production of ROS, hereby underscoring the differences in possible mechanisms of antifungal activity between these two defensins.

It has also been proposed that membrane permeabilization is an indirect side-effect induced by a signal cascading event after receptor binding of the defensin. This hypothesis further suggests that rapid cross-membrane Ca^{2+} and K^{+} flux are also indirect activities and that Ca^{2+} flux is not linked to membrane permeabilization. These claims are substantiated by Van der Weerden et al. (2008) who performed a range of fluorescence techniques and found that the defensin, NaD1 bound the hyphal cell walls of *F. oxysporum* hereby revealing the relevance of the fungal cell wall in the mode of action of defensins against fungi. This lead the authors to propose that defensin binding to cell wall is the first response to pathogen perception by the plant, followed by the rapid membrane permeablization of the fungal membrane allowing for defensin entry into the fungal cells leading to PCD as previously discussed (Van der Weerden et al., 2008).

2.5 Classification of antifungal plant defensins

The plant defensin family is well known for the great diversity in primary amino acid sequences of its members. Furthermore, this diversity is matched by the functional

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diversity of these antimicrobial peptides that remains problematic in linking sequence to function. Plant defensins can however be broadly divided into two groups mirroring the different effects that these peptides have on fungal growth. The first group was designated to be morphogenic defensins, characterized by inhibition of growth and morphologic abnormalities displayed by hyphae of fungi grown in the presence of these defensins. The morphologic hyphal abnormalities displayed by fungi treated with morphogenic defensins included the development of hyperbranched hyphae that appeared swollen and limited in length. Representative and well-characterized members of this group of defensins include Rs-AFP1, Rs-AFP2 and Hs-AFP1 (Osborn et al., 1995).

The second group of defensins is characterized by their ability to inhibit hyphal elongation of fungi, but does not cause hyphal deformities. These defensins are known as non-morphogenic defensins (Osborn et al., 1995) and include defensins such as Vv-AMP1 (*Vitis vinifera* antimicrobial peptide 1), Dm-AMP1, Dm-AMP2 and Ct-AMP1 (Broekaert et al., 1995; Osborn et al., 1995; De Beer and Vivier, 2008). Attempts have been made to link the three dimensional structures of plant defensins to their morphogenicity. It has therefore been proposed that the determining agents of the morphogenic antifungal activity of these defensins reside in the highly conserved γ-core motif in combination with regulating agents outside the γ-core motif (De Samblanx et al., 1997; Schaaper et al., 2001; Lay et al., 2003a, b; Sagaram et al., 2011). In an attempt to evaluate these postulations Sagaram et al. (2011) achieved the conversion of the antifungal activity of the morphogenic defensin, MsDef1, to near non-morphogenic activity by the substitution of the γ-core motif of MsDef1 with that of the non-morphogenic defensin, MtDef4 (Sagaram et al., 2011). However, future research regarding these hypotheses is required to substantiate the link between the γ-core motif and antifungal peptide morphogenicity.

Despite these attempts at linking defensin structure to their functionality, classification of these peptides was traditionally based upon the amino acid composition of the mature peptide domains (Harrison et al., 1997). According to this traditional classification, plant defensins could be broadly divided into two groups sharing 25% sequence homology. These groups, designated subgroup A and B, were further subdivided. Subgroup A contained a further four groups, subgroups A1-A4, and subgroup B further contained subgroup B1 and B2, all of which displayed at least 50% and 45% sequence homology within these subgroups, respectively (Figure 2.1).

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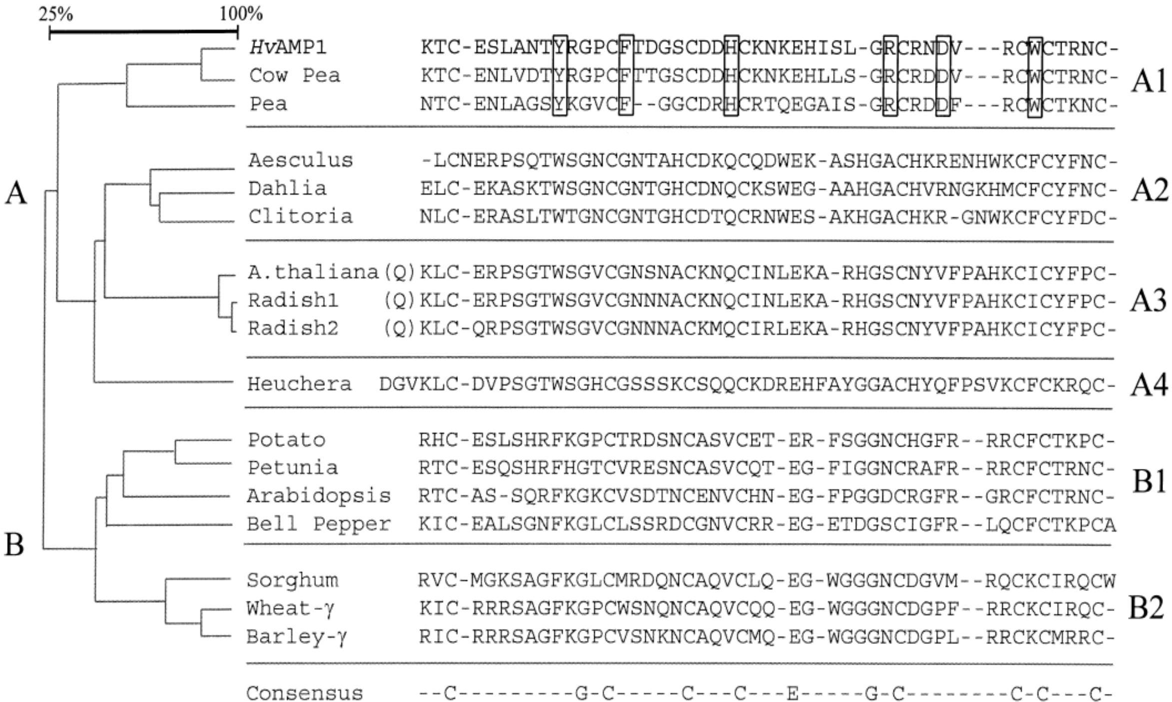

Figure 2.1 Dendrogram representing plant defensin sequence similarities and subgroup classifications according to Harrison et al., 1997. Plant defensin consensus sequences shows amino acid residues that are conserved within each member of the plant defensin family. Sequence subfamilies are indicated from A1 to B2.

To a certain extent, these subgroups could be linked to their mode of antimicrobial activities. Subgroup A2 could be characterized by members that were non-morphogenic in their antifungal activity (Broekaert et al., 1995), whereas members from group A3 also induced hyperbranching in fungal hyphae through morphogenic antifungal activity (Terras et al., 1992; Terras et al., 1993; Broekaert et al., 1995). Members of subgroup A4 are also morphogenic defensins, however, this subgroup shows significant differences in sequence homology to subgroup A3. Subgroup B is characterized by members that show a range of biological activities ranging from inhibition of α-amylase and protein inhibition and bactericidal activities (Osborn et al., 1995).

Even though some consensus sequences within the defensin family remains conserved, the steady increase of plant defensin sequences becoming available has proven the traditional classification system to be problematic with the increase in exceptions to the classification rules. Future development of a more efficient strategy for the classification of plant defensins would therefore be highly valuable in the world of plant defensin research and subsequent transgenic research.

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2.6 Transgenic crops with elevated resistance to fungal pathogens due to defensin manipulation

Despite the ability of plant defensins to inhibit the growth of pathogenic fungi, these peptides are non-toxic to most animal and plant cells (Osborn et al. 1995). These plant defensins are also generally the products of single genes, allowing the plant to deliver these molecules relatively rapidly and with minimal energetic expense to the plant upon pathogen attack (Thomma et al., 2002). Furthermore, plant defensins are known to interact with additional antimicrobial compounds in their biological context that further elevates host resistance to pathogenic attack (Oh et al., 1999; Chen et al., 2009). These factors contribute to making these antimicrobial peptides excellent candidates for genetic engineering strategies in the pursuit of alternative crop protection mechanisms. Subsequently, several research groups focused on plant disease resistance have identified plant defensins that elevate the level of pathogenic resistance when constitutively overexpressed in otherwise susceptible plants under controlled greenhouse conditions (Table 2.2) (Wang et al., 1999; Gao et al., 2000; Kanzaki et al., 2002; Turrini et al., 2004; Schaefer et al., 2005; Jha and Chattoo, 2009; Portieles et al., 2010).

Table 2.2 Plant defensins used as targets for engineering fungal resistance in crop plants.

Defensins	Source plant	Transgenic plant	Target fungal pathogen	Reference
DRR230-a	*Pisum sativum*	Canola	*Leptosphaeria maculans*	Wang et al., 1999
*Al*AFP	*Medicago sativa*	Potato	*Verticillium dahlia*	Gao et al., 2000
*Dm*AMP1	*Dahlia merckii*	Aubergine	*Botrytis cinerea*	Turrini et al., 2004
Mj-AFP1	*Mirabilis jalapa*	Tomato	*Alternaria solani*	Schaefer et al., 2005
Rs-AFP2	*Raphanus sativus*	Rice	*Rhizoctonia solani; Magnaporthe oryzae*	Jha and Chattoo, 2009
*Nm*Def02	*Nicotiana megalosiphon*	Potato	*Phytophthora infestans*	Portieles et al., 2010
Wasabi defensin gene	*Wasabia japonica*	"Egusi" melon	*Fusarium oxysporum* *Alternaria solani*	Ntui et al., 2010
BoDFN	*Brassica oleracea*	Broccoli	*Hyaloperonospora parasitica*	Jiang et al., 2012

However, reports of transgenic plants overexpressing plant defensin encoding genes through genetic engineering with increased resistance to pathogens under field conditions remain relatively limited (Gao et al., 2000; Portieles et al., 2010). In 2000, Gao et al. demonstrated that potato plants *(Solanum tuberosum*) transformed with the plant defensin, alfAFP, are more resistant to *V. dahlia* than their wild type counterparts under field conditions. Similarly, Portieles et al. (2010) engineered potatoes with elevated resistance to *P. infestanse* through overexpression of the *N. megalosiphon* defensin, NmDef02. These studies provided evidence that a single defensin peptide can reduce susceptibility in the production of commercially viable crop though genetic engineering.

Unfortunately, not all defensin overexpression strategies have proven to be quite so promising. Transformation of *S. lycopersicon* with the tomato defensin, DEF2 showed low transformation efficiency that lead to low levels of recovery of transformed plantlets. The growth of the recovered seedlings was further retarded, producing small leaves and fruits with defects in seed production when compared to wild type counterparts (Figure 2.2) (Stotz et al., 2009). Similarly *P. abeis* transformed with the Norway spruce defensin, Spi1,

showed low levels of successful germination (Elfstrand et al., 2001). Cotton plants transformed with NaD1 also produced substandard yields with small leaves, short internodes and reduced plant fertility (Anderson et al., 2009).

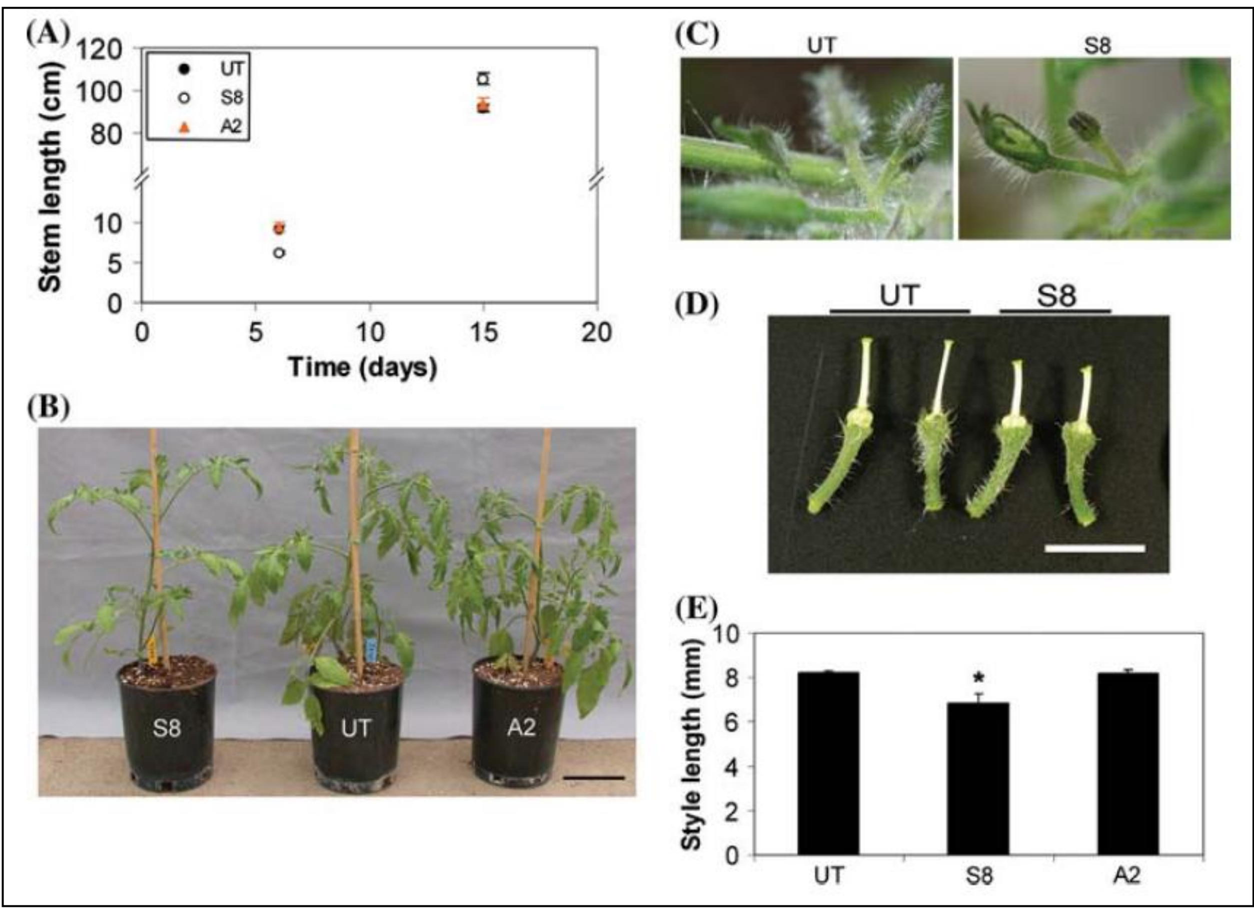

Figure 2.2 Effects of DEF2 expression on organ development and plant growth of *S. lycopersicon* as determined by Stotz et al., 2009. (A) Growth of an untransformed control (UT) and homozygous transgenic (S8 and A1) tomato plants. Error bars indicate standard error (n=7). (B) Image of 8 week old plants; bar, 10 cm. (C) Sepals of line S8 are shorter than those from UT tomato. (D) Carpels of flowers at anthesis; bar, 1 cm. (E) Style lengths as a function of genotype. UT, S8 and A2 tomato are compared. Error bars indicate standard error (n≥5). Asterisk indicates statistically significant differences from UT at P<0.05.

These reports indicate that some plant defensins may have deleterious effects on plant growth and reproductive development. Furthermore, current transgenic approaches have been predominantly focused on the constitutive expression of these plant defensins hereby expending unnecessary energy in the constant production of these peptides despite having no threat from pathogens. Because plant defensins possess such a wide array of potentially useful functions in transgenic crops such as resistance to biotic and abiotic stresses, new engineering strategies are being explored to implement these peptides in transgenic crops. These strategies explore the promoters that drive the

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expression of these peptides and how alterations of these promoters may lead to the production of commercially viable transgenic crops with no growth abnormalities.

In an attempt to restrict expression of defensin genes in transgenic plants, some promotors have gained particular attention due their inducibility upon pathogen attack (Rushton et al., 2002; Himmelbach et al., 2010). An example of these pathogen inducible promoters is the *GER4c* promotor from barley that is induced rapidly and strongly upon attack by a range of necrotrophic and biotrophic fungi (Himmelbach et al., 2010) and could therefore be applied in transgenic approaches involving defensin genes in monocotyledonous plants. Synthetic pathogen-inducible promotors have also been generated to target defensin gene expression to infection sites, however, these strategies remain to be evaluated (Rushton et al., 2002).

Another possible approach in the transgenic strategies regarding plant defensins includes the use of tissue specific promotors. Since many fungal pathogens target specific plant tissues exclusively, plant defensin expression could be targeted to that specific tissue to reduce infection. In maize, the ear rot disease caused by *Fusarium verticillioides*, targets the silk channel of the particular plant, therefore the use of the silk channel-specific promotor, SLG, to target defensin expression could potential limit the infection of this fungus (Liu et al., 2008). Numerous fungi similarly infect specific plant tissues exclusively and these tissue specific promotors could be successful in the targeting of plant defensin to specific tissues although these strategies are still being investigated.

Furthermore, it has also been reported by Anderson et al. (2009), that transformations generated by fusing the defensin NaD1 with a C-terminal domain that targets the defensin to the cell vacuole produces tomato and cotton plants with normal growth and increased resistance to *B. cinerea* and *F. oxysporum* (Anderson et al., 2009). These results prove that although genetic engineering strategies involving defensins require extensive future research, the potential implementation of these peptides for disease resistance remains promising.

Despite the overwhelming evidence of the economic importance of exploring genetic engineering strategies in the context of crop protection, the generation of genetically modified organisms (GMOs) remains a highly controversial topic. As a result, cisgenic engineering strategies have become increasingly more popular in that this strategy involves the generation of genetically altered plants that contains only genetic material

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from the host organism. Hereby, the intact functional gene controlled by its own promotor and regulatory signals is transferred to the target organism as a whole (Zhu and Jacobsen, 2012). Therefore, despite the cloud of controversy surrounding GMO research, it has been proposed that plants with cisgenic resistance genes should be exempt from GMO regulations by the United States Environmental Protection Agency (Reardon, 2011). Furthermore, the European Food Safety Authority compared the risk levels involved with cisgenic approaches to that of classical plant breeding strategies (EFSA, 2012). Future genetic engineering strategies involving defensin genes should shift focus towards cisgenics that may allow for the production of more commercially acceptable transgenic plants with elevated resistance against pathogens.

2.7 Grapevine defense

Grapevine is considered to be the most economically important fruit crop worldwide and cultivated in more than 60 countries (Vivier and Pretorius, 2002). However, no other crop is challenged by more fungal pathogens (Martelli and Boudon-Padieu, 2006). Almost all commercial grapevine production relies on the cultivars of the European grape, *Vitis vinifera*, which are highly susceptible to infection by various fungal pathogens since this species has very limited innate immunity against a multitude of necrotrophic and biotrophic pathogenic fungi. These fungal pathogens do not only cause substantial crop losses but lowers the quality of the grapes and subsequent grape related products. Furthermore, some fungal pathogens are known to produce mycotoxic compounds that pose health threats to the consumer.

Similar to the transgenic approaches taken in the disease resistance of various commercial crops previously discussed, attempts have been made at increasing grapevine resistance at the molecular level. However, because of the broad range of grapevine-specific fungal pathogens that threaten this crop, the mechanism of infection of various groups of fungal pathogens requires separate consideration before genetic engineering approaches will be discussed.

2.7.1 Pathogen lifestyle-specific infection

Of all grapevine diseases, infection by the biotrophic fungus that causes powdery mildew is most economically important (Winterhagen et al., 2008). Biotrophs spend the majority of

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their life-cycle in the intracellular spaces or as intracellular haustoria bound to the plasma membrane of their hosts. These pathogens depend on living plant tissue for their own growth and survival. Grapevine resistance against the grapevine powdery mildew fungus, *Erysiphe necator*, is known to consist of a two-layer defense response (Dry et al., 2009). These defense mechanisms include pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is considered to be the plant's first line of defense against powdery mildew infection as it involves the detection of the pathogen PAMP chitin by a plant membrane receptor-like kinase (Robatzek et al., 2006; Miya et al., 2007; Wan et al., 2008). This pathogen detection mechanism causes a cascade of signaling events that lead to the secretion of antimicrobial and cell-wall restructuring proteins in order to physically obstruct fungal penetration (Kwon, 2010). However, powdery mildew pathogens have developed host-specific resistance by means of the production of effector-molecules that can effectively suppress PTI. Therefore, the second layer of plant defense response involves the detection of these effector molecules, hereby triggering a second signaling cascade leading to numerous defense responses that include programmed cell death (PCD) (Peterhänsel et al., 1997).

A recent study evaluated the mechanisms of resistance to powdery mildew within the *Vitaceae* family (Feechan et al., 2011). This study revealed the various mechanisms of resistance to *E. necator* portrayed by the different grapevine species since it is well known that grapevine from North American descent show high levels of resistance to various grapevine pathogens. These species include *Vitis* species such as *Vitis riparia, V. aestivalis* and *V. rupestris* (Fung et al., 2008; Cadle-Davidson, 2011). The results of this evaluation revealed that various levels of resistance within this family were either associated with penetration resistance or PCD-associated resistance or a combination of these two mechanisms (Figure 2.3) (Feechan et al., 2011).

As previously discussed, it has been reported that programmed cell death (PCD) is involved in the inhibition activity of plant defensins (Aerts et al., 2006; Aerts et al., 2007; van Weerden et al., 2008; Aerts et al., 2009; Mello et al., 2011). Therefore, it would be reasonable to hypothesize that the overexpression of plant defensin encoding genes involved in the induction of PCD would result in a grapevine phenotype with enhanced resistance against *E. necator* infection.

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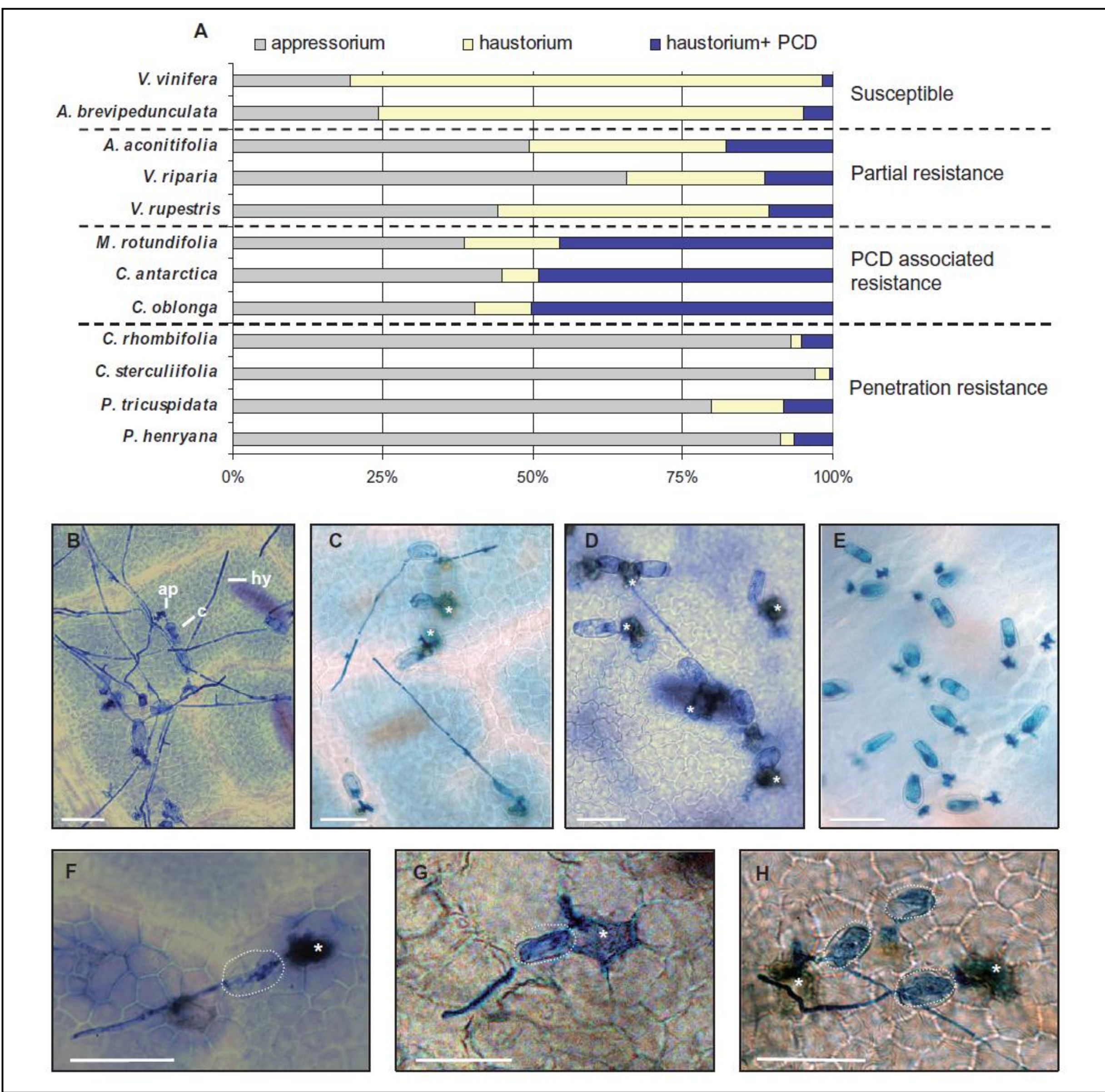

Figure 2.3 Susceptibility of various Vitaceae species to *Uncinula necator* infection as presented by Feechan et al. (2011). The results of *E. necator* infection on leaves at 48 hours post inoculation. (A) Frequency of *E. necator* penetration attempts on various Vitaceae members which lead to appressorium formation but no penetration, successful penetration and haustorium formation or a haustorium followed by PCD of the penetrated cell. Each data point is based on three biological leaf replicates on which a minimum of 100 germinated conidia were scored. (B-H_ Trypan blue staining following *E. necator* inoculation. (B) Susceptible *V. vinifera*. (C) Partial resistant *V. riparia*. (D) PCD-associated resistance of *Muscadinia rotundifolia*. (E) Penetration resistance in *Parthenocissus tricuspidata*. (F) PCD response in *M. rotundifolia*. (G) PCD response in *Cissus Antarctica*. (H) PCD response in *C. oblonga*. Asterisks indicate cells which have undergone PCD as stained by trypan blue. Broken white circles indicate the position of a conidium. Ap, appressorium; c, conidium; hy, hypha. Scale bars, 50 µm

It is well-known that North American grapevine species are much more resistant to *E. necator* than the European *V. vinifera* species and the underlying molecular basis of this resistance has been evaluated. One source of this resistance to powdery mildew has been identified as a single dominant locus designated *Run1* (Resistance to *U. necator* 1) (Pauquet et al., 2001; Dry et al., 2009). Functional analysis of *Run1* in *E. necator* resistance revealed PCD-associated defense responses linked to the *Run1*-mediated defense in known resistant cultivars such as *M. rotundifolia*. As discussed earlier, this PCD

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response involves apoptosis of epidermal cells penetrated by *E. necator*, hereby limiting the spread of this biotrophic fungus (Dry et al., 2009). Even though Run1 appeared to confer resistance against all *E. necator* isolates throughout Europe and Australia, the effectiveness of Run1 in the management of *E. necator* resistance have come under question after the identification of *E. necator* isolates that can overcome the activity of grapevine PR proteins (Ramming et al., 2011).

Wild Chinese *Vitis* species have also shown significant resistance to the grapevine powdery mildew fungus that has led to the identification of the single dominant locus *Ren4* (Resistance to *E. necator* 4) from *V. romanetii* (Wan et al., 2007; Mahanil et al., 2011; Ramming et al., 2011). Unlike *Run1*, *Ren4* has been found to confer a non-race-specific resistance to the *E. necator* fungus although the resistance mechanism has not yet been elucidated (Ramming et al., 2011).

Contrasting to the life-cycle of biotrophic fungi, necrotrophic fungi such as *B. cinerea* are known to rely on dead host cells for nutrition and reproduction. *B. cinerea* employs various molecular mechanisms in order to establish these optimal growth conditions by killing host plant cells. The pathogen achieves this through the secretion of phytotoxins that induces cell collapse, hereby aiding in successful host penetration and colonization (Colmenares et al., 2002). Additionally, *B. cinerea* produces reactive oxygen species that include hydrogen peroxide (H_2O_2) during the infection process (Schouten et al., 2002). To further escalate the process of cell death, plant cells themselves produce these reactive oxygen species as part of a hypersensitive response to pathogen attack (Dixon et al., 1994; Grant and Mansfield, 1999). These plant defense responses are driven by a signal transduction cascade that induces PCD through the production of AMPs in an attempt to isolate the pathogen from surrounding living, nutritious tissue as previously mentioned. However, this strategy proves to be counter-productive in the defense against *B. cinerea* that thrives on dead tissue. Therefore, the overexpression of defensin encoding genes proposed for the inhibition of biotrophic fungi could have no effect or even a beneficial effect on the growth of the necrotrophic *B. cinerea*.

Plant defensin can be considered as a group of antifungal peptides with pathogen specific inhibitory activity overlapping in antimicrobial spectra. Therefore, tailoring transgenic grapevine populations overexpressing combinations of antifungal peptide encoding genes that exhibit different modes of antifungal activity will prove to be most valuable in the pursuit of commercially viable transgenic grapevine crops. Evidence for the success of this

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genetic engineering strategy exists in the form of co-expression studies combining the antimicrobial activities of plant defensins and other antimicrobial proteins. In 2009, Chen et al. proved that co-expression of the defensin MsDef1 and a rice chitinases endowed tomato plants with elevated resistance against *B. cinerea* when compared to plants overexpressing either protein in isolation. Similarly, co-expression of the wasabi defensin and a *Streptomyces griseus* chitinase C provided transgenic tobacco with higher resistance to *F. oxysporum* than plants overexpressing either of these peptides alone (Ntui et al., 2011).

In grapevine, several attempts have been made to enhance pathogen resistance of this highly susceptible species through overexpression of PR proteins. In 2006, Vidal et al. performed functional analyses with grapevine overexpressing the small insect antimicrobial magainin peptide, mag-2, against the crown gall causing bacterial pathogen and *E. necator*. Of the nine uniquely transformed mag-2 grapevine lines, only two lines showed mild levels of resistance to *E. necator* (Vidal et al., 2006). In another study involving grapevine, overexpression of a rice chitinase gene (RCC2) showed two of the 20 uniquely transformed transgenic *V. vinifera* (cv. Neo muscat) showed increased resistance to *E. necator* (Yamamoto et al., 2000). These overexpression studies not only underscore the urgency in exploring antifungal resistance in grapevine as a means of crop protection, but the possible roles that overexpression of defensin genes may have on the improvement of grapevine fungal resistance.

2.7.2 The first grapevine defensin, Vv-AMP1

Since the grapevine genome became the first commercial fruit to be completely sequenced in 2007 (Jaillon et al., 2007; Velasco et al., 2007), attempts have been made to systematically identify genes that encode for defensin-like peptides (De Beer and Vivier, 2008; Giacomelli et al., 2012). The first antimicrobial peptide was identified in grapevine known as Vv-AMP1 (*Vitis vinifera* antimicrobial peptide 1) and this peptide have subsequently been isolated and characterized (De Beer and Vivier, 2008). Expression of the Vv-AMP1 encoding gene was found to be developmentally regulated, limited to berry tissue from the onset of ripening onwards. Expression of the *Vv-AMP1* gene was not inducible through external hormone stimulus, wounding or pathogenic infection. Upon further evaluation it was found that recombinant production of Vv-AMP1 yielded a highly heat-stable protein with a molecular mass of 5.495 kDa that accumulated primarily in the

apoplastic region of the plant cell. Furthermore, the recombinantly produced Vv-AMP1 peptide proved to inhibit growth of a wide range of plant pathogens at low concentrations through non-morphogenic antifungal activity (De Beer and Vivier, 2008). Subsequent *in vitro* antifungal assays confirmed the antifungal activity of Vv-AMP1 against a wide range of grapevine specific pathogens at exceptionally low peptide concentrations (Tredoux, 2011). These promising results prompted the overexpression and subsequent evaluation of this peptide in various biological systems.

Vv-AMP1 was overexpressed in tobacco and the putative transgenic population genetically characterized before detached leaf antifungal assays were performed with the necrotrophic fungus, *B. cinerea* (De Beer, 2008). In these transgenic tobacco lines, overexpression of Vv-AMP1 did not provide an increased resistance to *B. cinerea* and the authors attributed these results to the possible peptide instability or non-functionality in the heterologous environment (De Beer, 2008). Subsequently, Vv-AMP1 was constitutively overexpressed in its native host, hereby generating a genetically characterized transgenic population consisting of nine independently transformed transgenic *V. vinifera* (cv. Sultana) lines (Tredoux, 2011). Preliminary detached leaf antifungal assays with *B. cinerea* revealed only marginal resistance conferred by Vv-AMP1. However, the promising results obtained in the *in vitro* analysis of Vv-AMP1 provides enough support for the pursuit of establishing the *in planta* role of this peptide and its possible involvement in the production of transgenic grapevine with resistance to fungal pathogens.

2.8 Summary

Since the dawn of modern agriculture, the effective disease management of food crops has posed challenges to the rapidly growing world population. Due to the increased prevalence of pathogens with resistance towards traditional pesticide treatments, alternative strategies in disease control are being actively pursued. The latest strategies in the protection of crops against microbial pathogens are rooted in harnessing the highly complex innate immunity evolved by plants themselves through genetic engineering. At the center of plant innate immunity is the *de novo* production of defensin peptides within the antimicrobial peptide family.

The majority of plant defensins are known to possess some form of antimicrobial activity although the exact mechanism leading to the antifungal activity of plant defensins remains unconfirmed. It has however been established that defensins bind specific fungal

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membrane receptors, the sphingolipids, to facilitate fungal inhibition by permeabilization of the fungal membranes (Thevissen et al., 1999). These plant defensins have however been implicated in numerous biological functions that include plant responses to abiotic stresses and their involvement in plant reproductive development and growth. Furthermore, these defensin peptides are generally the products of single genes, allowing the plant to deliver these molecules relatively rapidly and with minimal energetic expense to the plant (Thomma et al., 2002). Defensins have also been found to be non-toxic to eukaryotic organisms. These factors contribute to making defensin peptides excellent candidates for genetic engineering strategies in the pursuit of alternative crop protection mechanisms.

Although several plant defensins have been successfully implemented in the production of transgenic crops with elevated resistance to fungal pathogens, increased resistance against fungal pathogens through defensin expression has not yet been attained in grapevine. Grapevine is considered to be the most economically important fruit crop worldwide and since the complete grapevine genome sequence became available in 2007, attempts have been made to identify defensin genes in this crop. These investigations lead to the identification of the first defensin from grapevine, Vv-AMP1 and subsequent studies proved this peptide to be highly active against fungal pathogens *in vitro. In planta* investigations of the resistance phenotype of Vv-AMP1 could reveal the possible applications of this plant defensin in the active pursuit of commercially viable transgenic crops.

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Chapter 3

Evaluation of the defense phenotype of transgenic *Vitis vinifera* overexpressing the defensin, Vv-AMP1

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Research Results

3.1 Introduction

Plants are continuously exposed to potentially harmful pathogens but despite their sessile nature, the prevalence of successful pathogen infection remains relatively infrequent. However, plant diseases caused by pathogens contribute to crop losses of an estimated 10% worldwide (Strange and Scott, 2005; Ronald and Adamchak, 2008). Therefore, one of the greatest challenges since the onset of modern agriculture is the successful disease management of these crops.

Similar challenges are addressed in the disease control of grapevine that is known as the most important and widely cultivated fruit crop in the world (Vivier and Pretorius, 2002). Almost all commercial grapevine production relies on the cultivars of the European grape, *Vitis vinifera*, which are highly susceptible to infection by various fungal pathogens since this species has very limited innate immunity against a multitude of necrotrophic and biotrophic pathogenic fungi. The severe pathogen pressure experienced by *V. vinifera* has been recently attributed to the domestication history through vegetative propagation of these grapevines (Myles et al., 2011).

Although disease management of these crops have depended mainly on the application of chemical fungicides (Pezet et al., 2004), attempts at biological control (reviewed in Compant et al., 2012) and challenging molecular breeding programs, a safe, economically viable crop protection strategy is still being actively pursued. Therefore, the latest strategies in the protection of crops against microbial pathogens are rooted in harnessing the highly complex innate immunity evolved by plants themselves through genetic engineering.

Among the highly specialized defense mechanisms in the plant's arsenal against pathogen attack is the *de novo* production of antimicrobial peptides (Osbourn, 1996; Ahn et al., 2002; van Loon et al., 2006; Ferreira et al., 2007).

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An example of these peptides is defensins that offer antimicrobial resistance to a range of microbial pathogens. The plant defensin family of peptides can be recognized based on the highly conserved three-dimensional structure that is shared between its members. This three dimensional structure comprises a single α-helix and three anti-parallel β-strands. At the core of this conserved structure is a cysteine-stabilized alpha-beta motif (CSαβ) (Bloch and Richardson, 1991; Fant et al., 1998; Almeida et al., 2002; Jansen et al., 2003). The plant defensins are highly basic, small peptides of ~5kDa or 45-54 amino acid residues characterized by typically eight cysteine residues linked through four disulfide bridges. Even though defensins display little homology on the amino acid level, they are considered to form part of a superfamily of similarly folded peptides with anti-microbial activity that predate the evolutionary divergence of animals and plants due to their representation among vertebrates, invertebrates, mammals and plants (Broekaert et al., 1995; Thomma et al., 2002).

The majority of plant defensins are known to possess some form of antimicrobial activity although the exact mechanisms remain unconfirmed. It has however been established that defensins bind specific fungal membrane receptors, the sphingolipids, to facilitate fungal inhibition by permeabilization of the fungal membranes (Thevissen et al., 1999). Furthermore, these defensin peptides are generally the products of single genes, allowing the plant to deliver these molecules relatively rapidly and with minimal energetic expense to the plant (Thomma et al., 2002). Defensins have also been found to be non-toxic to eukaryotic organisms. These factors contribute to making defensin peptides excellent candidates for genetic engineering strategies in the pursuit of alternative crop protection mechanisms.

Since the identification of the first antimicrobial peptide from grapevine known as Vv-AMP1 (*Vitis vinifera* antimicrobial peptide 1), this defensin peptide has been isolated and characterized (De Beer and Vivier, 2008). Expression of the Vv-AMP1 encoding gene was found to be tissue-specific and developmentally regulated, specifically limited to berry tissue from the onset of ripening and was not inducible through external hormone stimulus, wounding or pathogenic infection. Furthermore, the recombinant production of the Vv-AMP1 peptide in

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E. coli yielded a highly heat-stable protein with a molecular mass of 5.495 kDa. Recombinantly produced Vv-AMP1 was also evaluated for its antimicrobial activity *in vitro*. These assays proved the Vv-AMP1 peptide to inhibit growth of a wide range of grapevine pathogens, some at remarkably low concentrations through characteristic non-morphogenic antifungal activity (De Beer and Vivier, 2008; Tredoux, 2011). Vv-AMP1 was further overexpressed in tobacco, however, these transgenic lines showed no significant difference with regards to resistance to *Botrytis cinerea* in detached leaf infection assays, perhaps due to peptide instability or non-functionality in the heterologous environment (De Beer, 2008).

Recently, Vv-AMP1 was overexpressed in its native host, *V. vinifera* (cv. Sultana), hereby generating a putative transgenic *V. vinifera* (cv. Sultana) population that was subsequently genetically characterized (Tredoux, 2011). This transgenic population yielded nine unique, independently transformed plant lines with confirmed Vv-AMP1 expression and showed mild resistance to *B. cinerea* compared to an untransformed control line in a detached leaf infection assay (Tredoux, 2011).

Following the previous work, the present study aimed to evaluate the antifungal resistance phenotypes of these transgenic Vv-AMP1 Sultana lines *in planta* in order to establish whether these transgenic lines possessed reduced susceptibility against grapevine fungal pathogens. The above mentioned transgenic population of Sultana lines overexpressing Vv-AMP1 and untransformed Sultana wild type plants were established as a greenhouse population and morphologically characterized prior to infection assays. In order to establish a reproducible pathosystem for infection assays involving the biotrophic grapevine powdery mildew fungus, several preceding infection assays were performed for the purpose of optimizing the conditions for these experiments. Factors that were optimized included the ontogenic stages of leaves being infected and the various facets involved in the different methods of inoculation. Detached leaf infection assays were subsequently performed according to the newly optimized methods by challenging leaves at various developmental stages from the transgenic Vv-AMP1 Sultana lines and untransformed *V. vinifera* controls with *E. necator* conidia. Furthermore, a

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whole-plant infection assay with the necrotrophic fungus, *B. cinerea*, was performed according to a previously optimized pathosystem for infection assays with this pathogen.

Transgenic *V. vinifera* (cv. Sultana) overexpressing Vv-AMP1 showed elevated resistance to the biotrophic fungus, *E. necator*, despite showing no reduced susceptibility to the necrotrophic fungus, *B. cinerea* compared to the untransformed *V. vinifera* control line. These results expose the potential of the implementation of defensin peptides in alternative plant disease resistance strategies and warrants further exploration of the possible secondary functions of defensins *in planta*.

3.2 Materials and Methods

3.2.1 Plant growth conditions

The transgenic *V. vinifera* (cv. Sultana) population overexpressing Vv-AMP1 was obtained from the grapevine transformation and regeneration platform of the IWBT and maintained *in vitro* on Murashige and Skoog basal salt mixture (Murashige and Skoog, 1962) in a tissue culture growth room. The genetic characteristics of the transgenic population had been previously determined and are summarized in Table 3.1.

Uniquely transformed transgenic lines that were not represented in the *in vitro* population were re-initiated from a stable greenhouse population of transgenic Vv-AMP1 Sultana plants. These plantlets were cultivated on MS medium (Murashige and Skoog, 1962) supplemented with 10 µg/ml of 6 bensyl-aminopurine (BAP) to induce shoot formation. Developing shoots were transferred to MS medium supplemented with 2.6 µg/ml of naphthalenacetic acid (NAA) to induce root formation after which it was maintained on MS medium (Murashige and Skoog, 1962). The transgenic plantlets from each individual transgenic line, as well as untransformed controls, were multiplied *in vitro* to establish a working population for hardening off and subsequent experimentation.

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Table 3.1 Summary of the characterization of nine transgenic *V. vinifera* (cv. Sultana) lines overexpressing the Vv-AMP1 plant defensin as adapted from Tredoux (2011). Characterization included PCR screening, Southern and Northern blot analysis. Number of integrations refers to the number of copies of the Vv-AMP1 transgene integrated into the genome. A "+" indicates a positive result whereas a "-" denotes a negative result. Sultana WT indicates the untransformed *V. vinifera* cv. Sultana lines used as control.

Plant line	Sultana WT	6	7	8	9	10	14	17	18	19
PCR	-	+	+	+	+	+	+	+	+	+
Southern Blot	+	+	+	+	+	+	+	+	+	+
Northern Blot	-	+	+	+	+	+	+	+	+	+
Number of transgene integrations	0	2	6	2	3	2	3	2	2	5

Vegetatively propagated plantlets from each transgenic line, as well as several wild type *V. vinifera* (cv. Sultana) plants were then systematically hardened off in Grodan plugs (Grodan A/S, Denmark) supplemented with hydroponic Kompel nutrient solution (Chemicult Products Pty Limited, Camps Bay, South Africa). These plantlets were maintained at 25°C and moderate humidity in a greenhouse environment and subsequently transferred to potting soil (Double Grow, Durbanville, South Africa) and maintained under the same conditions. These plants were watered twice a week and supplemented with Nitrosol natural organic plant food (Envirogreen Pty Limited, Fleuron, Braamfontein, South Africa) once every four weeks.

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3.2.2 Morphological characterization of a transgenic *V. vinifera* (cv. Sultana) population overexpressing Vv-AMP1

After the process of hardening off the transgenic lines from their initial tissue culture environment, the plants were closely monitored for any observable morphological phenotypes under stable, non-stressed conditions. The morphological characters observed included general size of the plants, presence and appearance of tendrils and vegetative organs, size and general appearance of the leaves, and arrangements of the nodes and internode lengths.

3.2.3. Infection assays

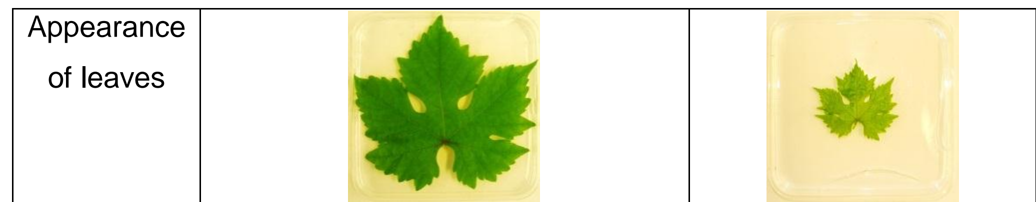
The transgenic population and untransformed controls were subjected to infection assays with a biotrophic and necrotrophic fungal pathogen according to the schedule outlined in Table 3.2. Details of the infection assays and the methods used to record the reaction of the plant and/or the pathogens are outlined below.

3.2.3.1 Detached leaf infection assays with the biotrophic fungus, *E. necator*

Grapevine powdery mildew (*E. necator*) was established and maintained on potted wild type *V. vinifera* (cv. Sultana) plants by inoculation with dry conidia obtained from naturally infected grapevine. These source plants were maintained under conditions favouring optimal growth and development of the grapevine powdery mildew fungus with regards to levels of relative humidity (RH), temperature and ultraviolet (UV) exposure (Delp, 1954; Doster and Schnathorst, 1985; Willocquet et al., 1996; Carrol and Wilcox, 2003). The inoculated plants were kept at 100% relative humidity (RH) in the dark for the first 24 hours after inoculation after which a light/dark cycle of 16/8 hours was maintained. At 48 hours post infection (hpi), the RH was lowered by removing the plants from the humidity chambers. Throughout the cultivation and maintenance of this fungus, a constant temperature of 25°C was maintained.

Table 3.2 Summary of the plant lines used for whole plant and detached leaf antifungal assays with necrotrophic and biotrophic pathogenic fungi. Indicated in brackets are the number of individual plants per line used in the whole plant infection assays and the number of detached leaves per line used in the detached leaf antifungal assays. The ontogenic stage of the leaves chosen for each assay is indicated and representative pictures were taken in 120 X 120 mm square culture dishes.

Plant Line	Necrotroph: *B. cinerea*	Biotroph: *E. necator*		
	Details of infection assay			
	Whole plant infection assay	Detached leaf: wet inoculation	Detached leaf: dry inoculation	Detached leaf: SEM analysis
6	-	-	+(7)	+(2)
8	-	-	-	+(2)
9	-	-	-	+(2)
10	+(3)	+(5)	+(7)	+(2)
14	+(3)	-	-	+(2)
17	-	-	-	+(2)
18	-	+(5)	+(7)	+(2)
19	+(3)	-	-	+(2)
Wild Type	+(3)	+(5)	+(7)	+(4)
Ontogenic leaf stage	Mature, fully expanded leaves		Young, glossy half expanded leaves (~6 cm diameter)	

Appearance of leaves	

3.2.3.1.1 Method optimization and description for E. necator detached leaf assays

In order to establish a repeatable and reproducible protocol for *in planta* infection assays with *E. necator,* several infection assays were performed for the purpose of optimizing the conditions for these experiments (results not

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shown). Factors that were optimized included the ontogenic stages of leaves being infected and the method of inoculation. Inoculation methods that were explored included an inoculation by sweeping of dry *E. necator* conidia onto healthy leaf surfaces (Feechan et al., 2011), as well as a spray inoculation method with a conidial suspension (Yamamoto et al., 2000). The optimal concentration of the conidial suspension, methods of cleaning the harvested conidia and the effect of Tween 20 as a wetting agent in the conidial suspension were also examined (results not shown). Furthermore, the method of application of the conidial suspension was optimized by spraying the conidial suspension onto the adaxial leaf surfaces with a UV-sterilized non-aerosol spray-attachment connected to a sterile 15ml falcon tube. During method optimization experiments, the efficacy of the spray attachment was evaluated to confirm that conidia were successfully emitted through the spray filter and that suspensions were evenly distributed on the leaf surface. This was achieved by spraying inoculum directly onto 1% water agar medium (which were used as support for the detached leaves) in square culture dishes (120 X120 mm) and observing conidial germination under a light microscope under 40X magnification. Furthermore, during inoculation with the *E. necator* conidial suspension, one spray was emitted onto each of the four quadrants of the culture dish surface at approximately 10 cm from the surface of the dish at a 90 degree angle, hereby ensuring homogenous distribution of inoculum amounting to approximately 0.5 ml per leaf.

The following method described for the preparation and inoculation of detached grapevine leaves with conidia of *E. necator* are therefore based upon the factors that proved to be optimal during preceding method optimization experiments.

3.2.3.1.2 *Preparation of leaves for infections*

All plants chosen for infection assays were healthy with no visible disease symptoms. The plant lines used in each infection assay were chosen due to the availability of ample biological repeats of each of these lines represented

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in the greenhouse environment. The lines chosen for each assay are summarized in Table 3.2.

The infection with a conidial suspension of *E. necator* was performed using five mature, fully expanded leaves of similar size per plant line, with three individual plants per line as outlined in Table 3.2. The infection assay with dry conidia of *E. necator* was performed on seven young, glossy leaves of approximately 6 cm in diameter per line, with three individual plants per line (Table 3.2). For the analysis with the Scanning Electron Microscope (SEM), two young, glossy leaves per transgenic Vv-AMP1 line and an untransformed wild type line were used for inoculation with dry *E. necator* conidia.

Following harvesting, the leaf surfaces were sterilized by soaking in 0.25% (w/v) $CaOCl_2$ solution for 5mins with three subsequent rinsing steps with dH_2O for 5mins each. The leaves were then patted dry between tissue paper and left to dry from remaining surface moisture in a laminar flow cabinet. The lower tip of the petiole of each leaf was removed, leaving a 1cm petiole section. The leaves were placed on 1% (w/v) water agar medium in square culture dishes (120 x 120 mm), adaxial side up, with the remaining petiole inserted in the medium. All preparation and wash steps were performed in a laminar flow cabinet. Leaves were then either inoculated with *E. necator* conidia in a suspension or as dry conidia (see below).

3.2.3.1.3 Detached leaf inoculation with a conidial suspension of E. necator

The *E. necator* conidia used for this infection assay were obtained from heavily infected leaves from source plants as previously described. Conidia were harvested from the leaf surfaces by rinsing leaf-cuttings in sterile distilled water containing 0.1% (w/v) Tween 20 as described by Yamamoto et al. (2000). The conidial suspension was subsequently cleaned by centrifugation at 250rpm for 5mins, followed by the replacement of the supernatant with fresh distilled water containing 0.1% (w/v) Tween 20.This cleaning step was repeated 3 times before the concentration of the conidia as determined using a Spencer Bright-Line haemocytometer and adjusted to a final concentration of $1x10^5$ $conidia.ml^{-1}$.

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The conidial suspension was then sprayed onto the adaxial leaf surfaces with a UV-sterilized non-aerosol spray-attachment connected to a sterile 15ml falcon tube by emitting one spray onto each quadrant of the culture dish as previously described during optimization steps. This amounted to approximately 0.5 ml of inoculum per leaf. All preparations and inoculations were performed in a laminar flow cabinet.

According to the optimal growth conditions determined for *E. necator* (Delp, 1954; Doster and Schnathorst, 1985; Willocquet et al., 1996; Carrol and Wilcox, 2003), the infected leaves were kept at 25°C at 100% RH, with the culture dish lids closed, in the dark for 24 hours after which a light/dark cycle of 16/8 hours was maintained without direct exposure to UV-radiation. 48 hours after initial infection the humidity was lowered by opening the culture dish lids once daily.

3.2.3.1.4 Detached leaf infection inoculation with dry E. necator conidia

Similar to the assay described above, prepared detached leaves in culture dishes were challenged with dry conidia of the powdery mildew fungus, *E. necator*. The *E. necator* conidia used for this infection assay were obtained from the same source of heavily infected plants maintained for the cultivation of this fungus as previously described.

The detached leaves were inoculated with *E. necator* conidia by gentle sweeping of the infected leaves over the healthy leaves, tapping off conidia onto the adaxial sides of the leaves in the culture dishes according to the method described by Feechan et al. (2011). The infected leaves were maintained as previously described.

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3.2.3.1.5 *Disease assessment of E. necator*

Microscopy

48 hours after inoculation with dry *E. necator* conidia, one leaf per plant line was removed from the infection and entirely stained in trypan blue solution for 1 hour in a boiling water bath as described by Koch and Slusarenko (1990). It was subsequently decolourized in a 2.5g/ml chloral hydrate solution for 30 mins before it was visualized and photographed under a light microscope at 40X magnification. Germinated conidia were counted and categorized according to each mechanism of infection as described by Feechan et al. (2011). These categories included conidia forming appressorium, haustorium or a combination of haustorium and programmed cell death (PCD). Percentages of each infection mechanism were calculated in order to scale the level of susceptibility of each plant line.

At 14 dpi this trypan blue staining method was repeated and results were observed and photographed as described above.

Two leaves representing each transgenic Vv-AMP1 line and four leaves representing a Sultana wild type line (Table 3.2) were observed under a Leo® 1430VP Scanning Electron Microscope at 48 hours post inoculation. Leaves were cut into 5 X 5mm squares and mounted with carbon tape for observation at 500X and 1000X magnification, respectively.

Plant phenotypic reaction

In order to describe the observable programmed cell death (PCD)-associated changes of the leaf surfaces in response to *E. necator* infection, a plant reaction scale (Table 3.3) was generated for the standardization of the descriptors used. The observable changes where scored daily from 4 to 14 dpi according to this scale.

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Table 3.3 The infection induced PCD scale generated for the standardization of descriptors referring to visual changes to grapevine leaf surfaces when challenged with *E. necator* in a detached leaf infection.

Index	Phenotypic characteristic descriptors	Visual representation
9	Green, healthy	
8	Majority green, healthy; small, slightly discoloured yellow patches (<1mm^2)	
7	Majority green, healthy; large slightly yellowing patches	
6	Majority covered in large yellow patches; rest of the tissue is slightly yellowing	
5	Majority covered in large yellow patches with small secondary necrotic lesions	
4	Entire leaf surface yellow with secondary necrotic lesions on ±25% of leaf surface	
3	Entire leaf surface discoloured with secondary necrotic lesions on ±50% of the leaf surface	
2	Entire leaf discoloured; majority of the leaf surface covered in secondary necrotic lesions	
1	Entire leaf dead	

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Evaluation according to the global resistance index

A stereomicroscope was used at 100X magnification to evaluate the development of fungal structures and sporulation of the inoculated *E. necator* conidia. These developments were then scored daily on a scale from 1 – 9 from 4 to 14 dpi as described by Miclot et al. (2012). The global resistance index is summarized in Table 3.4.

Table 3.4. Grapevine powdery mildew resistance indexes adopted from Miclot et al., 2012. Pictures were taken under a stereomicroscope at 100X magnification.

Resistance index	Description	Global response
9	Mycelium rare, no sporulation	
7	Mycelium scattered, no sporulation	
5	Mycelium widespread, low density, weak sporulation	
3	Mycelium widespread, dense, moderate sporulation	
1	Widespread, dense sporulation	

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Spore counting

At 14 dpi, four independent discs of 1cm in diameter were excised from each infected leaf recovered from the detached leaf assay inoculated with dry *E. necator* conidia. The leaf discs collected from each leaf were combined in 10ml sterile distilled water containing 0.1% (w/v) Tween 20. From each conidial suspension, five aliquots were taken and conidia were counted using a Spencer Bright-Line haemocytometer. The average conidial concentration of each leaf was calculated.

3.2.3.2 Whole-plant infection assay with the necrotrophic fungus, B. cinerea

The *B. cinerea* strain used as the fungal pathogen in this particular assay is considered to be a grapevine-related *Botrytis* strain that was originally isolated from a vineyard environment (Joubert et al., 2006). The strain was cultivated on sterile apricot halves (Naturlite, Tiger Food Brands Limited, South Africa) at 23°C until sporulation. Germination potential assays were performed of the developed spores by growing the spores on 0.8% (w/v) water agar.

A reproducible pathosystem for *B. cinerea* that had been established in prior infection studies (Moyo, 2011) were followed by using 1000 *B. cinerea* spores per infection spot. The spore concentration was determined microscopically by utilizing a haemocytometer and adapted to achieve an end concentration of 50% (v/v) red grape juice containing 1000 spores per 5µl spot.

Plants that have been hardened off and acclimatized to a greenhouse environment were used for the whole-plant infection assays. Plants chosen for this infection assay were of similar size and in good physical health and possessed several mature, fully expanded leaves to be infected with fungal spores (Table 3.2). These plants were sprayed with H_2O for three consecutive days to remove any excess dusting sulfur applied during the cultivation of the population and was left to settle between cleaning treatments to minimize handling stress before the infection assay was performed. 24 hours prior to the commencement of the infection assay, the prepared plants were pre-

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incubated in Perspex high humidity infection chambers to allow for ample acclimatization. Room temperature and a light/dark cycle of 16/8 hours were maintained before and during the entire infection assay.

Four leaves per plant (and three individual plants per line) were then infected with 3 spots each on the adaxial side. During spotting of the spores, the major leaf veins were avoided. The progression of the infection and the development of lesions on the leaf surface were monitored at 24 hour intervals by measuring the lesion diameter at each spot and the infection was allowed to progress for 7 days.

3.3 Results

3.3.1. Phenotypical analysis of transgenic grapevine overexpressing Vv-AMP1

Transgenic *V. vinifera* (cv. Sultana) lines overexpressing Vv-AMP1 were hardened off from an *in vitro* tissue culture environment to a greenhouse environment. The growth and developmental characteristics of these lines were monitored and recorded throughout maturation. Morphologically, these transgenic Vv-AMP1 lines behaved similar in growth speed, general plant size and appearance of the plants, however, subtle differences in leaf and internode morphology were recorded (Figure 3.1).

Transgenic Vv-AMP1 lines 8, 9, 18 and 19 consistently developed narrower leaves than the untransformed Sultana controls, whereas Vv-AMP1 lines 8, 9, 10, 14 and 19 developed longer leaves in comparison to their wild type counterparts (Figure 3.1A)

With regards to the internode length of the population, the measurements of Vv-AMP1 lines 8, 9 and 19 were shorter with statistical significance (Figure 3.1A), whereas lines 6, 10, 14 and 18 showed no statistically different average measurements as the untransformed Sultana controls (Figure 3.1A). In the comparison of internode/node arrangements of the vegetative organs of the transgenic population there was no difference recorded between the

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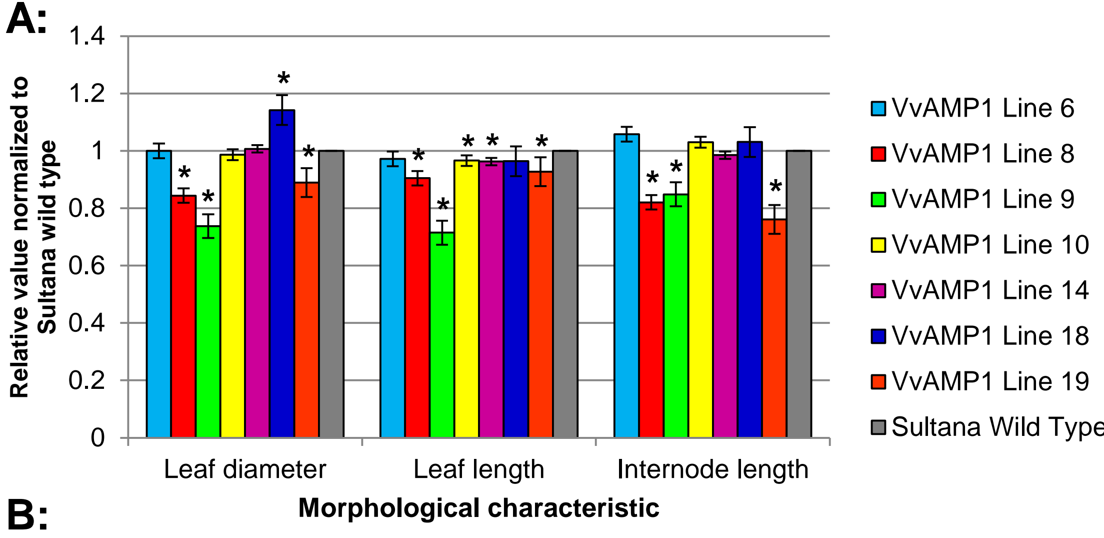
transgenic lines overexpressing Vv-AMP1 and the untransformed controls (data not shown).

When visually evaluating representative fully expanded leaves of each line within the transgenic Vv-AMP1 population, subtle differences in leaf morphology can be observed, specifically with regards to the leaf sinuses. Leaves from Vv-AMP1 lines 6, 10 and 14 can be more clearly distinguished from the rest of the Vv-AMP1 population and the untransformed Sultana wild type line, however, no drastic deviations from normal leaf development were observed (Figure 3.1B), given the fact that the plants were still in an overall juvenile growth phase.



B:

Vv-AMP1 Line 6	Vv-AMP Line 8	Vv-AMP1 Line 9	Vv-AMP1 Line 10
X: 12.61; Y:9.98	X: 10.64; Y: 9.29	X: 9.3; Y:7.34	X: 12.44; Y:9.92
Vv-AMP1 Line 14	**Vv-AMP1 Line 18**	**Vv-AMP1 Line 19**	**Sultana Wild Type**
X: 12.7; Y: 9.89	X: 14.4; Y:9.9	X: 11.21; Y:9.52	X: 12.61; Y:10.27

Figure 3.1 Summary of the morphological characteristics of a greenhouse population of transgenic Sultana plants overexpressing Vv-AMP1 in comparison to an untransformed Sultana population. (A) Values are an average of 10 measurements for each plant line represented in the population and are normalized to the Sultana wild type. All error bars indicate standard error and statistically different measurements from those of the Sultana wild type are indicated with an asterisk ($p<0.05$). (B) The morphological leaf characteristics of mature hardened off leaves representative of each transgenic *V. vinifera* (cv. Sultana) population overexpressing Vv-AMP1 and an untransformed Sultana wild type. X: Average leaf diameter (cm) (n=10); Y: Average leaf length (cm) (n=10).

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3.3.2. Detached leaf infection assays with the biotrophic fungus, *E. necator* in a wet inoculation with a conidial suspension

Mature, fully expanded leaves harvested from hardened off transgenic Sultana lines overexpressing Vv-AMP1 were challenged by a *E. necator* conidial suspension in a detached leaf antifungal assay in order to infer whether these lines possess improved resistance against this biotrophic pathogen. The evaluation considered both the infecting fungus, as well as the plant reaction to deduce the potential impact of the transgenic manipulation on disease resistance.

The development of the infection was monitored daily from 4 to 15 dpi and the development of fungal structures was observed under a stereomicroscope at 100X magnification. The fungal development was scored with the global resistance index (Table 3.4) and the data presented as a time-course to show the progression of disease over time (Fig 3.2A). From 6 dpi the first differences in the development of fungal structures could be observed between the various plant lines. Progression of powdery mildew infection was more rapid in the Sultana wild type lines, reaching an index value of four at the end of the assay (indicating susceptibility), whereas Vv-AMP1 lines 10 and 18 recorded resistant index values (8 on the scale). Moreover, by 12 dpi the Sultana wild type lines displayed mild sporulation whereas the transgenic lines never supported sporulation of the fungus (Figure 3.2A).

When considering the plants' reaction to the infection, the resistance levels obtained by considering the effect on the fungus was supported, again showing increased resistance in the transgenic lines. This was scaled according to the visual symptoms of PCD on the leaves as an indication of an active resistance mechanism (Figure 3.2B). By 4 dpi leaves originating from transgenic Vv-AMP1 lines began displaying yellow, chlorotic discolouration of the leaf tissue. By 12 dpi the discolouration progressed into large yellow patches represented in every leaf from transgenic origin with some developing necrotic tissue within the discoloured patches. The WT Sultana leaves did not show any visual symptoms of PCD for most of the time-course (Figure 3.3). By the end of the observation period at 15 dpi, four out of the five biological

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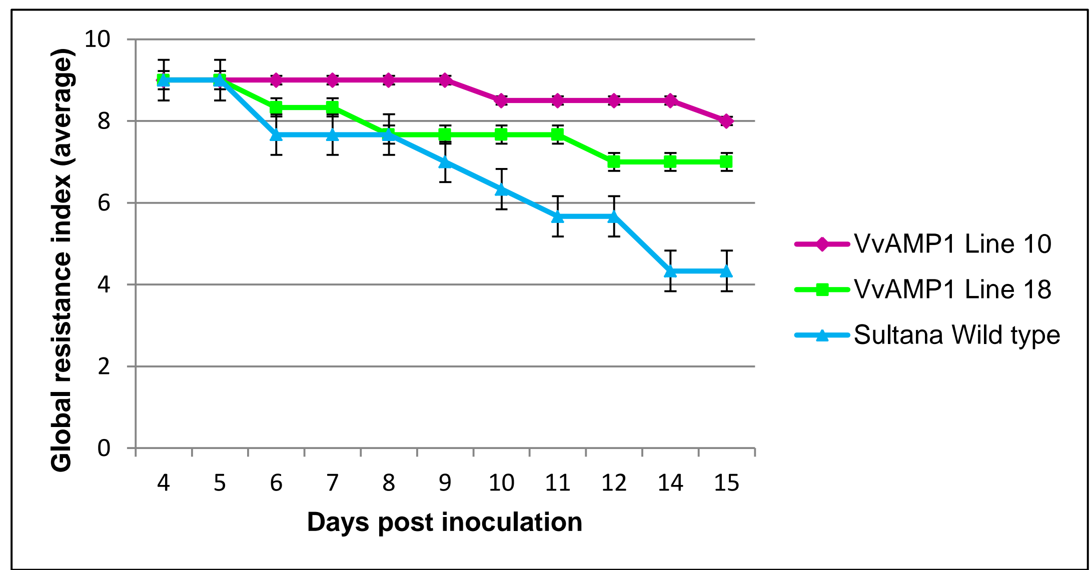
repeats of the untransformed Sultana lines began to display mildly yellowing spots (scaled at 8) whereas the transgenic Vv-AMP1 lines all displayed large yellowing patches with secondary necrotic lesions (scaled at 2 on the PCD scale) (Figure 3.2B).

A:



B:

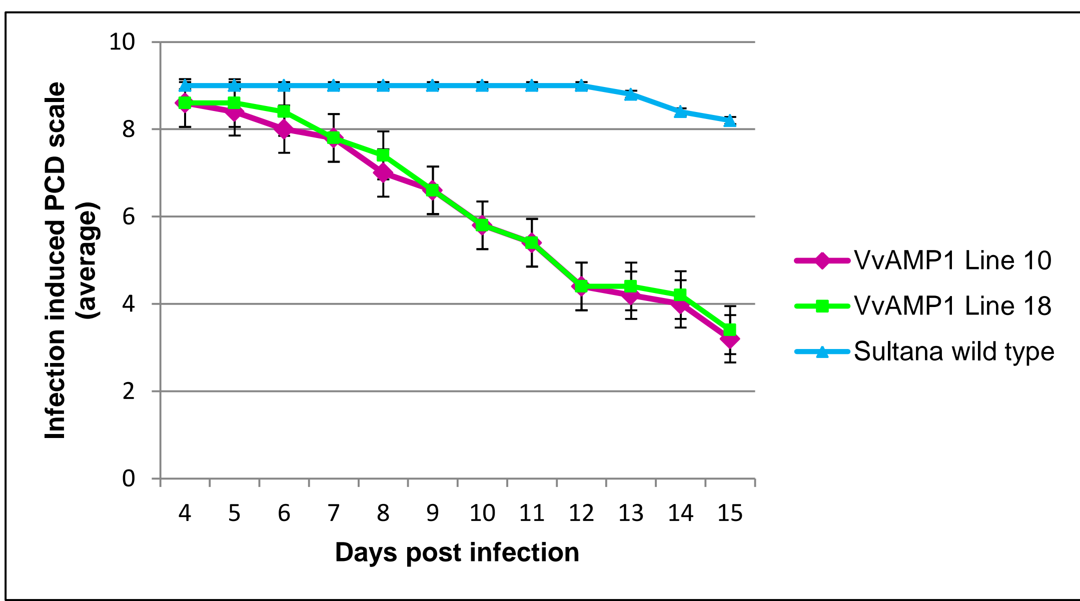


Figure 3.2 Comparison of grapevine powdery mildew infection with two transgenic grapevine lines overexpressing Vv-AMP1 and an untransformed control. (a) Time-course of the grapevine powdery mildew global resistance index (Miclot et al., 2012) of two transgenic grapevine lines overexpressing Vv-AMP1 in comparison to the Sultana wild type line in reaction to a wet inoculation with a *E. necator* conidial suspension 1X10^5 conidia/ml. (b) The time course comparison of the phenotypic changes between transgenic grapevine lines overexpressing Vv-AMP1 and untransformed control lines inoculated with a *E. necator* conidial suspension according to the newly established infection induced PCD scale. All bars indicate standard errors. All data shown are the average of five leaves.

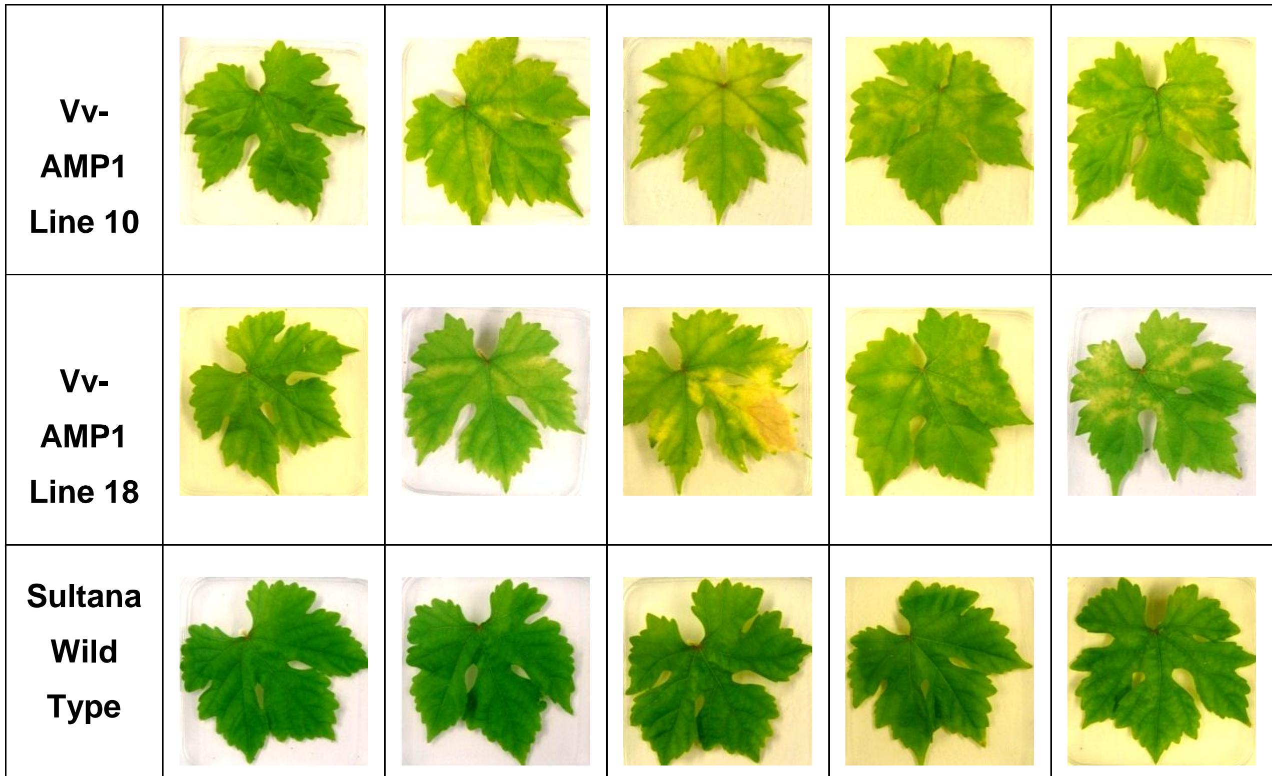


Figure 3.3 Visible programmed cell death symptoms of transgenic grapevine leaves overexpressing Vv-AMP1 as compared to *V. vinifera* (cv. Sultana) leaves in a detached leaf infection assay 12 days after inoculation with *E. necator* conidia in a suspension containing $1X10^5$ conidia/ml. All infected leaves per line are shown.

Additional noticeable observations included the formation of an initially unidentified white substance on the leaf surfaces. Upon further evaluation under a Scanning Electron Microscope the substance was identified to be calcium crystals through the solid state secondary electron detector and backscattered electron detector of the instrument that can be used to examine the specimen's composition, topography, crystallography, texture and other properties (Figure 3.4). These results proved that the crystals were composed of 71.47% calcium and 28.52% oxygen. When comparing the crystal formation on the leaf surfaces of the untransformed Sultana wild type line and the transgenic Vv-AMP1 leaves, clear differences in distribution patterns and quantity of these crystals could be distinguished (Figure 3.6). On the leaf surfaces of the untransformed Sultana wild type lines, the crystals appeared in dense patches along the main leaf veins and on the leaf lamina between veins. Contrastingly, these crystals found on the transgenic Vv-AMP1 leaves were consistently less dense and primarily distributed along the main veins, with rare observations on the lamina. The crystals found on the leaf surface of

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transgenic Vv-AMP1 leaves appeared to be marginally larger in size than those found on the untransformed control leaves (Figure 3.6).

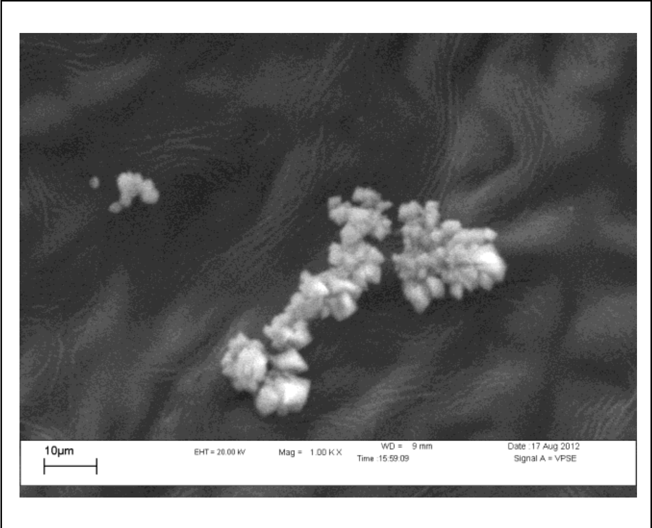

Figure 3.5 Scanning electron micrograph of a calcium crystal formation on the surface of a *V. vinifera* (cv. Sultana) leaf inoculated with a suspension of *E. necator* suspension at 12 dpi at a 1000X magnification.

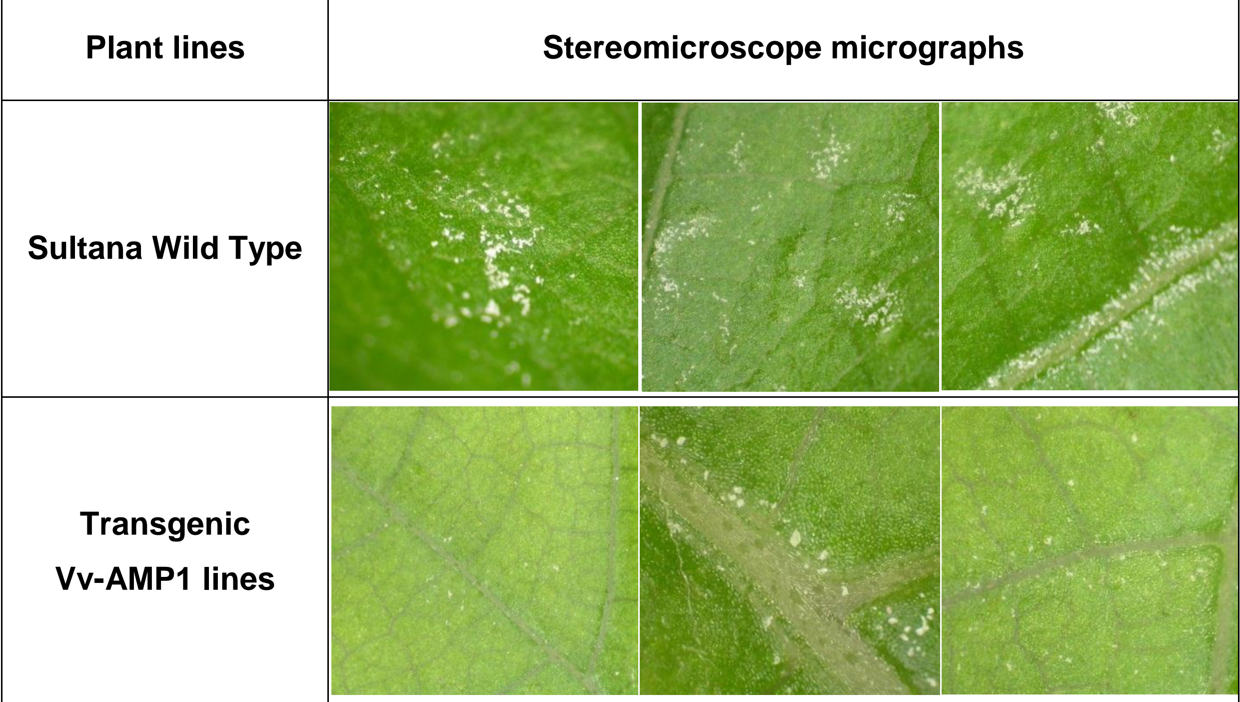

Figure 3.6 Comparison of density and distribution patterns of calcium crystals formed on the leaf surfaces of transgenic grapevine lines overexpressing Vv-AMP1 and an untransformed Sultana wild type line. Pictures were taken under a stereomicroscope at 100X magnification at 12 days post infection with an *E. necator* conidial suspension.

3.3.3. Detached leaf infection assay through inoculation with dry *E. necator* conidia

A separate infection assay, using more susceptible young, glossy, half expanded leaves and a dry inoculation of *E. necator* conidia was performed to corroborate the results obtained with the conidial suspension assay, and to compare the wet and dry inoculation methods. The reactions of both the pathogen and the plants were followed in a time-course.

3.3.3.1. Assessment of the development of E. necator infection in Vv-AMP1 transgenic lines

The development of the detached leaf infection was monitored daily from 3 to 15 dpi and the development of fungal structures was observed under a stereomicroscope at 100X magnification. The structure development was scaled according to a global resistance index as previously described (Table

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3.4). It was clear that the infection on the young leaves and with the dry inoculum was much more severe than on the older leaves inoculated with a conidial suspension (Fig 3.7). Moreover, initiation of the powdery mildew infection was more rapidly observable after inoculation in the Sultana wild type lines compared to the transgenic population. From the first day of observation the untransformed wild type line displayed scattered, clearly observable fungal structures on the leaf surfaces that rapidly progressed to form dense, weakly sporulating hyphae by 4 dpi. By 9 dpi the entire leaf surface of each replicate were densely covered in sporulating hyphae that could no longer be scaled according to the global resistance index (Figure 3.7).

The transgenic Vv-AMP1 lines 10 and 18 proved to experience a much less severe powdery mildew infection than the untransformed wild type when comparing fungal structure development. Vv-AMP1 line 10 remained resistant at the onset of the infection until 5 dpi when mycelium became scattered over the leaf surfaces. The first weakly sporulating hyphae could be observed by 8 dpi and by 15 dpi at the end of the infection assay that all the replicates representing Vv-AMP1 line 10 showed signs of densely sporulating hyphae (Figure 3.7). Vv-AMP1 line 18 showed similar resistance to the infection at 3 dpi but the infection became more severe from 3 to 6 dpi than that of Vv-AMP1 line 10. By the end of the infection assay at 15 dpi all the replicates of the transgenic Vv-AMP1 line 18 showed signs of densely sporulating hyphae (Figure 3.7).

Contrasting to the before mentioned transgenic lines, Vv-AMP1 line 6 showed a lower level of resistance to the *E. necator* infection from 3 dpi. The first signs of weak sporulation could be observed by 6 dpi and the infection could no longer be scaled due to densely sporulating hyphae at 12 dpi (Figure 3.7).

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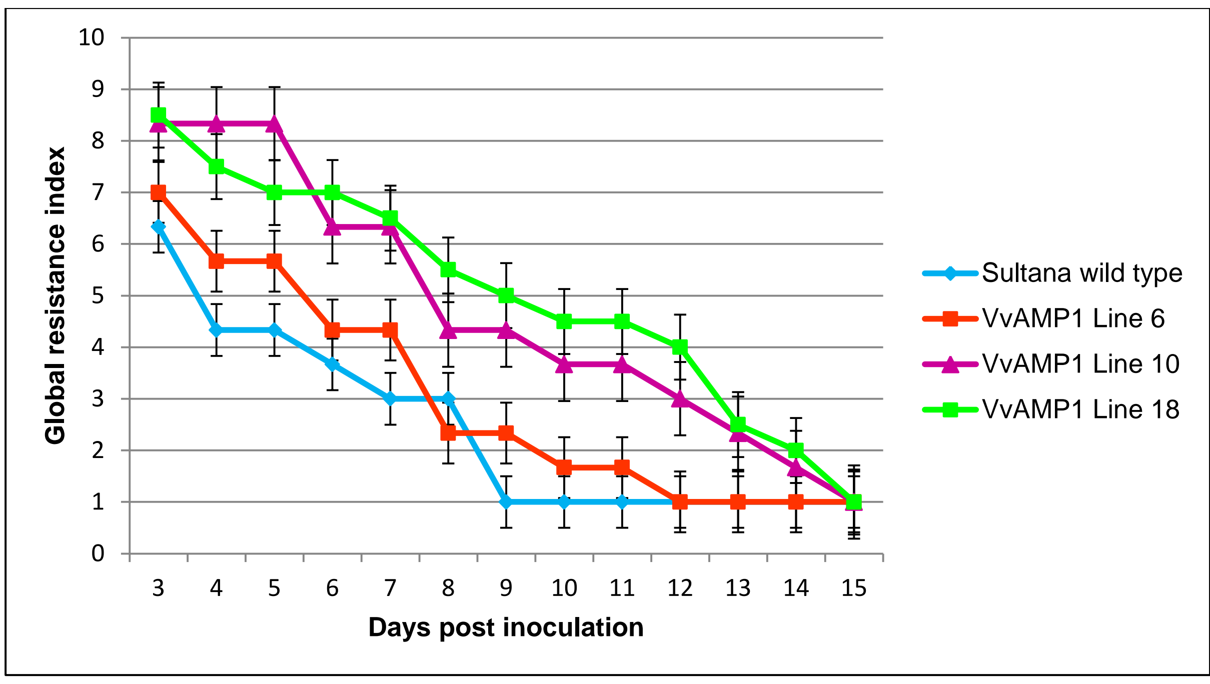

Figure 3.7 Time-course of the grapevine powdery mildew global resistance index (Miclot et al., 2012) of two transgenic grapevine lines overexpressing Vv-AMP1 in comparison to the Sultana wild type line in reaction to a dry inoculation with an *E. necator* conidial suspension 1X10^5 conidia/ml. All bars indicate standard errors. All data shown are the average of five leaves.

3.3.3.2. Characterization of resistance mechanisms of transgenic Vv-AMP1 lines to E. necator infection

Leaf material was harvested at 48 hrs after *E. necator* inoculation in order to determine the infection stage of the successfully germinated conidia through the identification of fungal appressoria and haustorium as well as the prevalence of PCD. Leaves were stained in trypan blue in order to distinguish fungal structures as well as dead host tissue. Germinated conidia were categorized according to their development of appressorium, haustorium or a combination of haustorium leading to PCD. The frequency of each infection mechanism was calculated in order to scale the level of susceptibility (Figure 3.8). According to this classification, the two types of *E. necator* resistance can be identified as either penetration resistance or induction of PCD. The wild type Sultana lines again showed high susceptibility to *E. necator* infection with 72% of germinated conidia leading to the development of haustoria and secondary hyphae after each infection attempt through appressorium formation. Only 16% of the germinated conidia on the untransformed wild type showed the development of an appressorium without

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successful penetration of the epidermal cells, whereas 9% of germinated conidia lead to PCD of the penetrated epidermal cells (Figure 3.8). At 14 dpi, a dense network of sporulating hyphae (Figure 3.9) was observed for the untransformed control.

Based on the method described by Feechan et al. (2011), two of the transgenic lines tested (lines 6 and 10) predominantly showed PCD associated resistance. The mechanism of resistance of these transgenic lines could be characterized by rapid host cell death following successful appressorium formation and epidermal cell penetration of the germinated conidium. Transgenic Vv-AMP1 lines 6 and 10 showed the prevalence of conidia leading to PCD to be 54 and 53%, respectively, whereas the successful penetration leading to formation of secondary hyphae was as low as 3 and 5%, respectively. The resistance to penetration characterized by the formation of an appressorium with no evidence of successful penetration displayed by these transgenic lines was also elevated in comparison to the untransformed Sultana lines (Figure 3.8).

Transgenic Vv-AMP1 line 18, although possessing the same number of transgene integrations as line 6 and 10, displayed subtle variations in the frequencies of resistance mechanisms employed. This line displayed a higher frequency of conidia showing penetration resistance (51%) as compared to the other two transgenic lines although the frequency of conidia developing into haustorium formation and subsequent secondary hyphae remains relatively similar between all the transgenic Vv-AMP1 lines at less than 7% (Figure 3.8). Despite the frequency of conidia that resulted in PCD in Vv-AMP1 line 18 being lower than that of the other transgenic lines, PCD related resistance in this line remains more than 3 times higher than that of the untransformed wild type Sultana line (Figure 3.8).

While microscopically observing the infection stage of the conidia on the various lines, a great number of conidia with appressoria that were detached from the leaf surface could be observed floating on the plant tissue of the Vv-AMP1 transgenic lines (data not shown). This observation indicates possible contribution to the frequency of penetration resistance of these lines although

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this could not be accurately quantified. Repetition of this assay at 14dpi showed sparsely distributed secondary hyphae formation with weak or no sporulation in the transgenic Vv-AMP1 host lines in comparison to the untransformed wild type Sultana line (Figure 3.9).

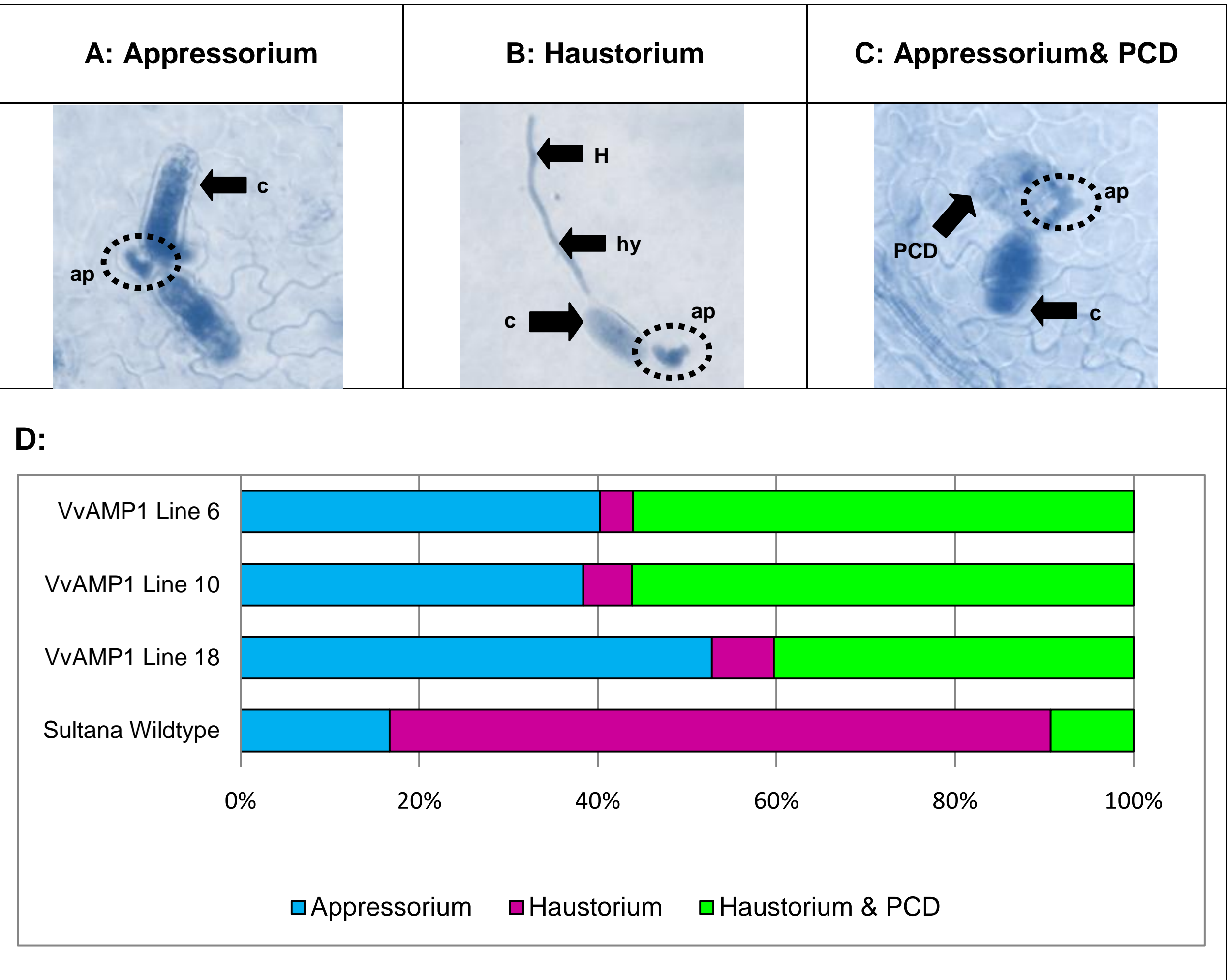

Figure 3.8 The susceptibility of various grapevine lines overexpressing Vv-AMP1 in comparison to the Sultana wild type in reaction to the infection with dry *E. necator* conidia in a detached leaf infection assay at 48hrs post-inoculation. The evaluation is based on the method described by Feechan et al., (2011). The samples had been stained in a trypan blue solution and visualized under a light microscope at 100X magnification. (A) Criteria according to which the infection mechanism of the conidia is classified as "appressorium" due to the visible formation of an appressorium but no successful penetration or subsequent fungal development. (B) Criteria according to which the infection mechanism of the germinating conidia is classified as "haustorium" due to the visible formation of an appressorium and subsequent formation of a haustorium and secondary hyphae. (C) Criteria according to which the infection mechanism of the germinating conidia is classified as "haustorium & PCD" due to the visible formation of an appressorium leading to successful penetration and subsequent programmed cell death. (D) The frequency of *E. necator* conidial penetration events resulting in appressorium formation but no successful penetration, successful penetration, haustorium formation and secondary hyphal development or the development of a haustorium followed by programmed cell death (PCD). Broken black circles indicate the position of an appressorium. ap, appressorium; c, conidium; hy, hypha; H, haustorium. The total number of germinated conidia counted for each line was as follows: Sultana wild type, 55; Vv-AMP1 line 6, 84; Vv-AMP1 line 10, 77; Vv-AMP1 line 18, 74.

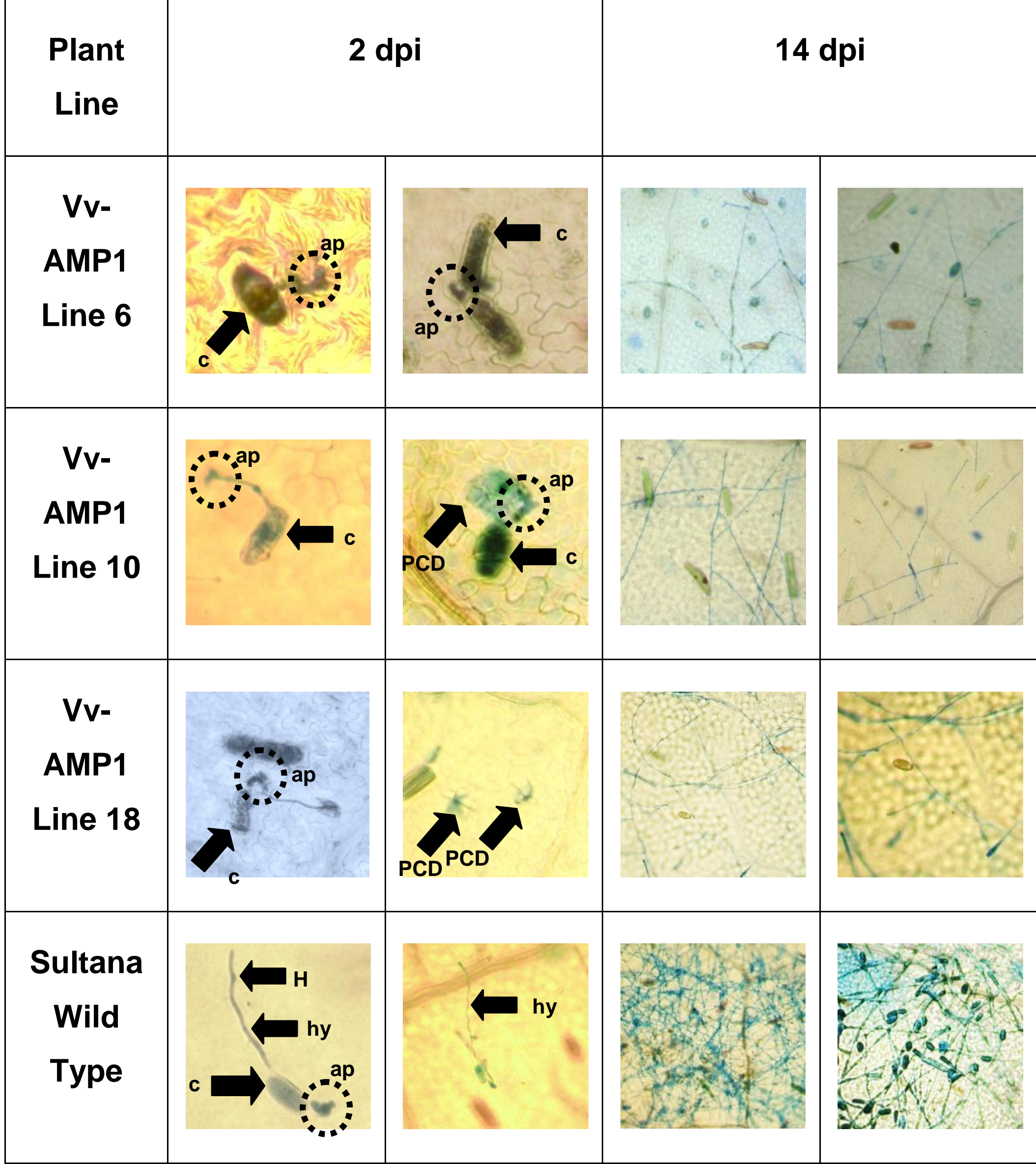

Figure 3.9 Comparison of infection stage of *E. necator* conidia on infected transgenic grapevine lines overexpressing Vv-AMP1 and an untransformed wild type line at 2 and 14 dpi. All pictures are taken under a light microscope at 40X magnification at various optical zoom ranges for optimal observation of infection structures. Broken black circles indicate the position of an appressorium. ap, appressorium; c, conidium; hy, hypha; H, haustorium.

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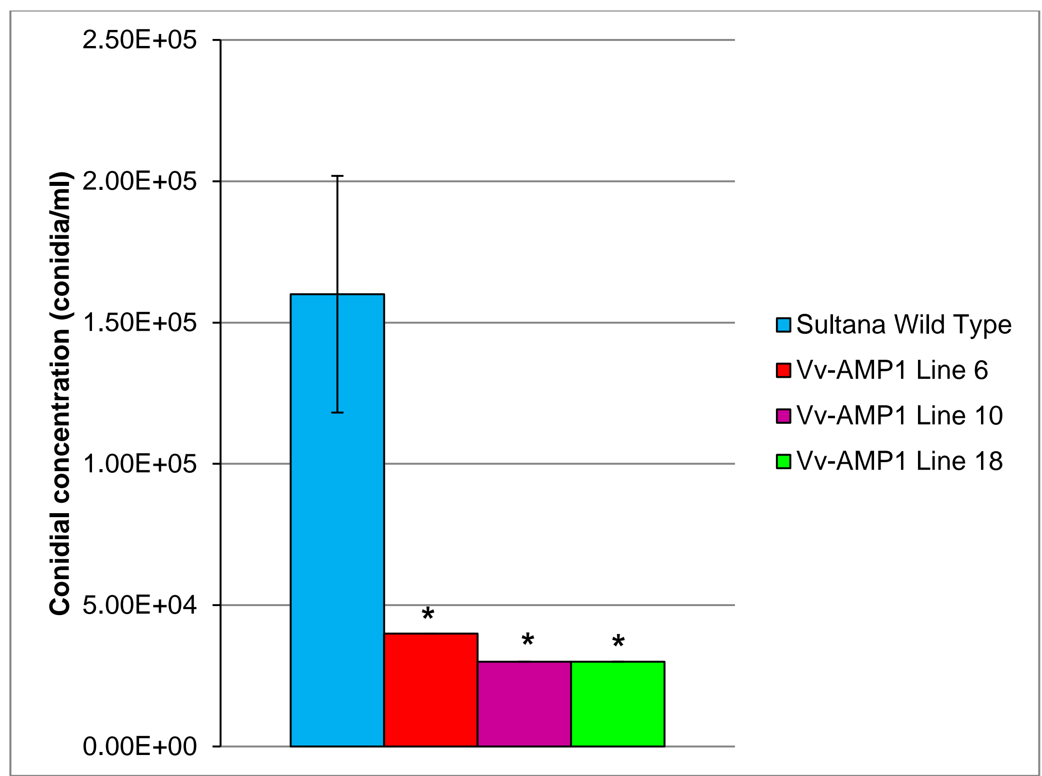

Figure 3.10. Comparison between spore concentrations obtained from three transgenic grapevine lines overexpressing Vv-AMP1 and the untransformed Sultana lines at 14 dpi with dry *E. necator* conidia in a detached leaf infection assay. Conidial concentration is the average conidial concentration of five technical repeats. Error bars indicate standard deviation between al detectable haemocytometer readings (n=3).

3.3.3.3. Determination of reproductive viability through determination of conidial concentration

In order to evaluate the reproductive viability of the powdery mildew fungi hosted on three transgenic Vv-AMP1 lines and the untransformed wild type line, conidial concentrations were determined using a haemocytometer. Five replicate counts were performed for each sample and the average conidial concentration produced on the untransformed wild type host were found to be at least three fold higher than that of the transgenic Vv-AMP1 lines (Figure 3.10). Transgenic lines 10 and 18 produced the same conidial concentration of 3 X 10^4 conidia/ml whereas line 6 produced a slightly higher concentration of 4 X 10^4 conidia/ml.

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3.3.3.4. Assessment of germination and penetration of E. necator conidia with the Scanning Electron Microscope

At 48 hrs post inoculation with dry *E. necator* conidia, leaves from every transgenic grapevine line overexpressing Vv-AMP1 and an untransformed control were evaluated under a Scanning Electron Microscope (SEM). Pictures were taken at 500X and 1000X magnification, respectively (Figure 3.11). Conidia on the leaf surfaces of the untransformed Sultana wild type showed observable differences to those on the transgenic Vv-AMP1 leaves. Conidia that had germinated, formed appressoria and successfully penetrated the leaf surface on the Sultana wild type leaves, were in more advance stages of secondary hyphae formation than those on the transgenic Vv-AMP1 lines. Grapevine lines overexpressing Vv-AMP1 displayed a high number of conidia that successfully formed appressorium but showed no secondary hyphae formation or further fungal development whereas the majority of the conidia germinating on the untransformed Sultana wild type leaves successfully penetrated the leaf surface and subsequently developed secondary hyphae at this time point.

Conidial germination on the wild type Sultana leaves could be characterized by a single germ tube protruding from the conidium surface, developing into a single multi-lobed appressorium at the point of leaf penetration. Similarly, appressoria developed on the leaf surfaces of all the transgenic Vv-AMP1 lines. However, some lines also showed the development of appressoria that appeared to have multiple penetration attempts or uncharacteristically long germ tubes. These lines include transgenic Vv-AMP1 lines 9, 10, 14, 18 and 19, respectively. Furthermore, Vv-AMP1 line 19 showed conidia that developed highly unusual appressoria with multiple penetration attempts and an abnormal morphology (Figure 3.11).

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Plant line	500X Magnification	1000X Magnification
Sultana Wild type		
Vv-AMP1 Line 6		
Vv-AMP1 Line 8		
Vv-AMP1 Line 9		
Vv-AMP1 Line 10		

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Plant line	500X Magnification	1000X Magnification
Vv-AMP1 Line 14		
Vv-AMP1 Line 18		
Vv-AMP1 Line 19		

Figure 3.11 SEM micrographs of germination stages and attachment of *E. necator* conidia on the leaf surfaces of transgenic grapevine overexpressing Vv-AMP1 and an untransformed control line at 48 hours post inoculation with dry conidia at 500X and 1000X magnification, respectively.

3.3.4. Whole-plant infection assays with the necrotrophic fungus, *B. cinerea*

Hardened off transgenic Sultana lines overexpressing Vv-AMP1 were challenged by *B. cinerea* spores in a 50% grape juice solution during a whole-plant antifungal assay in order to infer whether these lines possess improved resistance against this pathogen. 100% of the spore solution spots developed primary and subsequent secondary spreading lesions after being applied to the leaves. Although the onset of lesion formation was representative of that of the expected pathosystem as previously defined by similar studies (Moyo, 2011), the progression of the infection and lesion development throughout the designated time-course was more rapid in comparison. However, no notable

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difference could be observed between the lesion development of the transgenic Vv-AMP1 Sultana lines and those of the wild type Sultana control lines when comparing the lesion diameters of these lines from 2 days post infection. At 2 dpi lesions formed on the transgenic lines and untransformed controls showed no visible variation and the average lesion diameters (cm) were at an average of 0.76±0.015 (Figure 3.12). The lesion diameters of several leaves could no longer be accurately measured after 4 days and at the 6th day post infection, measurement of the lesions where ceased due to entirely overlapped lesions and abscission of most of the heavily infected leaves.

Even though only Vv-AMP1 line 14 showed statistically different lesion development from the Sultana wild types at 3 and 4 dpi, a notable phenotypic variation in lesion appearance can be identified in Vv-AMP1 transgenic line 19. This particular line, as well as the transgenic Vv-AMP1 line number 10, displayed the development of fungal reproductive structures as early as 3 dpi whereas the untransformed Sultana wild type showed the first signs of reproductive structures at 5dpi. The transgenic Vv-AMP1 line 14 did not appear to form any reproductive structures throughout the development of this infection assay. The unusual reproductive structures visible in Vv-AMP1 line 19 appear as multiple sporulation attempts of the fungus in an unusual radial pattern (Figure 3.13).

Plant line	0dpi	2dpi	3dpi	4dpi	5dpi	6dpi
Sultana Vv-AMP1 Line10						
Sultana Vv-AMP1 Line14						
Sultana Vv-AMP1 Line19						
Sultana Wild Type						

Figure 3.12 Whole-plant infection assays with 1000 *B. cinerea* spores per infection spot on *V. vinifera* (cv. Sultana) leaves

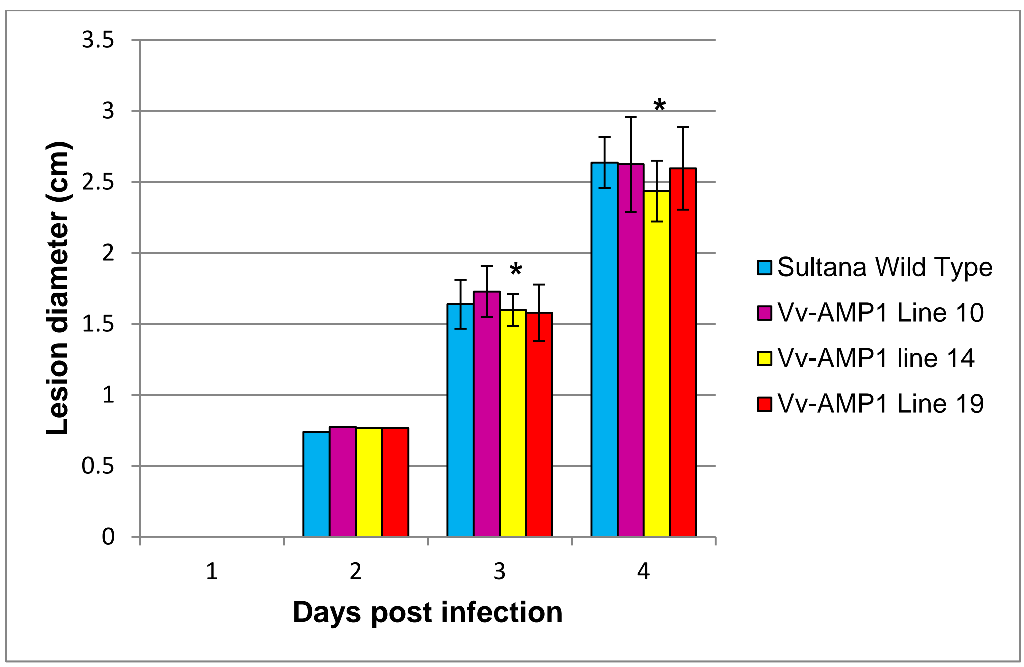

Figure 3.13 Lesion development of Sultana transgenic lines overexpressing Vv-AMP1 as compared to untransformed wild type Sultana lines in a whole-plant infection assay with *B. cinerea*. Three individuals from each plant line with four leaves per plant infected with three spots per leaf (1000 spores per spot). Error bars indicate standard deviation and asterisks indicate statistical difference from the Sultana wild type ($p<0.05$).

3.4 Discussion

Antimicrobial peptides from plants have attracted increasing scientific attention in the active pursuit of alternative plant disease control mechanisms (Ganz, 2005). These antimicrobial peptides offer promising potential in the production of commercially viable transgenic crops due to their great functional diversity, non-toxicity and the low energy expenditure required for plants to transcribe and translate from single antimicrobial peptide-encoding genes.

Attempts at genetic engineering strategies in grapevine are being made since the highly susceptible European grape, *V. vinifera*, is considered to be the most economically important crop worldwide (Vivier and Pretorius, 2002). The first antimicrobial peptide isolated from grapevine, Vv-AMP1, is a non-morphogenic plant defensin peptide within the antimicrobial peptide family that has been genetically characterized. Recombinantly produced Vv-AMP1 proved to be highly active against a wide range of plate-cultured fungal

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pathogens at remarkably low concentrations *in vitro* (De Beer, 2008; Tredoux, 2011). However, the *in planta* activity of this peptide against fungal pathogens remained to be explored in order to establish whether the overexpression of Vv-AMP1 can reduce the susceptibility of its native host.

An *in vitro* population of transgenic *V. vinifera* (cv. Sultana) lines overexpressing Vv-AMP1 was established and systematically hardened off to a greenhouse environment during which these plants were morphologically characterized. Comparison of the various uniquely transformed transgenic lines showed moderate variation of leaf morphology and internode length between lines as well as between individuals from the same line. No consistent significant differences between transgenic Vv-AMP1 lines and untransformed control lines could be established according to the characterization methods implemented in this study. Although morphological characterization methods with regards to leaf size, shape, lobes and sinuses are well established, these methods as described by the OIV (2009) would not be informative due to the juvenile nature of this transgenic Vv-AMP1 population. Not only are juvenile populations well known for morphological diversity, phenotypic variation is known to be significantly higher in plants from tissue culture generated populations than those of those grown under field conditions (Vuylsteke and Ortiz, 1996; Kuksova et al., 1997).

The defense phenotype of this transgenic *V. vinifera* (cv. Sultana) population overexpressing Vv-AMP1 was evaluated in detached leaf infection assays with the biotrophic powdery mildew grapevine fungus, *E. necator*. Vv-AMP1 provided the transgenic grapevine population with elevated resistance against *E. necator* infection through the implementation of a combination of PCD associated defense response and cell wall penetration resistance. Plant resistance against powdery mildew infection is known to consist of a two-layer defense response (Dry et al., 2009). These defense mechanisms include pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is considered to be the plant's first line of defense against powdery mildew infection as it involves the detection of the pathogen PAMP chitin by a plant membrane receptor-like kinase (Robatzek et al., 2006; Miya et al., 2007; Wan et al., 2008). This pathogen detection

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mechanism causes a cascade of signaling events that lead to the secretion of antimicrobial and cell-wall restructuring peptides in order to physically obstruct fungal penetration (Kwon, 2010). However, powdery mildew pathogens have developed host-specific resistance by means of the production of effector-molecules that can effectively suppress PTI. Therefore, the second layer of plant defense response involves the detection of these effector molecules, hereby triggering a second signaling cascade leading to numerous defense responses that include PCD (Peterhänsel et al., 1997). During the first detached leaf infection assay of mature, fully expanded grapevine leaves with an *E. necator* conidial suspension, the macroscopic PCD response of the transgenic grapevine overexpressing Vv-AMP1 could be observed soon after inoculation. Leaves from transgenic Vv-AMP1 lines displayed chlorotic discolouration spots on the leaf surfaces whereas untransformed Sultana wild type lines retained a green and healthy appearance throughout the entire infection assay.

The detached leaf assay with dry *E. necator* conidia lends further evidence of the PCD-associated resistance phenotype displayed by grapevine overexpressing Vv-AMP1. During this detached leaf assay, the fate of the *E. necator* conidia were quantified at 48 hrs post inoculation in order to establish the prevalence of either penetration resistance or PCD-associated resistance as previously explored by Feechan et al. (2011). Transgenic Vv-AMP1 Sultana lines showed elevated percentages of PCD associated resistance comparable to that of *Muscadinia rotundifolia*, a grapevine species known for strong resistance to *E. necator* infection (Dry et al., 2009).

When observing the infection attempts of the dry conidia inoculation with *E. necator* under a scanning electron microscope, appressorium deformities could be distinguished between conidia on the transgenic Vv-AMP1 population and that of the untransformed wild type control leaves. The transgenic Vv-AMP1 leaves showed germinated conidia with abnormal appressoria forming multiple penetration attempts whereas conidia germinating on wild type leaves could be characterized by a short germ tube with a multi-lobed appressorium and subsequent secondary hyphae development. These observations, although not quantified, supports findings

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by Schnee et al. (2008) who classified the level of grapevine powdery mildew resistance according to the number of penetration attempts performed by each conidium. Furthermore, the microscopic results following trypan blue staining from the present study proves the penetration resistance of grapevine overexpressing Vv-AMP1 to be at least 2 fold higher than that of the untransformed *V. vinifera* line included in this study. Therefore, although Vv-AMP1 overexpression lead to an elevated level of conidial penetration resistance in transgenic leaves, PCD associated resistance remains the primary defense mechanism employed by the transgenic population overexpressing Vv-AMP1.

When comparing the infection severity of *E. necator* on young leaves infected with dry conidia and mature leaves infected with conidia in suspension, leaves from the same transgenic Vv-AMP1 lines show a much more rapid and severe infection when inoculated with dry conidia. These observations may be attributed to factors regarding the infection methods. The first reason for a higher infection rate when performing a dry inoculation with *E. necator* conidia pertains to the suspension itself. Free water is known to have deleterious effects on *E. necator* conidia by retarding conidial germination and by causing some conidia to spontaneously lyse.

The second critical factor that may have led to a more rapid, aggressive powdery mildew infection with dry *E. necator* conidia pertains to the developmental stage of the leaves being infected. The young, glossy leaves that were infected with dry conidia were at the most susceptible stage of their development when infected for the purpose of this study (Doster and Schnathorst, 1985). Even though *V. vinifera* leaves never become entirely resistant to powdery mildew infection, susceptibility declines dramatically during leaf maturation although mature leaves frequently support vast quantities of this biotrophic fungus (Droster and Schnathorst, 1985). The relationship between inoculum density and the latent period of the infection as reported must also be considered (Rouse et al., 1984). The duration of the latent period of the infection were found to decrease as the number of conidia deposited per square millimeter was increased (Rouse et al., 1984). During the detached leaf infection assay performed with conidia in suspension, the

conidial concentration was predetermined at an estimated 1 X 10^5 conidia/ml. However, due to method of inoculation with dry conidia, the inoculum density was undetermined and could therefore have been significantly higher than the conidia in suspension, hereby leading to a more rapid onset and development of powdery mildew infection.

When considering the fungal reproductive viability through the production of *E. necator* conidia after infection with dry conidia, the differences between the concentrations of conidia produced on the leaf surfaces of the transgenic Vv-AMP1 lines and the untransformed control were significant. *E. necator* produced significantly more conidia on the untransformed Sultana leaves with a minimum of a 3-fold higher conidial concentration than on transgenic Vv-AMP1 lines. This dramatic difference indicates the high level of resistance that the transgenic lines overexpressing Vv-AMP1 have against *E. necator* infection, however, differences between infection severities scaled according to the resistance index were far less pronounced. These differences in results obtained from the same experiment underscore the limitations in using the global resistance index by over-simplifying a highly complex plant-pathogen interaction. Even though transgenic Vv-AMP1 lines were scored low according to the global resistance index for the presence of sporulating hyphae, the level of successful sporulation was extremely low in comparison. It is therefore imperative to perform several disease assessment methods in a more holistic approach to evaluating the complex interaction between plants and their pathogens.

SEM analysis provided further insight into the germination of *E. necator* conidia on the leaf surfaces of transgenic grapevine overexpressing Vv-AMP1. At 48 hrs post inoculation, the majority of conidia that had successfully germinated on the transgenic Vv-AMP1 leaves had formed appressoria, however, the development of secondary hyphae following successful leaf penetration were clearly limited in comparison to the untransformed wild type control leaves. The micrographs taken of the conidia germination on transgenic Vv-AMP1 line can be compared to those taken of a resistant *M. rotundifolia* (cv. Regale) population (Blanc et al., 2012) and in light of the newly determined PCD-associated resistance to this biotrophic pathogen it is

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reasonable to speculate that these conidia display characteristics indicative of a similar response. Furthermore, some appressorium abnormalities with regards to the number of penetration attempts and germ tube length could be observed on transgenic Vv-AMP1 lines that may indicate various degrees of penetration resistance projected by these transgenic lines. These observations further underscore the increased resistance that these transgenic *V. vinifera* (cv. Sultana) lines overexpressing Vv-AMP1 have against *E. necator* when compared to the untransformed grapevine line.

Although inoculation with a conidial suspension on mature grapevine leaves appeared to be a less effective mechanism in the evaluation of powdery mildew resistance phenotypes, it is important to consider the natural field conditions under which *E. necator* grows. Powdery mildew pathogens under field conditions are exposed to fluctuating natural temperatures, occasional exposure to direct UV radiation and excessively high precipitation; factors that contribute to sub-optimal growth conditions for this pathogen (Delp, 1954; Doster and Schnathorst, 1985; Willocquet et al., 1996; Carrol and Wilcox, 2003). This pathogen generally overwinters in the form of cleistothecia in most viticultural areas, discharging ascospores when wet by rain or irrigation in the spring. However, continued wetness is known to be detrimental to ascospore germination and infection (Gadoury and Pearson, 1990). The strict specificity of this pathosystem is therefore optimized during detached leaf assays hereby simulating optimal conditions for the pathogen to thrive. Furthermore, in combination with stimulating the natural wound response when harvesting the leaves and removing the leaves from their source of nutrition hereby placing the plant under stressed conditions, the pathogen proliferation is clearly favoured over that of the plant during these experiments. It is therefore important not to underestimate the effectiveness of resistance induced by the overexpression of Vv-AMP1 in these *V. vinifera* lines. Field conditions are expected to favour the plant over the pathogen and therefore the overexpression of Vv-AMP1 in grapevine has potential as an alternative crop protection mechanism. Regardless of the infection method, transgenic grapevine leaves of various ages overexpressing the plant defensin, Vv-

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AMP1, showed reduced susceptibility to grapevine powdery mildew through the implementation of primarily PCD associated defense mechanisms.

The transgenic population was further evaluated in a whole-plant infection assay with the necrotrophic fungus, *B. cinerea*. The transgenic lines did not show elevated resistance to this pathogen. This does however support what is well known with regards to defense response implemented by plants upon *B. cinerea* infection. Necrotrophic fungi such as *B. cinerea* are known to rely on dead host cells for nutrition and reproduction. *B. cinerea* employs various molecular mechanisms in order to establish these optimal growth conditions by killing host plant cells. The pathogen achieves this through the secretion of phytotoxins that induces cell collapse, hereby aiding in successful host penetration and colonization (Colmenares et al., 2002). Additionally, reactive oxygen species such as hydrogen peroxide (H_2O_2) are produced by *B. cinerea* during the infection process (Schouten et al., 2002). The rate of cell death is further escalated by the production of these reactive oxygen species by the plant itself as part of a hypersensitive response to pathogen attack. (Dixon et al., 1994; Grant and Mansfield, 1999). These plant defense responses are an effort at isolating the pathogen in dead tissue that forms part of a signal transduction cascade that induces programmed cell death through the production of antimicrobial peptides. However, this strategy proves to be counter-productive in the defense against *B. cinerea* that thrives on dead tissue.

Plant defensins are however known to facilitate fungal growth inhibition by binding sphingolipid receptors located in the fungal membrane, causing alteration of the membrane ion gradient hereby permeabilizing the fungal membrane (Thevissen et al., 1999). This permeabilization leads to the inhibition of fungal growth although the exact mechanism remains to be confirmed. Vv-AMP1 is not known to form part of the defense-related signaling pathways that leads to PCD. However, the Vv-AMP1 gene has been proven to be expressed exclusively in grape berries from the onset of ripening onwards (De Beer and Vivier, 2008). The transgenic Vv-AMP1 population currently being evaluated constitutively produces the Vv-AMP1 peptide continuously throughout all plant tissues hereby taking the activity of this well-

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studied peptide out of its natural biological context. Furthermore, plant defensins have been recently implicated in the prevalence of peptide promiscuity whereby different conditions such as pH or protein concentrations can cause a single peptide to assume different and unusual functions as reviewed by Franco (2011). Peptide promiscuity is therefore linked to the evolution of peptides with multiple functions related to plant defense against pathogens. These findings would warrant further investigation into the various possible biological activities of Vv-AMP1 that would provide insight into the currently unexplained calcium crystal phenomena. Although grapevine is well-known to produce calcium oxalate crystals as part of a calcium regulation mechanism, the production of these crystals have not been linked to responses to pathogen attack to date (Webb, 1999; Franceschi and Nakata, 2005).

Results generated from this study provide new insight into the plant disease resistance mechanisms inferred by the expression and translation of plant defensin-related genes. Plant defensins have once again proven to be successful targets in the ongoing pursuit of commercially viable transgenic food crops with enhanced resistance to economically relevant pathogens. Therefore, future studies focusing on Vv-AMP1 should evaluate the effect of this peptide on the full range of grapevine crop pathogens and consider the exploration of its possible secondary functions with regards to grapevine development and biotic and abiotic stresses that could contribute to the generation of engineered crops for the future.

3.5 Acknowledgements

Special acknowledgement is owed to the following individuals for their assistance and contributions to this study:

- Dr. Krishnan Vasanth, for performing the grapevine transformations.
- Mss. Charmaine Stander and Varsha Premsagar for assistance during the initial establishment of the transgenic *in vitro* grapevine population

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Chapter 4

In silico analysis of gene expression patterns of defensin-like peptides in *Vitis vinifera* L.

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Research Results

4.1 Introduction

Although plants are continuously exposed to microbial pathogens, the occurrence of successful microbial infection is relatively infrequent due to the many highly evolved mechanisms of protection that form part of the innate immunity of plants (Lamb et al., 1989). This innate immunity is considered to be an ancient defense strategy present in almost all living animals, plants, insects and fungi with origins predating the divergence of prokaryotes and eukaryotes (Javaux et al., 2001; Gao et al., 2009). These plant protection mechanisms include a wide range of physical and chemical responses upon pathogen perception. The physical responses include the restructuring and subsequent fortification of the plant cell wall in order to restrict pathogen invasion (Dixon et al., 1994; Grant and Mansfield, 1999; Heil and Bostock, 2002), whereas chemical responses include an oxidative burst as part of a hypersensitive response that leads to localized programmed cell death to limit the spread of impeding pathogens (Peterhänsel et al., 1997). However, among the chemical defenses, the *de novo* production of DEFL proteins is imperative to plant defense against pathogens (Bowles, 1990; Broekaert et al., 1997).

These defense-related proteins can be either constitutively produced or induced upon pathogen attack and includes proteins such as proteinase inhibitors, α-amylase inhibitors and various hydrolytic enzymes that facilitate the degradation of pathogenic fungal cell walls (Bowles, 1990; Bloch and Richardson, 1991; Broekaert et al., 1995; van Loon et al., 2006; Ferreira et al., 2007). These proteins also include a superfamily of small, cysteine-rich antimicrobial peptides.

The most frequently studied members of the antimicrobial peptide family are the plant defensins that are well known for their antifungal activity against a broad range of necrotrophic, biotrophic and oomycete pathogens. Even though it was initially believed that these peptides were members of small multigene families, it has now been established from model plant genomes that cysteine-rich peptide encoding genes are highly represented in plant genomes, contributing up to 3% of all genetic

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material in plants. Moreover, more than 300 of these defensin peptide-encoding genes have been identified in the *Arabidopsis* genome hereby further underscoring the importance of these peptides in not only plant survival against pathogens, but possibly other biological functions as well (Silverstein et al., 2005; Silverstein et al., 2007).

Despite showing remarkable diversity on the amino acid level, all antimicrobial peptides are well known for their small size of less than 160 amino acid residues, a C-terminal domain characterized by 4-16 cysteine residues and a conserved N-terminal that includes a secretion peptide signal (reviewed in Marshall et al., 2011). The high level of variation in possible biological functions has been related to the great diversity in primary structure displayed among these cysteine-rich antimicrobial peptides.

Despite numerous sources of information regarding plant antimicrobial peptides and their potential in plant engineering strategies, the defense-related peptide encoding genes of grapevine remains relatively unexplored.

Making use of the publically available gene expression data from microarray experiments in grapevine, the present study aimed to use an *in silico* data mining approach to evaluate previously identified putative grapevine defensin-like (DEFL) genes in the context of their wide array of possible biological functions. This was achieved by compiling a set of 16 DEFL genes, based on their primary sequences and deduced amino acid sequences. This set was then used to mine the available gene expression data sets to identify several clusters of co-expressing DEFL genes. The experimental conditions driving the co-expression of these putative DEFL genes were subsequently established and their potential biological functions were investigated further. This was achieved by identifying non-DEFL genes co-expressing with two of the defensin genes and exploring the conditional drivers of the putative co-expression. The expression of some DEFL genes was found to be driven by cultivar or tissue specificity whereas others show expression induced by biotic or abiotic stimuli. The grapevine defensin-encoding genes, Vv-AMP1 and Vv-AMP3 showed putative co-expression with genes that allowed inferences to be made regarding the involvement of these peptides in biotic and abiotic stress responses and their role in plant growth and development.

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4.2 Materials and Methods

4.2.1 Selection of *Vitis vinifera* putative DEFL genes

In order to determine the role of DEFL gene expression in grapevine, several genes were identified as targets for evaluation. The protein sequences of 79 genes identified as putative DEFL genes in grapevine in an earlier study (Giacomelli et al., 2012) were collected for the purpose of further analyses. Similarly, the protein sequences of 18 genes identified to be putative antimicrobial peptide genes in grapevine were obtained from a separate study (Tredoux, 2011; Abré De Beer, personal communication). These 97 putative DEFL grapevine genes were subjected to further evaluation before being used in the evaluation of grapevine transcription.

The first step in processing this initial set of genes was performed by removing 33 genes that were known to be pseudogenes and gene fragments (Giacomelli et al., 2012). Furthermore, with what is known about antimicrobial peptides and their DEFL primary nucleotide sequences, putative DEFL genes with sizes exceeding 400 bp were excluded from further analyses.

The remaining genes were then subjected to separate tBLASTn analyses using the BLAST algorithm provided by PLEXdb (Dash et al., 2012) to ascertain whether these genes would support successful binding on the VitisAffy Gene Chip (Gautier et al., 2004). Putative genes that did not provide a minimum of 98% identity with 100% query coverage to any probes were removed from the list of genes that were considered for further evaluation. These remaining genes were then used in separate tBLASTn analyses of the grapevine EST database using the BLAST algorithm provided by TIGR (http://compbio.dfci.harvard.edu/tgi/plant.html). Putative genes that provided unique EST data were further subjected to BLASTx using the BLAST algorithm provided by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The translated protein sequences of the putative genes that provided evidence of expression were compiled. Repeated entries of the same genes were removed and the remaining genes formed the final list of putative DEFL grapevine genes that would be included in the *in silico* analysis of the gene expression data to follow.

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4.2.2 Alignment of the identified DEFL genes

Deduced amino acid sequences of the final list of putative DEFL grapevine genes were obtained by performing BLASTx analyses using the BLAST algorithm of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The resulting protein sequences were evaluated for the presence of a signal peptide sequence with the Expasy SignalP tool (http://www.cbs.dtu.dk/services/SignalP/). In order to successfully categorize these DEFL peptides according to their sequence similarities, the signal peptide sequences were subsequently removed from each peptide sequence. An alignment of the DEFL genes was performed in Muscle (Edgar, 2004), the phylogeny performed with PhyML (Guindon et al., 2010) and the tree visualized with T-REX (Boc et al., 2012).

4.2.3 Collection of gene expression data from *V. vinifera* microarray experiments

The transcriptomic data used for this study was obtained from a collection of 18 publically available microarray expression studies. This data contains gene expression values generated from various experimental conditions, grapevine species and cultivars, tissues and developmental stages. The numbers of the studies as represented in the PLEXdb database (Dash et al., 2012) and their descriptions are summarized in Table 4.1.

Table 4.1 Summary of publically accessible *V. vinifera* microarray experiments included in the analysis of putative grapevine DEFL gene expression from www.plexdb.org (Dash et al., 2012).

Study identifiers	Description	Tissue
Vv1	Short term abiotic stress in Cabernet Sauvignon	Shoots with leaves
Vv2	Long-term salt and water stress in grapes	Grape berries
Vv3	Grape berry tissue differentiation	Grape berries

Table 4.1 (cont.)

Study identifiers	Description	Tissue
Vv5	Chardonnay and Cabernet Sauvignon's berry development	Grape berries
Vv7	Gene expression associated with compatible viral diseases in grapevine cultivars	Leaves
Vv9	High temperature effect on Cabernet Sauvignon berries	Grape berries
Vv10	Photoperiod regulation of grape bud dormancy	Buds
Vv11	Pinot Noir berry transcriptome during ripening.	Grape berries
Vv12	Powdery mildew-induced transcriptome in a susceptible Cabernet Sauvignon	Leaves
Vv13	Powdery mildew-induced transcriptome in a resistant grapevine “Norton”	Leaves
Vv14	Gene expression in grapevine in response to Bois noir infection	Leaves
Vv15	Expression data in individual grape berries during ripening initiation	Grape berries
Vv16	Grape skin in berries grown on the vine treated with exogenous abscissic acid	Grape berry skin
Vv17	Grape skin transcriptome in berries cultured *in vitro* treated with abscissic acid	Grape berry skin
Vv19	Gene expression patterns associated with grapevine resistance to downy mildew	Leaves

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Table 4.1 (cont.)

Study identifiers	Description	Tissue
Vv28	Gene expression associated with compatible viral diseases in berry	Grape berries
Vv29	Micro-propagated *Vitis vinifera* transferred to ex vitro conditions	Leaves
Vv31	Expression data from 35S::VvCBF4-overexpressing grapevines	All aerial tissues

4.2.4 Clustering methods

When analyzing microarray expression data, genes are associated with probes on the VitisAffy gene chip to which original cDNA samples were hybridized in order to generate expression data. However, some ambiguity exists between individual genes and their probe identities. Therefore, for the purpose of this analysis, only genes with no ambiguous probe associations where used for analysis. Probes that unambiguously bind the putative DEFL genes previously identified were used for further clustering analysis.

The publically available raw data files were collected from the PLEXdb website (Dash et al., 2012), combined into a directory and normalized, using rma from the biobase affy package (Gentleman et al., 2004) in R, to produce a complete data matrix containing probeset hybridization (gene expression) data and the experimental conditions driving the gene expression detected by each probeset, as log2 transformed expression values.

For the purpose of implementing a Markov Clustering algorithm, MCL (van Dongen, 2000), probesets were subjected to an all-against-all comparison in order to calculate an absolute value for each pairwise Pearson correlation. This similarity measure ranges from 0 to 1, where 0 is indicative of complete dissimilarity, whereas 1 indicates perfect similarity or correlation. These absolute correlation values for each pairwise comparison are used as measures of similarity in the Markov

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Clustering algorithm, in preprocess a correlation cut-off threshold was implemented to eliminate probes that were dissimilar with regards to their expression values.

To establish the expected granularity of the analysis when performing Markov Clustering, an inflation parameter was established. Higher inflation parameters are expected to result in a greater number of clusters. The clustering algorithm tries to group similar probes together while simultaneously ensuring that each cluster gets pushed away from each other. This is done by modeling stochastic flow of the column normalized similarity matrix, in this case correlation matrix, using successive alternation of matrix multiplication and a normalized haddamard product. Hereby, putatively co-expressed probes could be identified. Subsequent analyses aimed to identify the experimental conditions driving the putative co-expression by firstly transforming the data from the original data matrix to relative expression values by dividing each row of the matrix by the maximum element within that particular row, thus effectively performing row normalization, with the resultant transformed values now ranging between 0 and 1 respectively. This allowed us to define a threshold for significant expression, given a particular condition (indicated by the columns of the matrix). We used 0.9 as a threshold throughout our analysis, classifying a gene as significantly expressed under a condition if the normalized expression value, in that particular row and column, is greater than 0.9. This further allowed us to measure the degree of association a cluster has for a particular condition, which is done by determining the number and frequency of genes that are significantly expressed under this condition. We can therefore define a condition to be significantly associated with a cluster by using a frequency threshold, thus if more than a certain proportion of genes in a cluster is significantly expressed under that condition.

The above method was performed using various parameter values to determine the optimal threshold combination with which to perform further analyses. All pairwise correlations, Markov Clustering, matrix transformations and frequency determinations were kindly performed by Piet Jones (of the Computational biology group of the Institute for Wine Biotechnology).

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4.2.5 Experimental conditions driving gene expression clusters

Following the probe clustering analysis, the conditions driving co-expression clustering of the putative DEFL genes were evaluated. Results generated from the clustering analyses were in the form of sample code names linked to putatively co-expressed DEFL grapevine genes. Further information regarding each sample was subsequently obtained from the PLEXdb website (Dash et al., 2012).

The collected sample information included the grapevine species and cultivar from which each sample was obtained and the specific plant tissue from which the sample was taken. Furthermore, if samples were of grape berry origin, the specific developmental stage at which sampling was performed was also collected. With regards to conditions under which the experiment was performed, any treatments administered to the grapevine plants were recorded. These treatments included abiotic stress stimuli that involved controlled photoperiod, water deficit treatments, salinity stress, treatment with polyethylene glycol (PEG), as well as high and low temperature exposure. Biotic stress treatments administered to the grapevine during some experiments included infection with various grapevine pathogens such as the fungi that causes downy and powdery mildew, the leaf roll virus, GLRaV3, as well as Bios noir infections. An information matrix was then compiled of all available sample information. In order to group specific conditions for further evaluation, categories best describing the specific experimental conditions were compiled. These categories are summarized in Table 4.2.

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Table 4.2 Summary of experimental conditions/categories collected from combined publically available transcriptomic data for grapevine (Dash et al., 2012).

Category name	**Conditions included**
No treatment	Biological controls included in each experiment Samples taken at 0 hours post infection by any pathogen No treatment controls included in each experiment
Biotic stress treatments	Powdery mildew infections Downy mildew infection Leaf roll virus infections Bois noir infections
Abiotic stress treatments	Water deficit Polyethylene glycol treatment (PEG) Long or Short photoperiod Salinity stress Transgenic overexpression of VvCBF4

The category named “no treatment” would provide information regarding the putative co-expression of the defensin related genes with reference to their species, cultivar and tissue specific expression. This information will exclude the influence that experimental treatments may have on the expression of these putative genes. This category will further allow evaluation of the expression of these putative DEFL genes during berry development. Due to the numerous sources of the transcriptomic data, the stages of berry development were originally given several descriptions for the same stage of development. For the purpose of this study, descriptors of the stages of berry development were standardized according to the E-L stage classification (Coombe, 1995) and are summarized in Table 4.3.

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Table 4.3 Summary of the standardized descriptors of the stages of berry ripening adapted from Coombe (1995).

E-L numbering	Complete description of berry development	Standardized descriptors
31	Berries pea-size (7 mm diameter)	Pea size
32	Beginning of bunch closure, berries touching	Green touching
33	Berries still green and hard	Green hard
34	Berries begin to soften: Brix starts increasing	Green softening
35	Berries begin to colour and enlarge	Ripening
36	Berries with intermediate Brix values	Ripening
37	Berries not quite ripe	Ripening
38	Berries harvest-ripe	Ripe

Separate data matrixes were generated for each conditional category and the putatively co-expressed DEFL genes driven by these conditions. Graphs representing each conditional category were generated and visualized in Cytoscape (Shannon et al., 2003; Cline et al., 2007).

4.2.6 Identification of genes putatively co-expressed with DEFL genes driven by specific experimental conditions

Following the identification of putatively co-expressed DEFL genes driven by specific experimental conditions, further clustering analysis was performed to identify other genes represented in the grapevine genome that are putatively co-expressed with these DEFL genes under the same experimental conditions.

As previously described, pairwise correlations, Markov clustering, matrix transformations and frequency determinations were performed (Piet Jones, IWBT,

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personal communication). Probes that showed putative co-expression to the identified DEFL gene-probes were identified, data matrixes were generated and resulting graphs were visualized in Cytoscape (Shannon et al., 2003; Cline et al., 2007).

Identified genes were investigated by collecting all available GO annotations for these genes through workbench analyses on the Plaza 2.5 website (Proost et al., 2009; Van Bel, 2012). According to these GO-terms, putative *in planta* functions for the DEFL peptides in grapevine metabolism, growth and development could be identified. Redundant GO annotations and those that provided little information regarding the involvement of each gene (e.g. “Cell part”) were removed from the annotation lists. Matrixes including putatively co-expressed genes and their GO annotations were collected, graphs were generated and subsequently visualized in Cytoscape (Shannon et al., 2003; Cline et al., 2007).

4.3 Results

4.3.1 DEFL gene sequences

For the purpose of evaluating the expression of DEFL genes in grapevine, a total of 97 DEFL genes identified in earlier studies were evaluated as potential candidates for expression analysis. After these evaluations the remaining genes formed the final list of putative DEFL grapevine genes that were included in the *in silico* analysis of the gene expression data available for grapevine. These genes, their deduced amino acid sequences and descriptions of their closest homologs present in the *Arabidopsis thaliana* genome are depicted in Table 4.4.

Table 4.4 Deduced amino acid sequences of grapevine DEFL genes, their closest homologs represented in the *Arabidopsis thaliana* genome and their descriptions as depicted on the Plaza 2.5 website (Proost et al., 2009; Van Bel, 2012). Putative signal peptide sequences were determined with SignalP and are indicated in red.

Gene name	Amino Acid sequence	Closest Homolog in *A. thaliana* and description
DEFL1	MGSSKLQFTSLFILSLIFLSHSLGAMAQDP GSDCDFVGSCKNKADCAKPCGAKGHSPT AVLCVPNPNGGKRCCCIIA	AT5G37474; Encodes a defensin-like (DEFL) family protein; Putative membrane lipoprotein
DEFL10	MKSFLVILVLLFLGSGNEVRAEFNVCAKSE HLDNCTDPACAKLCVDKYGGQFHRDANG FCQPPSTCACTYLCSNVPSAAH	AT3G61177; Encodes a member of a family of small, secreted, cysteine-rich protein with sequence similarity to the PCP (pollen coat protein) gene family.; low-molecular-weight cysteine-rich 53
DEFL21	MAKSRSFGSFFLAMVVILSLVCKEVVSQQ DGRCCKDHPKLGHCAPGKDDDPNGGKC WTYCITKCSKGGLCKKLSGGRHVCHCYC	AT5G52605; Encodes a defensin-like (DEFL) family protein.;
DEFL26	MANLFTMCYFILFLLISSSADGKVCTDTFPC TTLGKCXNDCKVKHSTWLNSTCLGIPPHN PTSLQCLCYYNC	AT2G14935; Encodes a member of a family of small, secreted, cysteine-rich protein with sequence similarity to the PCP (pollen coat protein) gene family.; low-molecular-weight cysteine-rich 40
DEFL29	MAKLLGYLLSYALSFLTLFALLVSTEMVML EAKVCQSPSKTWSGFCGSSKNCDEFLQC KNWEGAKHGACHAKFPGVACFCYFNC	AT1G19610; Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin family
DEFL52	MKKMAVPLMLCLVILSSLLTFGQAQGGXF CTVTEHFPGKCPSENLGCFIEMSGKYGAS SMLHGCHCTQFXSDHTCACXAXCSPPL	AT4G15733; Encodes a member of a family of small, secreted, cysteine-rich proteins with sequence similarity to SCR (S locus cysteine-rich protein).;SCR-like 11
DEFL59	MKLYSCILVLFLLISSGTEMKEVKAARCME VLDPNGCILPSCKQRCLQEKNGNGVCVPN RNGGYECICYYNC	AT5G48905; Encodes a member of a family of small, secreted, cysteine-rich protein with sequence similarity to the PCP (pollen coat protein) gene family.; low-molecular-weight cysteine-rich 12

Table 4.4(cont.)

Gene name	Amino Acid sequence	Closest Homolog in *A. thaliana* and description
DEFL70	MXXLFXMCYFMLFLLISSSADAKVCTDTFPCTTLGKCTNDCKVKHSTWLNSTCLGVPPHNPTSLQCXCYYNCK	AT2G14935; Encodes a member of a family of small, secreted, cysteine-rich protein with sequence similarity to the PCP (pollen coat protein) gene family.; low-molecular-weight cysteine-rich 40
Vv-AMP1	MKGSQRLFSAFLLVILLFMATEMGPMVAEARTCESQSHRFKGTCVRQSNCAAVCQTEGFHGGNCRGFRRRCFCTKHC	AT2G02100; Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin family
Vv-AMP2	MASQETEARLCESQSHWFRGVCVSNHNCAVVCRNEHFVGGRCRGFRRRCFCTRNC	AT2G02130; Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin family
Vv-AMP3	MERKSLGFFFFLLLILLASQEMVVPSEARVCESQSHKFEGACMGDHNCALVCRNEGFSGGKCKGLRRRCFCTKLC	AT2G02130; Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin family
Vv-Snakin2	MKHLFPTLLLLSLLLHSCFSQPTTDGAGFCGLKCSKRCSQAAVLDEFLCMKYCGICCQECKCVPSGTYGNKHECPCYRDKKNSKGKPKCP	AT2G14900; Gibberellin-regulated family protein; involved in response to gibberellin stimulus
Vv-Snakin3	MQVLFNSIKLYKLSDCKSKCAYRCSKAGWHKLCLRACNTCCERCNCVPPGTAGNEDVCPCYAKMTTHGGRHKCP	AT4G09610; GAST1 protein homolog 2 (GASA2); Involved in response to gibberellin stimulus
Vv-Snakin6	MLSLSMMLLLLVQNNATITEAPTPQPQQSTNGFPMHGVTQGSLHPQECAPRCTTRCSKTAYKKPCMFFCQKCCAKCLCVPPGTYGNKQFCPCYNNWKTKRGGPKCP	AT1G74670; Gibberellin-regulated family protein
Vv-Snakin8	MAKVFALFLLALLAISMLHTTVLASHGHGGHHYDQKNYGPGSLKSFQCPSQCSRRCGKTQYHKPCMFFCQKCCKKCLCVPPGYYGNKAVCPCYNNWKTKEGGPKCP	AT5G15230; Gibberellin-regulated (GASA4);GAST1 protein homolog 4
Vv-Snakin13	MKLFSVFIISILLLQAFAEASLVISNAEHSLTSVDESRDEVALHKKSHPRKINCSYACSRRCRKASRKNVCSRACKTCCKRCHCVPPGTYGNKNMCPCYASLKTHGHKPKCP	AT1G22690; Gibberellin-regulated family protein

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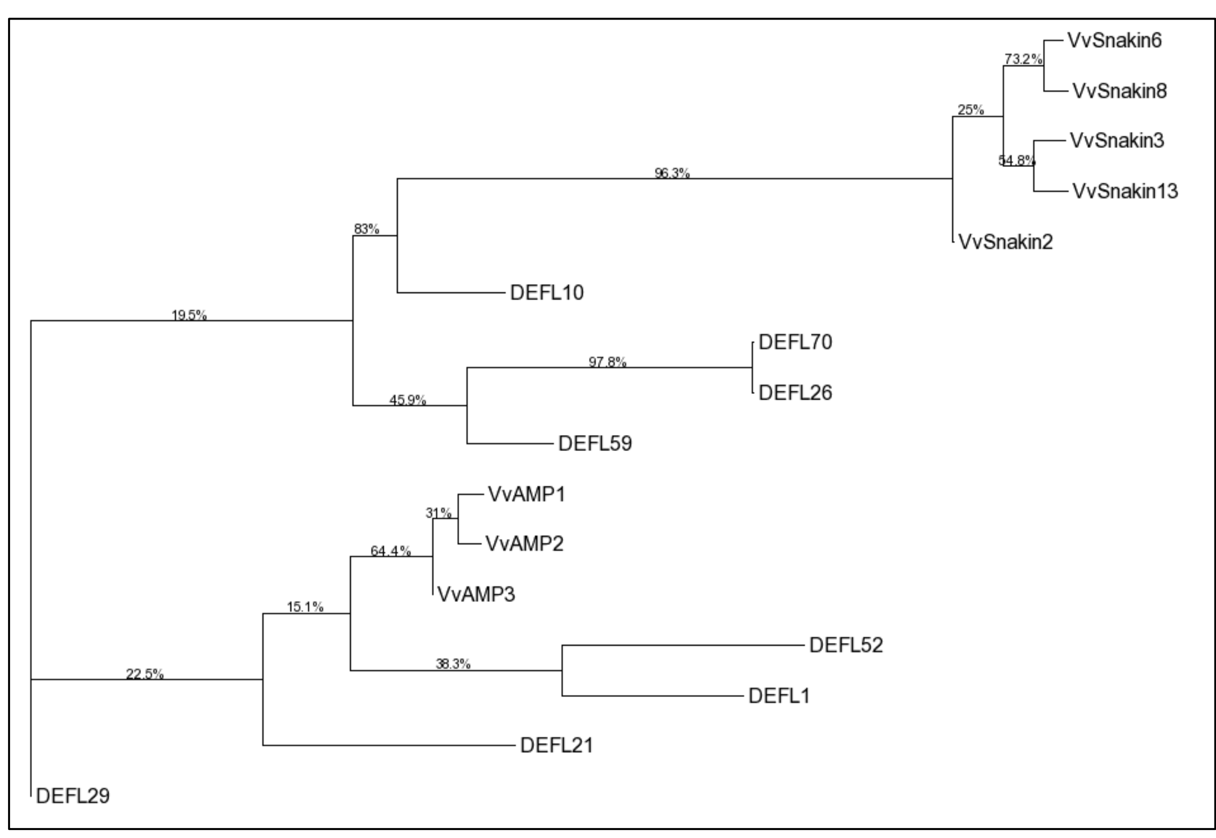
4.3.2 Alignment of putative DEFL peptides

The deduced amino acid sequences of the final DEFL genes selected for expression analysis were aligned using Muscle (Edgar, 2004). The phylogenic relationships as determined by PhyML (Guindon et al., 2010) are depicted in Figure 4.1.



4.1 Phylogenic relationship between the grapevine DEFL genes identified. Deduced amino acid sequences of these genes were aligned in Muscle (Edgar, 2004), the phylogeny performed with PhyML (Guindon et al., 2010) and the tree visualized with T-REX (Boc et al., 2012).

According to this alignment, there is high sequence similarity within the Vv-Snakin and Vv-AMP (defensin) groups, respectively. The DEFL genes identified by Giacomelli et al. (2012) form various groups with high sequence similarity, DEFL70, DEFL26, DEFL59 and DEFL10 were found to be more similar in sequence to the Vv-Snakin genes whereas DEFL52, DEFL1 and DEFL21 appear to be closer related to the grapevine defensins (Vv-AMPs). DEFL29 appear to form an entirely separate group based on its amino acid sequence.

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4.3.3 *In silico* analysis of transcriptomic data of grapevine

The effects of parameter thresholds on clustering results

Clustering methods were performed with various threshold combinations to establish the effect that these threshold combinations have on the quantity of data produced and at which threshold combinations these analyses would provide the most biologically informative results. These results are summarized in Figure 4.2. According to the comparisons of various threshold combinations, a correlation cut-off of 0.6 and a frequency cut-off of 0.13 yielded the highest number of combinations of putative DEFL grapevine genes (DEFL-genes) that showed significant expression driven by specific experimental conditions.

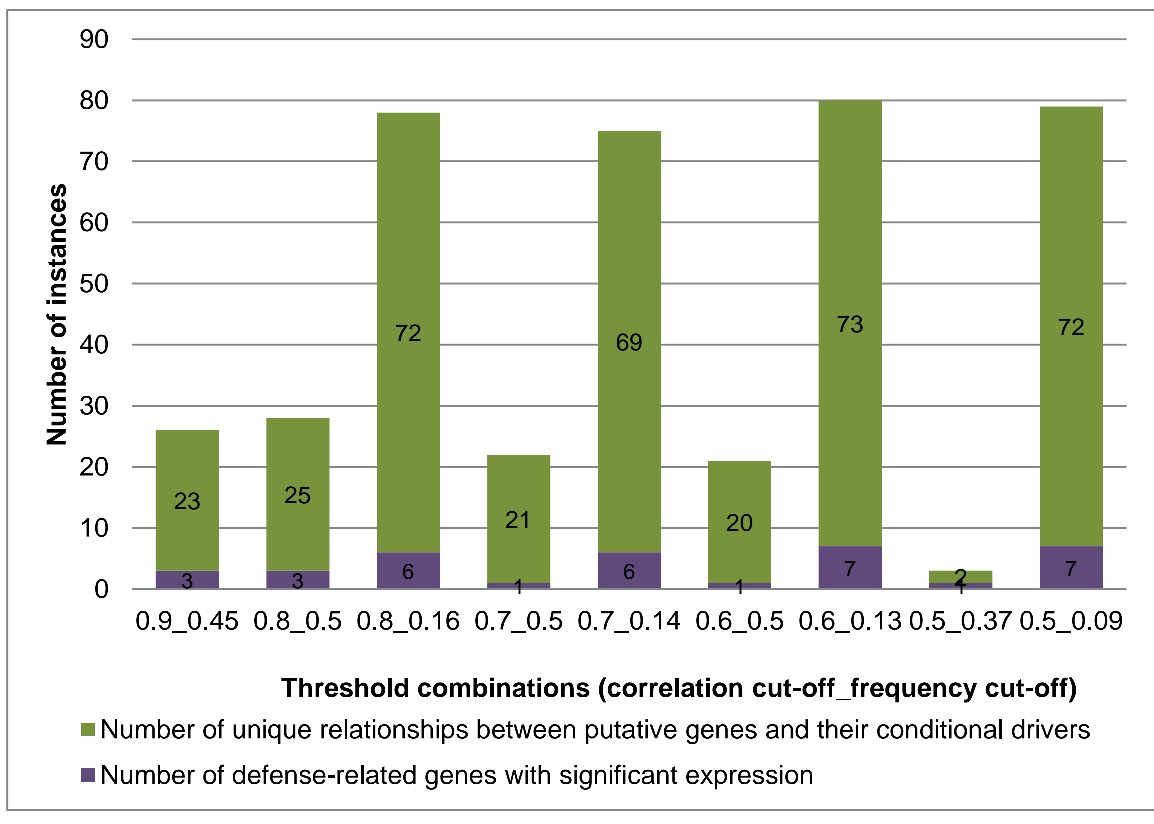

Figure 4.2 Effect of various parameter thresholds combinations on the quantity of data extracted with regards to the number of putative DEFL genes that show significant expression and the experimental conditions that drive expression. Inflation parameters and significance measure were at constant values of 7 and 0.9, respectively.

4.3.4 Gene expression patterns of grapevine DEFL genes

Tissue and cultivar specificity

The tissue and cultivar specific co-expression of the putative DEFL grapevine genes were identified with a correlation cut-off of 0.6, a Markov inflation cut-off of 7, an expression significance value of 0.9 and a frequency of 0.13. Seven DEFL genes showed putative co-expression driven by tissue and cultivar specificity under these parameter thresholds (Figure 4.3). Vv-AMP1 and Vv-AMP2 were exclusively putatively expressed in berries of various cultivars under no treatment conditions, whereas DEFL70 and Vv-Snakin3 showed putative expression in both berries and aerial tissues of various cultivars. Vv-AMP3 and Vv-Snakin8 were putatively co-expressed exclusively in aerial tissues of Cabernet Sauvignon whereas Vv-Snakin6 showed putative expression changes exclusively in aerial tissues of various cultivars (Figure 4.3).

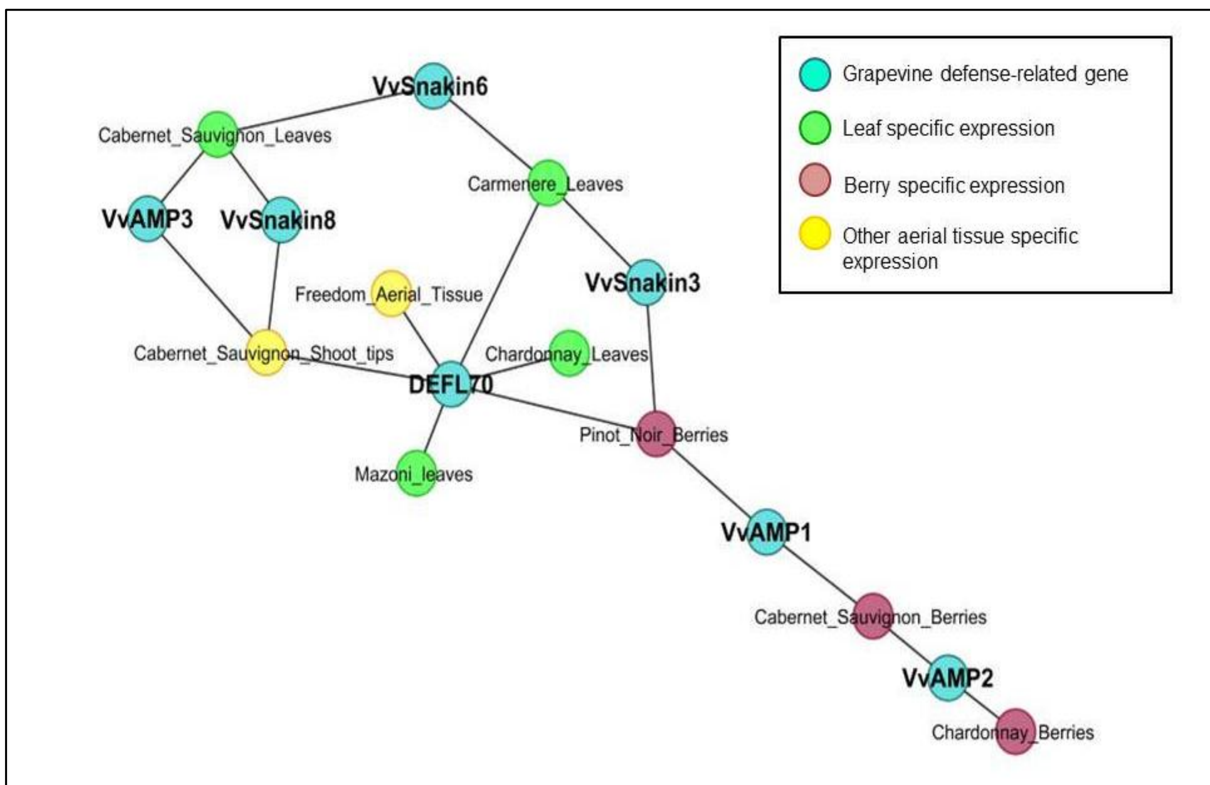

Figure 4.3 Visual representation of DEFL grapevine genes putatively co-expressed in a tissue-specific manner in various grapevine cultivars under no treatment conditions.

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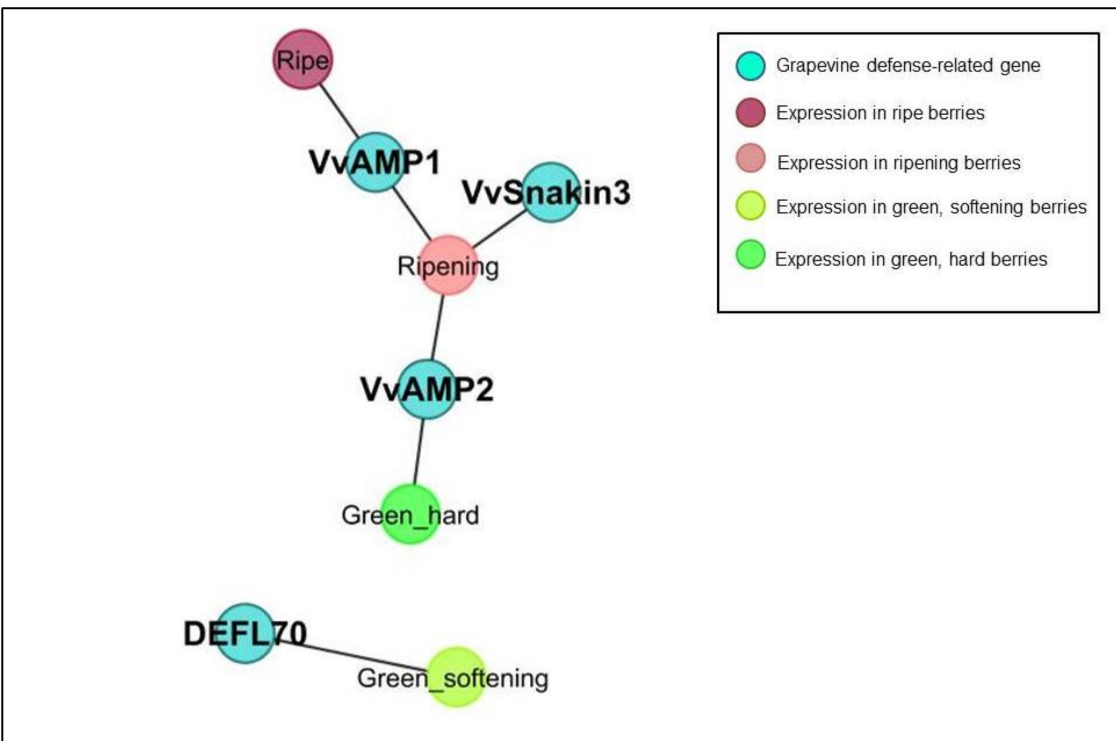
Berry development

Co-expression of the putative DEFL grapevine genes driven by berry developmental stages were identified with a correlation cut-off of 0.6, a Markov inflation cutoff of 7, an expression significance value of 0.9 and a frequency of 0.13. Four DEFL genes showed putative co-expression driven by berry developmental conditions under these parameter thresholds. These genes included Vv-AMP1, Vv-AMP2, Snakin3 and DEFL70 (Figure 4.4A).

Throughout both analyses Vv-AMP1 showed putative expression changes exclusively in ripening and ripe berries, whereas Vv-AMP2 showed putative expressing changes during various stages of grape development. DEFL70 showed putative expression changes in green, softening berries (Figure 4.4A, B). One of our analyses also showed Vv-Snakin3 to be putatively co-expressed with Vv-AMP1 in ripening berries (Figure 4.4A).

A:



B:

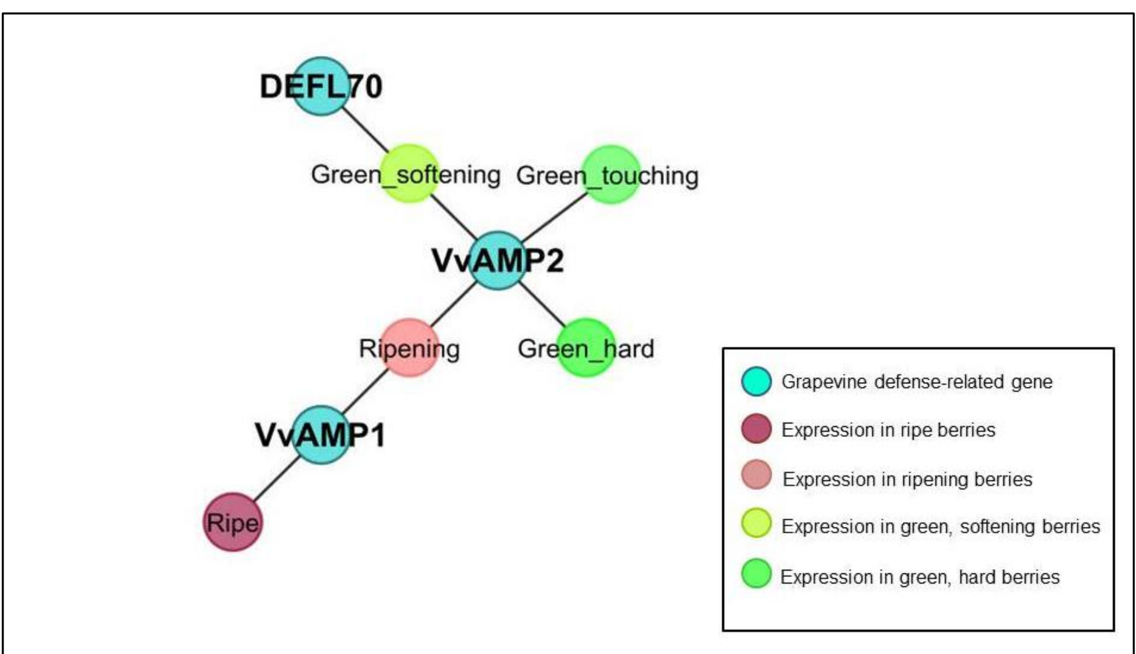


Figure 4.4 Visual representation of DEFL grapevine genes putatively co-expressed during various stages of grapevine berry development. (A) Results obtained from a correlation cut-off of 0.6, a Markov inflation cutoff of 7, an expression significance value of 0.9 and a frequency of 0.13. (B) Results obtained from a correlation cut-off of 0.7, a Markov inflation cutoff of 7, an expression significance value of 0.9 and a frequency of 0.14.

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Abiotic stresses

Co-expression of the putative DEFL grapevine genes driven by abiotic stress conditions were identified with a correlation cut-off of 0.8, a Markov inflation cutoff of 7, an expression significance value of 0.9 and a frequency of 0.9. Vv-AMP2, Vv-AMP3, Vv-Snakin6, Vv-Snakin8 and DEFL70 formed two co-expression clusters (Figure 4.5). The first cluster showed Vv-AMP3 and Vv-Snakin8 to be putatively co-expressed in reaction to salinity stress due to exogenous treatment with a salt solution. Under these thresholds, water deficit stress drove the putative co-expression of Vv-AMP1, Vv-AMP2 and DEFL70. Vv-AMP1 was also found to be significantly expressed in response to exogenous treatment with abscissic acid whereas DEFL70 also showed putative significant expression when exposed to a short daily photoperiod of 13 hrs as well as in transgenic grapevines overexpressing VvCBF4 (Figure 4.5).

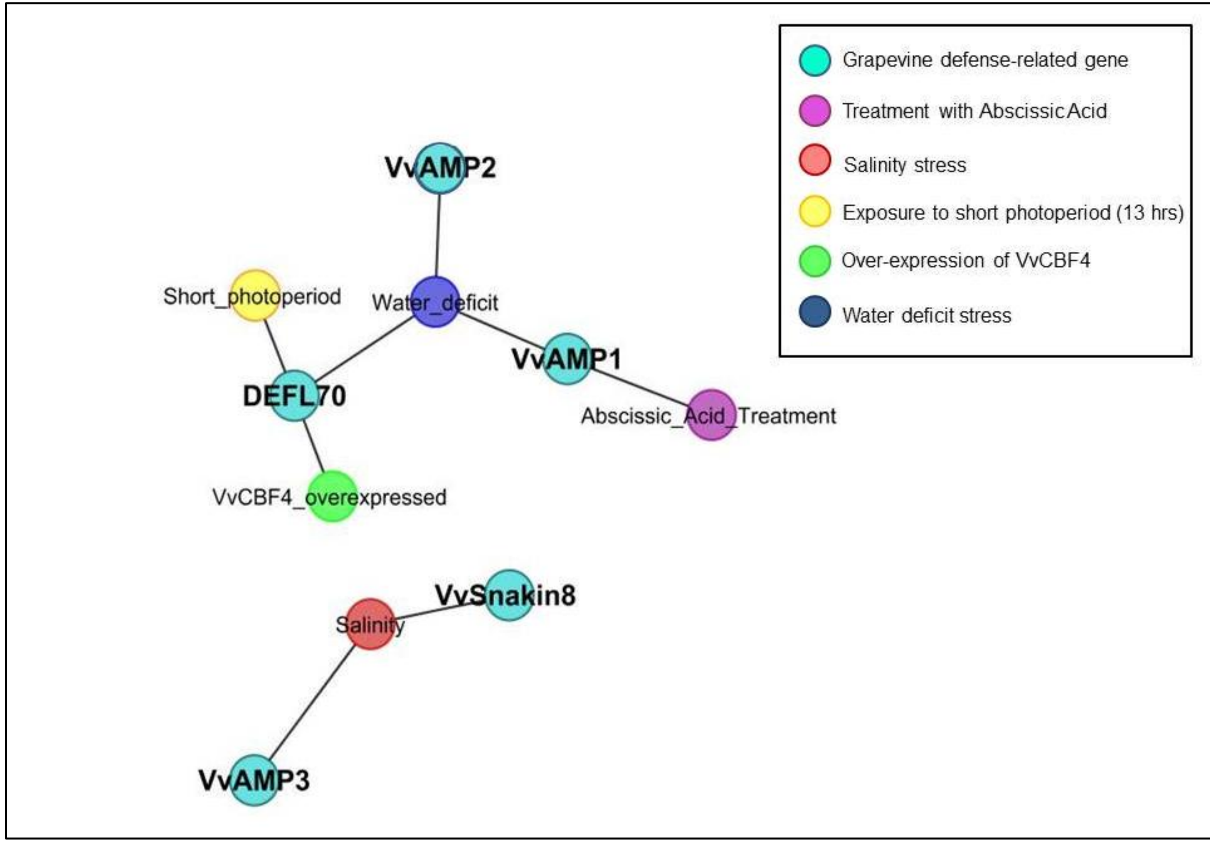


Figure 4.5 Visual representation of DEFL grapevine genes putatively co-expressed in response to various abiotic stress treatments.

Biotic stress

A correlation cut-off of 0.6, a Markov inflation cutoff of 7, an expression significance value of 0.9 and a frequency of 0.13 was used for the analysis showing Vv-AMP1, Vv-Snakin3, and DEFL70 forming two co-expression clusters (Figure 4.6). The first cluster showed Vv-AMP1 and Vv-Snakin3 to be putatively co-expressed in grapevine infected with the GLRaV3 virus, the cause of leaf roll, whereas the second cluster showed DEFL70 to be significantly putatively regulated by grapevine infection with the Bois noir phytoplasma.

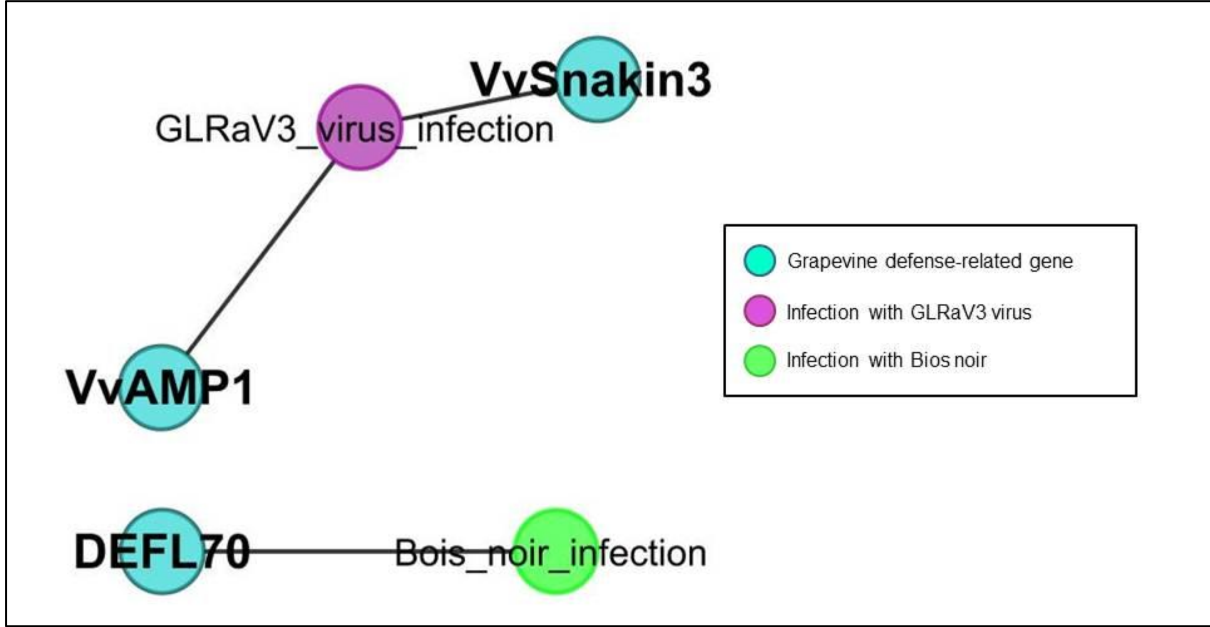


Figure 4.6 Visual representation of DEFL grapevine genes putatively co-expressed in response to biotic stresses induced by infections with grapevine pathogens.

Gene expression patterns of grapevine genes putatively co-expressing with DEFL genes

Clustering methods were performed with various threshold combinations to establish the effect that these threshold combinations have on the quantity of data produced and at which threshold combinations these analyses would provide the most biologically informative results. These results are summarized in Figure 4.7. According to the comparisons of various threshold combinations, a correlation cut-off of 0.8 and a frequency cut-off of 0.5 yielded the optimal amount of genes that are putatively co-expressed with the grapevine DEFL genes when considering the

number of proposed functions associated with each gene and were thus used to generate the results below.

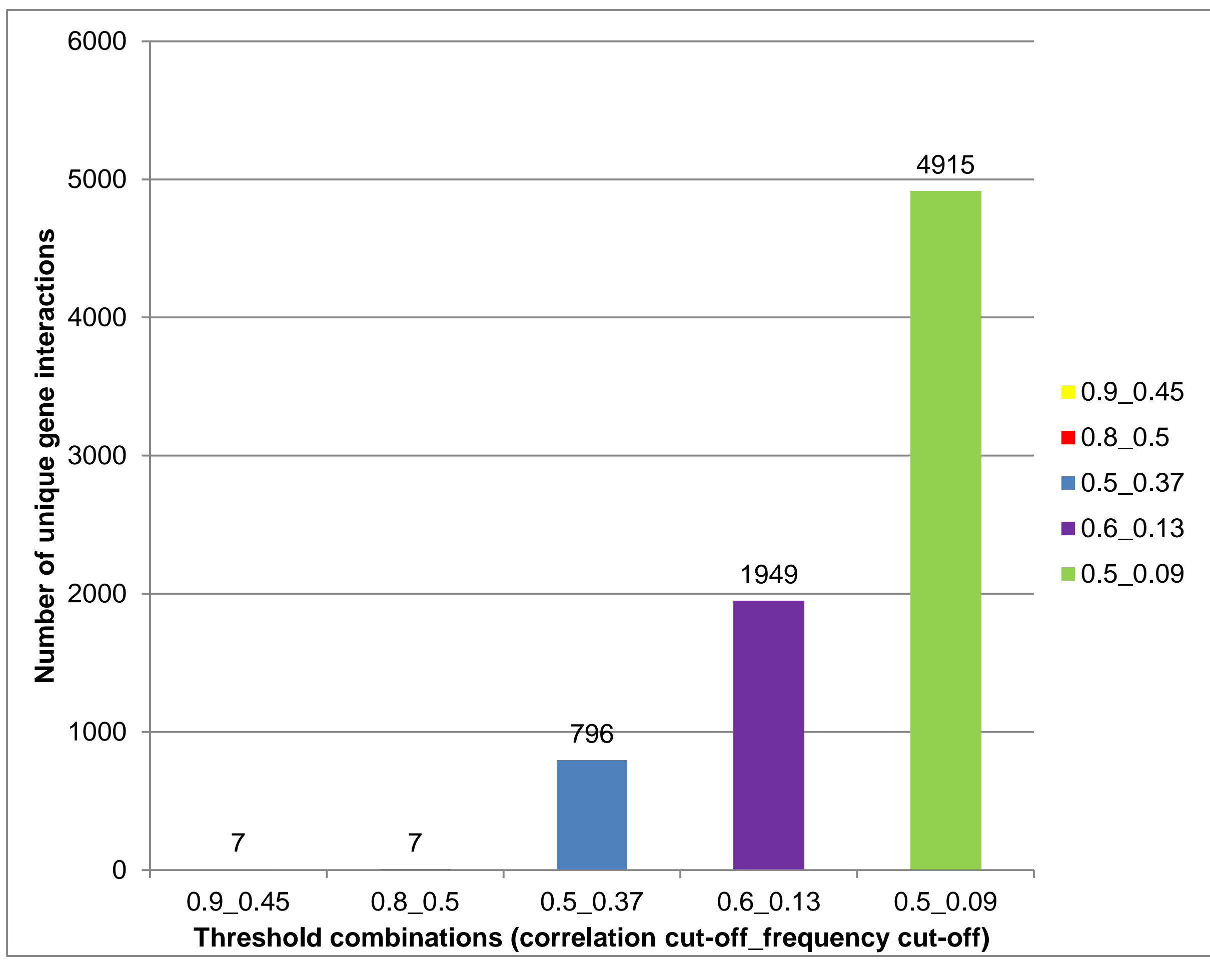
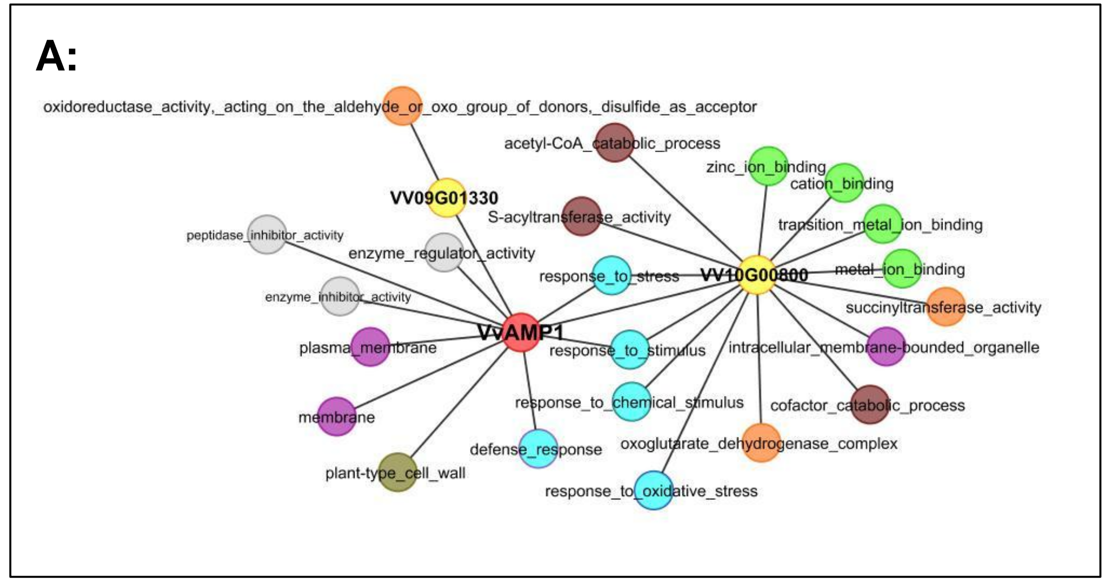


Figure 4.7 Effect of various parameter thresholds on the quantity of data extracted with regards to the number of grapevine genes that are putatively co-expressed with grapevine DEFL genes driven by specific experimental conditions. Inflation parameters and significance measure were at constant values of 7 and 0.9, respectively.

The analysis was performed with a correlation cut-off of 0.8, a Markov inflation cut-off of 7, an expression significance value of 0.9 and a frequency of 0.5. Vv-AMP1 and Vv-AMP3 showed co-expression with other genes present in the grapevine genome; for each of these genes that putatively co-expressed with either Vv-AMP1 or Vv-AMP3, GO annotations were collected, further classified according to their possible biological functions and colour coded for ease of identification (Figure 4.8).



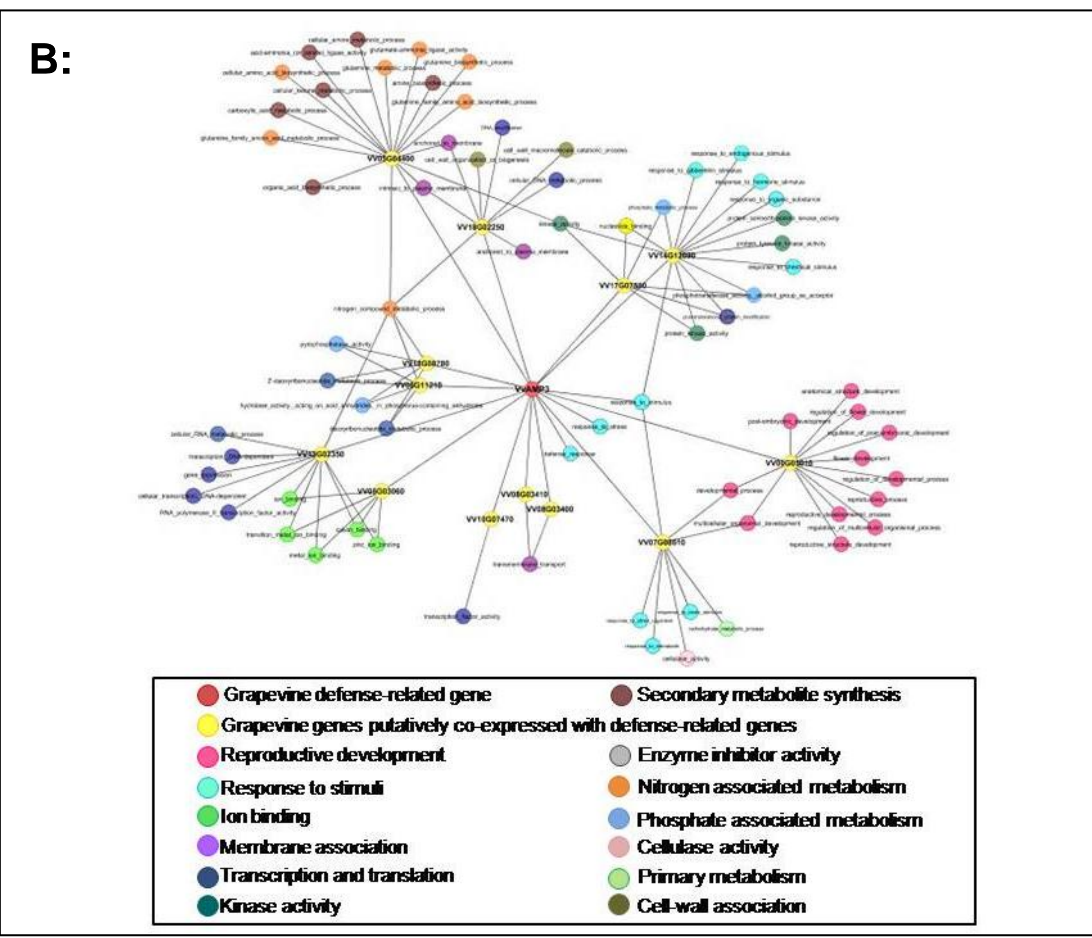


Figure 4.8 Visual representation of grapevine genes putatively co-expressed with DEFL genes and their associated GO terms (A) Co-expression network of grapevine defensin, Vv-AMP1 and associated GO terms (B) Putative co-expression network of grapevine defensinVv-AMP3 and associated GO terms. All co-expression was analyzed with a correlation cut-off of 0.8, a Markov inflation cutoff of 7, an expression significance value of 0.9 and a frequency of 0.5.

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Table 4.5 Summary of the possible biological functions associated with grapevine genes putatively co-expressed with DEFL-genes and previous publications that provide support for these functional associations.

Functions	**Activity**	**Associated DEFL-genes**	**References documenting the various activities and their link to defensin genes**
Defense	Enzyme inhibition	Vv-AMP1	Bowles, 1990; Broekaert et al., 1997; Colilla et al., 1990; Mendez et al., 1990
	Cellulase	Vv-AMP3	Balandin et al., 2005; Calderon et al., 1993
	Ion binding	Vv-AMP1; Vv-AMP3	Thevissen et al., 1999
	Membrane association	Vv-AMP1; Vv-AMP3	Thevissen et al., 1999
	Kinase activity	Vv-AMP3	Robatzek et al., 2006; Miya et al., 2007; Wan et al., 2008; Casagrande et al., 2011
	Phosphatases	Vv-AMP3	Widjaja et al., 2010
	Cell wall associated activity	Vv-AMP1; Vv-AMP3	Kwon, 2010
	Oxidative stress response	Vv-AMP1; Vv-AMP3	Aerts et al., 2006; Aerts et al., 2007; Mello et al., 2011; Aerts et al., 2009; van Weerden et al., 2008
Abiotic stress response	Zn-ion binding	Vv-AMP1; Vv-AMP3	Mirouze et al., 2006
Growth and development	Reproductive development	Vv-AMP3	Stotz et al., 2009
	Nitrogen metabolism	Vv-AMP1; Vv-AMP3	Espinoza et al., 2007; Lam et al., 1996, Pageau et al., 2006

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Thirteen genes showed putative co-expression with the defensin, Vv-AMP3. GO annotations for these genes were collected and similarly characterized as those for Vv-AMP1. These categories were colour coded according to their biological involvement and are visualized in Figure 4.8B. These functional categories, the DEFL genes putatively associated with these biological activities and previous publications that support these associations are summarized in Table 4.5. Due to the complexity of this network, the possible activities, three separate networks were generated focusing on the various combinations of this network in isolation (Figures 4.9, 4.10, 4.11).

The first network shows the putative co-expression of five genes with Vv-AMP3 that shows involvement in multiple activities regarding the developmental processes involving post-embryonic stages, flower development, biological responses upon external biotic stimuli as well as trans-membrane transport activities (Figure 4.9).

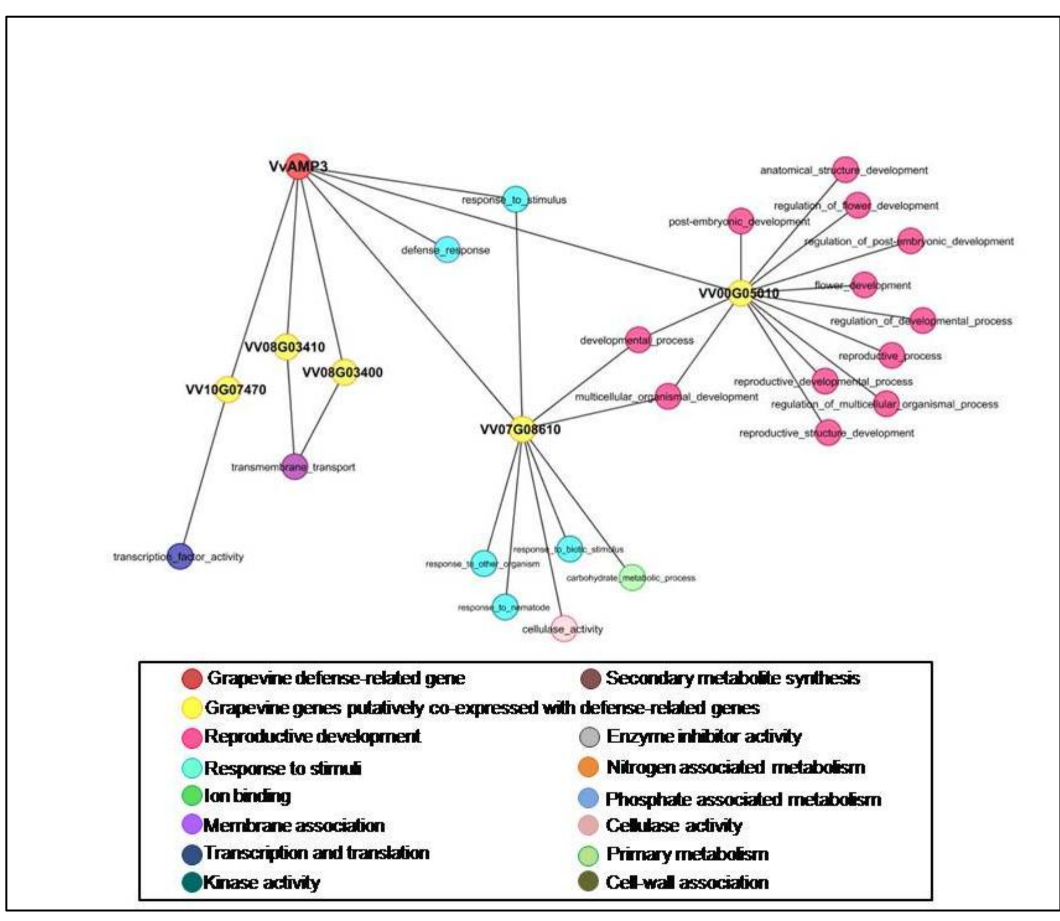

Figure 4.9 Visual representation of the first five grapevine genes putatively co-expressing with the grapevine defensin-encoding gene, Vv-AMP3 and their associated GO terms.

The second network depicts the putative co-expression of Vv-AMP3 with a further four genes. These genes show involvement in general cation binding, transition metal ion binding and zinc ion binding in particular as well as nitrogen-related metabolic processes, phosphate associated metabolism and processes involved in transcription and translation (Figure 4.10).

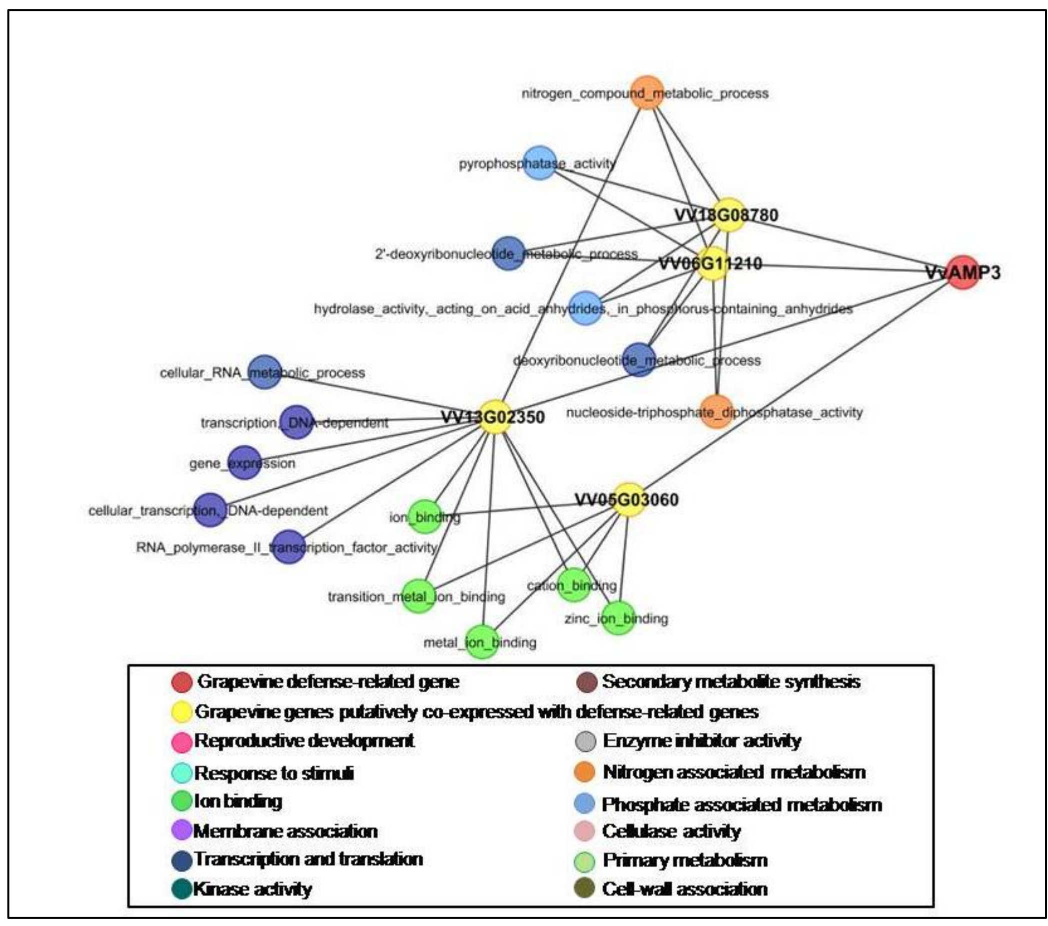

Figure 4.10 Visual representation of four of the grapevine genes putatively co-expressed with the grapevine defensin-encoding gene, Vv-AMP3 and their associated GO terms.

The third network is a representation of putative co-expression of Vv-AMP3 and the final four genes. Activities associated with the expression of these genes include involvement in protein kinase activity, post-translational protein modification and nucleotide binding activities. These genes are further associated with phosphate metabolic processes, response reactions to various chemical and hormonal stimuli that includes gibberellins. Further activities associated with the expression of these genes link them to plasma membrane localization as well as primary nitrogen compound metabolism, involvement in cell wall related metabolic processes and

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organization as well as DNA modification, involvement in multiple glutamine-associated reactions and secondary metabolism related functions (Figure 4.11).

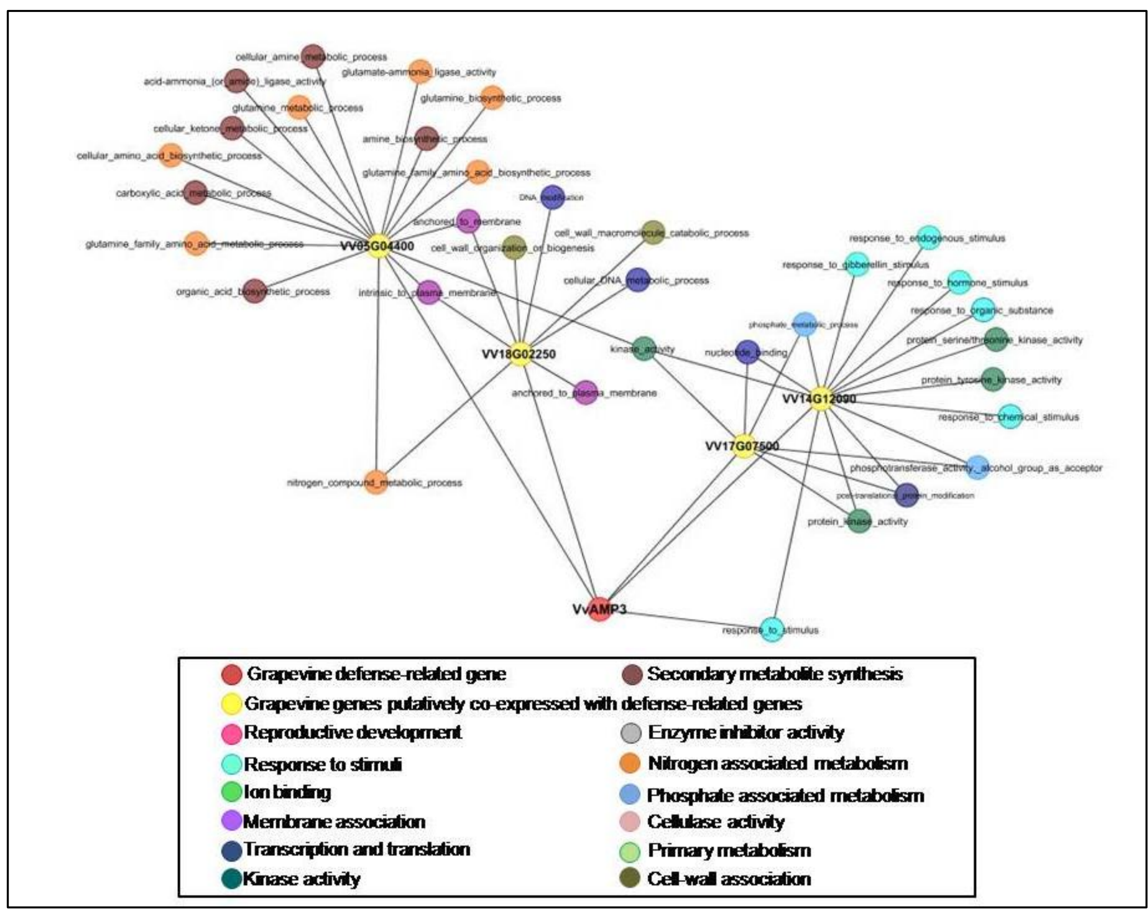

Figure 4.11 Visual representation of the final four grapevine genes putatively co-expressed with the grapevine defensin-encoding gene, Vv-AMP3 and their associated GO terms.

4.4 Discussion

The completion of the *V. vinifera* genome sequence, and the increasing number of molecular profiling tools and datasets becoming available for grapevine as a consequence, has made it possible to evaluate the presence and importance of DEFL peptides in this species. The purpose of this investigation was therefore to accumulate insight regarding DEFL peptides in grapevine through the expression patterns exhibited by the genes that encode for these peptides. This was attempted through evaluating the DEFL genes, their putative co-expression patterns and their possible involvement in grapevine biological functions.

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Classification of DEFL genes

The plant antimicrobial peptide family is well known for the great diversity in primary amino acid sequences of its members. Furthermore, this diversity is matched by the functional diversity of these antimicrobial peptides that remains problematic in linking sequence to function. Classification of these peptides was traditionally based upon the amino acid composition of the mature peptide domains (Harrison et al., 1997).

Our results reveal the phylogenetic relationships between 16 DEFL grapevine genes based on their deduced amino acid sequences. Vv-AMP1 is the first and best-characterized grapevine defensin and due to the close sequence similarity between this defensin and Vv-AMP2 and Vv-AMP3, these DEFLs are similarly classified as plant defensins. According to our classification method, DEFL1, DEFL21 and DEFL52 are more closely related in amino acid sequences to the plant defensins than to any other group included in our investigation. Although the functions of these DEFL genes remain to be tested, DEFL1 and DEFL52 have been found to be upregulated in inflorescences and the seeds of green berries according to qPCR analyses performed by Giacomelli et al. (2012). Even though Vv-AMP1 is well known for its exclusive presence in berries from the onset of ripening, this sustained presence of DEFLs in berries underscores the well-known role of plant defensins in the protection of plant reproductive structures (Osborn et al., 1995; Song et al., 1995; Almeida et al., 2000; Chen et al., 2002). Similar to DEFL1 and DEFL52 expression, other defensins have also been isolated from inflorescences from other species and have been implicated in a regulatory role of floral development and reproduction (Karunanandaa et al., 1994; Lay et al., 2003; Stotz, 2009).

The Vv-Snakins included in this investigation show close relationships in their deduced amino acid sequences and DEFL70, DEFL26, DEFL59 and DEFL10 appear to be more closely related to these snakin-encoding genes than to any other DEFL genes included in this analysis. Once again, limited information is available regarding these DEFL genes but qPCR analysis by Giacomelli et al. (2012) revealed that DEFL59 shows upregulation in grapevine inflorescences. These results are in keeping with findings of the limited plant snakin studies that have identified snakins in potato tubers and *Arabidopsis* inflorescences (Shi et al., 1992; Herzog et al., 1995;

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Ben-Nissan and Weiss, 1996; Segura et al., 1999; Garcia-Olmedo, 2001; Berrocal-Lobo et al., 2002; Kovalskaya and Hammond, 2009).

Our alignment analysis further revealed that DEFL29 do not form part of a clear group within our set of analyzed DEFL genes according to amino acid sequences. Furthermore, qPCR analysis performed by Giacomelli et al., (2012) showed this gene to be upregulated in all grapevine tissues with the exception of inflorescences at various developmental stages. Therefore, this gene can be considered to be constitutively expressed and may form part of the preformed defense mechanisms as part of the innate immunity of grapevine.

Even though some consensus sequences within the antimicrobial peptide family remains conserved, the steady increase of AMP sequences becoming available has proven the traditional classification system to be problematic with the increase in exceptions to the classification rules. It is important to develop a more efficient strategy for the classification of plant defensins.

Tissue specific expression of DEFL genes in various grapevine cultivars

Antimicrobial peptides and defensins in particular are known to be either constitutively expressed or induced upon external stimulus in a tissue-specific manner (Bowles, 1990; Ryan and Jagendorf, 1995; Broekaert et al., 1997; Epple et al., 1997; Thomma et al., 1998; Ryan and Moura, 2002; De Beer and Vivier, 2008). The tissue and cultivar specificity of the expression patterns of the putative grapevine DEFL genes that formed part of this study were therefore evaluated under unstressed conditions. Although the relevance of the genetic variation between the different cultivars within the genus should be considered when comparing expression patterns (Myles et al., 2011), results from this evaluation proved tissue specificity to be a much stronger driver for putative co-expression of DEFL genes. The well-studied grapevine defensin, Vv-AMP1, along with putative defensinVv-AMP2 shows putative co-expression exclusively in berry tissue. These results confirm those obtained by De Beer and Vivier (2008) who identified and isolated the Vv-AMP1 peptide from *V. vinifera* (cv. Pinotage) berry tissues. A recent study reported this peptide to be present in not only berries, but also leaves, roots and flowers of Pinot Noir. The authors attributed these findings to cultivar-specific expression (Giacomelli

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et al. 2012). However, our findings further corroborate initial analyses localizing Vv-AMP1 expression exclusively to berries (De Beer and Vivier, 2008) with no indication of different expression patterns in different cultivars. qPCR performed by Giacomelli et al. (2012) did, however, reveal extremely low expression levels of Vv-AMP1 in tissues other than berries with a 15-fold higher expression level in ripening berry flesh than in roots, leaves or inflorescences. Such low expression values are not necessarily grounds for characterizing the Vv-AMP1 encoding gene to be cultivar-specific in its expression, since normalization to the expression of another house-keeping gene could have proven the same Vv-AMP1 gene expression to be negligible. These values were normalized to the actin expression levels even though it has recently been proven that specific grapevine genes such as VATP16 that codes for a V-type proton ATPase genes are much more efficient house-keeping gene candidates for qPCR normalization of grapevine DEFL genes involved in plant-pathogen interactions (Gamm et al., 2011).

To further contribute to the hypothesis of tissue-specificity driving putative co-expression of these DEFL genes, Vv-Snakin6 shows significant expression in the leaves of both *V. vinifera* cultivars Cabernet Sauvignon and Carmenere, but in no other tissues form the same cultivars. Snakin peptides are plant antimicrobial peptides that are known for possessing both fungicidal and bactericidal activity (Segura et al., 1999; Garcia-Olmedo, 2001; Berrocal-Lobo et al., 2002; Kovalskaya and Hammond, 2009). These DEFL peptides are expected to occur in both monocotyledonous and dicotyledonous plants and have been found to share structural motifs with kristin from snake venom (Segura et al., 1999). These snakin genes are predominantly constitutively expressed, however, some are known to be upregulated by pathogenic attack, wounding and gibberelic acid (GA) stimulus such as the GAST encoding gene from tomato (*Lycopersicon esculentum*) and *GASA1* and *GASA4* from *Arabidopsis* (Shi et al., 1992; Herzog et al.,1995; Ben-Nissan and Weiss, 1996). Furthermore, while most snakins have been associated with the protection of plant reproductive structures, some have been found to be expressed in leaves (*A. thaliana*) (Herzog et al., 1995; Aubert et al., 1998). Our results further underscore the diversity in expression regulation within the plant snakin family. Although grapevine cultivar-specific expression of plant snakins has not been elucidated to date, our results indicate possible cultivar-specific expression of Vv-

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Snakin8, whereas Vv-Snakin6 appears to be expressed exclusively in leaf tissue irrespective of the cultivar. Vv-Snakin3 appears to be constitutively expressed in various tissues and cultivars.

Similar to Vv-Snakin6, expression of DEFL70 does not appear to be restricted by cultivar or tissue. DEFL70 shows expression in berries, leaves, shoot tips and all other aerial tissues of both *V. vinifera* and non-vinifera cultivars. Furthermore, the cultivar described as 'Freedom' is a *Vitis champinii* grapevine of North American descent and is well-known for a high level of resistance to nematodes and microbial pathogens. Due to the constitutive expression pattern of this DEFL70 gene, it is probable that this peptide forms part of grapevine's innate immunity, involving constitutive expression of antimicrobial peptides. These findings are in keeping with the predominantly constitutive expression of plant snakins that show high sequence similarity to DEFL70. It would therefore be interesting to determine the defense role of this antimicrobial peptide in the cultivars that are known for resistance to microbial pathogens and whether overexpression of this DEFL70 would lead to increased resistance to pathogens in otherwise susceptible cultivars.

DEFL gene expression during grape berry development

The developing and ripening grape berry has been the focus of numerous transcriptomic analyses in recent years (Grimplet et al., 2007; Pilati et al., 2007; Lund et al., 2008; Koyama et al., 2010;Lijavetzky et al., 2012). Defensins are well known for their involvement in the protection of plant reproductive structures due to their overwhelming presence in seeds, seedpods and fruits of various plant species (Osborn et al., 1995; Song et al., 1995; Almeida et al., 2000; Chen et al., 2002).

When evaluating the expression patterns of DEFL genes in grapevine, our results revealed that putative expression of these genes is developmentally regulated in unstressed grapevine berries. Despite findings of the Vv-AMP1 peptide in pre-véraison, véraison and ripening berry stages in EST analyses (Goes da Silva et al., 2005; Giacomelli et al., 2012), our results support the initial findings that limited expression of Vv-AMP1 exclusively in grapevine berries from the onset of ripening onwards (De Beer and Vivier, 2008). During ripening the grape berry serves as an accumulation point for sugars that are transported from surrounding plant organs

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(Coombe and McCarthy, 2000), hereby making berry tissues particularly vulnerable to fungal attack. Furthermore, from the onset of berry ripening, malic and tartaric acid levels begin to decrease, hereby providing optimal conditions for defensin activity that are known to function optimally at pH levels higher than 4 (De Beer and Vivier, 2008; Wang et al., 2009).These factors contribute to the possible defense role of not only Vv-AMP1 but Vv-Snakin3 that are putatively co-expressed under ripening conditions.

In two separate analyses we found the putative defensin, Vv-AMP2 to be expressed in various stages of berry ripening from the green hard stage to the ripening stages of berry development. Despite the high level of homology between this defensin and Vv-AMP1, Vv-AMP2 shows a much more general expression pattern with regards to berry development perhaps indicating a more tissue-specific than developmentally regulated expression pattern when considering our findings in tissue-specificity.

Furthermore, both Vv-AMP1 and Vv-AMP3 showed putative co-expression with genes involved in response reactions to various stresses. These stress conditions include chemical, oxidative stresses, responses to hormone stimuli as well as biotic stresses that include responses to nematodes and general defensin responses. Oxidative stress responses associated with a gene putatively co-expressed with Vv-AMP1 can be linked to the levels of reactive oxygen species (ROS) in developing berries since we have established Vv-AMP1 to be present exclusively in berries from the onset of ripening. In recent years, a dual role of ROS has been identified in plants that implicate ROS as toxic by-products of aerobic metabolism, as well as growth, development and defensin regulators. Although the topic of oxidative stress during grape berry development has been somewhat controversial, the H_2O_2 content in berries seem to correspond to the stage of véraison, reaching a maximum concentration at about 2 weeks post-véraison and then gradually decreasing towards the ripe stages through enzymatic detoxification (Pilati et al., 2007). These findings could therefore explain the prevalence of oxidative stress related genes putatively co-expressed with Vv-AMP1.

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DEFL genes involved in plant physiology and development

Two genes putatively co-expressed with the Vv-AMP3 defensin show biological functions relating to developmental processes and reproductive differentiation. A recent study involving a tomato defensin, DEF2, proposed a new biological activity to the plant defensin family that involves serving as a signal for plant development. The experiments leading to this claim included several expression studies that confirmed the necessity of DEF2 in *S. lycopersicon* (tomato) flowers during early flower development as well as the necessity of the inactivation of DEF2 expression during pollen development (Stotz et al., 2009).

Furthermore, the significant change in gene expression associated with the several glutamate associated activities can be linked to primary nitrogen metabolism of plants. Nitrogen assimilation is at the center of plant growth and development and relies on the assimilation of inorganic nitrogen into five key amino acids to fulfill the purpose of nitrogen transport. These amino acids include glutamine, asparagine and aspartate (Lam et al., 1996). One of the most important enzymes responsible for glutamine biosynthesis is glutamine synthetase (GS), an enzyme that's expression is greatly influenced by pathogen attack and stress-induced hormone production (Lam et al., 1996, Pageau et al., 2006). A study focusing on the regulatory effects of pathogenic attack, stress hormones and reactive oxygen species on GS in *Nicotiana tabacum* leaves found a significant decrease in expression of chloroplastic GS (*GS2*) and an increase in cytosolic GS (*GS1*) under all stress conditions evaluated (Pageau et al., 2006). These findings indicate a shift in primary nitrogen management as a response to stress, hereby indicating the significance of expression changes of these nitrogen metabolism associated genes that show putative co-expression with both Vv-AMP1 and Vv-AMP3 in our results. A later study confirmed these findings by the expression patterns of GS encoding genes in response to GLRaV-3 in *Arabidopsis* and grapevine plants (Espinoza et al., 2007). Similarly, Vv-AMP1 showed significant changes in expression in response to the same virus hereby further establishing the putative co-expression of nitrogen metabolism associated genes with plant defensins in response to pathogen attack.

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Expression of DEFL genes in response to abiotic stress

The expression patterns of putative DEFL genes in grapevine were evaluated in response to a range of abiotic stresses. Members of the plant defensin family have been found to be inducible through treatment with various endogenous plant hormones such as methyl jasmonate, salicylic acid and abscissic acid (ABA) to name a few. Our *in silico* results indicate that expression of the grapevine defensin, Vv-AMP1, could be induced through treatment with exogenous ABA. This is in contrast what was found by De Beer and Vivier (2008) when studying the expression and induction pattern of Vv-AMP1 in *V. vinifera* (cv. Pinotage). It is possible that cultivar variability could influence the inducibility of these peptides, but this aspect warrants further evaluation.

Vv-AMP1 further showed putative co-expression with Vv-AMP2 and DEFL70 under water deficit stress conditions. Similarly, the induction of CADF1 (*C. annuum* defensin 1) in leaves had been reported in response to drought and salinity stress (Do et al., 2004). Furthermore, Vv-AMP3 is putatively co-expressed with Vv-Snakin8 when stressed by high salt concentrations. A strong link between dehydration stress and ABA metabolism has been confirmed in a wide range of plant species. Similarly, a great overlap was identified between genes up- and downregulated by exogenous ABA treatment and water deficit in Cabernet Sauvignon berries (Koyama et al. 2010). In *Arabidopsis* a link between ABA, dehydration stress and defensins was found when mutants that lack the hos10 transcription factor encoding gene were studied. These mutants have a deficiency in ABA biosynthesis and also cannot express a plant defensin (At1g75830), leading to a hypersensitivity to dehydration and salinity (Zhu et al., 2005). It is now accepted that plant defensins are not only involved in plant protection against pathogens but play a critical role in abiotic stress response as well, a fact clearly supported by the gene expression data obtained in this study.

DEFL70 showed significant changes in putative expression levels in *V. vinifera* (cv. "Freedom") overexpressing the grapevine C-repeat binding factor (CBF) gene. The VvCBF4 gene has been implicated in elevated tolerance levels to freezing temperatures when overexpressed in non-cold acclimatized grapevine (Tillett et al., 2012). Interestingly, DEFL70 also showed significant changes in putative expression

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levels when exposed to a short daily photoperiod. These environmental changes are instrumental cues in the process of overwintering in woody perennials such as grapevine (Wake and Fennell, 2000; Welling et al., 2004). This indicates a possible role of DEFL70 in the adaptation of grapevine to allow for timely acclimatization to these adverse conditions.

Several genes involved in ion binding and transport activities have shown to be putatively co-expressed with both defensins Vv-AMP1 and Vv-AMP3. Since zinc ions are specifically included in these ion-related activities, the possible defensin function related to ion transport could involve the proposed contribution of plant defensins in heavy metal tolerance. Heavy metals and zinc in particular, have detrimental effects on the growth of numerous plant species. Therefore, mechanisms to increase zinc tolerance in plants have been investigated. In a study performed by Mirouze et al. (2006), the molecular mechanism of zinc tolerance in the zinc hyper-accumulating plant, *A. halleri*, was evaluated. A cDNA library of this plant was expressed in *Saccharomyces cerevisiae* and zinc tolerant strains were selected by incubation on medium containing toxic concentrations zinc. Of the nine cDNAs selected, four were found to encode for similar peptides with remarkable sequence similarity to plant defensins (Mirouze et al., 2006). Although the exact mechanism of the possible zinc tolerance conferred by plant defensins are not known, these findings substantiates the possible role that defensins and their co-expression patterns may have in the level of heavy metal tolerance in grapevine.

Expression of DEFL genes in response to biotic stimuli

The natural plant defense mechanisms have been honed and fine-tuned over millennia through the ongoing evolutionary arms race between plants and their microbial pathogens. These highly complex defense strategies of plants involve structural and biochemical defense mechanisms that can either be induced upon pathogen attack or constitutively maintained (Bowles, 1990; Broekaert et al., 1997). Although a wide range of experiments involving biotic stress stimuli were included in our investigation of grapevine DEFL gene expression, our results yielded limited putative co-expression of these genes under the predefined set of thresholds that were implemented. Once again Vv-AMP1 showed putative co-expression with Vv-Snakin3 when infected with the leaf roll associated closterovirus-3 (GLRaV-3). This

Ampelovirus is considered to be one of the most prolific of the more than 40 grapevine-specific viruses and are known to be transmitted through grafting and infection with mealybugs (Martelli, 1993; Ling et al., 2004). Even though plant defensins have not yet been directly implicated in the inhibition of viral replication *in vivo* (Carvalho and Gomes, 2009), it would be useful to consider the numerous reported insecticidal activities of plant defensins. Plant defensins are known to inhibit the activity of insect digestive enzymes such as α-amylases and proteases, thereby limiting the prevalence of insect herbivory (Bowles, 1990; Colilla et al., 1990; Mendez et al., 1990; Broekaert et al., 1997). Since the GLRaV-3 virus depends on an intermediate insect host for transmission to its grapevine host, it is possible that the Vv-AMP1 defensin responds to the external stimulus of an intruding insect pathogen, rather than the GLRaV-3 virus itself. This response may have developed as a result of the co-evolutionary arms race between the virus and the defensin-responses of its two host organisms.

Due to the consistent putative co-expression of Vv-AMP1 with Vv-Snakin3 under numerous conditions, it is possible that the expression of these genes could be regulated by the same transcription factor. Even though little evidence exists for the involvement of snakin peptides in plant defense against viruses, the combinatorial effect of co-expression of plant snakin and defensin peptides in plant defense against other microbial pathogens have been proven *in vitro* (Kovalskaya and Hammond, 2009). Despite their different spectra of antibiotic activity, the synergistic and additive effects of the plant defensin, PTH1, and the snakin, SN1 both isolated from potato, have been identified against *Collectotrichum coccoides* and *Pseudomonas syringae* cultures (Kovalskaya and Hammond, 2009). However, further evidence of the synergistic activities of plant defensins and snakins against viral pathogens remains to be established. Furthermore, plant defensins very rarely display both antifungal activities and inhibit α-amylase activity (Osborn et al., 1995), so it would be interesting to evaluate the roles of Vv-AMP1 and Vv-Snakin3 more carefully in the grapevine, leafroll and insect vector interaction.

The putative DEFL70 encoding gene has also been implicated in the response reaction against the grapevine-specific Bois Noir phytoplasma. Phytoplasmas are wide-spread prokaryotic organisms responsible for a variety of diseases in plants

that are generally described as "yellows". Bois noir is responsible for the greatest number of incidences of yellows in the European grape, *V. vinifera* (Martelli and Boudon-Padieu, 2006). Although many attempts have been made to limit the spread of this infection through targeting its leaf-hopper vector and the phytoplasma itself, no known method of effective restriction of this disease has been achieved to date (Borgo and Angelini, 2002). Similar to the gene expression in response to the GLRaV-3 pathogen, DEFL70 may show significant expression changes in response to the transmission vector insect of the Bois noir pathogen, rather than the pathogen itself. The DEFL70 peptide has been linked to several potential functions and seems an excellent candidate for more in-depth functional analysis.

As previously mentioned, several genes identified to be putatively co-expressed with both Vv-AMP1 and Vv-AMP3 were associated with ion binding activities. Although the exact mechanism of defensin antifungal activity has not yet been established, it is well known that plant defensins cause the dissipation of the intracellular Ca^{2+} concentration gradient required for fungal growth through a rapid Ca^{2+} influx and simultaneous K^{+} efflux directly following fungal attack. Thevissen et al. (1999) were the first group to explore the possibility that the fungal plasma membrane could be the target of plant defensin activity. They identified a rapid influx of Ca^{2+} and efflux of K^{+} and the subsequent changes in membrane potential caused by the plant defensins, RsAFP2 (*Raphanus sativus*) and Dm-AMP1 (*Dahlia merckii*) upon interaction with the pathogenic fungus, *Neurospora crassa*.

Putative co-expression of Vv-AMP1 and Vv-AMP3 further involved genes that encoded for peptides associated with the cell membrane. This observation contributes to what is well known regarding the innate immunity of grapevine and other plants. Plant resistance against fungal infection is known to consist of a two-layer defense response (Dry et al., 2009). These defense mechanisms include pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is considered to be the plant's first line of defensin against fungal infection as it involves the detection of the pathogen PAMP chitin by a plant membrane receptor-like kinase (Robatzek et al., 2006; Miya et al., 2007; Wan et al., 2008; Casagrande et al., 2011). Several genes involved in kinase activity were further identified to be putatively co-expressed with Vv-AMP3. This

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pathogen detection mechanism involving protein kinases causes a cascade of signaling events that leads to the secretion of antimicrobial and cell-wall restructuring peptides in order to physically obstruct fungal penetration (Kwon, 2010). Processes involved in cell wall associated organization were also identified in the putative co-expression patterns of both Vv-AMP1 and Vv-AMP3. This combination of membrane, cell wall and protein kinase associated activity underscores the importance of these grapevine defensins in the successful implementation of the innate immune systems of grapevine. Furthermore, phosphatase activity in combination with transcription regulation involvement has been identified in two genes putatively co-expressed with Vv-AMP3. Although phosphatases were not initially considered to be associated with DEFL gene expression in plants, it has recently been implicated in the induction of plant defensins in response to pathogen infection (Widjaja et al., 2010).

Furthermore, one of these putatively co-expressed genes show response reactions to several biotic stimuli and cellulase activity, both activities which contributes to known defensin functions. The role of plant defensins in the protection of seeds have been well-studied (Terras et al., 1993; Carvalho et al., 2006) and it has further been proposed that defensins are involved in protection of the developing plant embryo (Balandin et al., 2005) whereas cellulases are known to stimulate the production of resveratrol and the hypersensitive response in grape cell cultures (Calderon et al., 1993). The hypersensitive response forms part of the multi-layered gene-expression cascade inherent in the plant innate immunity upon pathogen attack as earlier discussed.

Other genes that were putatively co-expressed with either Vv-AMP1 or Vv-AMP3 showed involvement in secondary metabolite production. Certain secondary metabolites such as phytoalexins have been implicated in plant resistance to pathogens (Spiteller, 2008). However, more information regarding the specific secondary metabolites produced is necessary to generate hypotheses regarding the link between grapevine defensins and secondary metabolite expression.

Conclusion

Despite the low genome coverage of the Affy GeneChip and the limit that the available data places on the number of DEFL genes that could be evaluated, this

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investigation provided insight into the expression of these DEFL genes and the possible functions of the peptides they encode for. Analyses of a group of grapevine DEFL genes revealed putative co-expression of some of these genes with each other as well as with other genes in the grapevine genome. Some DEFL genes proved to be highly regulated by tissue-specificity whereas others displayed cultivar specific expression. These data provides further evidence for the developmental role that these plant defensins play in the reproduction and ripening of grape berries and identifies the DEFL genes that are expressed in response to abiotic stresses. The role that these grapevine DEFL genes play in the innate immunity of plants were further accentuated and this data reveals the potential involvement of these genes against insect herbivory. Grapevine genes found to be putatively co-expressed with two grapevine defensins revealed several mechanisms possibly involved in the functioning of these defensins. These functions involved nitrogen assimilation, ion binding and membrane associated activities and the regulation of plant growth and development. The complexity of these co-expression gene networks clearly indicates that the successful functioning of the innate immunity of plants rely on expression of DEFL genes themselves, but also a host of gene products that facilitate successful functioning of these DEFL genes as well. The approach used provided an *in silico* base from which to generate and test hypotheses with regards to DEFL genes and their possible *in planta* functions.

4.5 Acknowledgements

I would like to express sincere gratitude to Daniel Jacobson and Piet Jones of the Computational biology group of the Institute for Wine Biotechnology at Stellenbosch University for performing all pairwise correlations, Markov Clustering, matrix transformations and frequency determinations and their general contribution to the work. I would also like to acknowledge Abré de Beer for the identification of the grapevine antimicrobial peptide encoding genes and for his assistance in the analysis during the generation of the complete set of DEFL genes included in this *in silico* analysis.

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Chapter 5

General discussion and conclusions

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General discussion and conclusions

5.1 General discussion and conclusions

The rapidly increasing human population has managed to place tremendous pressure on modern agriculture for the production of economically sustainable, high quality food crops in sufficient quantities. These goals are, however, constantly threatened by the myriad crop pathogens that cause annual crop losses of an estimated 10% (Strange and Scott, 2005). Therefore, the success of modern agriculture relies greatly upon the effective disease management of the economically important crops that include for example corn, soy, rice and grapevine.

The latest strategies in the protection of crops against microbial pathogens are rooted in harnessing the highly complex innate immunity evolved by plants through genetic engineering. At the centre of this innate immunity in plants lies the *de novo* production of antimicrobial peptides that offer resistance against plant microbial pathogens.

Since the grapevine genome became the first commercial fruit to be completely sequenced in 2007 (Jaillon et al., 2007; Velasco et al., 2007), attempts have been made to systematically identify genes that encode for antimicrobial peptides in grapevine (Tredoux et al., 2011; Abré De Beer, personal communication; Giacomelli et al., 2012). Subsequently, the first antimicrobial peptide was identified in grapevine and is known as Vv-AMP1 (*Vitis vinifera* antimicrobial peptide 1). Extensive research focused on Vv-AMP1 was subsequently undertaken that lead to the isolation and complete characterization of this plant defensin peptide (De Beer, 2008; De Beer and Vivier, 2008; Tredoux, 2011). Recombinantly produced Vv-AMP1 was also evaluated for its antimicrobial activity *in vitro* and proved to inhibit growth of a wide range of grapevine pathogens, at remarkably low concentrations (De Beer and Vivier, 2008; Tredoux, 2011). Although attempts have been made to confirm the antifungal activity of Vv-AMP1 *in planta*, using a transgenic tobacco population overexpressing the peptide, inconclusive data was obtained (De Beer, 2008). To functionally analyze Vv-AMP1 in its native host, the gene was transformed under

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control of a strong constitutive promoter to *Vitis vinifera* cv. Sultana. A transgenic population was generated and characterized to confirm transgene presence, pattern and number of integration events per line, as well as transgene expression (Tredoux, 2011). The defense phenotype of this population was, however, still relatively unexplored and prompted follow-on work (this study).

The purpose of this study was therefore to evaluate the antifungal resistance phenotypes of grapevine overexpressing Vv-AMP1, hereby supplementing the previously established information regarding this defensin peptide (De Beer and Vivier, 2008; De Beer, 2008; Tredoux, 2011). These evaluations included the characterization of the defense response of grapevine overexpressing Vv-AMP1 when challenged by grapevine-specific necrotrophic and biotrophic fungal pathogens in *in planta* analyses. The putative defensin-like (DEFL) genes in grapevine were further studied in an *in silico* analysis to collect information regarding the expression patterns of these plant defensins.

Infection assays confirm Vv-AMP1 defensins to protect grapevine against powdery mildew infection when overexpressed

As outlined in Chapter 1, a fully genetically characterized *in vitro* population of *V. vinifera* (cv. Sultana) constitutively overexpressing Vv-AMP1 in all plant tissues was obtained as the result of extensive prior research focused on this grapevine defensin (De Beer, 2008; De Beer and Vivier, 2008; Tredoux, 2011). This population consisted of eight uniquely transformed transgenic lines and the untransformed *V. vinifera* (cv. Sultana) control that were systematically hardened off and monitored to identify any possible morphological abnormalities that developed as a result of Vv-AMP1 overexpression. Moderate variation of leaf morphology and internode length between lines as well as between individuals from the same line could be observed, however, no substantial differences between transgenic Vv-AMP1 lines and untransformed control lines could be established according to the characterization methods implemented in this study. These findings are of particular interest since previous studies that overexpressed defensin genes produced plants with reduced reproductive viability and retarded growth phenotypes as demonstrated by Stotz et al. (2009). However, our investigation did not include mature plants for the investigation of their reproductive development and yield, since grapevine have an

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extended youth phase and do not readily form reproductive structures under normal green house conditions. Within the scope of our study, we focused on the characterization of a juvenile population and these juvenile populations are well known for morphological diversity. Furthermore, phenotypic variation is known to be significantly higher in plants from tissue culture generated populations than of those grown under field conditions (Vuylsteke and Ortiz, 1996; Kuksova et al., 1997). Therefore, the establishment of a mature transgenic grapevine population overexpressing Vv-AMP1 under field conditions would generate more accurate associations between the morphological plant characteristics and the effects of Vv-AMP1 overexpression. This goal would require the establishment of a transgenic field trial that is subject to strict regulatory processes and approvals and falls outside the scope of the current study.

The transgenic grapevine population was used in *in planta* antifungal assays for determining whether the overexpression of Vv-AMP1 could induce an elevated resistance against fungal pathogens. Due to the promising results obtained from the *in vitro* antifungal assays and a preliminary detached leaf assay when challenged by *B. cinerea*, we included a whole-plant infection assay with this necrotrophic pathogen according to a well established pathosystem. For the purpose of infection assays with a biotrophic fungus, a method for the cultivation and infection with the powdery mildew causing fungus, *Erysiphe necator* was optimized to generate a reproducible pathosystem for this fungus on grapevine. Detached leaf infection assays with various leaf ages and methods of inoculation were therefore performed according to the newly optimized method for powdery mildew infections.

The antifungal assay with the necrotrophic fungus revealed that the transgenic grapevine lines overexpressing Vv-AMP1 had no elevated resistance against *B. cinerea* in comparison to their wild type counterpart. Contrastingly, the transgenic Vv-AMP1 lines displayed elevated resistance to the biotrophic *E. necator* fungus associated with programmed cell death (PCD) in leaves at every ontogenic stage regardless of the method of inoculation. This level and mechanism of resistance to *E. necato*r can be compared to that of the highly resistant grapevine species, *Muscadinia rotundifolia.* When this grapevine species is crossed with *Vitis* species in an attempt to harness this high level of resistance in *V. vinifera*, the offspring

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produced has low viability and therefore, this resistant phenotype has been inaccessible through traditional breeding programmes. One source of this high resistance level against the powdery mildew fungus have been identified as a single dominant locus designated *Run1* (Resistance to *U. necator* 1) (Pauquet et al., 2001; Dry et al., 2009) that is involved in a PCD-associated defense response as revealed by functional analyses (Dry et al., 2009), similar to our findings regarding the antifungal activity of Vv-AMP1. Run1 appeared to confer resistance against all *E. necator* isolates throughout Europe and Australia through PCD associated resistance, hereby warranting further investigation of the specific mechanism of Vv-AMP1 resistance to *E. necator*.

However, as previously mentioned, introducing transgenic plant populations overexpressing peptides to biotic and abiotic stresses involved in field grown conditions have in some cases exposed those populations to display negative effects on plant growth and reproductive viability (Elfstrand et al., 2001; Anderson et al., 2009; Stotz et al., 2009). Whether or not the grapevine population will maintain the observed protective effects under field conditions, without an impact on growth, flower and fruit formation remains to be established in context of the cultivation conditions of commercial grapevine.

In silico analysis provides insight into the possible biological functions of grapevine DEFL genes, as well as the underlying mechanisms involved

During our investigation of the previously identified DEFL genes in grapevine, our results revealed the phylogenetic relationships between 16 DEFL grapevine genes based on their deduced amino acid sequences. According to our analyses, three broadly categorized DEFL groups could be identified that grouped genes with sequence similarity to plant defensins and plant snakins, respectively. The third group is characterized by an unknown DEFL gene that requires further functional evaluation.

In the *in silico* analysis of these grapevine DEFL genes, their co-expression patterns were evaluated in an attempt to determine the possible alternative functions and biological associations of these genes. These *in silico* analyses are of great value in the pursuit of gene expression information in plant species that show greater

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complexity and lower predictability than model organisms such as *Arabidopsis*. Grapevine is further known to be difficult to transform and has an extremely low transformation efficiency (Reustle and Buchholtz, 2009). Therefore, determining putative functions of DEFL genes *in silico* before attempting the time-consuming and expensive procedure of grapevine transformation can allow for more efficient and economical management of grapevine improvement strategies.

The results of our analyses revealed putative co-expression of some of these genes with each other as well as with other genes in the grapevine genome. Some DEFL genes proved to be highly regulated by tissue-specificity whereas others displayed cultivar specific expression. These data provides further evidence for the developmental role that these DEFL peptides play in the reproduction and ripening of grape berries and identifies the DEFL genes that are expressed in response to abiotic stresses. The role that these grapevine DEFL genes play in the innate immunity of plants were further accentuated and these data reveal the possible involvement of these genes against insect herbivory, all aspects worthy of further studies.

Grapevine genes found to be putatively co-expressed with two of the grapevine defensins, Vv-AMP1 and VvAMP3, revealed several putative mechanisms involved in the functioning of these defensins. These functions involved enzyme inhibition, nitrogen assimilation, ion binding and membrane associated activities and the regulation of plant growth and development. Due to the complexity of these co-expression gene networks it can be proposed that the successful functioning of the innate immunity of plants do not only rely on expression of DEFL genes themselves but the gene products that facilitate successful functioning of these associated non-defense-related genes as well.

The limitation placed on our investigation by the low level of grapevine genome coverage by the VitisAffy Gene Chip may have lead to an under-representation of grapevine DEFL genes as candidates for resistance to biotic and abiotic stresses in the datasets we used. Nimblegen provides an alternative Gene Chip with a higher grapevine genome coverage, however, as of yet limited microarray studies performed with this chip is currently publically available. Most recently, a grapevine expression atlas became available that provides 91% coverage of all predicted

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grapevine genes (Fasoli et al., 2012). This publication presents evidence for global transcriptomic reprogramming during maturation, unique to woody perennial plants such as grapevine and could provided more insights into the co-expression of DEFL genes and their possible functions in grapevine, an aspect that could be pursued in further studies.

Expression analysis using publically available data relies on the assumption that all microarray experiments are performed under identical conditions and that the applied treatments would be the only drivers of the regulation of gene expression. In reality, many unknown variables influence these analyses and should therefore be considered when making inferences regarding changes in gene expression. The *in silico* mining of available data is particularly useful to establish trends and hypothesis, but always need to be followed with confirmatory studies to establish the validity of the predictions. It does however provide powerful conceptual capacity, as demonstrated in the current study. Furthermore, a future aspect to explore is the mapping of the DEFL genes to the grapevine genome to evaluate the representation of these genes in the grapevine genome. This could provide insights into the prevalence of these genes, as well as possible co-positioning with known markers, or quantitative trait loci.

Conclusions and future perspectives

In conclusion, the morphological characterization of a genetically characterized transgenic *V. vinifera* (cv. Sultana) population overexpressing the grapevine defensin, Vv-AMP1, revealed no substantial growth abnormalities compared to the untransformed control lines in a juvenile population. After optimization of an effective infection method, this transgenic Vv-AMP1 population displayed an elevated resistance to infection with the biotrophic powdery mildew fungus, *E. necator* in detached leaf assays, but showed no difference to the untransformed control lines when challenged with the necrotrophic fungus, *B. cinerea* during whole-plant infection assays. These results reveal the possible association of Vv-AMP1 with a PCD-associated response to fungal pathogen attack.

In silico analyses further revealed multiple possible biological functions nested within the putative co-expression networks of 16 identified grapevine defense-related

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genes. These analyses implicated the grapevine defensins, Vv-AMP1 and Vv-AMP3, in a wide range of biological functions that may prove these peptides to be indispensible candidates for genetic engineering of transgenic grapevine with not only enhanced resistance to pathogens but beneficial attributes in the plant's response to other biotic and abiotic stresses as well. Similarly, the co-expression patterns highlighted DEFL70 to be a promising candidate to be targeted for functional analysis. When considering the results obtained from both the *in planta* and *in silico* analyses included in this study, future functional characterization studies of Vv-AMP1 should be performed in the context of the possible involvement of this peptide in grapevine responses to insect and viral pathogens as well.

The promising results obtained through this study reveal the potential of Vv-AMP1, and the grapevine DEFLs in general, as candidates in genetic engineering strategies. Future studies will be required to elucidate whether Vv-AMP1 can confer resistance to a wider range of grapevine pathogens *in planta.* Further explorations should also focus on the molecular mechanism of grapevine resistance to necrotrophic fungi and how the combinatorial overexpression of DEFL genes could enhance resistance to all the major grapevine fungal pathogens simultaneously. Hereby, subsequent research could contribute to the enhanced resistance of grapevine as a commercially viable transgenic crop, or contribute to the identification of functional markers in marker-assisted breeding programmes of grapevine.

Despite the overwhelming evidence of the economic importance of exploring genetic engineering strategies in the context of crop protection, the generation of genetically modified organisms (GMOs) remains a highly controversial topic. However, the use of plant defensin peptides in the engineering of disease resistant crops has proven to be highly successful in field trials in some cases (Gao et al., 2000; Portieles et al., 2010). The successful implementation of plant defensins as a means of resistance to fungal pathogens also relies on an in-depth understanding of plant-pathogen interactions to develop reasonable strategies for plant improvement. The current study contributed to all these aspects and provides further evidence that plant defensins provide exciting opportunities understanding plant stress responses.

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