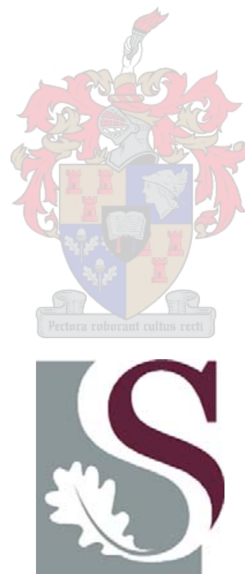
Isolation and Characterization of Antifungal Peptides from Plants

By

Abré de Beer



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Promoter:
Prof. Melané A. Vivier

DECLARATION

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

Abré de Beer **Date**

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SUMMARY

Over the last decade research has shown the importance of small antimicrobial peptides in the innate immunity of plants. These peptides do not only play a critical role in the multilayered defense systems of plants, but have proven valuable in the engineering of disease resistant food crops towards the ultimate aim of reducing the dependency on chemical fungicides. As the lists of isolated and characterized peptides grew, it became clear that other biological activities, in addition to the antimicrobial capacity, could be linked to some of these peptides; these alternative activities could have important applications in the field of medicine. This has made the defensin encoding genes prime targets for the agricultural and medical biotechnology sectors.

To this end we set out to evaluate South African flora for the presence of plant defensin sequences and to isolate plant defensin genes that might be useful in biotechnology applications. Moreover, by isolating and characterizing these novel peptides, also in an *in planta* environment and in interaction with fungal pathogens, important knowledge will be gained of the biological role and importance of the peptides in the plant body.

The plant host targets were South Africa *Brassicaceae* species including indigenous species, as well as *Vitis vinifera*, as the most important fruit crop in the world and since no defensins have been isolated from this economically important crop plant. The *Brassicaceae* family has been shown to be abundant in defensin peptides and several of the best characterized peptides with potent activity have been isolated from this family. Based on initial activity screens conducted on selected South African *Brassicaceae* spp. we concluded that these spp. contain promising antifungal peptide activities, warranting further efforts to isolate the genes and encoding peptides and to characterize them further. The preliminary activity screens used a peptide-enrichment isolation strategy that favored the isolation of basic, heat-stable peptides; these properties are characteristic features of plant antimicrobial peptides. These peptide fractions showed strong antifungal activities against the test organisms. A PCR-amplification strategy was subsequently designed and implemented, leading to the isolation of 14 novel defensin peptide encoding genes from four South African *Brassicaceae* spp., including the indigenous South African species *Heliophila coronopifolia*.

Amino acid sequence analysis of these peptides revealed that they are diverse in amino acid composition and share only 42% homology at amino acid level. This divergence in amino acid composition is important for the identification of new biological activities within closely related plant defensins. Single amino acid changes have been contributed with the divergent biological activities observed in closely related plant defensin peptides. Phylogenetic analysis conducted on the deduced amino acid sequences revealed that all the new defensins share a close relationship to other *Brassicaceae* members of the plant defensin superfamily and was furthest removed from the defensins isolated from the families *Solanaceae* and *Poaceae*. Classification

analysis of these peptides showed that they belong to subgroup A3 of the defensin superfamily.

A putative defensin sequence was also isolated from *V. vinifera* cultivar, Pinotage, and termed *Vv-AMP1*. Genetic characterization showed that only a single gene copy of this peptide is present within the *V. vinifera* genome, situated on chromosome 1. Genetic characterization of this peptide encoding gene within the *Vitis* genus showed that this gene has stayed conserved throughout the divergent evolution of the *Vitis* genus. Expression studies of *Vv-AMP1* revealed that this gene is expressed in a tissue specific and developmentally regulated manner, being only expressed in grape berries and only at the onset of vèraison. Induction of Vv-AMP1 in grapevine leaf material could never be achieved through the external application of hormones, osmotic stress, wounding, or pathogen infection by *Botrytis cinerea*.

Deduced amino acid analysis showed that *Vv-AMP1* encoded for a 77 amino acid peptide consisting of a 30 amino acid signal peptide and a 47 amino acid mature peptide, with putative antifungal activity. The Vv-AMP1 peptide grouped with the subclass B type defensins, which have been documented to have both antifungal and antibacterial activities. The Vv-AMP1 signal peptide directed the green fluorescent protein (GFP) reporter gene to the apoplastic regions in cells with high levels of accumulation in the vascular tissue and the guard cells of the stomata.

Recombinant Vv-AMP1 peptide was successfully purified from a bacterial host and shown to have a size of 5.495 kDa. Recombinant Vv-AMP1 showed strong antifungal activity at low concentrations against a broad spectrum of fungal pathogens, which included *Verticillium dahliae* (IC_{50} of 1.8 $\mu g\ mL^{-1}$) and the necrotrophic pathogen *Botrytis cinerea* (IC_{50} of 12-13 $\mu g\ mL^{-1}$). Antifungal activity of Vv-AMP1 did not induce morphological changes in fungal hyphae, but its activity was associated with induced membrane permeabilization in treated hyphae.

Vv-AMP1 was successfully introduced into *Nicotiana tabacum* as confirmed by Southern blot analysis and 20 individual lines were generated. Genetic characterization confirmed the integration and expression of the gene in the heterologous tobacco environment. The peptide was under control of its native signal sequence which has been shown to direct its product to the apoplastic regions of cells. The transgenic lines were analyzed to determine the presence and activity of the grapevine defensin peptide. Western blot analyses of partially purified plant extracts detected a signal of the expected size in both the untransformed control and the transgenic lines. Comprehensive analysis of EST databases identified three highly homologous sequences from tobacco that probably caused the background signal in the control. These crude protein extracts were able to inhibit the growth of *V. dahliae in vitro* when tested in a microtiter plate assay, but the inhibition could not be conclusively linked to the presence of the transgenic peptide, since non-expressing transgenic lines, included as controls, also showed inhibition. Similar results were obtained with infection studies, clearly showing that despite successful integration and expression of the transgene, the peptides was either not functional in the heterologous environment, or perhaps unstable

under the particular regulatory conditions. This peptide belongs to a subclass of peptides known for associated activities that might activate tight control by plant hosts if threshold levels are reached. These aspects need further investigation, specifically since it is in stark contrast to previous results obtained with defensins from a different subclass.

This study has also yielded significant other related resources that would be instrumental for further possible biotechnology exploitation of some of the novel peptides, but also to provide genetic constructs and plant material that would be invaluable to address fundamentally important questions such as the regulation and mode of action of defensin peptides, specifically in interaction with pathogen hosts. The novel peptides have been transformed to various hosts, including grapevine and these transgenic populations are available to facilitate the next rounds of research into this extremely promising group of antifungal peptides.

OPSOMMING

In die laaste dekade het navorsing die belangrike rol van klein antimikrobiese peptiede in plantweerstandsmeganismes beklemtoon. Hierdie peptiede speel nie alleenlik 'n belangrike rol in die komplekse lae van plantweerstandstelsels nie, maar het ook hulle ekonomiese potensiaal getoon in die manipulering van siekteweerstandbiedendheid in voedselgewasse met die oorkoepelende doel om landbougewasse minder afhanklik te van chemiese spuitstowwe te maak. Soos wat die hoeveelheid geïsoleerde en gekarakteriseerde peptiede toeneem, het dit duidelik geword dat ander biologiese aktiwiteite, bykomend tot die antimikrobiese kapasiteit, met sommige van dié peptiede verbind kan word; hierdie alternatiewe aktiwiteite het belangrike toepassing in veral die mediese veld. Dit het die defensin-koderende gene kernteikens vir die landbou- en mediese biotegnologiesektore gemaak.

In die studie is daar begin om die Suid-Afrikaanse blommeryk te evalueer vir die teenwoordigheid van plantdefensingene en om dié gene te isoleer wat van ekonomiese belang vir die biotegnologiebedryf kan wees. Deur die *in vitro*- én *in planta* karakterisering van die unieke plantdefensinpeptiede word daar gemik daarna om belangrike inligting in te win oor die biologiese rol van die peptiede binne die plantligggaam.

Die plantgashere wat geteiken, is sluit in die Suid-Afrikaanse *Brassicaceae*-spesies, insluitende inheemse spesies, asook *Vitis vinifera*, wat as die belangrikste vrugtegewas ter wêreld beskou word . Die *Brassicaceae*-familie is welbekend daarvoor dat dit 'n ryk bron van plantdefensinpeptiede is en verskeie van die bes gekarakteriseerde antifungiese defensinpeptiede is van dié familie afkomstig. Aanvanklike aktiwiteitstoetse het getoon dat die Suid-Afrikaanse *Brassicaceae*-spesies belowende antifungiese aktiwiteit toon, wat die verdere isolering en karakterisering van dié gene en hul peptiedprodukte regverdig. Die aanvanklike aktiwiteitstoetse het 'n selektiewe peptiedverrykingstrategie gevolg wat die isolering van basiese, hittestabiele peptiede bevoordeel het; hierdie eienskappe is baie kenmerkend van plant-antimikrobiese peptiede. Die peptiedfraksies wat met hierdie metode geïsoleer is, het sterk antifungiese aktiwiteit teen die toetsorganismes getoon. Die resultate het gelei tot die ontwikkeling en toepassing van 'n polimerasekettingreaksie-strategie, wat daartoe gelei het dat 14 nuwe defensingene van vier Suid-Afrikaanse *Brassicaceae*-genera, insluitend die inheemse spesie *Heliophila coronopifolia*, geïsoleer kon word.

Afgeleide aminosuurvolgorde-analises van die nuwe defensinpeptiede het gewys dat hulle slegs 42% homologie het. Hierdie diversiteit in aminosuurvolgorde is belangrik vir die identifisering van nuwe biologiese aktiwitiete binne die groep van verwante peptiede. Navorsing het verder getoon dat enkel-aminosuurverskille bydra tot die diverse spektrum van biologiese aktiwiteite binne 'n groep van verwante defensinpeptiede. Filogenetiese analise van die aminosuurvolgordes het getoon dat al die nuwe defensinpeptiede 'n sterk verwantskap met plantdefensinpeptiede, wat van

ander *Brassicaceae*-spesies geïsoleer is, toon. Daarteenoor het dit die kleinste verwantskap getoon met plantdefensinpeptiede wat van die *Solanaceae*- en *Poaceae*-families geïsoleer is. Klassifikasiestudies het bewys dat die nuwe peptiede saam met subgroep A3 van die plantdefensin-superfamilie groepeer.

'n Moontlike plantdefensingeen, genaamd *Vv-AMP1*, is ook van die *V vinifera*-kultivar, Pinotage, geïsoleer. Genetiese karakterisering het aangedui dat slegs 'n enkele kopie van die geen in die *V. vinifera*-genoom teenwoordig en op chromosoom 1 geleë is. Genetiese karakterisering van *Vv-AMP1* binne die *Vitus*-genus het gewys dat die geen binne die genus evolusionêr gekonserveerd is. Uitdrukkingstudies van *Vv-AMP1* het verder bewys dat die geen uitgedruk word op 'n weefselspesifieke, ontwikkelingsgekoppelde wyse, naamlik slegs in druiwekorrels en slegs tydens rypwording. *Vv-AMP1*-uitdrukking kon nooit geïnduseer word in wingerdblare deur die uitwendige toediening van hormone, osmotiese stres, wonding of patogeeninfeksie deur *Botrtys cinerea* nie.

Ontleding van die afgeleide aminosuurvolgorde het gewys dat *Vv-AMP1* kodeer vir 'n 77-aminosuurpeptied, wat uit 'n 30-aminosuurseinpeptied en 'n 47-aminosuur-aktiewe peptied met voorspelde antifungiese aktiwiteit bestaan. Die Vv-AMP1-peptied is gegroepeer met subgroep B van die plantdefensin-superfamilie, 'n subgroep wat vir beide antifungiese en antibakteriese aktiwiteit gedokumenteer is. Die Vv-AMP1-seinpeptied het die groen fluoressensie-indikatorproteïen (GFP) na die apoplastiese areas van die plantselle gelei, met hoë vlakke van lokalisering in die vaatbundelweefsel en sluitselle van die huidmondjies.

Die rekombinante Vv-AMP1-peptied is suksesvol geproduseer en uit 'n bakteriese produksieras gesuiwer, en het 'n molekulêre massa van 5.495 kDa gehad. Die gesuiwerde peptide het by lae konsentrasies 'n sterk aktiwiteit getoon teen 'n breë spektrum van fungiese patogene, wat *Verticllium dahliae* (IC_{50} van 1.8 $\mu g\ mL^{-1}$) en die nekrotrofiese patogeen, *B. cinerea* (IC_{50} van 12-13 $\mu g\ mL^{-1}$), ingesluit het. Vv-AMP1-aktiwiteit het geen ooglopende morfologiese veranderinge in die fungi-hifes veroorsaak nie, maar hulle aktiwiteit is verbind met 'n verhoogde membraandeurdringbaarheid in behandelde fungi-hifes.

Suksesesvolle intergrasie van *Vv-AMP1* in die *Nicotiana tabacum*-genoom is deur Southern-kladontledings bevestig en 20 individuele transgeniese lyne is ontwikkel. Genetiese karakterisering van die transgeniese lyne het gewys dat *Vv-AMP1* suksesvol geïntegreer is en ook in die transgeniese tabakomgewing uitgedruk word. Die peptied is uitgedruk onder beheer van sy eie seinpeptied, wat die aktiewe produk na die apoplastiese areas van die plantselle teiken. Die transgeniese tabaklyne is ook ontleed om te bepaal of die wingerdpeptied suksesvol geproduseer word en sy aktiwiteit in die transgeniese omgewing behou. Western-kladanalise van semi-gesuiwerde plantproteïenekstrakte het 'n positiewe sein gelewer in beide die kontroleplante en die transgeniese plantlyne. Bestudering van tabakgeenuitdrukkings-databasisse het drie nukleotiedvolgordes opgelewer wat homologie met *Vv-AMP1* toon en moontlik verantwoordelik kan wees vir die positiewe sein in die ongetransformeerde

kontroleplante. Kru proteïenekstrakte van die transgeniese tabaklyne het *in vitro*-aktiwiteit teen *V. dahliae* getoon. Geen oortuigende ooreenkoms kon egter gevind word tussen *V. dahliae*-inhibisie en die teenwoordigheid van die transgeniese Vv-AMP1-peptied nie, aangesien kontroleplante wat Southern-klad-positief is, maar nie geenuitdrukking toon nie, ook inhibisie van *V. dahliae* veroorsaak het. Soortgelyke resultate is met infeksiestudies verkry. Alle resultate dui daarop dat, al is daar suksesvolle intergrasie en uitdrukking van die geen in tabak verkry, dat die Vv-AMP1 peptied óf onaktief óf onstabiel in die transgeniese tabakomgewing is. Die peptied behoort aan 'n subgroep peptiede met aktiwiteite wat, sodra sekere vlakke van peptied oorskry word, die moontlik streng kontrole op proteïenvlak in die gasheerplant kan uitlok. Sekere aspekte van die studie sal verder bestudeer moet word, aangesien die data teenstrydig is met data wat verkry is met soortgelyke plantdefensinpeptiede wat aan 'n ander subgroep behoort.

Die studie het baie hulpbronne gegenereer wat vir die biotegnologiesektor belangrik kan wees, veral op ekonomiese gebied. Verder is die geenkonstrukte en plantlyne wat ontwikkel is waardevol om fundamentele vrae rondom die regulering en meganisme van aksie van defensinpeptiede, spesifiek plantpatogeeninteraksie, te beantwoord. Die nuwe plantdefensingene is na verskeie gasheerplante, insluitende wingerd, getransformeer waar die transgeniese lyne die volgende rondte van navorsing oor die bestudering oor die belangrike groep van antifungiese peptiede, sal aanvul.

This dissertation is dedicated to
Hierdie proefskrif is opgedra aan

Anika en my ouers

BIOGRAPHICAL SKETCH

Abré de Beer was born in Bellville, South Africa, on 23 February 1976, and later moved to Strand. He matriculated from Strand High in 1994. In 1995, Abré enrolled for a BSc degree in Microbiology and Biochemistry at Stellenbosch University, which he obtained in 1997. In 1998 he received a BScHons degree in Wine Biotechnology and in 2001 an MSc degree in Wine Biotechnology, both through the Institute for Wine Biotechnology at Stellenbosch University. He enrolled for his PhD (Wine Biotechnology) at the same institute in 2002.

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PREFACE

This dissertation is presented as a compilation of 6 chapters including two Addendums. Each chapter is introduced separately and is written according to the style of Plant Physiology. Chapter 4 has been submitted for publication to BMC Plant Biology.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Plant Defensins

Chapter 3 **Research Results**
A PCR-Isolation Strategy Yields 14 New Antifungal Peptide Encoding Genes from South African *Brassicaceae* species

Chapter 4 **Research Results**
A Ripening Induced Gene from *Vitis Vinifera* Shows Sequence Homology to the Superfamily of Plant Defensins

Chapter 5 **Research Results**
In Planta Analysis of Vv-AMP1, a Ripening-Specific Defensin Isolated from *Vitis Vinifera* L. cv. Pinotage

Chapter 6 **General Discussion and Conclusions**

I hereby declare that I was the primary contributor with respect to the experimental data presented on the multi-author manuscripts presented in Chapters 3, 4 and 5. My supervisor, Prof MA Vivier was involved in the conceptual development and continuous critical evaluation of this study.

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Chapter 1

INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Currently, agriculture relies on an arsenal of chemical pesticides to protect economically important food crops against disease and herbivore destruction. The negative impact of these chemicals on human health and the natural environment has led to a public outcry against the use of chemical fungicides and pesticides resulting in a demand for more environmentally friendly and even organically grown food crops (Barr et al., 2004). Legislation, designed around consumer concerns, have forced farmers to use lower concentrations of pesticides. This, together with the emergence of fungicide resistant plant pathogens (Chapeland et al., 1999; Yourman and Jeffers, 1999; Hayashi et al., 2002), leaves the farmer with a limited and sometimes ineffective means of protecting our valuable food resources.

Over the last two decades scientists have been searching for replacements to chemical fungicides that would lessen the impact on human health and the environment. These strategies included the identification of biological control agents (Tronsmo and Ystaats, 1980; McLaughlin et al., 1992; Elad et al., 1993; Elad, 1996; Kohl et al., 1998), breeding for new resistant cultivars and the use of genetic engineering to design more resistant crop species with similar or enhanced nutritional value (Epple et al., 1997; Shah, 1997; Broekaert et al., 1999; Banzet et al., 2002; Choon Koo et al., 2002; Jeandet et al., 2002; Sawada et al., 2004).

Some of these strategies have proven successful in combating certain insect pests. Genetic engineering of various crops with Bt toxin from *Bacillus thuringiensis* has led to enhanced protection against some worm and insects pests (Dempsey et al., 1998). This toxin is very specific due to its narrow spectrum of activity. When it comes to designing new strategies for protecting crops against microbial pathogens scientist have however been less successful.

New strategies for the protection of crops against microbes are centred round the utilization of natural defense systems against these pathogens that are already present in plants or other living organisms. These natural plant defense systems have been refined over millennia in the constant battle for supremacy between pathogen and host. In general the plant defense systems can be divided into the constitutive or preformed and induced defense systems. These defense systems can be further divided into structural, chemical and biochemical defense mechanisms (da Cunha et al., 2006).

The structural defense components, such as the cuticle and plant cell wall, form the first barrier of defense in what is perceived as the passive defense system (Ferreira et al., 2007). The preformed defense system contains both chemical and biochemical compounds that accumulate at basal levels within peripheral plant tissue to form microbial barriers and are most prevalent in nutrient-rich plant organs such as

flowers and seeds (Broekaert et al., 1995; Osbourn, 1996; Stahl and Bishop, 2000; Ferreira et al., 2007).

One of the most important mechanisms of plant defense is the production of proteinaceous compounds with antimicrobial activity in response to pathogen attack (Ahn et al., 2002; Nurnberger et al., 2004; Koga et al., 2006; van Loon et al., 2006; Ferreira et al., 2007). These compounds can range from fungal cell wall degrading enzymes to small antimicrobial peptides. In plant-fungal interactions, most of these proteinaceous compounds have traditionally been classified into two groups, namely pathogenesis related proteins (PR-proteins) and antifungal proteins, with some cross classification occurring for some of the antifungal defense related proteins (van Loon et al., 2006; Ferreira et al., 2007). According to Ferreira et al. (2007), PR proteins are plant proteins that are newly produced upon pathogen attack or related situations, excluding proteins that are present as low, but detectable levels in healthy plant tissues before induction by pathogen attack. According to this classification, 17 families of PR proteins have been identified (Table I).

Table I. Classification of pathogenesis-related (PR) proteins (adopted from Ferreira et al., 2007)

Family	Type member	Biochemical properties	MW (kDa)
PR-1	Tobacco PR-1a	Unknown	15-17
PR-2	Tobacco PR-2a	β-1,3-glucanase	30-41
PR-3	Tobacco P,Q	Chitinase class I, II,IV, VI, VII	35-46
PR-4	Tobacco R	Chitin-binding proteins	13-14
PR-5	Tobacco S	Thaumatin-like	16-26
PR-6	Tomato Inh 1	Proteinase inhibitor	8-22
PR-7	Tomato P_{69}	Endoproteinase	69
PR-8	Cucumber chitinase	Chitinase class III	30-35
PR-9	Tobacco lignin peroxidase	Peroxidase (POC)	50-70
PR-10	Parsley 'PR-1'	Ribonuclease-like	18-19
PR-11	Tobacco class V chitinase	Chitinase class V	40
PR-12	Radish Rs-AFP3	Defensins	5
PR-13	*Arabidopsis* THI-2.1	Thionins	5-7
PR-14	Barley LTP4	Lipid transfer proteins	9
PR-15	Barley OxOa (germin)	Oxalate oxidase	22-25
PR-16	Barley OxOLP	Oxalate oxidase-like protein	100
PR-17	Tobacco PRp27	Unknown	ND

The antifungal proteins have been classified into 13 families and also contain proteins isolated from organisms other than plants (Ferreira et al., 2007). These proteins have been classified according to their antifungal activity and mode of action (Table II).

Table II. Classes of antifungal proteins (adopted from Ferreira et al., 2007)

Class	Source	Characteristics	Mechanism of action
PR-1 proteins	Plants	15-17 kDa	Unknown
β-glucanases	Microorganisms, plants, invertebrates and vertebrates	β-1,3-endoglucanase activity	Hydrolysis of structural 1,3-β-glucan present in fungal cell walls
Chitinases	Viruses, bacteria, fungi, snails, fish, plants, insects, mammals and amphibians	Chitinase activity 26-43 kDa	Cleave cell wall chitin polymers *in situ*
Chitin binding proteins	Bacteria, plants, insects and crustaceans	3.1-20 kDa	Binding to chitin
Thaumatin-like proteins	Plants	~22 kDa	Alter fungal cell membrane permeability Bind 1,3- glucan, exhibit β-1,3-glucanase activity
Defensins/thionins	Fungi, insects, plants and mammals	Low molecular weight, cysteine-rich proteins	Fungal inhibition probably occurs through an ion efflux mechanism
Cyclophilin-like proteins	Bacteria, fungi, plants and animals	Example: mungin	Unknown
Glycine/histidine-rich proteins	Insects	Glycine and histidine comprise up to 80% of the protein	Unknown
Ribosome inactivating proteins (RIPs)	Fungi and plants	RNA *N*-glycosidases that depurinates rRNA	Inactivates fungal ribosomes *in vitro* and presumably *in situ*
Lipid transfer proteins (LTPs)	Bacteria, fungi, plants and mammals	~8.7 kDa	Unknown
Killer proteins (killer toxins)	Yeast	Toxin	Varied mechanisms of action
Protease inhibitors	Microorganisms, plants and animals	Inhibitors of serine and cysteine proteases	Unknown
Other proteins	Plants	Examples: viridin and snakin1	Unknown

The aim of designing disease resistant crops species is to transfer the resistance phenotype of a donor organism to the susceptible host crop species via genetic engineering. Until recently candidate defense genes, which mainly belong to the PR proteins or antifungal proteins, have shown little promise in the engineering of disease resistant crops at field trail level. These failures can be contributed to loss of expression of the transgene or reduced performance of the transgenics with regards to the manipulated trait under field conditions.

Small antifungal peptides (PR protein classes 12-14) have been successfully used to engineer disease resistant potatoes and rice. Potatoes were protected, in field trails, against the wilting disease causing agent *Verticilium dahliae* (Gao et al., 2000). The defense gene used in this strategy encoded for a small antifungal peptide belonging to a group known as plant defensins. This work was followed by the genetic engineering of rust fungus resistant rice in 2002 by means of another plant defensin gene (Kanzaki et al., 2002; Kawata et al., 2003). Plant defensins are represented in both the PR and antifungal protein families and play an important role in the protection of the reproductive structures of plants (Broekaert et al., 1995; Thomma et al., 2002; Lay and Anderson, 2005; Ferreira et al., 2007). More recent results have also linked specific plant defensin genes to resistant phenotypes observed in resistant cultivars of some crop species (de Zélicourt et al., 2007).

Recent bioinformatical analysis of the *Arabidopsis* and rice genome and expressed sequence tag (EST) databases revealed that defensin genes, and related cysteine-rich peptide encoding genes, could comprise up to 3% of the genetic material of the model organisms (Silverstein et al., 2005; Silverstein et al., 2007). It was also shown that these genes are over represented in the reproductive structures of certain plant species (Silverstein et al., 2005; Silverstein et al., 2007). This suggests an important role for these peptides in plant defense and perhaps even general plant physiology.

These results and others have sparked a global interest in the plant defensin peptide family as possible candidates for the engineering of disease resistant crops. As a consequence numerous reports have been published on new plant defensins. Some of these publications merely report on the isolation and cryptic characterisation of the peptides, without providing any further biological data or functional analysis of the peptides. Several peptides have also been patented (Broekaert et al., 1999). The genetic screens of genomes and expression databases, as well as the isolation reports, serve the purpose of confirming the importance of these peptides in plant defense. The evolutionary control over peptide gene inheritance, as well as the *in vivo* mode of action of the various peptide classes arguably poses the most interesting scientific questions that still remain to be answered. To study evolutionary control imparted on the antifungal peptides, it is also useful to isolate defensin genes from plant species with a sequenced genome in an effort to add genomic screening data to isolation reports. Similarly, the *in planta* and *in vivo* activities, mode of actions

and regulation of the peptide encoding genes will provide the much needed information to intelligently evaluate the prospects of these peptides in manipulated disease resistance strategies.

1.2 PROJECT AIMS

The project was initiated to isolate and characterise novel antifungal peptide genes from plants. These genes had to be novel and not protected by patents. Moreover, a further objective was to not only isolate the encoding new peptides, but to characterise them further regarding their antimicrobial activities, inhibition spectra, mode of action and regulatory control in their host(s). The ultimate outcome of the project was seen as novel genetic material (isolated peptide encoding genes) and tested genetic resources (transgenic plant lines) that could be used to understand more of the activities and mode of action of the antifungal peptides. The associated research to develop these "products" would ultimately produce new knowledge on these peptides and the function of peptides in general in plant defense.

The search for new peptide encoding genes with antifungal activity was directed to indigenous *Brassicaceae* spp. to exploit the unique and rich flora present in South Africa. The *Brassicaceae* family has been shown to contain very potent antifungal peptides and several of the typical antimicrobial peptides have been isolated from various species in this family. *Vitis vinifera*, as the most important fruit crop, was also targeted since its genome has recently been sequenced and no reports of defensin peptide activity exist.

The specific aims of this project were as follows:

1. To design and use a rapid screening method for the identification and isolation of plant defensin genes present in South African *Brassicaceae* spp.
    a. Evaluation of the antifungal activities of the peptide fractions in crude protein extracts from the local *Brassicaceae* species;
    b. Evaluation of the level of gene homology that exist within the known plant defensins isolated from *Brassicaceae* species and the designing of a PCR amplification strategy;
    c. Identification and isolation of new defensin genes from *Brassicaceae* species present in South Africa; and
    d. Characterization of the newly isolated defensin genes by using an *in silica* approach.

2. To isolate and fully characterize a plant defensin encoding gene(s) and peptide from *Vitis vinifera*.
    a. Isolation of putative defensin encoding gene(s) from *V. vinifera* by using a PCR approach and/or screen of the genomic sequence;
    b. Characterization of the genomic organization and expression profile of the isolated gene(s) and the targeting of the peptides; and
    c. Purification, biochemical characterization and *in vitro* analyses of the antifungal activities of the putative peptides and the production of antibodies.
3. To functionally evaluate the putative *Vitis vinifera* defensin(s) for *in planta* antifungal activities against necrotrophic and biotrophic plant pathogens.
    a. Overexpression of the isolated *Vv-AMP1* gene under its native secretion signal in tobacco and molecular analysis of the transgenic population; and
    b. Evaluation of the possible resistance phenotypes of the transgenic tobacco lines against *Botrytis cinerea* and *V. dahliae* in infection assays.

Aim 1 is addressed in chapter 3 with aim 2 and 3 addressed in chapters 4 and 5, respectively.

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Chapter 2

LITERATURE REVIEW

Plant Defensins

LITERATURE REVIEW

2.1 INTRODUCTION

Plants are constantly challenged by pathogens, but the onset of plant disease is the exception rather than the rule. The reason for this being that the constant arms race between pest and plant host has led to the development of an efficient plant defense system. The interaction of a pathogen with a plant can lead to one of two responses. In a compatible interaction the pathogen evades the plants defense system and establishes disease, while in an incompatible interaction the plant recognizes the pathogen and elucidates a defense response through various signal cascades.

The general plant defense system can be broadly divided into a preformed defense system and an induced defense system. The preformed defense mechanism is a major component of the non-host defense system and may play an important role in determining the host range of certain plant pathogenic fungi (Morrissey and Osbourn, 1999; de Zélicourt et al., 2007).

The preformed defense systems are the first line of defense against pathogen infection and consist of preformed biochemical and proteinaceous molecules with antimicrobial activity (Broekaert et al., 1995; García-Olmedo et al., 1998; Morrissey and Osbourn, 1999; Heath, 2000). These antimicrobial molecules form protective barriers, which prevent the onset or spread of pathogen infection. These antimicrobial barriers are usually present in the outer cell layers of plant organs, which represent the first plant cells to elicit a defense response upon pathogen infection (Osbourn, 1996). The most noticeable preformed defense barriers are present in roots and seeds, where they protect the plant material from the pathogen-rich soil environment (Osbourn et al., 1994; Terras et al., 1995; Osbourn, 1996; Papadopoulou et al., 1999).

The induced defense system relies upon recognition of the plant pathogen and the elucidation of defense mechanisms activated by a signalling cascade. Early events in the induced defense usually involve hypersensitive cell death and activation of the structural defense (Devlin and Gustine, 1992; Greenberg and Yao, 2004; Kiraly et al., 2007). The structural defense involves the alteration of the plant cell wall and its structural sub components upon pathogen recognition, either to prevent establishment of disease or to prevent further spread of the disease. The structural defense usually involves the strengthening and thickening of the plant cell wall through the deposition of lignin (Zabala et al., 2006).

A major component of preformed and induced defense involves the *de novo* synthesis of proteinaceous compounds with antifungal activity. Some of the best characterized proteinaceous compounds that plays a role in plant defense are the pathogenesis related (PR) proteins. PR proteins have been classified into 17 families based on their amino acid sequence and biological activity (Table I) (Ferreira et al., 2007) and include cell wall degrading enzymes such as chitinase and

glucanase (Stintzi et al., 1993; Jongedijk et al., 1995) as well as non enzymatic proteins with antimicrobial properties.

Table I. Classification of pathogenesis-related (PR) proteins.

Family	Type member	Biochemical properties	MW (kDa)
PR-1	Tobacco PR-1a	Unknown	15-17
PR-2	Tobacco PR-2a	β-1,3-glucanase	30-41
PR-3	Tobacco P,Q	Chitinase class I, II,IV, VI, VII	35-46
PR-4	Tobacco R	Chitin-binding proteins	13-14
PR-5	Tobacco S	Thaumatin-like	16-26
PR-6	Tomato Inh 1	Proteinase inhibitor	8-22
PR-7	Tomato P_{69}	Endoproteinase	69
PR-8	Cucumber chitinase	Chitinase class III	30-35
PR-9	Tobacco lignin peroxidase	Peroxidase (POC)	50-70
PR-10	Parsley 'PR-1'	Ribonuclease-like	18-19
PR-11	Tobacco class V chitinase	Chitinase class V	40
PR-12	Radish Rs-AFP3	Defensins	5
PR-13	Arabidopsis THI-2.1	Thionins	5-7
PR-14	Barley LTP4	Lipid transfer proteins	9
PR-15	Barley OxOa (germin)	Oxalate oxidase	22-25
PR-16	Barley OxOLP	Oxalate oxidase-like protein	100
PR-17	Tobacco PRp27	Unknown	ND

A large component of the proteinaceous defense consists of small antimicrobial peptides and over the last 15 years the importance of these peptides in plant defense has been highlighted (Florack and Stiekema, 1994; Broekaert et al., 1997; Garcia-Olmedo et al., 1998; Thomma et al., 2002; Castro and Fontes, 2005; Lay and Anderson, 2005; Pelegrini and Franco, 2005; Stec, 2006). The best characterized of these small peptides is a family known as plant defensins.

2.2 BIOLOGICAL ROLE OF PLANT DEFENSINS IN DEFENSE

Plant defensins are a major component of the chemical defense system of plants. The *de novo* synthesis of these peptides can be constitutive, resulting in the formation of protective barriers around specific plant organs, or between different tissue types within a plant organ (Figure 1) (Broekaert et al., 1995).

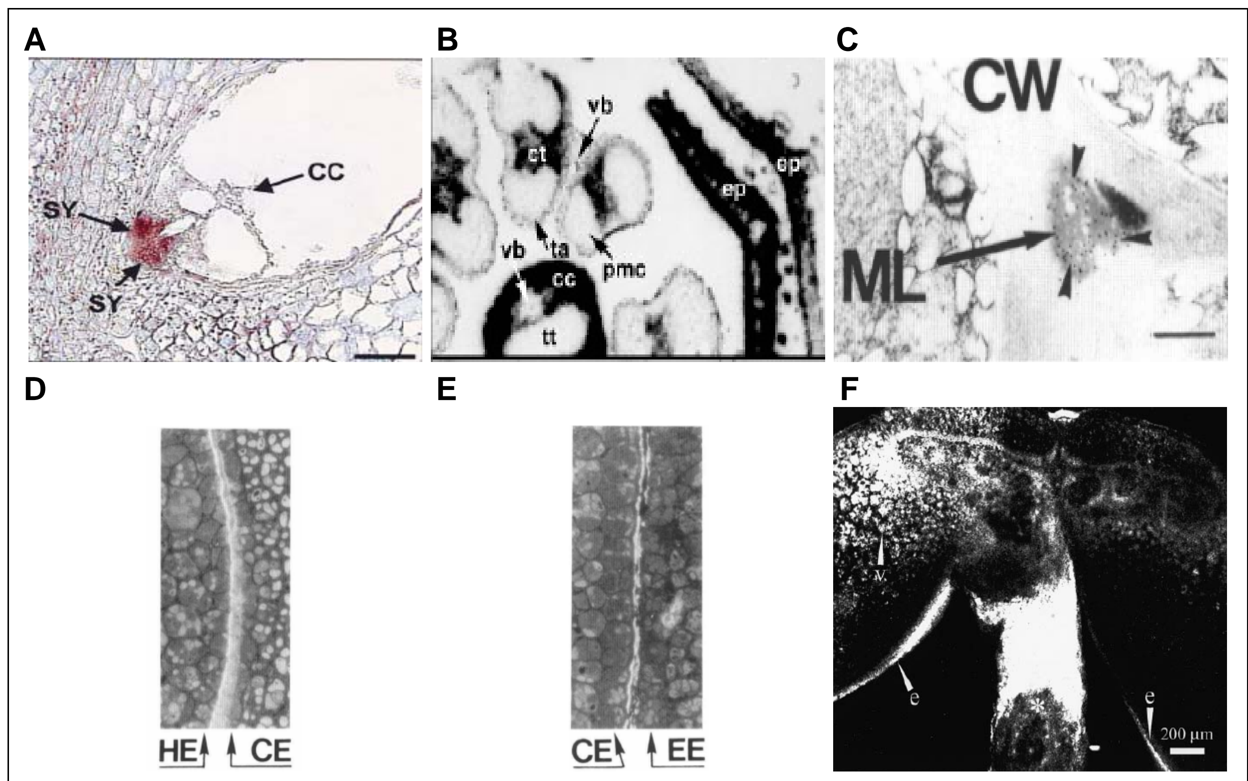

Figure 1. Localization of different plant defensins as determined by *in situ* hybridization studies. (A) Expression of *ZmES*, indicated in purple, in the egg apparatus of maize; **CC**, central cell; **SY**, synergid (Cordts et al., 2001). (B) *In situ* location (black) of *NaD1* mRNA in flower buds; epidermal cells (**ep**), petal (**pe**), sepal (**se**), cortical cells (**cc**), style (**st**), connective tissue (**ct**), anther (**a**), pollen mother cells (**pmc**), tapetum (**ta**), vascular bundle (**vb**), and transmitting tissue (**tt**) (Lay et al., 2003a). (C) An immunogold localization of radish seed defensins (Rs-AFPs); cell wall (**CW**), middle lamella (**ML**) (Terras et al., 1995). (D-E) Immunofluorescent localization of Rs-AFPs within radish seeds; cotyledon epidermis (**CE**), endosperm epidermis (**EE**), hypocotyl epidermis (**HE**) (Terras et al., 1995). (F) Immunofluorescent localization of the pea defensin PsD2 in pea pod tissue; epidermis (**e**), vascular tissue (**v**) (Almeida et al., 2000).

The first plant defensins identified and isolated were from plant seeds (Colilla et al., 1990; Mendez et al., 1990; Terras et al., 1992; Terras et al., 1993). Extensive analysis of the plant defensins isolated from radish seeds proposed a role for these peptides in the protection of germinating seeds and the young seedlings (Terras et al., 1995). The radish defensin content of a radish seed is only 0.5% (w/w) of the total seed proteins, but it contributes up to 30% (w/w) of proteins released during seed germination. This amounted to 1 μg of plant defensin peptide released by each individual seed. This induction of radish defensin is only observed when maceration of the seed coat occurs and could be artificially induced by mechanical damage of the radish seed coat with a scalpel. These results suggests that production of radish defensins are induced and released when the seed coat is ruptured by the radical of the germinating seed embryo, forming a protective halo around the germling to protect it from soil borne fungi (Figure 2A) (Terras et al., 1995).

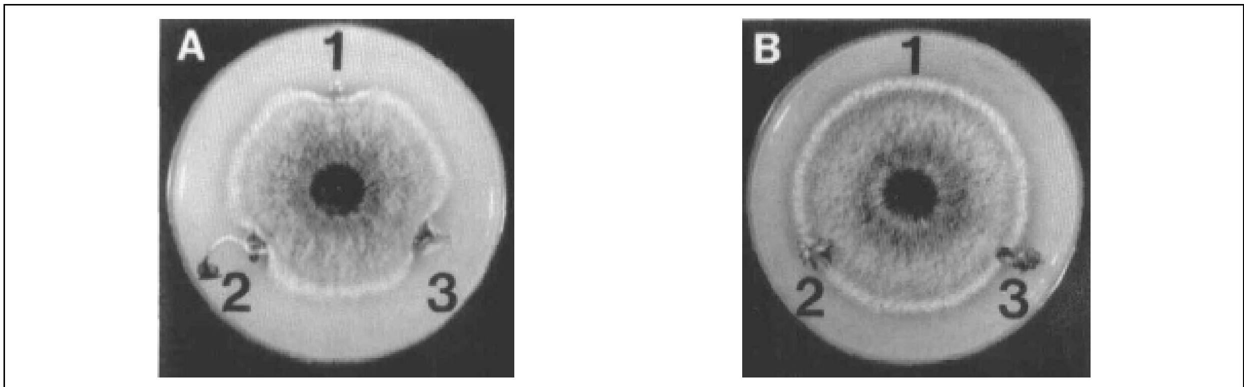

Figure 2. Release of antifungal compounds by germinating radish seeds. One microgram of purified Rs-AFP1 was applied at the positions indicated by the number 1. Radish seeds at the positions 2 had an intact seed coat, whereas seeds at positions 3 had an incised seed coat (along half of the seed periphery). The fungus *P. tritici-repentis* was used in this assay. (A) Assay plates containing five cereal agar. (B) Assay plates containing five cereal agar supplemented with 50 ng mL^{-1} Pronase E (Terras et al., 1995).

Plant defensins are not however restricted to the preformed defense system and can also be induced by external environmental stimuli (Table II), which include pathogen attack, environmental stress, herbivore damage and plant hormones (Figure 3).

Table II. Identified environmental stimuli able to induce plant defensin genes.

Simuli	**Defensin**	**Origin**	**Tissue**	**Reference**
Pathogen	DRR230a-c Rs-AFP3-4	*Pisum sativum* *Raphanus sativum*	Leaves Leaves	(Lai et al., 2002) (Terras et al., 1992)
Wounding	PgD1	*Picea glauca*,	Cell Culture	(Pervieux et al., 2004)
Zn^{+}	AhPDF1.1	*Arabidopsis halleri*	Shoot	(Mirouze et al., 2006)
Jasmonic acid	PDF1.2	*Arabidopsis thaliana*	Leaves	(Thomma et al., 1998)
Salicylic acid	CADEF1	*Capsicum annum*	Leaves	(Mee Do et al., 2004)
Abscisic acid	tgas118	*Lycopersicon esculentum*	Flower	(van den Heuvel et al., 2001)
Drought	CADEF1	Capsicum annum	Fruit	(Mee Do et al., 2004)
Cold	Tad1	*Triticum aestivum*	Crown	(Koike et al., 2002)

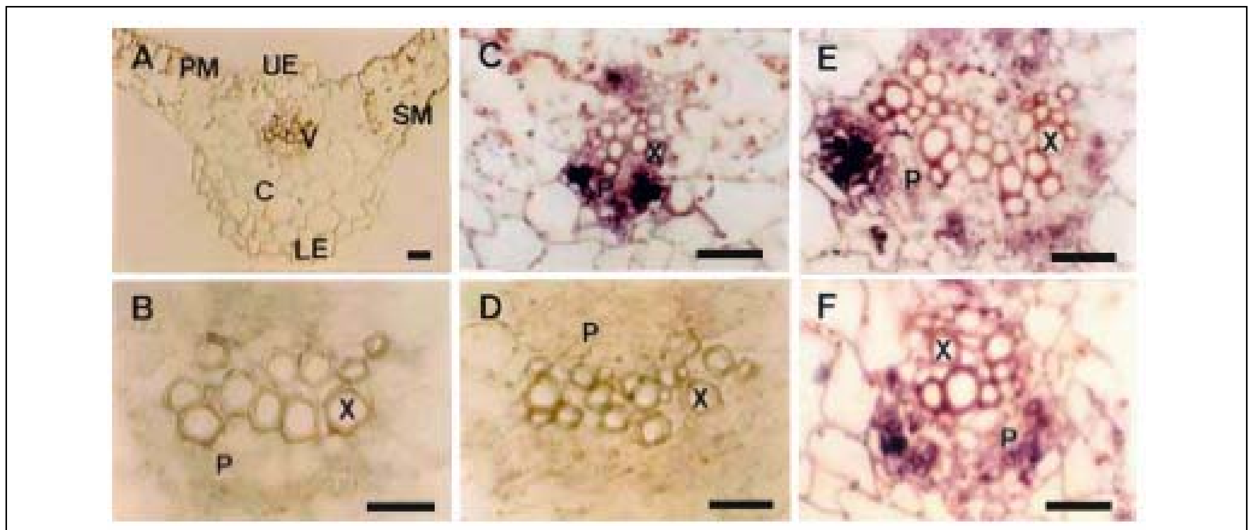

Figure 3. *In situ* localization of *CADEF1* mRNAs in pepper leaf tissues treated with various abiotic elicitors. (A) Untreated leaf tissues, (B) vascular bundles of untreated leaf tissues, (C) 5 mM salicylic acid, (D) 5 $\mu l\ l^{-1}$ ethylene, (E) 100 μM methyl jasmonate and (F) 100 μM abscisic acid. Each sample was obtained at 18 h after treatment and hybridized with digoxigenin-labeled pepper *CADEF1* cDNA. P, phloem; X, xylem. PM, pallisade mesophyll cell; SM, spongy mesophyll cell; UE, upper epidermis; Vs, vascular bundle; Co, collenchyma cell.
Scale bars = 30 μM (Mee Do et al., 2004).

Recently a role for plant defensins in protection of its host against parasitic plants has been identified (de Zélicourt et al., 2007). Expression profiling of a susceptible and resistant sunflower cultivar towards the parasitic plant boomrape, revealed that elevated levels of plant defensin transcript were present the roots of the resistant cultivar compared to the susceptible cultivar. The defensin transcript in the roots of the resistant cultivar was even higher than the levels of transcript induced upon infection by boomrape on the roots of the susceptible cultivar.

The defensin in question was the root specific defensin Ha-DEF1 (de Zélicourt et al., 2007). Activity assays conducted with purified Ha-DEF1 against the boomrape species *Orobanche cumana* and *O. ramosa* revealed that this peptide causes extensive damage to the roots of the parasitic boomrape seedlings (Figure 4). Microscope analysis conducted after FDA staining revealed that treatment of boomrape roots with Ha-DEF1 ultimately resulted in death of the root meristimatic cells after 24 h, with most of the root dying after 6 days in the presence of Ha-DEF1 (Figure 4i-j) (de Zélicourt et al., 2007).

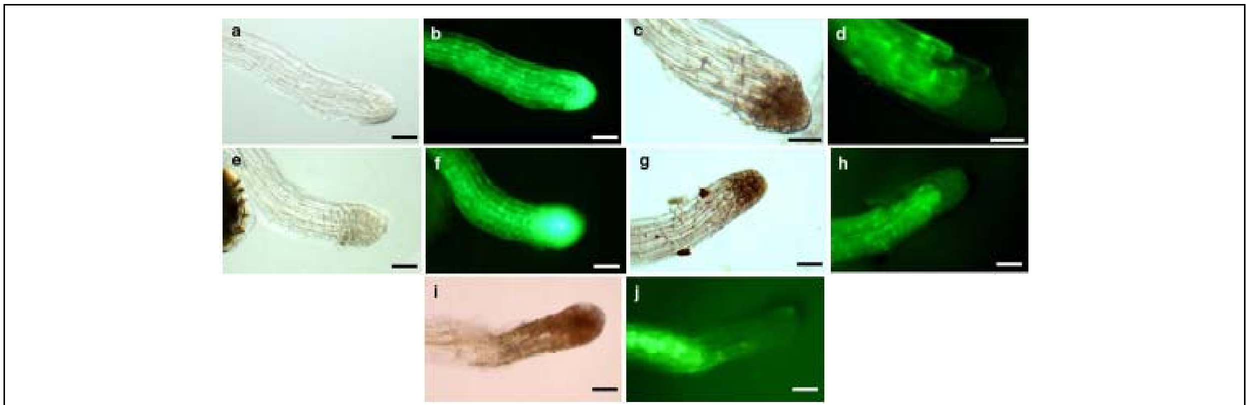

Figure 4. The effect of Ha-DEF1 on *Orobanche* seedlings. Pictures were taken after 24 h (a–h) or 6 days (i–j) of incubation of *O. cumana* (a–d, i,j), *O. ramosa* (e–h) seedlings with water (a, b, e, f) or with 10 μg ml^{-1} Ha-DEF1 (c, d, g–j). Observations were done after 10 min of FDA staining under light (a, c, e, g, i) or fluorescence (b, d, f, h, j) microscope (de Zélicourt et al., 2007).

The best evidence of a role for plant defensins in plant defense is the ability of these peptides to confer resistance against pathogens in susceptible plant hosts. Many plant defensins have been able to confer resistance to different plant species in transgenic strategies, especially the two defensins Rs-AFP2, from radish (Terras et al., 1995) and the defensin from wasabi (Figure 5.) (Kanzaki et al., 2002; Sjahril et al., 2006).

Figure 5. Infection studies conducted on transgenic clone #2 of *Phalaenopsis Wataboushi* '#6.13' to evaluate resistance towards bacterial soft rot caused by *Erwinia carotovora*. The plants were evaluated one week after the inoculation. Black arrows indicate the inoculation sites. The control plant (left) showed clear symptoms of soft rot, whereas no disease symptoms were observed in the transformant (right) (Sjahril et al., 2006).

2.3 MOLECULAR CHARACTERIZATION OF PLANT DEFENSINS

When first isolated, plant defensins were thought to be a new subclass of the plant peptide family of thionins and were thus termed γ-thionins. This term was given to plant defensins because they shared a similar size with thionins of 5 kDa, were cysteine-rich and contained four disulfide bridges in accordance with the α- and β-thionins. (γ-thionins were later renamed to plant defensins due to their structural and functional relation to insect and human defensins (Terras et al., 1995).

Analysis of plant genomes and EST databases have revealed that plant defensin encoding genes are highly represented within the genomes of most plants and are present as multigene families. Annotation of the Arabidopsis genome and transcriptome suggest the presence of more than 300 defensin genes (Silverstein et al., 2005), while large multigene families have also been identified in *Medicago truncatula* and *Oryza sativa* (Silverstein et al., 2007). Research have shown that these peptides are highly over represented in plant genomes and EST databases analyzed, suggesting the importance of these peptides in plant physiology (Silverstein et al., 2007).

2.3.1 Classification of plant defensins

Classification of plant defensins has traditionally been based on the amino acid composition of the mature defensin domain (Harrison et al., 1997). This early classification system divided the plant defensin superfamily into two subgroups sharing only 25% homology (Figure 6).

Subgroup A and B was further subdivided into four and two groups respectively, with the sequences within each group sharing at least 50% homology for subgroup A and 45% homology for subgroup B. This classification system also associated certain characteristic of defensin activity with the different subgroups. Members of subgroup A3 induced morphological changes on treated hyphae, usually inducing hyperbranching of the fungal hyphae (Terras et al., 1992; Terras et al., 1993), while subgroup A2 induced no morphological changes on treated hyphae and only inhibited hyphae elongation (Osborn et al., 1995). Members of subgroup B showed an array of activities including anti-bacterial and α-amylase inhibitory activity, with some members also able to inhibit protein synthesis.

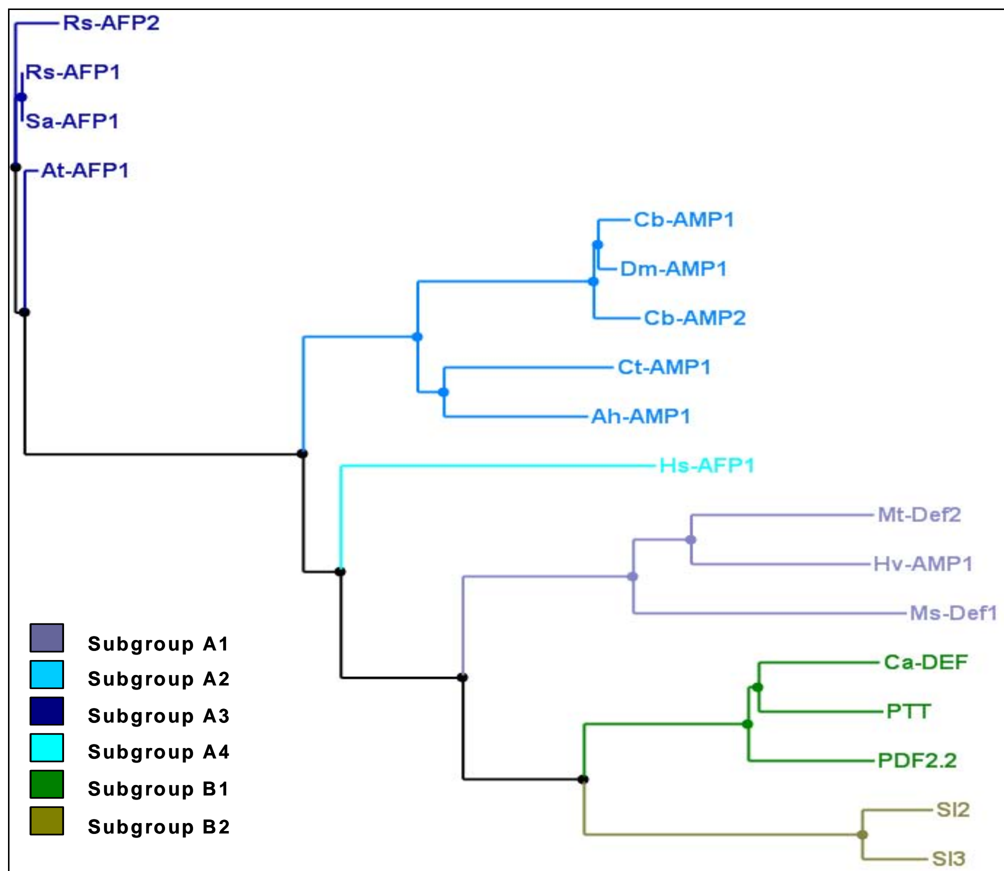

Figure 6. The phylogenetic relationship of the different members of plant defensins constituting the two major subgroups as proposed by (Harrison et al., 1997). Subgroup A is represented as shades of blue, with subgroup B in shades of green. The phylogenetic tree was created with Clustal X (Thompson et al., 1997) and edited in Arbodraw (Canutescu and Dunbrack Jr, 2006).

Although this classification system is still used today it is becoming less relevant as the number of new defensin sequences isolated increase. Currently more than 350 defensin sequences, isolated from 68 different plant species, have been deposited in the protein database at the National Centre for Bioinformatics (NCBI). Alignment analysis of these defensin sequences has revealed that the subgroups proposed by Harrison fall apart and only some of the consensus sequences described by Broekaert and colleagues (Terras et al., 1992; Broekaert et al., 1995) remain intact (Thomma et al., 2002). These include the cysteine residues defining the defensin backbone and the glycine at position 34 (numbering according to Rs-AFP1) (Figure 8). Today no new attempts have been made to subdivide the different members of the plant defensin superfamily into new subgroups based on their amino acid composition.

2.3.2 Amino acid composition and precursor structure

Plant defensins can be divided into two groups based on their precursor protein structure, with the majority of plant defensins sharing a common precursor structure consisting of a 29 or 30 amino acid secretion signal peptide, followed by a mature

active defensin domain of between 45 and 54 amino acids (Broekaert et al., 1995; Garcia-Olmedo et al., 1998; Thomma et al., 2002; Lay and Anderson, 2005). The second group of plant defensins, thus far only identified in *Solanaceae* species, differ from the basic precursor peptide structure observed for most defensins with the addition of a C-terminal prodomain of approximately 33 amino acids (Figure 7).

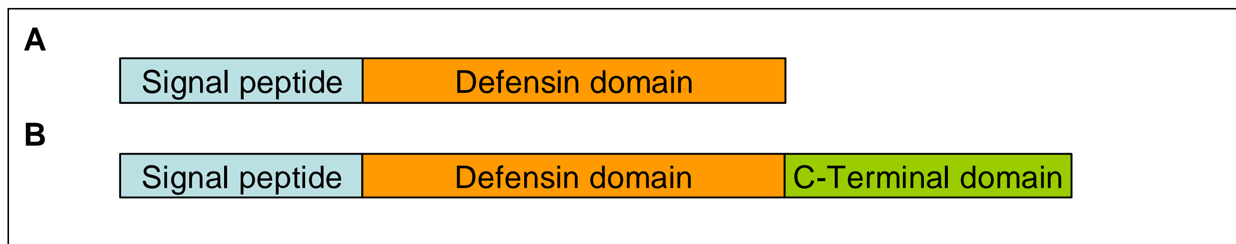

Figure 7. The basic structures observed in plant defensin precursor proteins (A) the common precursor structure observed for most defensin peptides. (B) The deduced precursor structure for some plant defensin cDNA sequences isolated from *Solanaceae* species, showing the additional C-terminal domain (Lay and Anderson, 2005).

Characterization of the C-terminal prodomains observed in the solanaceous defensins revealed that they are acidic in nature, with the exception of Ha-DEF1, and have a high representation of hydrophobic amino acids. It was observed that the acidic net charge of the C-terminal domain is able to counteract the basic nature of the defensin domain, resulting in an overall neutrally charged defensin peptide (Table III). It is hypothesized that the neutralization of the defensin peptide is part of a chaperone process, preventing the defensin from interacting with cellular components while being processed by the secretary pathway of the plant cell (Lay and Anderson, 2005).

Table III. Characterization of the different domains observed in the precursor protein structure of the solanaceous defensins.

Defensin	**Defensin domain**			**C-terminal domain**			**References**
	Basic	Acidic	**Net charge**	Basic	Acidic	**Net charge**	
NaD1	+11	-4	**+7**	+3	-9	**-6**	(Lay et al., 2003a)
FST	+11	-4	**+7**	+3	-9	**-6**	(Gu et al., 1992)
NeThio2	+12	-4	**+8**	+2	-9	**-7**	(Amano et al., 1997)
NpThio1	+11	-3	**+8**	+1	-8	**-7**	(Komori et al., 1997)
PhD1	+11	-4	**+7**	+2	-7	**-5**	(Lay et al., 2003a)
PhD2	+10	-4	**+6**	+1	-8	**-7**	(Lay et al., 2003a)
CcD1	+13	-5	**+8**	+1	-7	**-6**	(Aluru et al., 1999)
Ha-DEF1	+10	-6	**+4**	+8	-6	**+2**	(de Zélicourt et al., 2007)

Another hypothesis is that the C-terminal prodomain is involved in sub-cellular localization of the defensin peptide, since the high level of acid and hydrophobic amino acid residues are indicative of vacuolar sorting signals. This hypothesis is further strengthened by the presence of high levels of NaD1 defensin in the tobacco vacuole as revealed by *in situ* hybridization (Lay et al., 2003a).

Despite the clear differences observed in the precursor structure of plant defensin there is a conserved sequence defining the mature defensin domains of most defensin peptides. Most plant defensins contain a conserved sequence (Figure 8), consisting of the 8 cysteine residues, an aromatic amino acid at position 11, two glycine residues at position 13 and 34 and a glutamate at position 39 (numbering according to Rs-AFP1) (Broekaert et al., 1995; Terras et al., 1995).

```
gi|59798992 Rs-AFP1      --QKLC-ERPSGTWSGVCGNNNACKNQCINLEKARHG-SCNYVFPAHKCICYFPC
gi|1703206  Rs-AFP2      --QKLC-QRPSGTWSGVCGNNNACKNQCIRLEKARHG-SCNYVFPAHKCICYFPC
gigi|15225243 PDF1.2b    --QKLC-EKPSGTWSGVCGNSNACKNQCINLEGAKHG-SCNYVFPAHKCICYVPC
gi|1049479  Ct-AMP1      ---NLC-ERASLTWTGNCGNTGHCDTQCRNWESAKHG-ACHKRGN-WKCFCYFNC
gi|1049478  Ah-AMP1      ----LCNERPSQTWSGNCGNTAHCDKQCQDWEKASHG-ACHKRENHWKCFCYFNC
gi|2147320  Dm-AMP1      ---ELC-EKASKTWSGNCGNTGHCDNQCKSWEGAAHG-ACHVRNGKHMCFCYFNC
gi|1049482  Hs-AFP1      DGVKLC-DVPSGTWSGHCGSSSKCSQQCKDREHFAYGGACHYQFPSVKCFCKRQC
Hv-AMP1                  ---KTC-ESLANTYRGPCFTDGSCDDHCKNKEHISLG-RCRND---VRCWCTCNC
gi|20139322 PSD1         ---KTC-EHLADTYRGVCFTNASCDDHCKNKAHLISG-TCHN----WKCFCTQNC
gi|544136   D230         ---NTC-ENLAGSYKGVCF--GGCDRHCRTQEGAISG-RCRDD---FRCWCTKNC
gi|11387095 SA21         ---RVC-RRRSAGFKGLCMSDHNCAQVCLQ-EGWGGG-NCDG--VMRQCKCIRQC
gi|1173437  SIA3         ---RVC-RRRSAGFKGLCMSDHNCAQVCLQ-EGWGGG-NCDG--VIRQCKCIRQC
gi|2501196  THG1         ---RVC-RRRSAGFKGVCMSDHNCAQVCLQ-EGYGGG-NCDG--IMRQCKCIRQC
gi|135793   THG_H        ---RIC-RRRSAGFKGPCVSNKNCAQVCMQ-EGWGGG-NCDG--PLRRCKCMRRC
gi|129350   P322         ---RHC-ESLSHRFKGPCTRDSNCASVCET-ERFSGG-NCHG--FRRRCFCTKPC
gi|11387188 THGF         ---RTC-ESQSHKFKGTCLSDTNCANVCHS-ERFSGG-KCRG--FRRRCFCTTHC
CONSENSUS                     C    :  a G C     C   C   E    G  C        C C   C
```

Figure 8. Amino acid alignment of plant defensins representing the different subgroups of plant defensin superfamily as described by Harrison et al (1997). The conserved amino acids are indicated in colour. The cysteines are indicated in yellow, aromatic residue in green, glycines in blue and the glutamate in grey. Alignment was created in ClustalX (Thompson et al., 1997).

2.3.3 Structural composition of plant defensin peptides

Structurally plant defensins belong to a superfamily of peptides that contain a unique motif known as the cysteine stabilizing motif (CSαβ motif). The structural determinants of this motif are encoded within six cysteine residues, with the following sequence arrangement: C1…C2XXXC3…C4…C5XC6 (Figures 9 and 10). This arrangement of cysteines was first observed by Bontems et al (1991), when comparing the structural motifs present in scorpion toxins and insect defensins. The CSαβ structural motif was however named by Cornet et al (1995) after solving the three dimensional structure of insect defensins A.

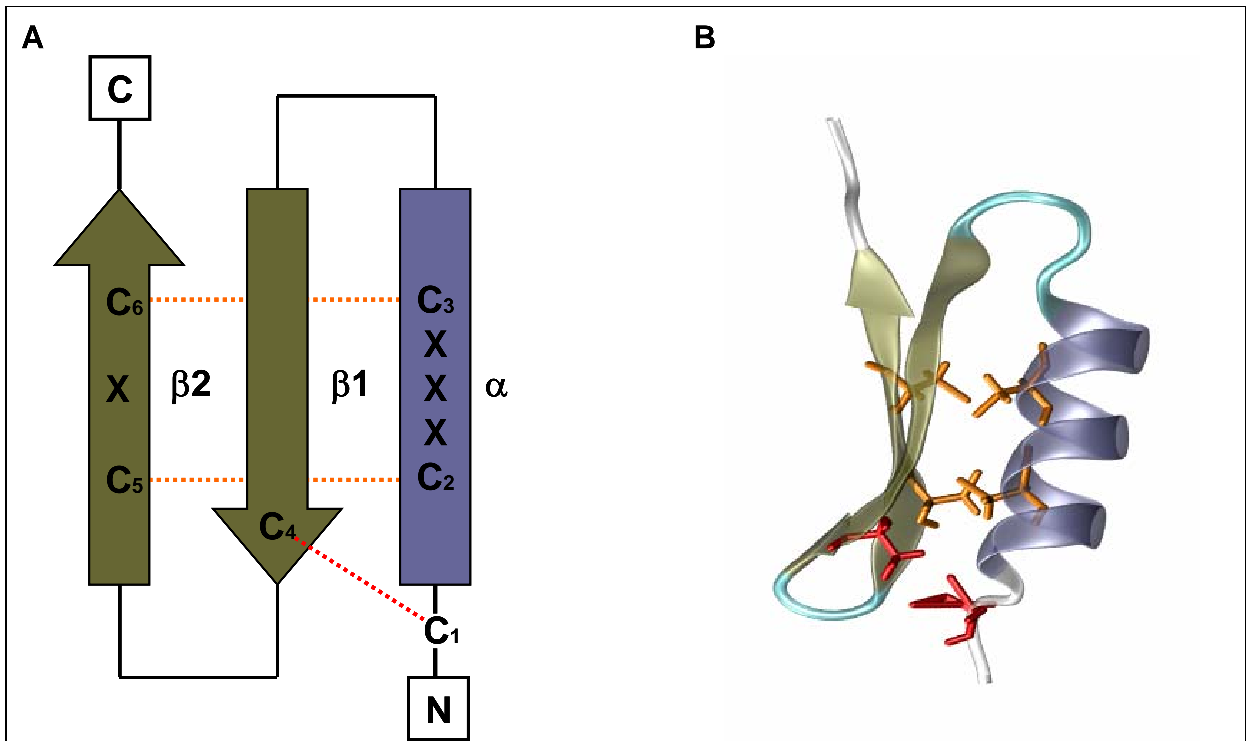

Figure 9. (A) The consensus arrangement of the cysteine residues and their respective disulfide bridges within the CSαβ motif. The disulfide bridges are represented by the dotted lines. (B) A three dimensional representation of the CSαβ motif. The cysteines are indicated as stick models and the colours indicate which cysteines pare up to form a disulfide bridge (Cornet et al., 1995; Zhu et al., 2005).

On a tertiary level the CSαβ motif consists of a single α-helix that is connected to a β-sheet consisting of two anti parallel strands (Figure 9B.) The arrangement of the cysteines within the tertiary structure is very important for maintaining the global fold of all CSαβ motif containing proteins. The CXXXC segment of the CSαβ motif is located in the α-helix, with the fourth cysteine situated in the first β-strand and CXC located in the second β-strand. The whole motif is further stabilized by three disulfide bridges. The first bridge connects C1 with C4, thus linking the N-terminal with the first β-strand. The second and third bridge is maintained between C2/C5 and C3/C6 linking the α-helix with C-terminal β-strand, to form the CSαβ motif (Tamaoki et al., 1998).

Figure 10. Amino acid alignment and disulphide bridge formation of defensin sequences clearly showing the amino acids involved in the defining the tertiary structure of the CSαβ motif (red) and the fourth bridge (black) present in most defensin peptides. The eight cysteine residues are indicated in yellow. Alignments were created with ClustalX (Thompson et al., 1997).Donor rorganisms are; Rs-AFPs from *Raphanus sativum*, Sa-AFPs from *Sinapsis alba*, At-AFP1 from *Arabidopsis thaliana,* Cb-AMPs from *Cnicus benedictus*, Dm-AMP1 from *Dahlia mercii*, Ct-AMP1 from *Clitorea ternatea,* Ah-AMP1 from *Amaranthus hypocondriacus*, Hs-AFP1 from *Heuchera sangunea*, SIαs from *Sorghum bicolor*.

Most plant defensins, however contain eight cysteine residues, which leads to the formation of a fourth disulfide bridge in addition to the three bridges contained in the core CSαβ motif (Figure 9). Structurally plant defensins contain one α-helix and one β-sheet (Figure 11A) consisting of three antiparallel strands (Fant et al., 1998; Almeida et al., 2002; Zhao et al., 2002; Lay et al., 2003b) and are described as being pseudo-cyclic peptides, due to the disulfide bridge linking the cysteine residue at the C-terminus (C8) with the cysteine residue at the N-terminus (C1) (Lay and Anderson, 2005).

There is however plant defensin peptides (PhD1 and PhD2 from *Petunia hybrida*) that contain 5 disulphide bridges (Janssen et al., 2003). The fifth disulfide bridge links the first β-strand with the α-helix, further reinforcing the structure, but not altering the position of the α-helix in relation to the β-sheet when compared to the four disulfide bridge defensins (Janssen et al., 2003) (Figure 11B).

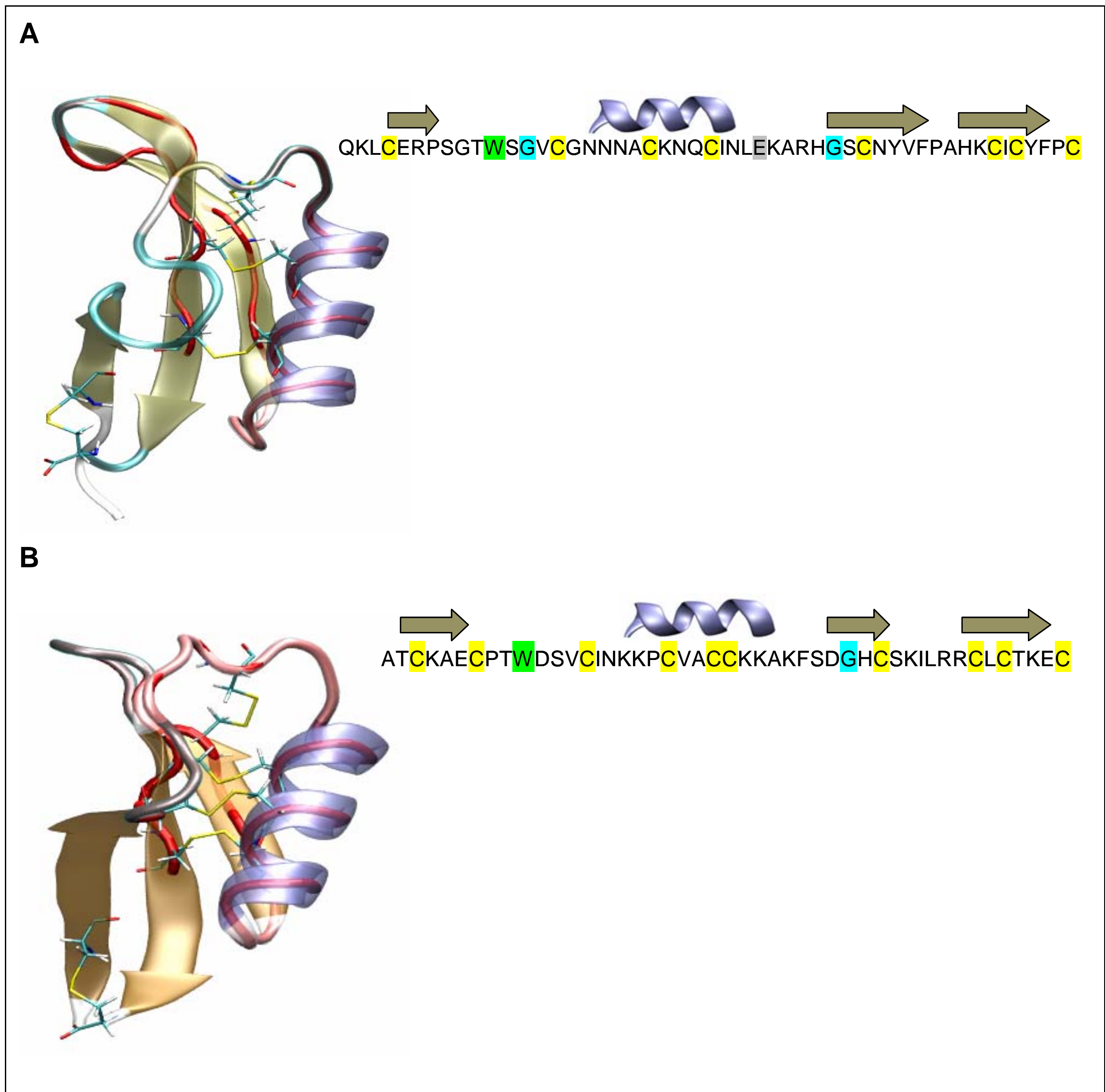

Figure 11. (A) A three dimensional representation of Rs-AFP1 (PDB id 1AYJ) showing the characteristic structure of plant defensins (Fant et al., 1998). (B) The global structure of PhD1 (PDB id 1N4N) from *Petunia hybrida* (Janssen et al., 2003). The CSαβ motif is represented as a solid red tube structure, the α-helix and β-sheet are represented in purple and tan colours, respectively. The internal disulphide bridges stabilizing the whole structure is indicated as yellow stick models. The amino acids responsible for the formation of the tertiary structures are indicated next to each structure on the right. Colouring is according to Figure 8 and conserved amino acids are also indicated.

2.4 BIOLOGICAL ACTIVITIES OF PLANT DEFENSINS

Plant defensins have been described as some of the most functionally diverse CSαβ motif peptides isolated to date (Zhu et al., 2005). Most of the functional diversity of these peptides are encoded within the amino acid residues that forms the loops connecting the α-helix and β-strands (Zhu et al., 2005). Biological activities observed

in these peptides are listed in Table IV and range from antimicrobial activity to the inhibition of HIV reverse transcriptase.

Table IV. Biological activities observed within the plant defensin superfamily.

Biological activity	Example	Plant source	Reference
Antifungal	Rs-AFP1 Br-AFP1	*Raphanus sativa* *Brassica rapa*	(Terras et al., 1992; Terras et al., 1993)
Antibacterial	Tad1 Fabatin-1 Pth-St1 Cp-thionin II	*Triticum aestivum* *Vicia faba* *Solanum tuberosum* *Vigna unguiculata*	(Koike et al., 2002) (Zhang and Lewis, 1997) (Moreno et al., 1994) (Franco et al., 2006)
Insecticidal	VrCRP	*Vigna radiata*	(Chen et al., 2002)
Proteinase inhibition	Cp-thionin I	*Vigna unguiculata*	(Melo et al., 2002)
Protein synthesis inhibition	Hv-AMP1	*Hardenbergia violacea*	(Harrison et al., 1997)
α-Amylase inhibition	SIα1-3	*Sorgum bicolor*	(Bloch Jr and Richardson, 1991)
Sodium channel inhibition	γ1- zeathionin γ2-zeathionin	*Zea mays*	(Kushmerick et al., 1998)
Anti-plant	Ha-DEF1	*Helianthus annuus*	(de Zélicourt et al., 2007)
Metal tolerance	AhPDF1.1	*Arabidopsis halleri*	(Mirouze et al., 2006)
Anti-HIV	Vulgarinin Sesquin	*Phaseolus vulgaris* *Vigna sesquipedalis*	(Wong and Ng, 2005a; Wong and Ng, 2005b)

Although plant defensins exhibit an array of potential economical and medical important activities, not a lot of research has focussed on elucidating the underlying mechanisms resulting in the observed mode of action. Over the last 10 years most of the research has focussed on unravelling the mechanisms underlying the antifungal activity of the defensin peptides and their ability to inhibit certain enzymes.

When plant defensins interact with fungi to cause inhibition, two different morphological changes can be observed in fungal growth morphology. The defensin can either cause severe hyperbranching of the fungal hyphae, or it can arrest the elongation of the fungal hyphae. Based on these changes induced in fungal hyphae morphology, plant defensins have thus been classified into morphogenic defensins (Type I) and non-morphogenic defensins (Type II) respectively (Figure 12). There are also a third grouping of plant defensins that exert no antifungal activity (Figure 12). For most plant defensins the mechanism of action is still unknown and no fungal molecular targets have yet been identified. The exception is the defensins Rs-AFP2 (morphogenic defensin) and Dm-AMP1 (non-morphogenic defensin) for which a few fungal membrane targets have been identified and a proposed mode of action has been hypothesized.

```
Type I                 1    5    10   15   20   25   30     35   40   45   50
  Rs-AFP1           --ZKLC-ERPSGTWSGVCGNNNACKNQCINLEK-ARHGSCNYVFPAHKCICYFPC
  Rs-AFP2           --ZKLC-QRPSGTWSGVCGNNNACKNQCIRLEK-ARHGSCNYVFPAHKCICYFPC
  Sa-AFP1           --QKLC-ERPSGTWSGVCGNNNACKNQCINLEK-ARHGSCNYVFPAHKCICYFPC
  Sa-AFP2           --QKLC-QRPSGTWSGVCGNNNACRNQCINLEK-ARHGSCNYVFPAHKCICYFPC
  At-AFP1           --QKLC-ERPSGTWSGVCGNSNACKNQCINLEK-ARHGSCNYVFPAHKCICYFPC
  Hs-AFP1           DGVKLC-DVPSGTWSGHCGSSSKCSQQCKDREHFAYGGACHYQFPSVKCFCKRQC

Type II
  Cb-AMP1           ---ELC-EKASKTWSGNCGNTKHCDDQCKSWEG-AAHGACHVRNGKHMCFCYFNC
  Cb-AMP2           ---ELC-EKASKTWSGNCGNTKHCDNKCKSWEG-AAHGACHVRSGKHMCFCYFNC
  Dm-AMP1           ---ELC-EKASKTWSGNCGNTGHCDNQCKSWEG-AAHGACHVRNGKHMCFCYFNC
  Ct-AMP1           ---NLC-ERASLTWTGNCGNTGHCDTQCRNWES-AKHGACHKR-GNWKCFCYFNC
  Ah-AMP1           ----LCNERPSQTWSGNCGNTAHCDKQCQDWEK-ASHGACHKRENHWKCFCYFNC

Type III
  SIα2               --RVC-MKGSAGFKGLCMRDQNCAQVCL-QEG-WGGGNCDGVM--RQCKCIRQC
  SIα3               --RVC RRRSAGFKGLCMRDHNCAQVCL-QEG-WGGGNCDGVI--RQCKCIRQC
```

Figure 12. Amino acid alignment of defensins showing differential antifungal activities. Type I groups the morphogenic defensins, while Type II consists of plant defensins with antifungal activity without causing hyperbranching. Type III includes the defensins without antifungal activity. Amino acids that could play a possible role in antifungal activity are coloured in grey. Alignments were created with ClustalX (Thompson et al., 1997).

2.4.1 Interaction of the morphogenic defensin, Rs-AFP2 with fungal hyphae

Most of the scientific work done on elucidating the mode of action of Rs-AFP2 has been conducted on the model systems of *Saccharomyces cerevisiae*, *Pichia pastoris* and *Candida albicans*. It was observed that Rs-AFP2 show very little activity against the yeast *S. cerevisiae*, with a minimum inhibitory concentration (MIC) >40 μM, but inhibited *P. pastoris* at 2.0 μM and *C. albicans* at 2.5 μM. Analysis of the lipids present in the membranes of *S. cerevisiae* and *P. pastoris* revealed that *S. cerevisiae* had no glucosylceramide (GlcCer) (Figure 13) present in its membrane, while GlcCer was abundant in the membrane of *P. pastoris*. This identified GlcCer as a possible target for Rs-AFP2 in the yeast membrane of *P. pastoris*.

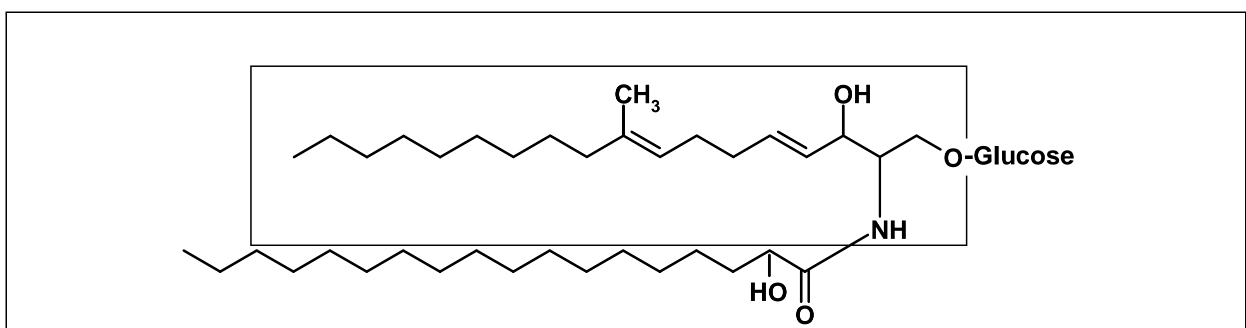

Figure 13. The neutral glycosphingolipid, glucosylceramide. The sphingoid backbone (9-methyl-4,8-sphingadienine) is boxed (Thevissen et al., 1996).

Screening of a *P. pastoris and C. albicans* mutant deletion library confirmed this hypothesis when mutants were identified with the same resistance as *S. cerevisiae* towards Rs-AFP2. These deletion mutant was identified as lacking the gene GCS encoding for the enzyme UDP-glucose:ceramide glucosyltransferase. UDP-glucose:ceramide glucosyltransferase catalyses the last step in the synthesis of GlcCer.

Binding assays conducted with Rs-AFP2 and purified GlcCer from *P. pastoris,* soybean, humans and monogalactosyldiacylglycerol from plants revealed that Rs-AFP2 selectively interacted with GlcCer from *P. pastoris* and not those from soybean or humans (Figure 14) (Thevissen et al., 2004).

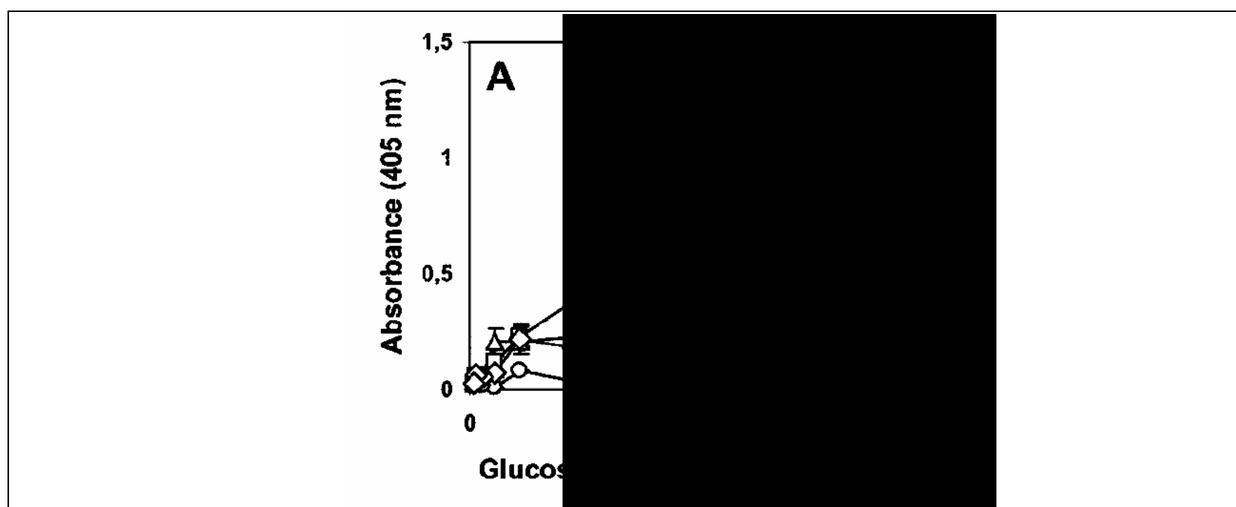

Figure 14. An interaction assay of Rs-AFP2 with glucosylceramides from *P. pastoris* (□), human spleen (Δ), soybean (◊) and monogalactosyldiacylglycerol from plants (O), clearly showing the interaction of this peptide with the glucosylceramides from *P. pastoris*, but not those from humans or plants (Thevissen et al., 2004).

In contrast to Dm-AMP1, no enhancement of GlcCer binding was observed for Rs-AFP2 when the fungal sterol ergosterol was added to the assay, suggesting no role for sterol as a target for Rs-AFP2. The hypothesis that GlcCer is the target for Rs-AFP2 is further strengthened by the results of a competition assay where the GlcCer sites on a wild-type *P. pastoris* strain could be blocked with antibodies raised against GlcCer, resulting in a four fold decrease in sensitivity towards Rs-AFP2.

Mutational analysis of Rs-AFP2 was also conducted to identify possible amino acids involved in antifungal activity. The amino acids in Rs-AFP2 were mutated to the amino acids of the defensins SIα2 and 3 that show no antifungal activity. The mutational study revealed that 13 amino acids play a role in the antifungal activity of the plant defensin Rs-AFP2 (De Samblanx et al., 1997). The results of individual mutational analysis are listed in Table V.

Table V. A summary of the mutations made on Rs-AFP2 and their effect on antifungal activity as observed by De Samblanx et al (1997).

Amino acid	Mutation	Change in Amino acid charge	Effect on activity
Gly-9	G9R	Neutral to charged	↑150%
Thr-10	T10G	Polar to neutral	↓ 80%
Ser-12	S12R	Neutral to charged	↓50%
Leu-28	L28R	Hydrophobic to charged	↓80%
Ala-31	A31W		↓80%
Tyr-38	**Y38G**	**Polar to neutral**	**↓90%**
Val-39	V39R	Hydrophobic to charged	↑150%
Phe-40	F40M	Hydrophobic to neutral	↓75%
Pro-41	P41Δ	Deletion	↓90%
Ala-42	A42R	Hydrophobic to charged	↓50%
Lys-44	K44Q	Charged to polar	↓70%
Ile-46	I46R	Hydrophobic to charged	↓72%
Phe-49	F49R	Hydrophobic to charged	↓60%

It was observed that increasing the overall charge of Rs-AFP2 resulted in higher antifungal activity, but that the increase in charge should be assigned to positions 9 and 39. It was also noted that the amino acid important for antifungal activity grouped together on the tertiary structure level to form distinct sites associated with antifungal activity. The first site was situated at the type VI β-turn connecting β-strand 2 and β-strand 3 (Figure 15) and consisted of the amino acids 38, 40-42, 44 and 46.

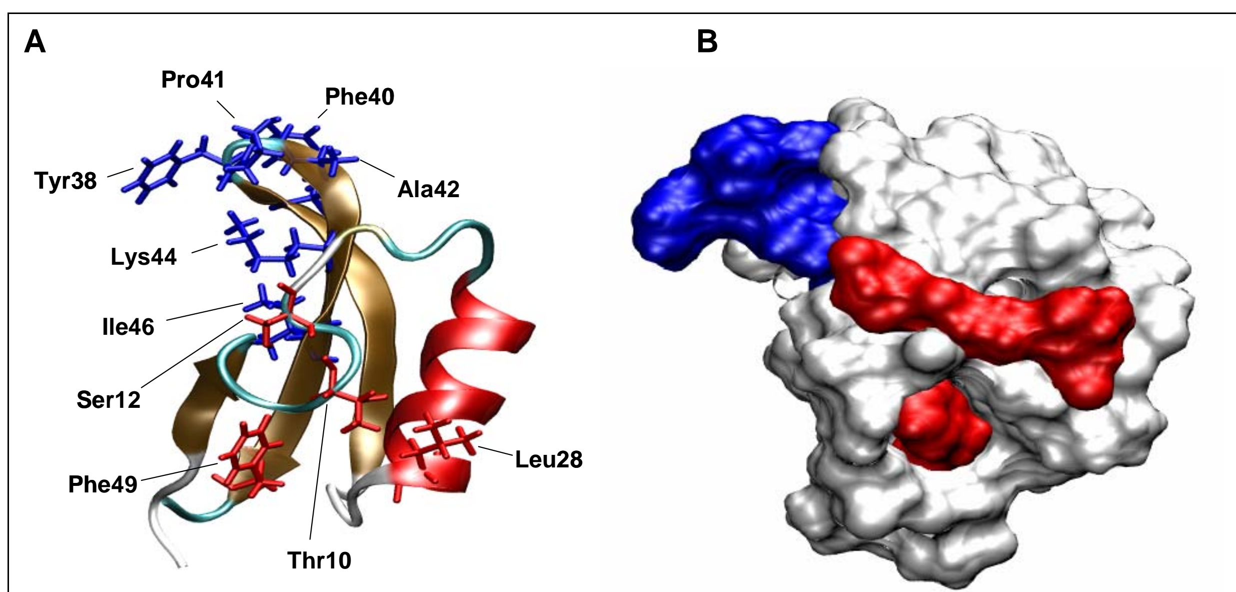

Figure 15. (A) Position of the amino acids important for creating the active antifungal sites in Rs-AFP2. (B) Three dimensional surface representation of Rs-AFP2 showing the active the active sites important for antifungal activity in Rs-AFP2; active site 1 (blue) and active site 2 (red). Adapted from the data of De Samblanx et al (1997).

The second site was located at the loop connecting the α-helix and the first β-strand (amino acids 10 and 12) and included residues present on the α-helix and β-strand 3 (amino acids 28 and 49 respectively) (De Samblanx et al., 1997).

Antifungal activity assays and GlcCer binding assays conducted with the mutant peptide Rs-AFP2(Y38G) against *P. pastoris,* revealed that this mutant peptide is inactive against *P. pastoris*, but is still able to bind GlcCer to same extent as native Rs-AFP2 (Figure 16). This result suggests that binding of GlcCer by Rs-AFP2 is not directly linked to its activity, but that it is essential for the antifungal activity of Rs-AFP2, since *gcs* yeast mutants lacking GlcCer in their membrane are resistant to Rs-AFP2 (De Samblanx et al., 1997).

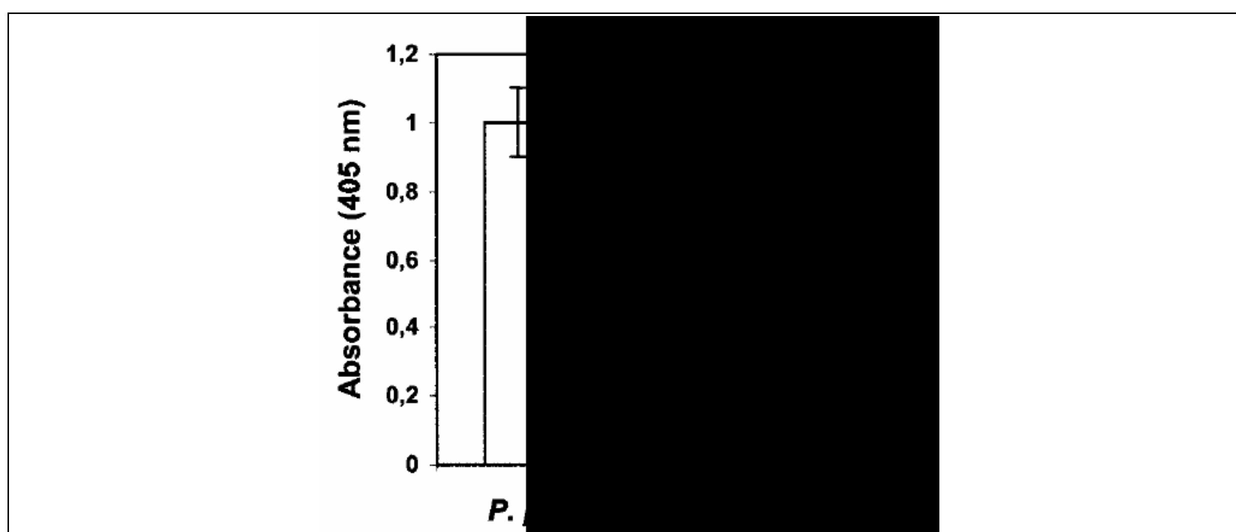

Figure 16. A glucosylceramide binding assay showing the ability of the Rs-AFP2(Y38G) mutant peptide to bind glucosylceramide (De Samblanx et al., 1997). Clear bar = WT Rs-AFP2; black/white = Rs-AFP2(Y38G) mutant.

A two step model has been proposed for the interaction of Rs-AFP2 and its membrane targets. The first involves the binding of Rs-AFP2 to GlcCer in the fungal membrane, followed by the interaction of a yet unknown target, resulting in fungal membrane permeabilization.

2.4.2 Interaction of the non-morphogenic defensin, Dm-AMP2 with fungal hyphae

The non-morphogenic defensin Dm-AMP1, in contrast to Rs-AFP2, show strong antifungal activity against the yeast *S. cerevisiae*, suggesting that it has a different membrane target than Rs-AFP2, the latter binding to GlcCer. Using a genetic complementation approach, a gene was identified that conferred resistance to Dm-AMP1 in the yeast *S. cerevisiae.* This gene was identified as IPT1 which encodes for an enzyme inositol phosphotransferase (Thevissen et al., 2000). This enzyme is involved in the last step of the synthesis of the sphingolipid mannosyldiinositolphosphorylceramide ($M(IP)_2C$) (Figure 17)

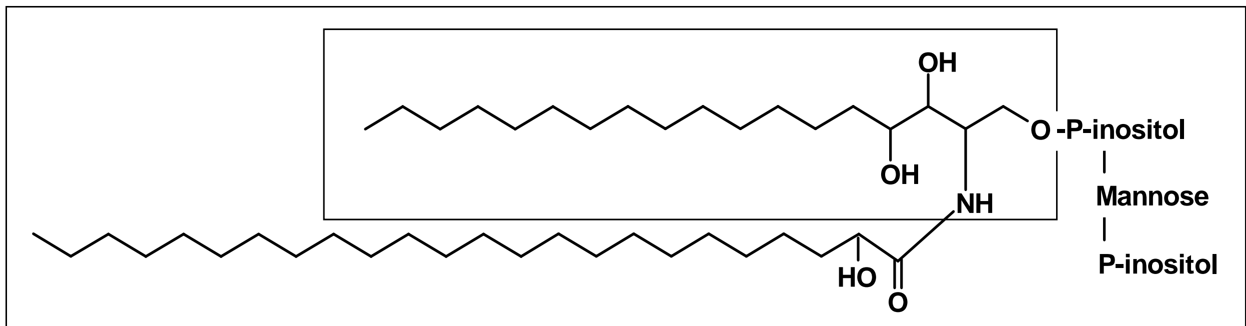

Figure 17. The structure of mannosyldiinositolphosphorylceramide M(IP)2C. The sphingoid backbone (phytosphingosine) is boxed and represents the most complex GIPC present in the yeast *S. cerevisiae* (Thevissen et al., 2003b).

A *S. cerevisiae* strain containing a non-functional allele of IPT1 was shown to bind less Dm-AMP1 compared to the sensitive wild-type strain. Furthermore, an *ipt1* null mutant grown under nutrient starvation condition showed sensitivity towards Dm-AMP1 to the same extent as the wild-type strain. Analysis of the membrane lipid content revealed that a small amount of ($M(IP)_2C$) was being produced via a yet unidentified pathway, confirming ($M(IP)_2C$), and not IPTI as the target for Dm-AMP1. The binding of Dm-AMP1 to ($M(IP)_2C$) was also confirmed with ELISA-based binding assays (Figure 18A). The association of Dm-AMP1 to ($M(IP)_2C$) could also be enhanced by the addition of the fungal sterol ergosterol at physiological concentrations present in membranes (Figure 18B) (Thevissen et al., 2003a).

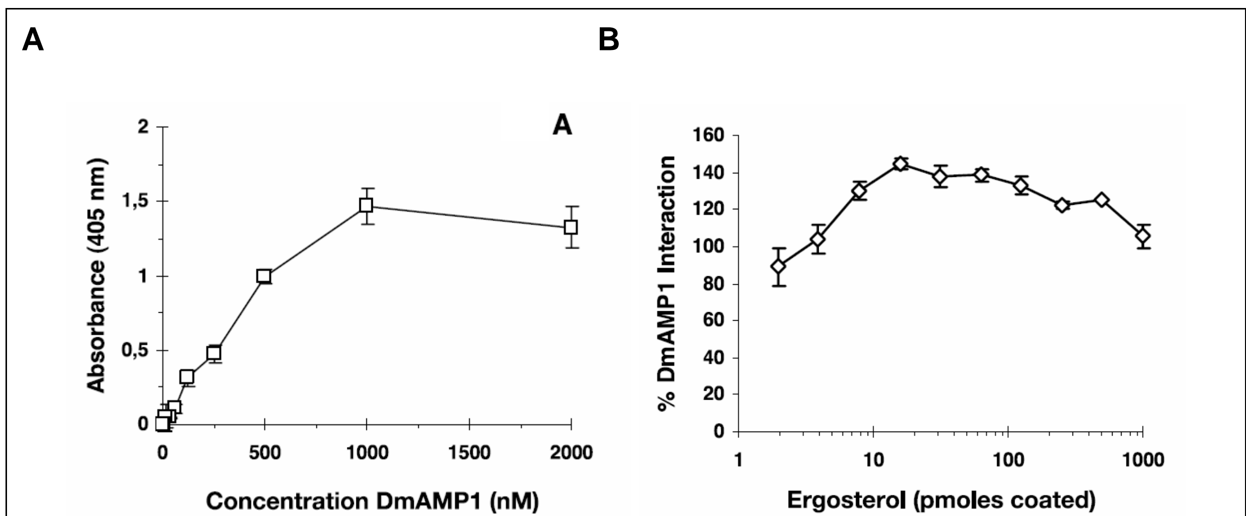

Figure 18. The interaction of DmAMP1 with sphingolipids isolated from *S. cerevisiae* and the fungal sterol ergosterol. (A) A dose-response curve showing the interaction of DmAMP1 with 15 pmol of coated sphingolipids (B) The additive effect of ergosterol on the interaction of 1 μM DmAMP1 with 15 pmol of *S. cerevisiae* sphingolipids (Thevissen et al., 2003a).

A mode of action has been proposed for Dm-AMP1 by Thevissen et al (2003a) that directly involves ($M(IP)_2C$) and ergosterol. Fungal membranes are asymmetrical in nature with the phosphoglycolipids in the inner leaflet and the sphingolipids and

sterols on the outer leaflet. It is known that sphingolipids and sterols can be closely associated to form what is known as lipid rafts. It has been shown that these rafts play a role in transport, apoptosis and signal transduction. Interaction of Dm-AMP1 with these rafts would result in a high concentration of this peptide localized to a small area on the fungal membrane. Binding of Dm-AMP1 to the fungal membrane results in membrane permeabilization, whether this permeabilization is the result of insertion of Dm-AMP1 into the fungal membrane, or due to the activation of a sphingolipid linked pathway is still unclear.

This permeabilization effect of the fungal membrane results in the rapid uptake of Ca^{2+} and rapid efflux of K^{+} in both Dm-AMP1 and Rs-AFP2 treated fungal hyphae (Figure 19). Whether the permeabilization of the fungal membrane by Dm-AMP1 or Rs-AFP2 is the causal mechanism of antifungal activity is still unclear. It can not be ruled out that these peptides might have internal cellular targets and that the interaction of Rs-AFP2 and Dm-AMP1 with these targets result in fungal growth inhibition (Thevissen et al., 2003a and 2003b).

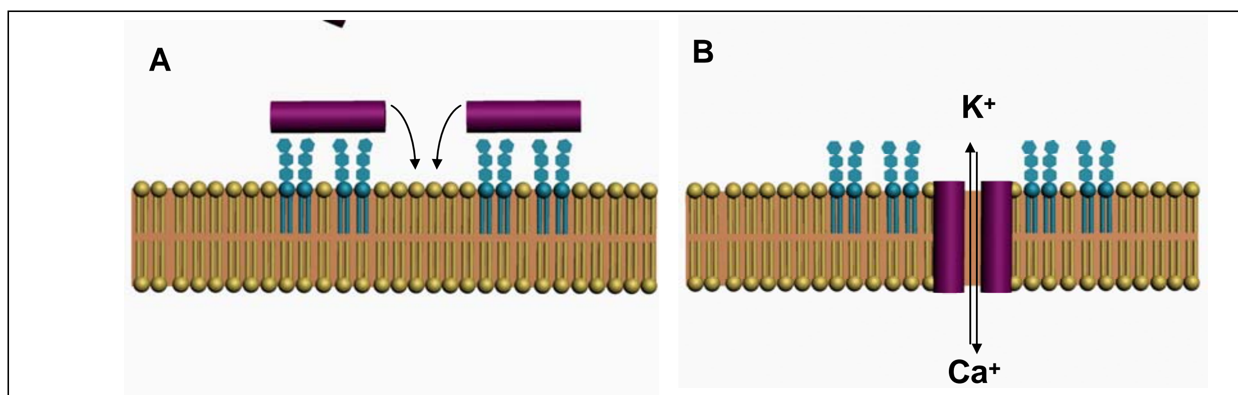

Figure19. A model for the mode of action of plant defensins proposed by Thevissen et al. (2003b): (A) Plant defensins, represented in purple bind to membrane rafts composed of sphingolipids (represented in blue) where after membrane integrity is altered (B) Alteration of membrane permeability results in an increased Ca^{2+} uptake and K^{+} efflux.

2.4.3 Interaction of the Psd1 with internal fungal target molecules

In early 2007 the first interaction of a plant defensin with an internal fungal target was reported (Lobo et al., 2007). Microscopical studies done on *Fusarium solani* hyphae, treated with fluorescently labelled *Pisum sativum* Defensin1 (Psd1), a defensin from the common garden pea, revealed that this peptide entered the fungal cell and associated with the fungal nucleus (Figure 20). Screening of a *Neurospora crassa* conidal cDNA library using the yeast two hybrid system and Psd1 as the bait protein, led to the isolation of 11 possible clones with nine being identified as nuclear proteins.

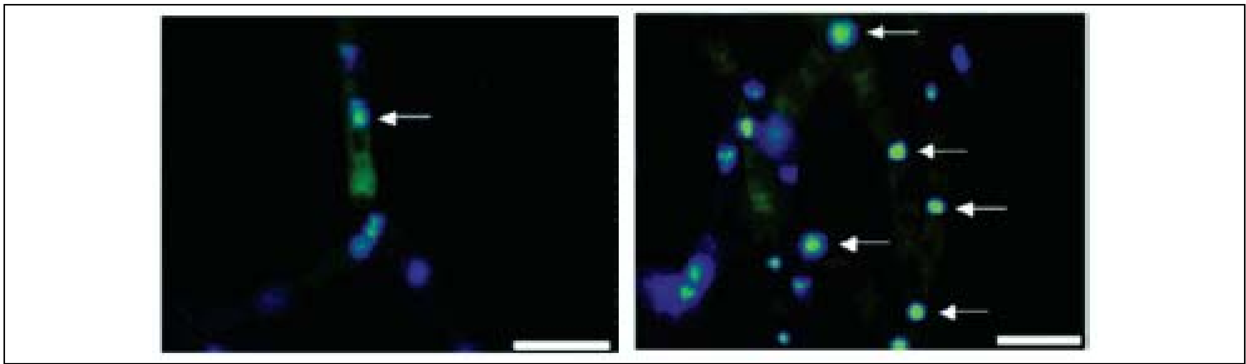

Figure 20. Fluorescence microscopic analysis of *Fusarium solani* hyphae treated with FITC-conjugated *Ps*d1 (green fluorescence) and DAPI-stained DNA (blue fluorescence). The co-localization of the Psd1 and the nucleic DNA can be clearly seen (indicated by white arrows) (Lobo et al., 2007).

Further analysis using GST pull-down assays revealed that Psd1 closely associated with the nuclear protein cyclin F. Cyclin F plays a functional role in the mitotic cell cycle and general cell cycle control. Cyclin F expression coincides with the transition of the S phase of the cell cycle to the G2 phase and from G2 to the M phase (Lobo et al., 2007).

Cell cycle studies conducted in the neonatal retina tissue of rats revealed that Psd1 is able to retard cell nucleus migration, by blocking the role of cyclin F during S to G2 phase transition. Nucleus migration is important events during yeast daughter cell formation and also occurs during fungal hyphae tip extension. Unlike some other peptide antibiotics, Psd1 does not disrupt the cytoplasmic micro tubulin network, which would result in a loss of nuclear migration. The hypothesis is rather that Psd1 interacts with cyclin F thereby disrupting its role as chaperone for cyclin B1, which requires cyclin F to be transported to the nucleus. Cyclin B1 is an important component involved in G2 to M phase transition during the cell cycle and overexpression of cyclin B1 have been associated with tumour cell proliferation. Interference with cyclin B results in endoreplication and an increase in nuclear DNA. This could explain the increased DNA content of *N. crassa* conidia treated with Psd1 (Lobo et al., 2007).

2.4.4 Plant defensins as enzyme inhibitors

The enzyme inhibitory activity of plant defensins was first identified in 1991 (Bloch Jr and Richardson, 1991) with the isolation of the α-amylase inhibitory defensins from *Sorghum bicolour*. Since then only a few plant defensins have been identified with the ability to inhibit certain enzyme reactions. These include the inhibition of insect α-amylases (Bloch Jr and Richardson, 1991; Liu et al., 2006; Shiau et al., 2006), the protease trypsin (Wijaya et al., 2000; Melo et al., 2002) and HIV reverse transcriptase (Wong and Ng, 2003; Ngai and Ng, 2005; Wong and Ng, 2005a and 2005b). Despite the huge potential of these peptides in the fight against HIV and cancer, it is the

ability of these peptides to inhibit insect α-amylases and trypsin that have received the most attention by research community.

Although the inhibition of α-amylase by the plant defensins SIα1, 2 and 3 was first reported in 1991, not a lot of research has focused on elucidating the exact mechanism involved in the inhibitory activity of these peptides. Research have shown that these peptides are able to bind Ca^{2+} ions and it is suggested that these peptide chelate certain metal ions necessary for the activity of the α-amylase enzyme, thus leading to its inhibition.

More recent research focussed on VrD1 and VrD2 from *Vigna radiate* and their interaction with the α-amylase from *Tenebrio molitor* larvae (TMA). The defensin VrD1 has strong α-amylase inhibitory activity, while VrD2 has no inhibitory activity. Analysis of the two peptides at amino acid level showed that VrD1 and VrD2 shared little homology (Figure 21A), although both share a similar tertiary structure (Figure 21 B and C). Importantly these two peptides differed significantly in the amino acids comprising loop 3 (L3) of their tertiary structures (Figure 21A). Differences in amino acids present in the tertiary loops of the plant defensins have been linked to the diverse activities observed for this peptide family (De Samblanx et al., 1997; Spelbrink et al., 2004; Zhu et al., 2005; Lui et al., 2006; Lin et al., 2007).

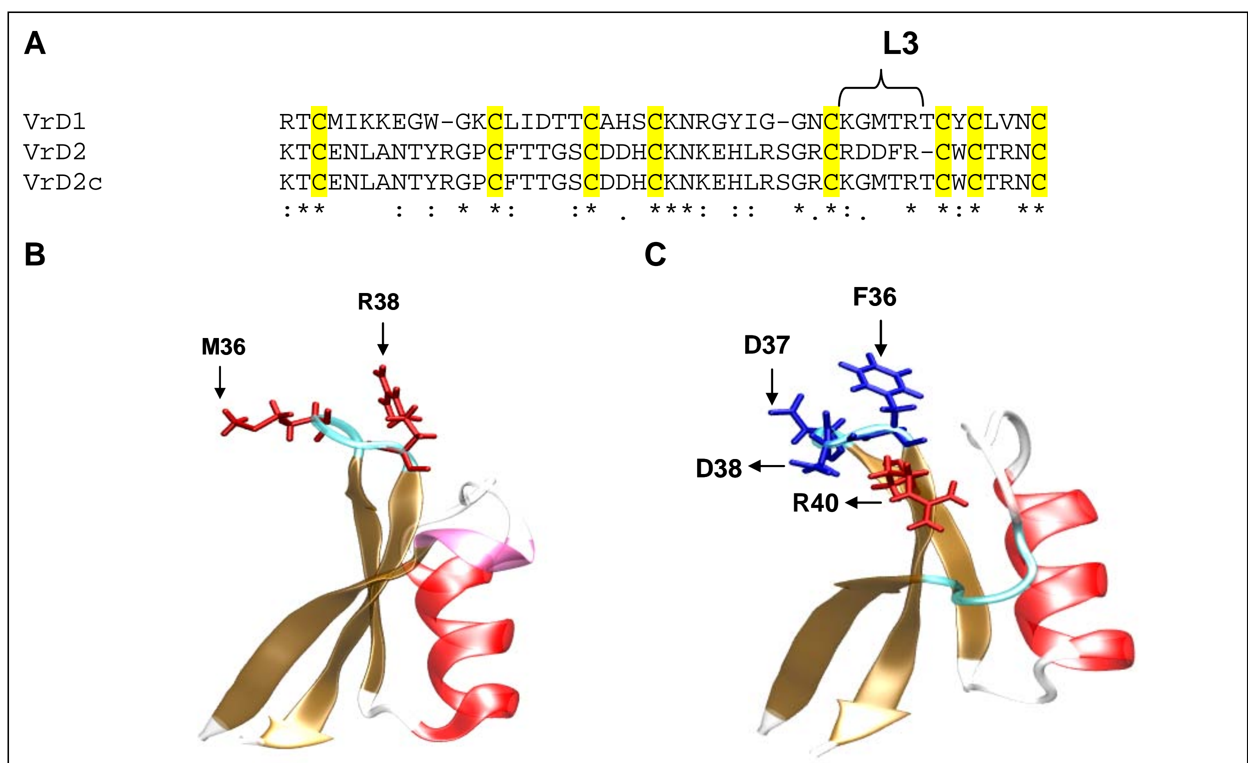

Figure 21. Comparative analysis of the α-amylase inhibitory peptide VrD1 and VrD2 lacking inhibitory activity. (A) Amino acid alignment of VrD1, VrD2 and the chimera peptide VrD2c. The amino acids responsible for the formation of the active peptide loop (L3) is indicated. (B-C) Tertiary structure comparison of VrD1 (B) and VrD2 (C). The positively charged residues important for the inhibitory activity of VrD1 (M36 and R38, in red) is indicated. The negatively charged residues of VrD2 (D37 and D38 in blue) occupying the active loop L3 inhibiting its activity with α-amylase (Lui et al., 2006; Lin et al., 2007).

Previous work done on VrD1 suggested that the length and charge of L3 is important for its inhibitory activity (Liu et al., 2006), suggesting a loop length of 3-5 amino acids, containing a positively charged residue as optimal (Figure 21B). However, mutational studies conducted on L3 of VrD2 to adjust its length and charge did not result in α-amylase inhibitory activity. Only by replacing L3 of VrD2 with L3 of VrD1, creating a chimera peptide (VrD2c), were the α-amylase inhibitory activity restored in VrD2 (Lin et al., 2007).

The inhibitory activity of VrD1 and VrD2c involves the direct insertion and interaction of L3 with the active site of TMA, thus blocking the site and preventing substrate binding (Figure 22). This interaction is dependant on the size of the loop and the interaction of the positively charged residues ARG38 and M36 in the case of VrD1 (Figure 21B) and VrD2c. Native VrD2 have no inhibitory activity because L3 is too short and the negatively charged amino acids, D37 and D38 occupy L3 (Figure 21B) (Lin et al., 2007).

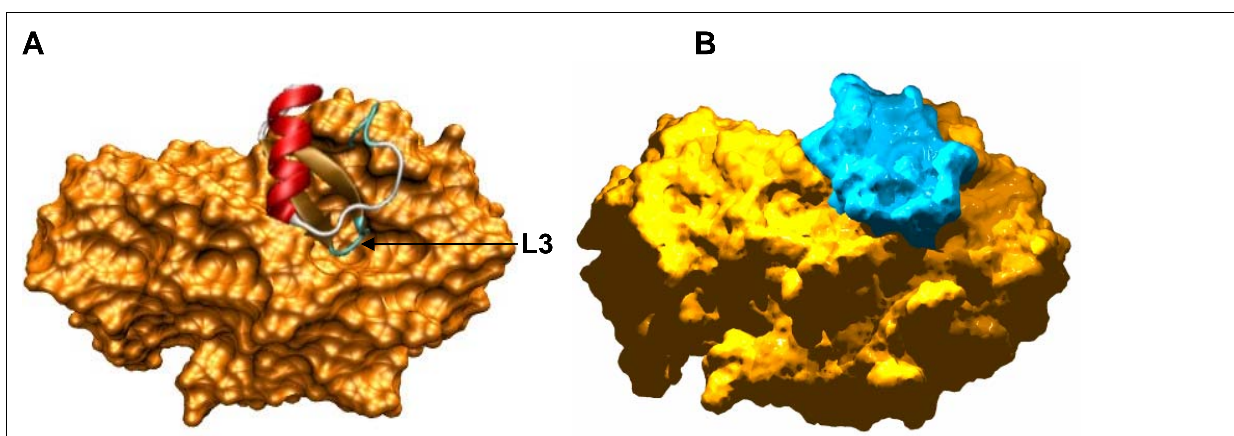

Figure 22. The interaction of VrD2c with the α-amylase from *Tenebrio molitor* larvae (TMA). (A) The orientation and interaction of VrD2c (represented as a ribbon structure) with the α-amylase TMA. VrD2c positions loop L3 to occupy the active site of TMA, thus blocking substrate binding. (B) A surface representation of the interaction of VrD2C (blue) with TMA (orange), showing the occupation of the TMA active site by VrD2c (Lin et al., 2007).

Only two plant defensin peptides with proteinase inhibitory activity have thus far been identified, namely Cp-thionin I, isolated from *Vigna unguiculata* (Melo et al., 2002), and CfD1 isolated from *Cassia fistula* (Wijaya et al., 2000). These two peptides share very little homology at amino acid level (Figure 23) and differ in the position of the amino acids involved in their inhibitory activity, although the amino acid lysine has been implicated in the inhibitory activity of both peptides. Of the two peptides Cp-thionin I has been best characterised in terms of its inhibitory activity. Cp-thionin I is a plant defensin of 47 amino acids and its interaction with bovine pancreatic trypsin (BPT) has been well studied (Melo et al., 2002).

```
CfD1           KTCEVLSGKFGGACSTIINGPKCDKTCKNQEHYISGTCKSD-FKCWCTKNC
CfD2           KTCEKPSKFFSGGCIGTTGNKQCDYLCRRGEGLLSGACKG--LKCVCTKAC
Cp-thionin I   RVCESQSHGFKGACT---GDENCALVCRN-EGFSGGNCRGFRRRCFCLTKC
```

Figure 23. Amino acid alignment created with ClustalX (Thompson et al., 1997) of the two defensins from *Cassia fistula* (CfD1 and CfD2) with Cp-thionin I from *Vigna unguiculata*. CfD1 and Cp-thionin I have proteinase inhibitory activity, whereas CfD2 has no proteinase inhibitory activity (Melo et al., 2002).

Inhibition and docking studies done with BPT revealed that the inhibition activity of Cp-thionin I is encoded within one amino acid, namely Lys11. Lys11 occupies the S1 active site of BPT, where it interacts with Asp189 of BPT, as part of a canonical style interaction. Docking studies have revealed that Lys11, although occupying the S1 site of BPT, is not close enough to Asp189 to directly interact with this amino acid and that the interaction of these two amino acids is facilitated by the presence of a water molecule a characteristic shared with bovine pancreatic trypsin inhibitor (Melo et al., 2002).

2.5 INDUSTRIAL IMPORTANCE OF THE PLANT DEFENSIN PEPTIDES

Over the last decade the economic potential of these peptides have been realized. Early studies have focused on the broad spectrum of antifungal activity exerted by these peptides and the possible impact they could have in protecting commercial crop species against fungal-borne diseases. To date various defensin peptides have been expressed in model plant systems, as well some commercial crop species in a effort to evaluate the potential of plant defensins to protect crops against fungal disease (Table VI).

The potential of plant defensins to protect crops using a biotechnology approach was realized in 2000 with the development of disease resistant potatoes by using a plant defensin (Gao et al., 2000). Gao and colleagues were able to confer resistance to potatoes towards the wilting disease-causing agent, *Verticillium dahliae* by overexpressing the alfAFP defensin from *Medicago sativa*. Levels of *V. dahliae* in the transformed potato plants were reduced six-fold compared to the untransformed wild-type potato plants. The protection conferred by the alfAFP transgene was not only observed under greenhouse conditions, but was also maintained in field trails over several years and in different geographical locations (Gao et al., 2000). Furthermore, the level of *Verticillium* wilt resistance in the transgenic potato plants was comparable or greater than, the level of resistance observed in the wild-type potato plants grown in fumigated, non-infested soil.

Table VI. Plant defensin peptides expressed in host plants for protection against biological pests.

Transgene	Source plant	Recipient plant(s)	Increased resistance against test organism(s)	Reference
Rs-AFP2	Radish	Tobacco	*A. longipes*	(Terras et al., 1995)
Rs-AFP2	Radish	Tomato, oil rape	*A. solani, F. oxysporum, P. infestans, R. solani, V.dahliae*	(Koike et al., 2002)
AlfAFP	Alfalfa	Potato	*V. dahliae*	(Gao et al., 2000)
DRR230-a *DRR230-c*	Pea	Tobacco	*F. oxysporum, A. pinodes, T. reesei, A. lentis, F. solani, L. maculans, A. pisi, A. alternata*	(Lai et al., 2002)
BSD1	Chinese cabbage	Tobacco	*P. parasitica*	(Park et al., 2002)
WT1	Wasabi	Rice Maize	*M. grisea*	(Kanzaki et al., 2002)

The successful development of wilting disease resistant potatoes was followed with the development of disease resistant rice in 2002 (Kanzaki et al., 2002) and 2003 (Kawata et al., 2003) (Figure 24) by using the defensins from wasabi and *Brassica campestris*, respectively.

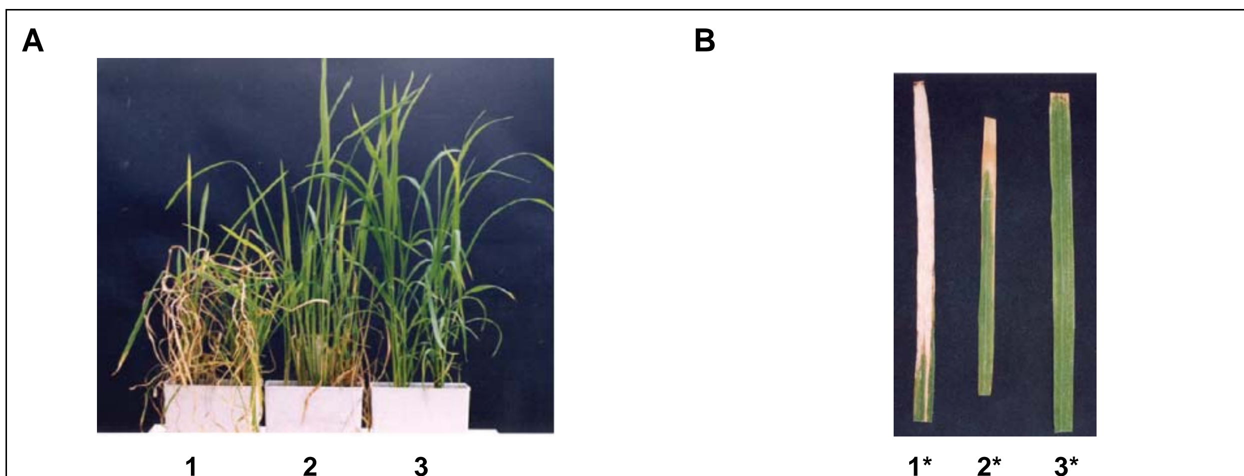

Figure 24. (A) The rice blast fungus and (B) leaf blight resistance phenotype observed in transgenic rice overexpressing the plant defensin from *B. campestris* (1 = WT; 2 and 3 transgenic lines; 1* = Wt, 2* = native Rs-AFP2 and 3* = mutated Rs-AFP2 (Kawata et al., 2003).

With the isolation and manipulation of plant defensins with insecticidal activity the possibility of creating insect resistant crops can not be excluded, but this trait have not yet been shown in transgenic crops.

With the identification of peptides with anti-cancer and anti-HIV activity and the ability to manipulate the spectrum of defensin activity through protein engineering these peptides are prime candidates for the design of new therapeutic drugs. This makes the isolation and characterization of plant defensins from new plant species important for the identification of defensins with new and unique spectrums of activity.

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Chapter 3

RESEARCH RESULTS

A PCR-isolation Strategy Yields 14 New Antifungal Peptide Encoding Genes from South African *Brassicaceae* species

Chapter 3 will be combined with data obtained from Addendum A and submitted for publication

RESEARCH RESULTS

A PCR-isolation Strategy Yields 14 New Antifungal Peptide Encoding Genes from South African *Brassicaceae* species

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3.1 ABSTRACT

Antifungal plant peptides and their encoding genes are abundant in most plant species. Although several classes of antifungal peptides have been identified, within the different classes high sequence homology and conservation of core motifs and structural features occur. Antifungal peptides have a recognized biotechnology potential and several promising peptides have been patented for various applications. South Africa has a vast and unique floral resource and this study focused on some native *Brassicaceae* spp. for the potential isolation of novel antifungal peptides. As a first screen, an enrichment purification strategy was used to determine if the small, basic, heat-stable peptide fractions from *Heliophila coronopifolia*, *Lunaria biennis* and *Lobularia maritima* seeds had any antifungal activities. From this screen, peptide fractions with a molecular weight between 5-6 kDa were obtained and shown to have antifungal activities characteristic of a peptide family known as plant defensins. A homology-based PCR strategy was implemented to isolate putative defensin encoding genes from the tested *Brassicaceae* spp. This method allowed isolation of 14 new plant defensin sequences from four genera of the *Brassicaceae* family present in South Africa. Bioinformatical analysis conducted on the deduced amino acid sequences showed that these peptides contained N-terminal signal peptides putatively directing the mature peptides to the apoplastic regions. The newly isolated peptides are predicted to be highly basic and have a molecular weight of ± 5 kDa. Phylogenetic analysis conducted on the deduced amino acid sequences revealed that all the new defensins share a close relationship to other *Brassicaceae* members of the plant defensin superfamily and was furthest removed from the defensins isolated from the *Solanaceae* and *Poaceae* families. Classification analysis of these peptides showed that they belong to subgroup A3 of the defensin superfamily. Members of this group are well known for their strong antifungal activity, but activities such as metal tolerance and inhibition of protein translation might also occur. The outcomes of the study confirmed that simple PCR amplification, based on the extensive sequence homology of antifungal peptides, is a useful means of isolating additional defensin genes. The unique genetic resources obtained in this study have entered our

biotechnology program where improved disease resistance of grapevine is the main aim.

3.2 INTRODUCTION

Plants are constantly challenged by their potential microbial pathogens, but infection and the subsequent establishment of disease is the exception rather than the rule. The effective combating of plant disease is the result of a plant defense system that has evolved over millennia in the constant arms race between pathogen and host. The interaction of a plant pathogen with its host can follow two routes: the interaction can either be compatible, resulting in disease, or be incompatible (Bell et al., 1986). In the incompatible interaction, the plant activates its defense systems to inhibit infection or limit the spread and damage of an established pathogen.

Some of the early responses include an oxidative burst associated with hypersensitive cell death (Dixon et al., 1994; Grant and Mansfield, 1999; Flors et al., 2005; Flors and Nonell, 2006; Flors et al., 2006), thus trapping the pathogen in an area of dead tissue, or strengthening of the plant cell wall in the areas surrounding the site of infection, thus inhibiting the spread of the disease (Ferreira et al., 2007). In addition to the early hypersensitive response, the plant also activates other chemical responses, which includes the production of phytoalexins, phytoanticipins, oxidized phenols and saponins (Bennett and Wallsgrove, 1994; Osbourn et al., 1994; Benhamou, 1995; Osbourn, 1996; Osbourn, 1999). An important component of the chemical defense response is the *de novo* synthesis of proteinaceous compounds (Nurnberger et al., 1997; Fellbrich et al., 2000; Kroj et al., 2003; van Loon et al., 2006). The proteinaceous component of the plant defense system, termed pathogenesis related proteins, includes proteinase inhibitors (Korsinczky et al., 2001; Melo et al., 2002; Zhao et al., 2002; Shatters et al., 2004), alpha amylase inhibitors (Bloch Jr and Richardson, 1991; Chagolla-Lopez et al., 1994; Liu et al., 2006; Shiau et al., 2006), and hydrolytic enzymes that are able to degrade the fungal cell wall (Stintzi et al., 1993; Zhu et al., 1994; Jongedijk et al., 1995; Vannini et al., 1999; Aziz et al., 2003). A major component of the proteinaceous defense system involves the production of low molecular weight cysteine-rich proteins with antifungal activity. Over the last 15 years nine different families of cysteine-rich peptides have been identified and research has shown that these peptides are major contributors to the innate immune system of plants (Thomma et al., 2002; Lay and Anderson, 2005).

Collectively the family of cysteine-rich peptides include plant defensins (Broekaert et al., 1995; Terras et al., 1995; Thomma et al., 2002; Lay and Anderson, 2005), thionins (Bohlmann and Apel, 1991; Florack and Stiekema, 1994; Broekaert et al., 1995), lipid transfer proteins (Molina et al., 1993; Garcia-Olmedo et al., 1995; Garcia-Olmedo et al., 1998), hevein-type peptides (Broekaert et al., 1990a; Choon Koo et al., 2002), knottin-type peptides

(Chagolla-Lopez et al., 1994; Garcia-Olmedo et al., 1998), MBP1 (Duvick et al., 1992), ibMBP (Tailor et al., 1997; Patel et al., 1998), snakins (Segura et al., 1999; Berrocal-Lobo et al., 2002) and plant cyclotides (Craik et al., 1999; Jennings et al., 2001; Trabi and Craik, 2004). This array of proteinaceous compounds can either be constitutively expressed, contributing to the preformed defense system, or be induced upon pathogen infection to contribute to the defensive barriers activated post-infection (da Cunha et al., 2006; van Loon et al., 2006; Ferreira et al., 2007).

In the search for alternatives to chemical fungicides, scientist have studied the possibility to manipulate the chemical defense system of plants to improve the resistance of crop species to pathogen attack (Cornelissen and Melchers, 1993; Shah, 1997; Dempsey et al., 1998; Broekaert et al., 1999; Choon Koo et al., 2002; Jeandet et al., 2002; Sawada et al., 2004). In this regard the small cysteine-rich peptides have huge potential since they show strong antimicrobial activity and unlike the organic chemical components of the defense system, is the result of a single gene product, thus having less energy requirements for production (Lay and Anderson, 2005).

Recent studies have shown that cysteine-rich peptides are highly represented within nucleotide databases containing information on 33 plant species, including the model plant *Arabidopsis thaliana* and crop plant *Oryza sativa* (Silverstein et al., 2007). It is predicted that cysteine-rich peptide encoding genes can contribute up to 3% of the total genetic material of a plant; in *Arabidopsis*, for example, more than 300 plant defensin genes have been identified (Silverstein et al., 2005).

The best characterized of the cysteine-rich peptide family is the plant defensins, which show a broad range of antifungal activity against necrotrophic, biotrophic and oomycete plant pathogens (Terras et al., 1993; Park et al., 2002; Solis et al., 2006). Plant defensins has been especially effective in combating plant pathogens in transgenic crops and are currently the only single gene products able to confer economical field trail resistance to commercial crop species (Gao et al., 2000; Kanzaki et al., 2002). This has led to huge interest in these small peptides by commercial biotechnology companies and these peptides are currently being isolated and patented at a rapid rate for the commercial sector.

This study set out to isolate novel defensin genes from South African plant species. In the end, the effort was directed at *Brassicaceae* spp., including the indigenous species *H. coronopifolia*, since this family has been shown to contain potent antifungal peptides (Terras et al., 1993; Terras et al., 1995; Kanzaki et al., 2002). After confirming that the peptide fractions from the targeted species indeed have defensin-like antifungal activities, a bioinformatical approach on available nucleotide sequences present in databases was used to implement a PCR amplification strategy. The outcome of this approach was that 14 new putative antifungal peptide encoding sequences were obtained and further characterized.

3.3 MATERIALS AND METHODS

3.3.1 Plant material and microbial strains

Seeds for *Lunaria biennis*, *Lobularia maritima* and *Matthiola incana* were obtained from Ball Straathof Seed Company, Stellenbosch, South Africa. Seeds for *Heliophila coronopifolia* were obtained from Silverhill Seed Company, Cape Town, South Africa. Plants for *L. maritima, M. incana* and *H. coronopifolia* were established from seeds in peat and were grown under greenhouse conditions of 25°C, 55% humidity and natural light. *Escherichia coli* strain DH5α was used in all routine cloning strategies. *Botrytis cinerea* was obtained from the Department of Plant Pathology, Stellenbosch University and maintained and prepared for spore production as described in Carstens et al. (2003).

3.3.2 Screening of South African *Brassicaceae* species for antifungal peptide activity

A selective extraction protocol was followed that enriched for the isolation of small, basic, heat-stable peptides. Ten gram seed material from *H. coronopifolia*, *L. biennis* and *L. maritima*, respectively, were ground to a fine powder in the presence of liquid nitrogen and extracted with 30 ml extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, 1.5% polyvinylpolypyrrolidone, 1 mM phenylmethylsulfonylfluoride, 2 mM thiourea) according to Terras et al. (1992). The supernatant was collected, heat treated at 80°C for 15 min and the denatured proteins removed by centrifugation at 10 000 rpm for 30 min. MES buffer pH 6.0 (Sigma, St Louis, USA) was added to the samples and the volume adjusted to 100 ml to give a final protein sample containing 50 mM MES, pH 6.0. The samples were loaded on a SP-Sepharose column (Amersham Biosciences, NJ, USA) equilibrated with 50 mM MES buffer pH 6.0 at a flow rate of 2 ml min^{-1} and eluted at 1 ml min^{-1} with a salt gradient of 0-1 M NaCl over a period of 100 min.

Twenty μl of each fraction obtained from the *H. coronopifolia* seeds were assayed for antifungal activity against *Botrytis cinerea* in a microplate assay as described by Broekaert et al. (1990b). Each fraction was assayed three times. For the fractions obtained from *L. biennis* and *L. maritima* only the peak with the highest protein concentration were assayed against *B. cinerea*. Microscopic photographs were taken after 48 h of incubation in the presence of crude peptide fractions.

3.3.3 Genomic DNA isolation and cDNA synthesis

Genomic DNA was isolated from *L. maritima, M. incana* and *H. coronopifolia* leaf material and from *L. biennis* seed material. Plant material was collected, flash frozen in liquid nitrogen and ground to a fine powder. One gram of tissue was extracted with 10 ml of extraction buffer according to Steenkamp et al. (1994).

cDNA was synthesized from the different plant organs of *H. coronopifolia*. Total RNA was isolated from roots, leaves, flowers, stems and seedpod material. Tissue was collected and ground to a fine powder in the presence of liquid nitrogen. Total RNA was extracted from 100 mg of plant tissue with Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, USA). cDNA was prepared from 1 μg of total RNA using the Superscript III cDNA synthesis Kit (Invitrogen, Carlsbad, USA).

3.3.4 Isolation of *Brassicaceae* defensin genes

Representative members of defensin genes isolated from the *Brassicaceae* family were identified in the NCBI Genbank database. The complete coding sequences of these genes were aligned with ClustalX (Thompson et al., 1997) (Figure 1). Areas with high levels of homology were identified and primers designed for these areas. SPDef-5' (5-ATGGCTAAGTTTGCTTCCATCAT-3') together with the degenerate reverse primer Def-3' (5-ACAWGGRAARTARCAGATACAHYTGTG-3') were designed to PCR amplify defensin genes present in the tested *Brassicaceae* species. These primers would recognise the complete coding region of the putative defensin sequences.

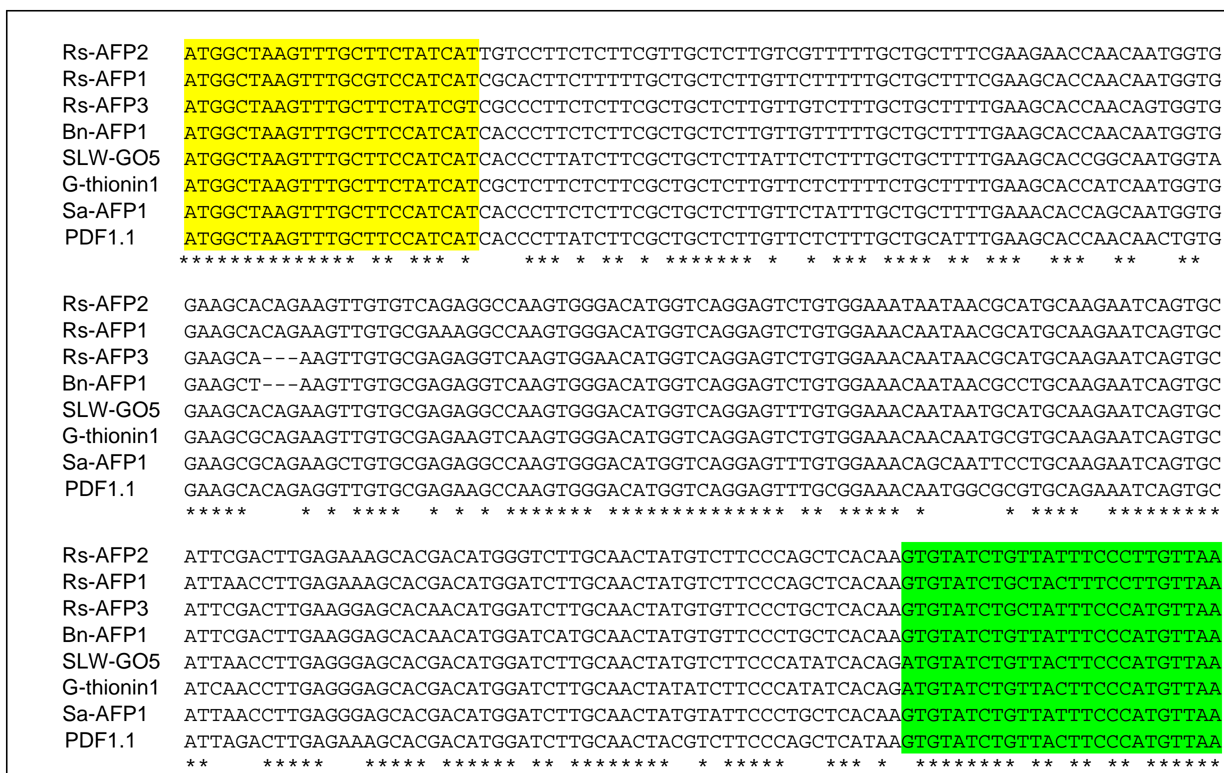

Figure 1. Alignment of nucleotide sequences of plant defensins from the *Brassicaceae* family (ClustalX, Thompson et al., 1997). *Rs-AFP1* (gb|U18557.1); *Rs-AFP2* (gb|U18556.1); *Rs-AFP3* (emb|X97319.1) from *Raphanus sativa*. *Bn-AFP1* (gb|U59459.1) from *Brassica napus*. *SLW-GO5* (gb|DQ226632.1) from *Boechera divaricarpa*. *G-thionin1* (dbj|AB012871.1) from *Wasabia japonica*. *Sa-AFP1* (gb|AY998243.1) from *Sinapis alba*. *PDF1.1* (gb|AY961376.1) from *Arabidopsis halleri*. * indicates homology and the positions of primers SPDef-5' and Def-3' are indicated in yellow and green, respectively.

Genomic DNA and synthesized cDNA served as template for the amplifications. PCR reactions were performed on 10 ng template DNA with the Expand high fidelity PCR enzyme (Roche Diagnostics GmbH, Mannheim, Germany) in 50 µl reactions (1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 µM SPDef-5', 2.5 µM Def-3'). PCR reactions were performed on a Biometric thermocycler with the following program: 94°C for 5 min; 94°C for 1 min, 53°C for 30 sec, 72°C for 1 min, 35 cycles; 72°C for 5 min. PCR products obtained were cloned into the pGEM-T easy vector (Promega Corporation, Madison, USA). Four colonies of each PCR isolation were sent for sequening. Sequences were analyzed using the BLASTN algorithm at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and clones showing homology to the superfamily of plant defensins were termed *Lb-AFPs* for *L. biennis*, *Lm-AFPs* for *L. maritima*, *Mi-AFPs* for *M. incana*, *Hc-AFPs* for *H. coronopifolia*.

3.3.5 Bioinformatical analysis of newly isolated defensin genes

In an effort to determine the intron splice sites of the newly isolated genomic defensin sequences it was subjected to intron splice site analysis using the SPIDEY software (http://www.ncbi.nlm.gov/spidey/) at NCBI, with Rs-AFP2 from radish serving as cDNA template (Terras et al., 1992). After intron splice site analysis the predicted mRNA coding sequences were assembled using Microsoft Word. The deduced coding sequences, together with the newly isolated cDNA defensin sequences from *H. coronopifolia* were subjected to sequences alignment analysis against other members of the defensin super family. Alignment analysis was performed with the alignment software ClustalX. Alignment results were also used to determine the phylogenetic relationship between the newly isolated defensin genes and existing members of the defensin super family. Phylogenetic trees were generated with Arbodraw (Canutescu and Dunbrack Jr, 2006).

Bioinformatical analysis of the predicted protein sequences, encoded by the newly isolated defensin nucleotide sequences, were achieved by converting the coding sequences into protein sequences with the translation tool at the Expasy proteomics server (http://us.expasy.org/tools/). The deduced amino acid sequences were subjected to alignment analysis against known protein sequences of the defensin super family, including other *Brassicaceae* peptides. Biochemical properties such as predicted molecular weight and isolelectric point were assessed with the protein analysis tool from Vector NTI 9.0 (Invitrogen, Carlsbad, USA). Predicted signal peptides and sub-cellular localization of the new defensin peptides was achieved with the Expasy tools SignalP and PSORT, respectively. Disulphide bridge prediction was preformed with DIpro (http://contact.ics.uci.edu/bridge.html).

3.4 RESULTS

3.4.1 Antifungal activity of *H. coronopifolia, L. biennis* and *L. maritima* seed extracts

The peptide enrichment method was very successful in isolating small antimicrobial peptide fractions from the seeds tested. Further purification, involving cation exchange chromatography of the heat-stable fractions of *H. coronopifolia* seed, yielded eight fractions with activity against *B. cinerea*, with the most active fraction showing 48% inhibition of *B. cinerea* hyphal growth (Figure 2). SDS-PAGE analysis revealed that this fraction contained a peptide of a molecular mass between 5 and 6 kDa (Figure 2).

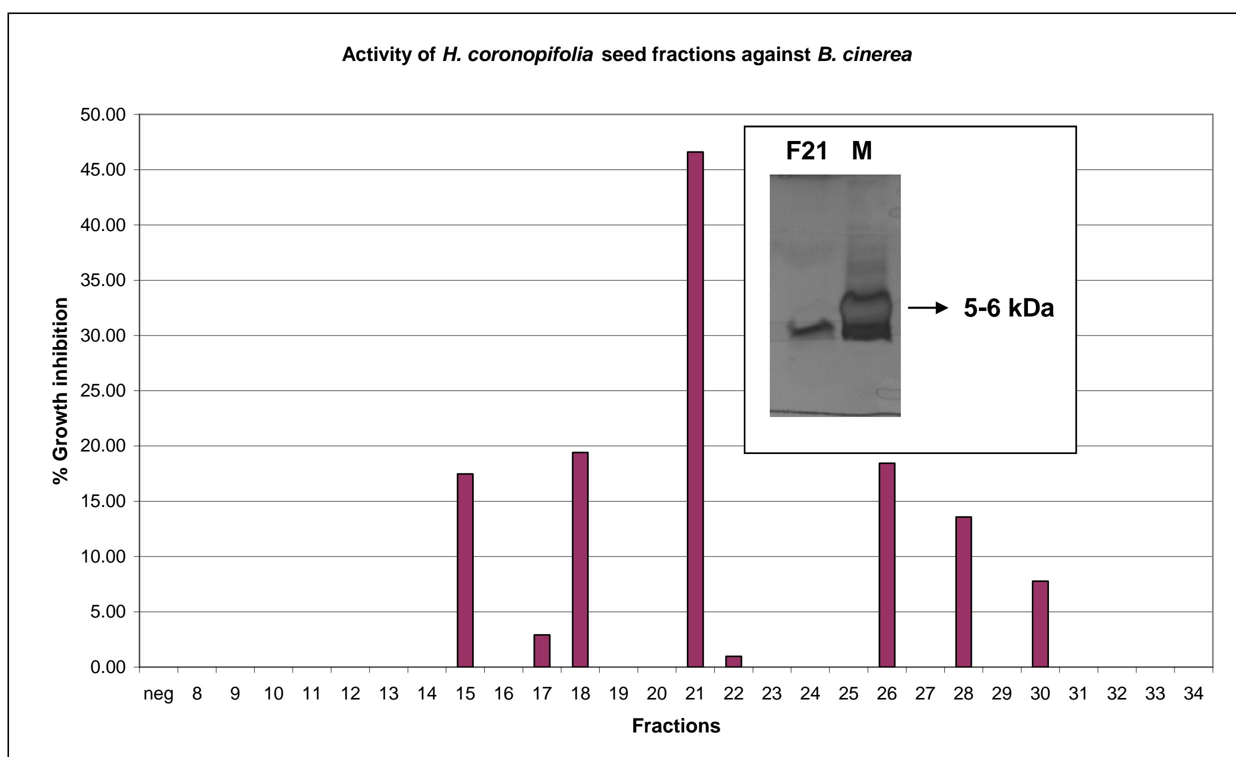

Figure 2. Antifungal activity of the different protein fractions obtained after cation exchange chromatography of the basic, heat-stable protein fraction from *H. coronopifolia* seeds. The insert (top right) shows the SDS-PAGE analysis of fraction 21, which caused a reduction of 48% in *B. cinerea* hyphal development, M = low molecular weight marker (Sigma, St Louis, USA).

Cation exchange chromatography conducted on the basic, heat-stable fraction from *L. biennis* yielded only a single peak (maximum absorbance, fraction 12) that eluted between 100 and 200 mM NaCl (Figure 3). Mass-spectrometry conducted on fraction 12 showed that it contained a mixture of peptides with molecular weights ranging from 5.6-5.8 kDa (Figure 3). The peptide with a mass of 5.6 kDa constituted the major peptide present in this fraction.

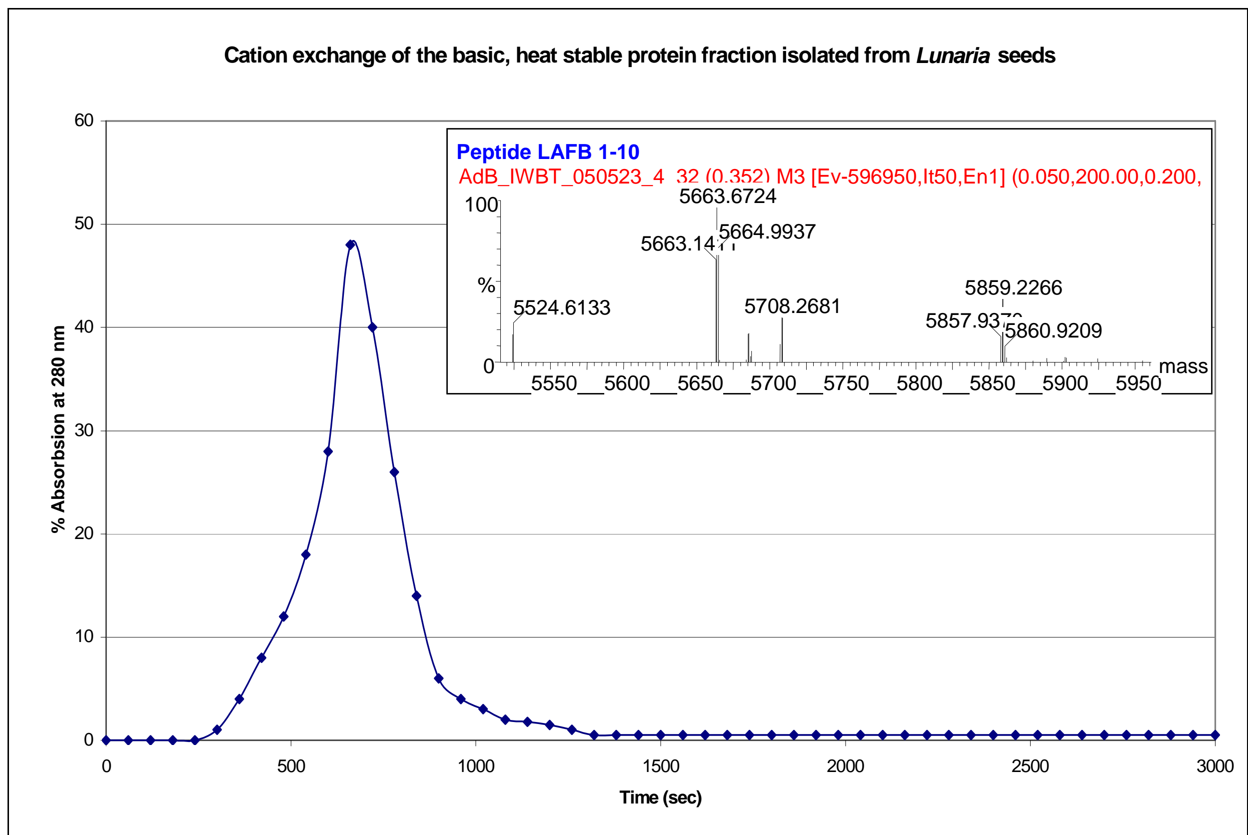

Figure 3. Cation exchange chromatography of the basic, heat-stable protein fraction from *L. biennis* seeds. A single peak eluted between 100 and 200 mM NaCl and was captured in fraction 12 of the elution profile. The insert (top right) is the mass-spectrometry analysis of fraction 12, which showed the presence of peptides with masses between 5-6 kDa.

Antifungal activity assays conducted against *B. cinerea* spores during a spore germination and hyphal development assay, revealed that the peptides from fraction 12 were able to inhibit the growth and development of *Botrytis* (Figure 4A). Fifty $\mu g\ ml^{-1}$ of this peak fraction was able to inhibit the growth of *B. cinerea* by 50%. Microscopical analysis revealed that the observed activity is associated with hyphal tip swelling and a reduction in the elongation of hyphae. No hyperbranching effect, typical of morphogenic antifungal peptides, was observed (Figure 4B).

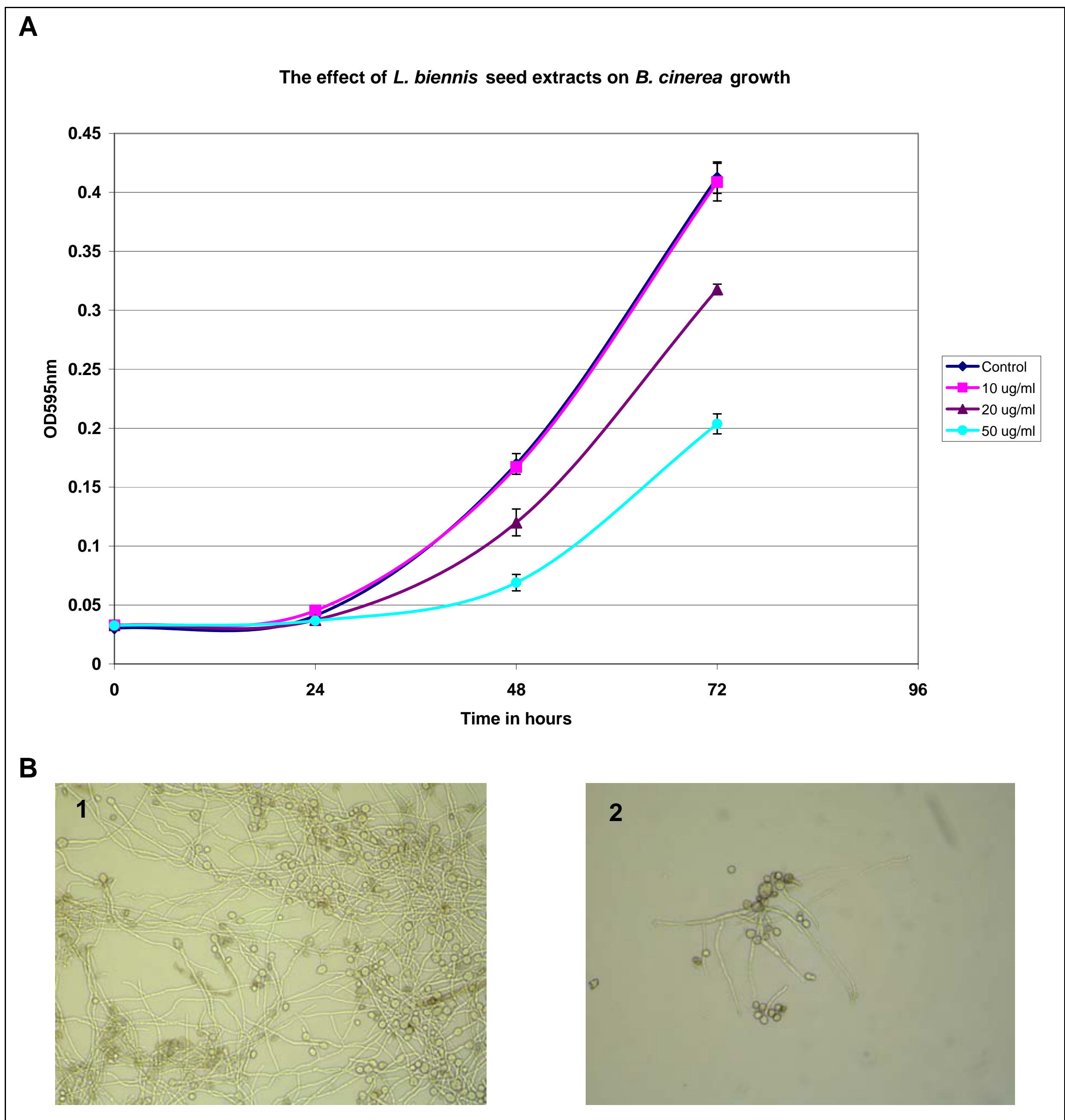

Figure 4. Antifungal activity of the major basic, heat-stable peptide fraction isolated from *L. biennis* on *B. cinerea*. (A) Growth and development of *B. cinerea* in the presence of increasing concentrations of fraction 12 peptides. 50% growth inhibition was observed at a concentration of 50 μg ml^{-1}. The analysis was based on a microtiter-plate assay as described by Broekaert et al. (1990) (B) Microscopical analysis of the effect of the peptide fraction on *Botrytis* after 48 h. (B1) The control reaction, showing *B. cinerea* development in the absence of any peptide. (B2) Severe inhibition of spore germination and hyphal development in the presence of 50 μg ml^{-1} of peptide fraction 12.

Cation exchange chromatography of the basic, heat-stable seed proteins isolated from *L. maritima* yielded a single peak that eluted with 100-200 mM of the salt gradient (Figure 5). SDS-PAGE analysis revealed that this peak contained peptides with a molecular mass of 5-6 kDa. This peptide fraction showed strong

antifungal activity at 30 μg ml^{-1} against *B. cinerea* (Figure 6A). Microscopical analysis revealed that the observed antifungal activity is associated with severe alteration of *B. cinerea* hyphal morphology. These peptides induced noticeable hyperbranching and leakage of the cytoplasmic components (Figure 6B).

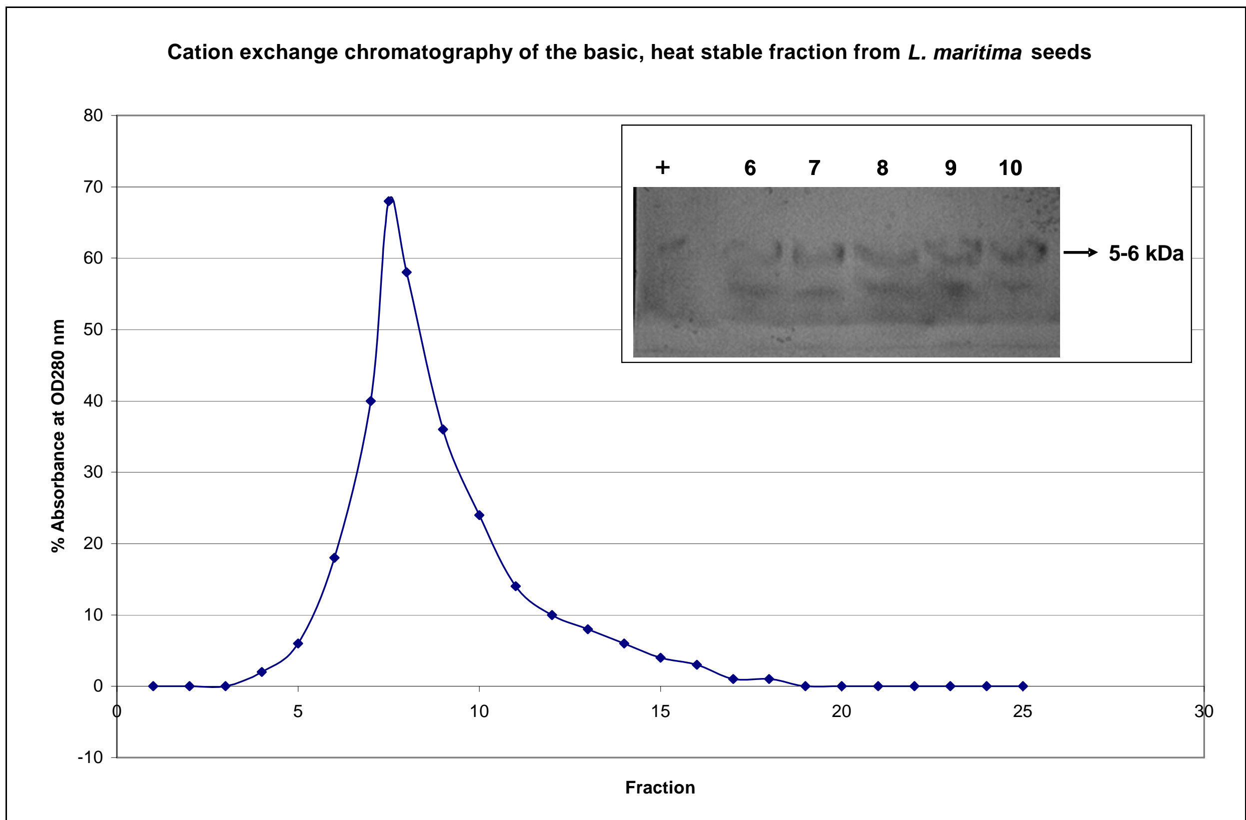

Figure 5. Cation exchange chromatography conducted on the basic, heat-stable protein fraction isolated from *L. maritima* seeds. The insert (top right) shows a SDS-PAGE analysis and silver-staining of a positive control and fractions 6-10. The sizes of the low-molecular marker are indicated.

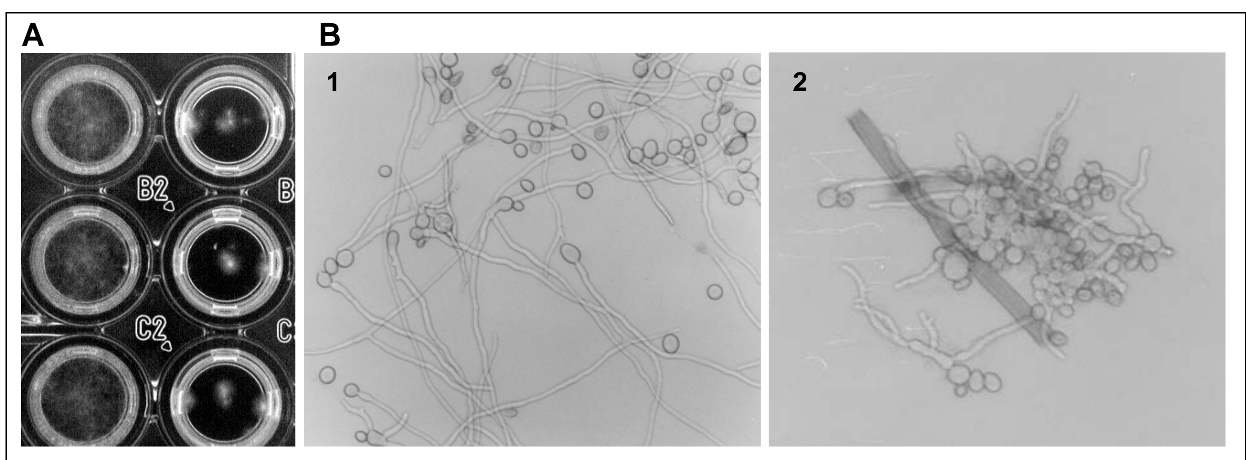

Figure 6. Antifungal activity of the basic, heat-stable peptides isolated from *L. maritima* seeds. (A) Activity of the peptides against *B. cinerea* in a microtiter-plate assay 48 h after inoculation (column 1 = no peptide, column 2 = peptides at 30 μg ml^{-1}; each column contains three technical repeats. (B) Microscopical analysis of the effect of the peptide fractions 6-10 on *B. cinerea* hyphae (B1) in the absence of peptide, and (B2) in the presence of 30 μg ml^{-1} peptide fraction.

3.4.2 Isolation and bioinformatical characterization of *Brassicaceae* defensin genes

The PCR-based gene isolation strategy allowed for the isolation of 14 new defensin genes from *Brassicaceae* species present in South Africa. The 14 new defensin sequences were distributed over all four *Brassicaceae* species tested. *L. maritima, M. incana* and *H. coronopifolia* yielded two, four and five new defensins respectively, whereas *L. biennis* yielded three new defensin sequences.

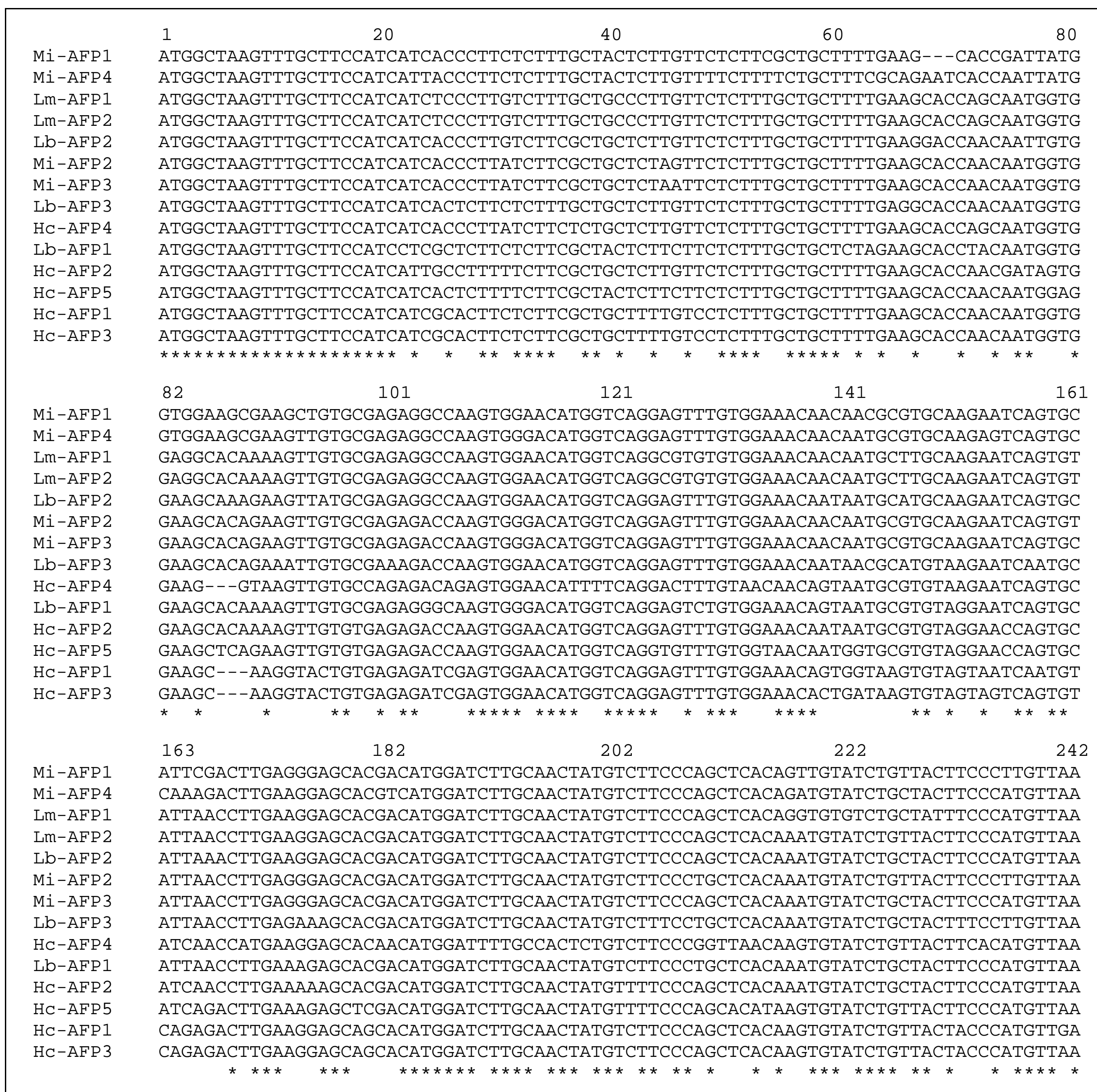

Figure 7. ClustalX (Thompson et al., 1997) alignment of the plant defensin nucleotide sequences isolated from the *Brassicaeae* species in South Africa. *Lb-AFPs* from *L. biennis*, *Lm-AFPs* from *L. maritima*, *Mi-AFPs* from *M. incana*, *Hc-AFPs* from *H. coronopifolia*. * Denotes homology.

Comparative analysis between the genomic sequences and the cDNA sequences isolated revealed that the nucleotide sequences encoding for the defensin signal peptide were interrupted by an intron at position 64 relative to the start codon.

This was found for all the genomic copies of the newly isolated defensins (see table I for summary of genetic characteristics). Alignment analysis of the newly isolated defensin coding sequences showed that the newly isolated defensin genes shared 56% homology at nucleotide level (Figure 7), compared to 48% when the sequences used to design the primers for isolation were included.

The phylogenetic tree created during the alignment of the new defensins with those used to design the primers showed that Lb-AFP1 and 3 were more closely related to the defensins from radish, while the peptides from *H. coronopifolia* mostly grouped with PDF1.1 from *Arabidopsis*; the exception was Hc-AFP4, which grouped with Sa-AFP1 from onion (Figure 8). Mi-AFP2 and 3 showed a close relationship to the *Boechera divaricarpa* defensin SLW-GO5 and the gamma thionin from *Wasabia japonica.* The rest of the new defensins showed no clear relationship to any of the other defensin genes used to design the primers and were more related to each other.

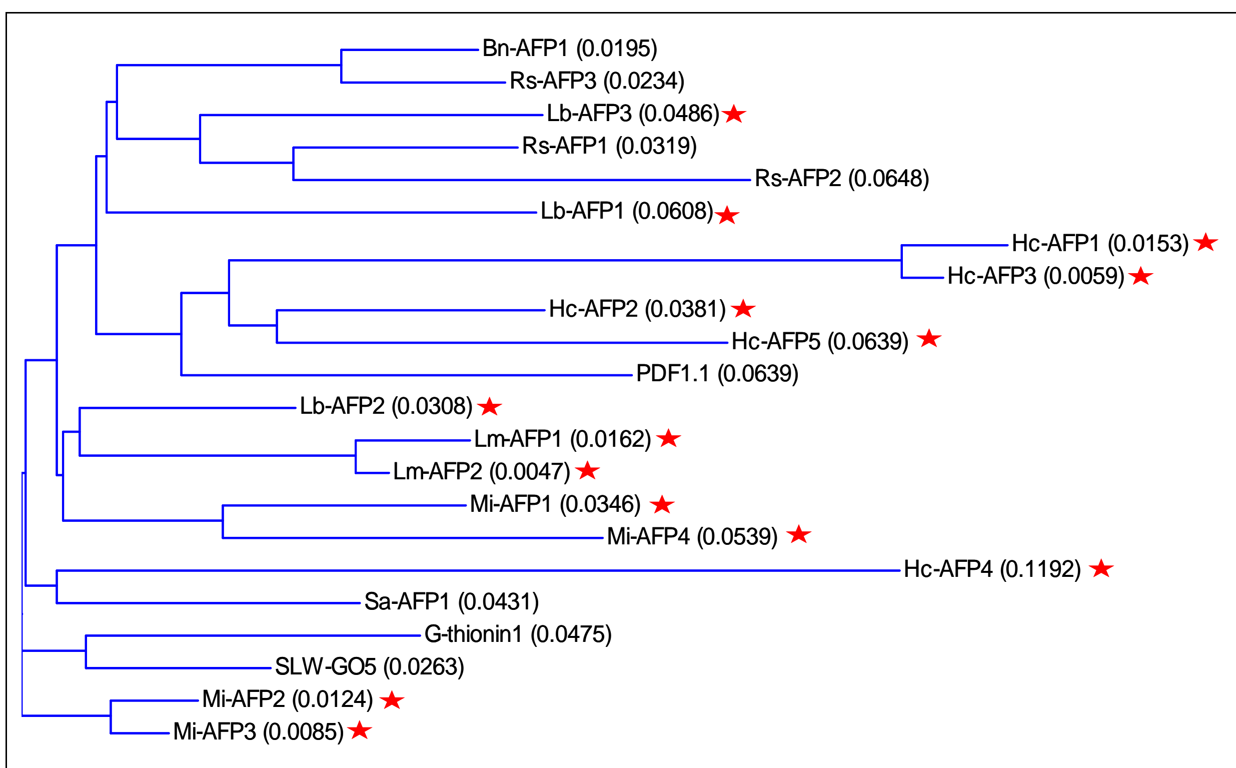

Figure 8. The phylogenetic relationship of the new defensins sequences (★) with the *Brassicaceae* defensins used to design the primers for defensin gene isolation. *Rs-AFP1* (gb|U18557.1); *Rs-AFP2* (gb|U18556.1); *Rs-AFP3* (emb|X97319.1) from *Raphanus sativa*. *Bn-AFP1* (gb|U59459.1) from *Brassica napus*. *SLW-GO5* (gb|DQ226632.1) from *Boechera divaricarpa*. *G-thionin1* (dbj|AB012871.1) from *Wasabia japonica*. *Sa-AFP1* (gb|AY998243.1) from *Sinapis alba*. *PDF1.1* (gb|AY961376.1) from *Arabidopsis halleri*. *Lb-AFPs* from *L. biennis*, *Lm-AFPs* from *L. maritima*, *Mi-AFPs* from *M. incana*, *Hc-AFPs* from *H. coronopifolia*.

Table I. Bioinformatical characterization of the newly isolated defensin sequences and their deduced amino acid sequences

		Genetic characteristics					**Predicted peptide characteristics**				
Plant species	**Defensin**	**Gene/size**	**Source Tissue**	**Sp[a] size**	**Intron size**	**Mp[b] size**	**Peptide (aa[c])**	**Sp (aa)**	**Mp (aa)**	**MW (Da)**	**pI**
H. coronopifolia	*Hc-AFP1*	cDNA: 240 bp	Stem	87 bp	none	153 bp	79	29	50	5482.57	8.50
	Hc-AFP2	gDNA: 331 bp	Nd[d]	87 bp	88 bp	156 bp	80	29	51	5722.19	8.73
	Hc-AFP3	cDNA: 240 bp	Leaf	87 bp	none	153 bp	79	29	50	5527.60	8.20
	Hc-AFP4	cDNA: 240 bp	Seedpod	87 bp	none	153 bp	79	29	50	5542.06	8.51
	Hc-AFP5	cDNA: 243 bp	Seedpod	87 bp	none	156 bp	80	29	51	5735.23	8.94
L. biennis	*Lb-AFP1*	gDNA: 635 bp	Nd	87 bp	392 bp	156 bp	80	29	51	5697.13	8.74
	Lb-AFP2	gDNA: 507 bp	Nd	87 bp	264 bp	156 bp	80	29	51	5637.17	8.90
	Lb-AFP3	gDNA: 370 bp	Nd	87 bp	127 bp	156 bp	80	29	51	5694.18	8.72
L. maritima	*Lm-AFP1*	gDNA: 359 bp	Nd	87 bp	116 bp	156 bp	80	29	51	5637.04	8.51
	Lm-AFP2	gDNA:359 bp	Nd	87 bp	116 bp	156 bp	80	29	51	5623.06	8.51
M. incana	*Mi-AFP1*	gDNA: 393 bp	Nd	87 bp	153 bp	153 bp	79	29	50	5495.91	8.51
	Mi-AFP2	gDNA: 340 bp	Nd	87 bp	97 bp	156 bp	80	29	51	5623.06	8.51
	Mi-AFP3	gDNA: 341 bp	Nd	87 bp	98 bp	156 bp	80	29	51	5623.06	8.51
	Mi-AFP4	gDNA: 349 bp	Nd	90 bp	106 bp	153 bp	80	30	50	5552.96	8.74

[a] Sp = Signal peptide

[b] Mp = Mature peptide

[c] aa = amino acids

[d] Nd = Not determined

Analysis of the deduced amino acid sequences of the new defensin genes revealed that all the peptides shared the common characteristics of defensin peptides (Table I). The peptides had an N-terminal signal peptide of 29 or 30 amino acids followed by a mature peptide of 50 or 51 amino acids. The deduced peptides had a predicted size of 5-6 kDa and are highly basic in nature with isoelectric points (pI) above 8. Prediction of sub-cellular localization placed the peptides in the apoplastic regions between plant cells.

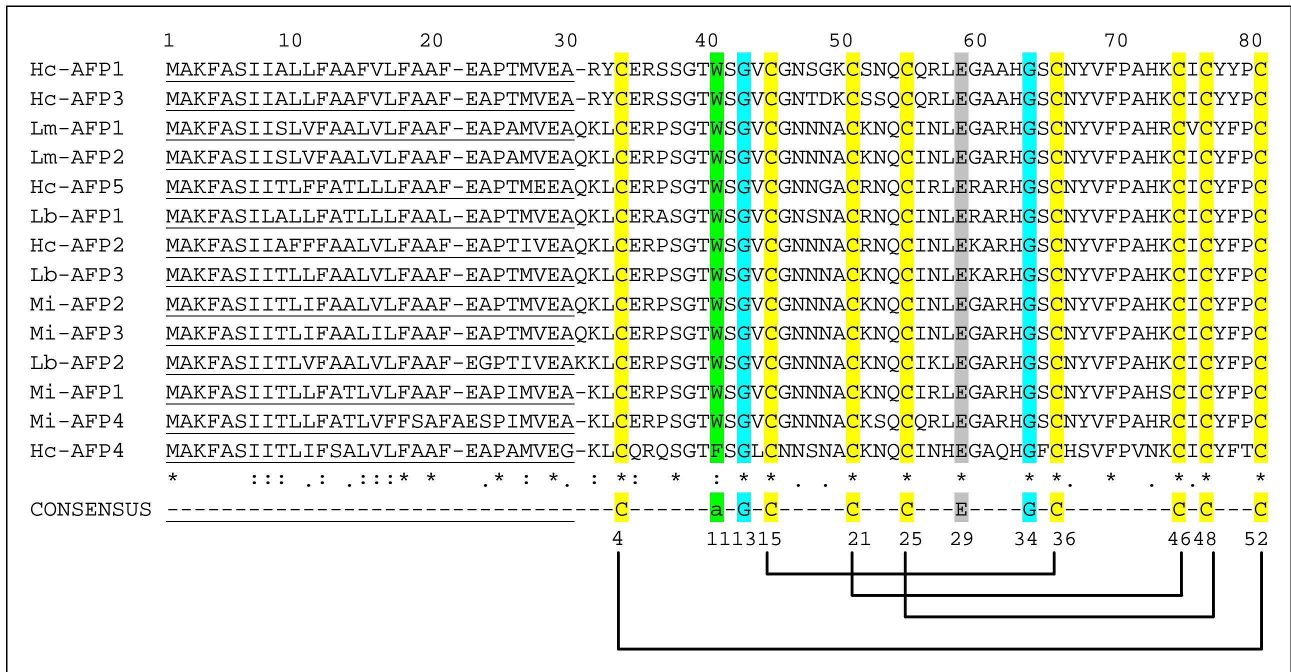

Figure 9. Alignment analysis (ClustalX, Thompson et al., 1997) of the newly isolated defensin sequences revealing the same pattern of conservation as observed for the defensin superfamily. The amino acid sequences of the N-terminal signal peptide are underlined. The predicted disulphide bridge formation as determined with DIpro is illustrated below the consensus sequence. Numbering of the consensus sequence is according to Rs-AFP1 (Terras et al., 1992).

Amino acid alignment showed that the new peptides shared 42.5% homology at the deduced amino acid sequences level and shared the conserved amino acids present in most defensin peptides (Figure 9). These included an aromatic residue at position 11, two glycine residues at position 13 and 34 respectively and a glutamate at position 29, together with the eight cysteine residues at positions 4, 15, 21, 25, 36, 46, 48, 52, present in all plant defensins (numbering according to the mature peptide of Rs-AFP1, (Terras et al., 1992). Disulphide bridge analysis done with DIpro predicted the same pattern of disulphide bridge formation as observed for Rs-AFP1 and 2, (Figure 9) with cysteine 4 linked to cysteine 52, cysteine 15 linked with cysteine 36, cysteine 21 linked to cysteine 46 and cysteine 25 bridged with cysteine 48 (numbering according to Rs-AFP1).

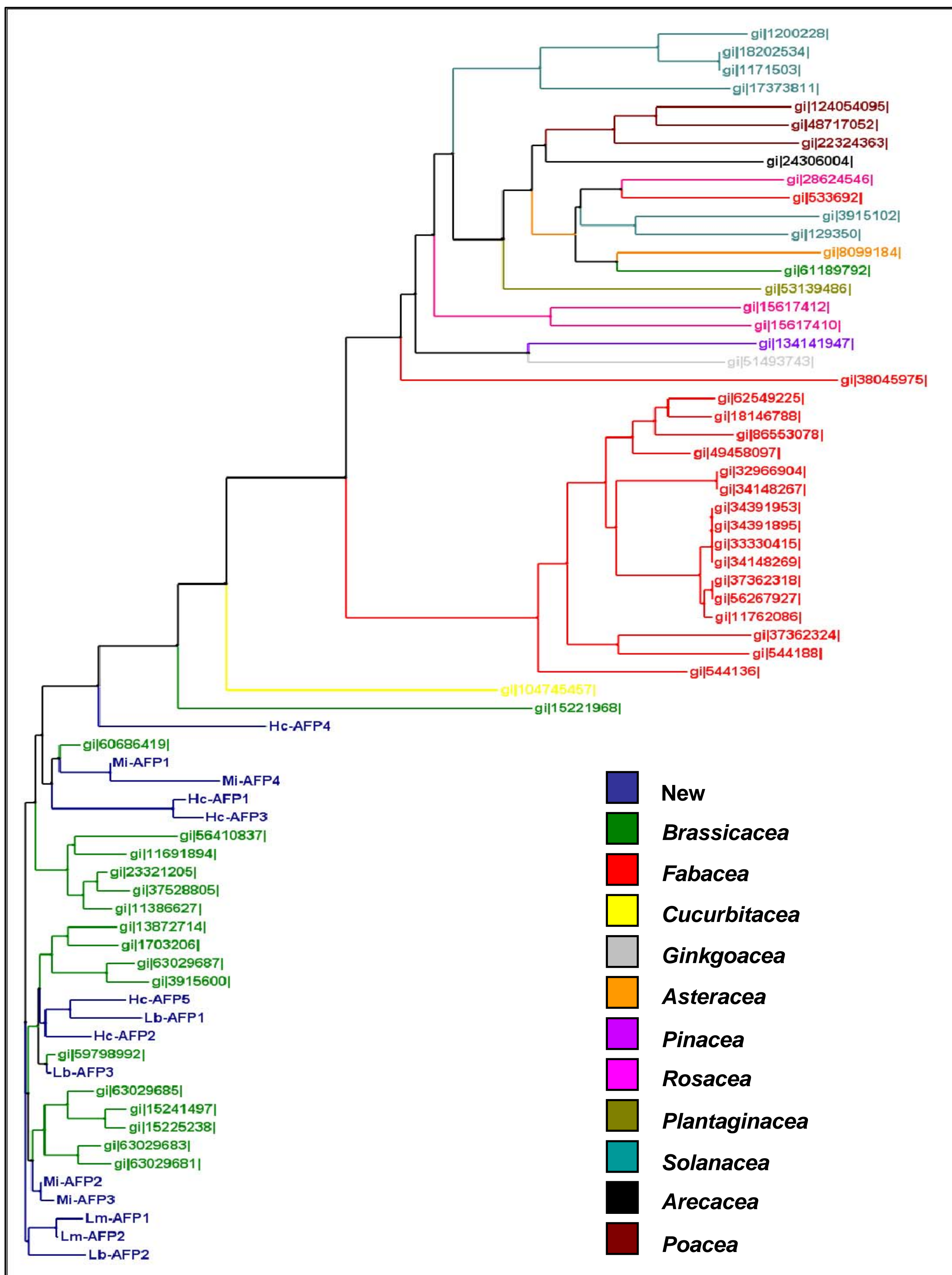

Figure 10. The phylogenetic relationship of the newly isolated deduced defensin peptide sequences. The phylogenetic tree was generated with ClustalX (Thompson et al., 1997) and edited in Arbodraw (Canutescu and Dunbrack Jr, 2006).

Alignment and subsequent phylogenetic analysis of the deduced amino acid sequences with other members of the plant defensin super family revealed that all the new defensins have a close relationship to other plant defensin members isolated from *Brassicaceae* and was furthest removed from the defensins isolated from *Solanaceae* and *Poaceae* (Figure 10). Comparison of the new defensins with defensins indicative of the defensin superfamily subgroups (Harrison et al., 1997), revealed that most of the defensins belong to subgroup A3, with Hc-AFP1, 3 and 4 showing some divergence and seem to fall in their own grouping (Figure 11), positioning themselves between subgroup A3 and subgroup A2.

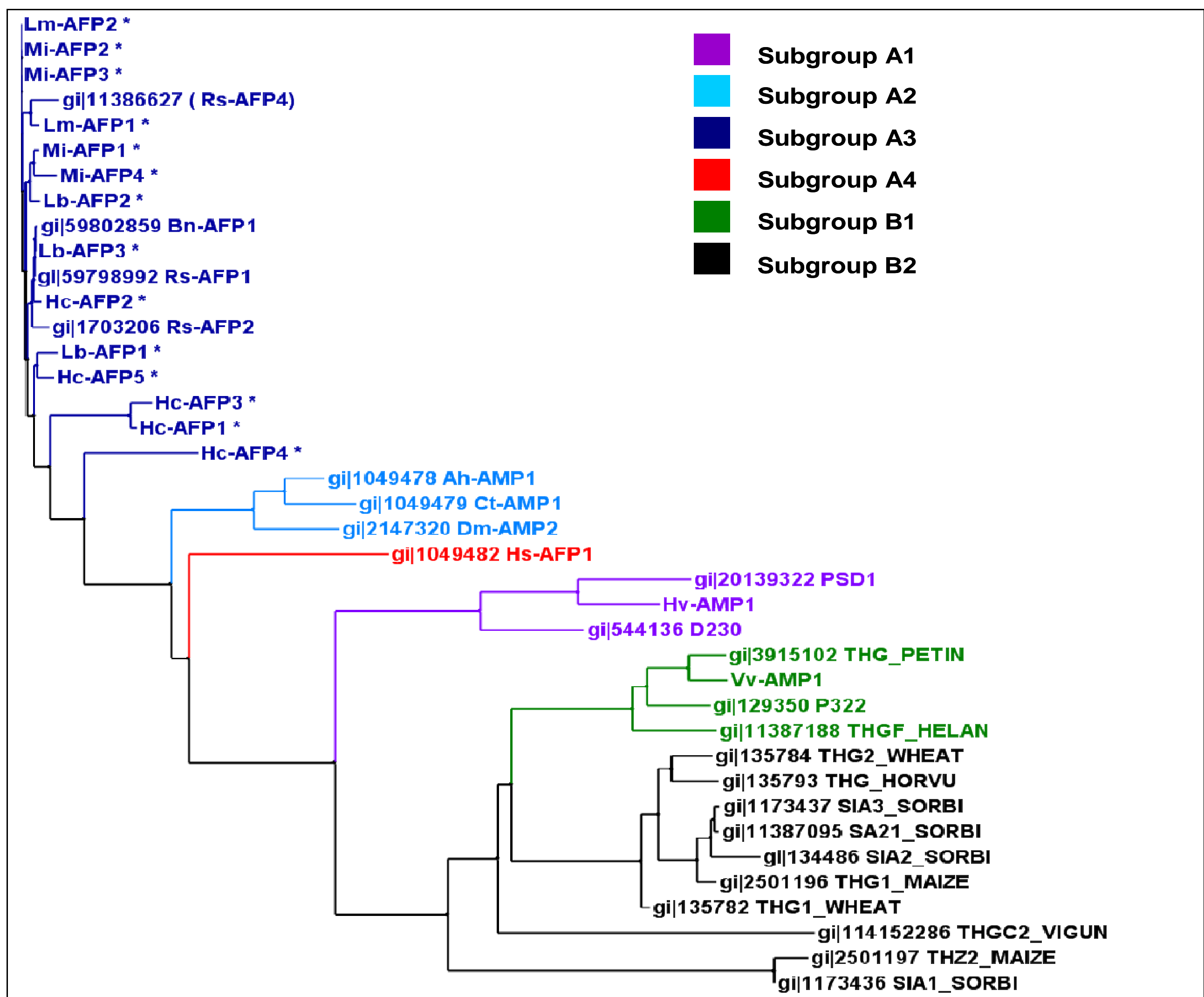

Figure 11. The phylogenetic relationship of the newly isolated defensins (marked with a *) with characterized members of the different defensin subgroups present in the defensin superfamily (Harrison et al., 1997). Subgroup A is indicated in shades of blue, red and purple whereas subgroup B is shown in green and black. The phylogenetic tree was generated with ClustalX (Thompson et al., 1997) and edited in Arbodraw (Canutescu and Dunbrack Jr, 2006).

Tertiary structure comparison between Rs-AFP1 (Fant et al., 1998) and the predicted structures of the new defensins, showed that the new defensins shared a similar tertiary structure than Rs-AFP1, consisting of a α-helix and a β-sheet comprising of three antiparallel β-strands (Figure 12).

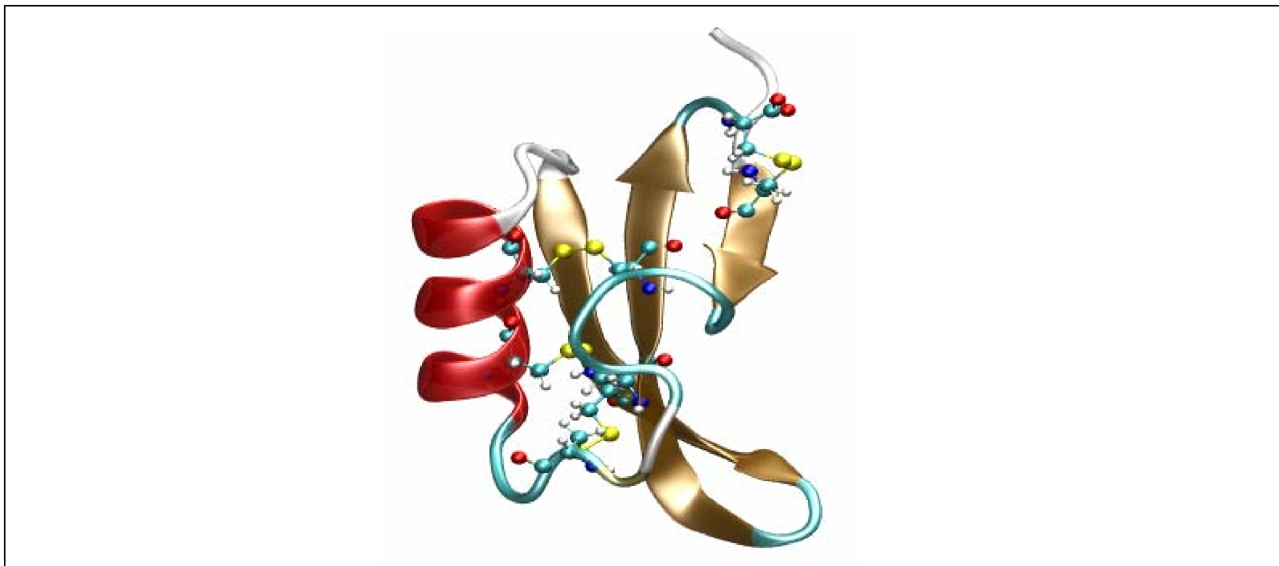

Figure 12. A three dimensional representation of Rs-AFP1 (PDB id 1AYJ) showing the characteristic structure of plant defensins (Fant et al., 1998). The α helix is indicated in red with the β-sheet indicated in brown. The internal disulphide bridges stabilizing the whole structure is indicated in yellow.

3.5 DISCUSSION

Small antifungal peptide encoding genes are very common in plant genomes (Silverstein et al., 2007). These peptides might be an evolutionary conserved mechanism in plant defense and although several classes of antifungal peptides exist, they share characteristic features that are conserved and linked to their activities (Harrison et al., 1997). *Brassicaceae* species present in South Africa were targeted as source material for novel antifungal peptide encoding genes. The strategy was to first perform a peptide-enrichment protein isolation from seed, favouring the isolation of heat-stable and basic peptides. These properties are characteristic of antifungal defensin peptides and the isolation strategy should eliminate most other PR-proteins activities. The method successfully isolated peptide fractions from three *Brassicaceae* species. The fractions contained peptides with molecular weights between 5-6 kDa, the typical sizes of most isolated plant defensin peptides and had antifungal activities against *B. cinerea*. Moreover, the antifungal activity screen also indicated that the peptide fractions displayed characteristic defensin-type activities, causing hyphal tip swelling, inhibiting hyphal elongation and giving rise to hyperbranching. The size of the

peptides coupled with their observed antifungal activities and induced morphological changes on fungal hyphae suggested that they could belong to the peptide family of plant defensins. These results warranted a search for defensin peptide encoding genes in the South African *Brassicaceae* spp.

The homology based PCR isolation method used identified 14 new putative defensin genes. The high level of conservation within the signal peptide encoding region of plant defensin genes allowed for the isolation of the complete coding sequences of these genes with one set of primers from various *Brassicaceae* genera. The successful amplification of defensin sequences from *H. coronopifolia* using this method confirmed the high level of conservation within the *Brassicaceae* family, specifically since this species has been shown to evolve independently from the other genera and is exclusive to the Western Cape region of South Africa. Sequence alignment of the newly isolated defensin sequences reveal 56% homology at nucleotide level, which translated into 42.5% homology at deduced amino acid level. Phylogenetic analysis conducted on the nucleotide sequences confirmed that these peptides are more closely related to other defensins isolated from the *Brassicaceae* family than those isolated from the *Solanaceae*, *Fabaceae* and *Poaceae* families, as could be expected.

Bioinformatical analysis of the deduced amino acid sequences suggest that these peptides share the basic characteristics as Rs-AFP1 and 2 isolated from *Raphanus sativus* (radish), with a signal peptide of between 29 or 30 amino acids and a mature peptide of 50 to 51 amino acids (Terras et al., 1992). Sub-cellular localization predictions suggest that the signal peptide region will direct the mature peptide towards the secretary pathway of plant cells depositing the mature region on the outside of the plant cells. All the new peptides are predicted to be highly basic with pI values above 8.2 and a molecular weight of ± 5 kDa. Alignment analysis of the deduced amino acid sequences revealed that these peptides shared the common consensus sequence present in most plant defensin peptides (Figure 9) and the common disulphide bridge pattern observed for the other defensin peptides.

Alignment analysis and subsequent phylogenetic analysis suggests that the newly isolated peptide sequences are restricted to subgroup A3 of the plant defensin superfamily, unlike *Arabidopsis thaliana*, also a *Brassicaceae*, which have peptides that are spread across subgroup A and B. Grouping of the newly isolated peptides with subgroup A3 predicts that these peptides will have a strong antifungal activity, characterized by extreme hyperbranching of the fungal hyphae and little or no antibacterial activity (Harrison et al., 1997). The predicted antifungal and morphogenic activities were observed for the peptide fractions isolated by the initial peptide enrichment purification, although these fractions and their activities might not be representative of (all) the isolated peptide sequences. Heterologous overexpression of the individual genes and in-depth functional and biochemical characterization will be

needed to conclude on the characteristics per putative defensin (see Addendum A and B for progress with these aspects). Other biological functions can not however be excluded, since plant defensins exhibit a wide variety of activities ranging from metal tolerance to anti-HIV activity (Lay and Anderson, 2005; Mirouze et al., 2006) all encoded within the amino acids of the loops connecting the α-helix and β-sheet structure of the defensin peptide (Zhu et al., 2005). Preliminary indirect findings could indicate that some of the peptides might have antibacterial activities, although this is not normally associated with peptides of the A3 subclass. Bacterial overexpression of some of the peptides consistently failed due to bacterial cell lysis (results discussed in Addendum A). It is interesting to note that it was the isolated peptides least restricted to the A3 subclass (Hc-AFPs) that caused severe cell lysis in the bacterial overexpressing cultures (see Addendum A). As mentioned previously, Hc-AFP1, 3 and 4 formed their own grouping between subclasses A2 and A3 (Figure 13), both groups known for their strong antifungal activity, but causing different morphological effects on fungal hyphae (Terras et al., 1993; Osborn et al., 1995; Harrison et al., 1997).

Analyses of genome and gene expression databases have revealed that cysteine-rich peptides can account for up to 3% of the active gene pool of model plant species like *Arabidopsis* and rice and their expression are overrepresented in reproductive structures of some of the 33 plant species present in genomic and expression databases (Silverstein et al., 2005; Silverstein et al., 2007). This suggests an important role for cysteine-rich peptides in general plant physiology. With 3200 species present within the *Brassicaceae* family, this isolation method would allow for quick screening of defensin coding regions with possible unique biological activities that can contribute to the fields of agricultural and medical biotechnology.

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Chapter 4

RESEARCH RESULTS

A Ripening Induced Gene from *Vitis Vinifera* Shows Sequence Homology to the Superfamily of Plant Defensins

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RESEARCH RESULTS

A Ripening Induced Gene from *Vitis Vinifera* Shows Sequence Homology to the Superfamily of Plant Defensins

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4.1 ABSTRACT

A berry specific cDNA sequence designated *Vv-AMP1*, *Vitis vinifera* antimicrobial peptide 1, was isolated from *Vitis vinifera*. *Vv-AMP1* encodes for a 77 amino acid peptide that shows sequence homology to the family of plant defensins. *Vv-AMP1* is expressed in a tissue specific, developmentally regulated manner, being only expressed in berry tissue at the onset of berry ripening and onwards. Treatment of leaf and berry tissue with biotic or abiotic factors did not lead to increased expression of *Vv-AMP1* under the conditions tested. The predicted signal peptide of Vv-AMP1, fused to the green fluorescent protein (GFP), showed that the signal peptide allowed accumulation of its product in the apoplast, most notably surrounding the guard cells of the stomata and the areas around the vascular stem tissue. Vv-AMP1 peptide, produced in *Escherichia coli*, had a molecular mass of 5.495 kDa as determined by mass-spectrometry. Recombinant Vv-AMP1 was extremely heat-stable and showed strong antifungal activity against a broad spectrum of plant pathogenic fungi, with very high levels of activity against the wilting disease causing pathogens *Fusarium oxysporum* and *Verticillium dahliae*. The Vv-AMP1 peptide did not induce morphological changes on the treated fungal hyphae, but instead strongly inhibited hyphal elongation, confirming that the peptide groups with the subfamily of non-morphogenic plant defensins. A propidium iodide uptake assay suggested that the inhibitory activity of Vv-AMP1 might be associated with altering the membrane permeability of the fungal membranes.

4.2 INTRODUCTION

Plants are constantly subjected to microbial attack, especially phytopathogenic fungi and use various defense strategies to protect themselves against disease. These defenses include the strengthening of the physical cell wall barriers (Dixon et al., 1994) and the production of chemical and proteinaceous antimicrobial compounds (Kuc, 1990 and 1992; Osbourn, 1996; Prost et al., 2005; van Loon et al., 2006). Over the last 15 years it has become evident that small, basic, cysteine-rich peptides also form part of the overall defense of plants against phytopathogens, contributing significantly to the innate immunity of plants (Bohlmann

and Apel, 1991; Thomma et al., 2002; Lay and Anderson, 2005; Stec, 2006). It has been suggested that all plants possess such a peptide defense system (Lay and Anderson, 2005). The peptides range from 2-9 kDa in size and are classified into nine families of which thionins and defensins have been the best characterized (Bohlmann and Apel, 1991; Terras et al., 1993; Florack and Stiekema, 1994; Broekaert et al., 1995; Osborn et al., 1995; Garcia-Olmedo et al., 1998; Thomma et al., 2002; Lay and Anderson, 2005). When first isolated, defensins were classified as γ-thionins, but were later renamed to plant defensins due to their structural and functional similarities to insect and human defensins (Terras et al., 1995; Aerts et al., 2005; Aerts et al., 2007).

Plant defensins are a family of basic, cysteine-rich peptides of between 45-54 amino acids in size. Structurally they consist of one α-helix and one β-sheet, comprising three antiparallel (-strands, and stabilized by the formation of disulfide bridges between the cysteine residues (Fant et al., 1998; Almeida et al., 2002; Janssen et al., 2003; Liu et al., 2006). Although plant defensins are structurally conserved, their overall homology at the amino acid level is low. However, all plant defensins contain eight cysteine residues linked by four disulfide bridges, an aromatic residue at position 11, two glycines at positions 13 and 34 and a glutamate at position 29 (numbering according to Rs-AFP1 Terras et al. (1995)).

Most plant defensins exhibit some antimicrobial activity, inhibiting the growth of fungi, oomycetes and Gram positive bacteria *in vitro*. The exact mechanisms underlying the antifungal activity exerted by plant defensins is not known, but there is evidence that plant defensins bind to a specific receptor in the fungal membrane, rather than random binding and integration into the phospholipid bilayer of the fungal membranes (Thevissen et al., 1997; Thevissen et al., 2000a; Thevissen et al., 2000b; Im et al., 2003; Thevissen et al., 2003; Thevissen et al., 2004; Thevissen et al., 2005; Aerts et al., 2006). Other biological activities such as proteinase and α-amylase inhibition (Bloch Jr and Richardson, 1991; Melo et al., 2002; Liu et al., 2006), metal tolerance (Mirouze et al., 2006), as well as the inhibition of protein translation and HIV proliferation have also been reported for some of the isolated plant defensins (Wong and Ng, 2003, 2005a, 2005b).

The majority of defensins have been isolated from plant seeds, (Osborn et al., 1995; Almeida et al., 2000; Chen et al., 2002; Wong and Ng, 2003; Song et al., 2004; Chen et al., 2005; Song et al., 2005; Wong and Ng, 2005a and 2005b), but defensins have also been isolated from leaves (Kragh et al., 1995; Terras et al., 1995), flowers (Karunanandaa et al., 1994; Urdangarin et al., 2000; Park et al., 2002; Tregear et al., 2002; Lay et al., 2003), tubers (Moreno et al., 1994), seedpods (Almeida et al., 2000), as well as from fruits (Meyer et al., 1996; Oh et al., 1999; Wisniewski et al., 2003). Although plant defensins play an important role in the preformed defense, some members of the defensin family are also upregulated upon pathogen attack or by environmental stimuli, while the expression of others are strictly developmentally regulated (Meyer et al., 1996;

Manners et al., 1998; Oh et al., 1999; van den Heuvel et al., 2001; Ahn et al., 2002; Koike et al., 2002; Hanks et al., 2005; Mirouze et al., 2006; de Zélicourt et al., 2007).

Here we report the isolation and characterization of the first plant defensin from *Vitis vinifera.* The peptide encoding gene shows a strict tissue-specific and developmentally regulated expression pattern. The antifungal activity and inherent characteristics of the peptide were analyzed subsequent to heterologous overexpression and purification.

4.3 MATERIALS AND METHODS

4.3.1 Plant materials and microbial strains

Escherichia coli strain DH5α were used for all cloning experiments, while *E. coli* strain BL21 (Rosetta-gami pLys S) DE3 (Novagen (Madison, WI, USA) were used for recombinant protein production. *Botrytis cinerea*, *Fusarium oxysporum* and *Fusarium solani* were obtained from the Department of Plant Pathology (DPP), Stellenbosch University. *Alternaria longipes* (ATCC 26293), *Fusarium oxysporum* (ATCC 10913) and *Verticillium dahliae* (ATCC 96522) were obtained from the American Type Culture Collection. All of the above fungal strains were maintained on potato dextrose agar at 25°C until sporulation. Tobacco seeds were obtained from Lehle Seeds, Round Rock TX 78681, USA and tobacco plants were maintained on Murashige Skoog (MS) medium (Murashige and Skoog, 1962) in a growth room with a temperature of 25°C and a 16 h photoperiod. Pinotage berry material were collected from farms in the Stellenbosch area. Pinotage leaf material was obtained by budding dormant cane material in a growth room at 25°C and a 16 h photoperiod.

4.3.2 Primer design and defensin gene isolation

The EST database of *V. vinifera* at TIGR (www.tigr.org) was screened using the BLAST algorithm. The database was screened with the γ-thionin sequence (PPT, gb|L27173.1) from *Petunia inflate* (Karunanandaa et al., 1994). Primers were designed from the EST clone TC69032 to recognize the complete coding sequence encoded within the EST.

Total RNA was isolated from 1 g *V. vinifera* cv. Pinotage root, leaf and berry tissue using a sodium perchlorate method (Davies and Robinson, 1996). cDNA was synthesized from total RNA using the SuperscriptIII cDNA synthesis kit (Invitrogen, Carlsbad, USA).

Genomic DNA was isolated from Pinotage leaves. Leaf tissue was collected, flash frozen in liquid nitrogen and ground to a fine powder. One gram of tissue was extracted with 10 ml of extraction buffer according to an established method (Steenkamp et al., 1994).

The genomic and cDNA isolated from *V. vinifera* cv. Pinotage were used as template in a PCR strategy to isolate possible *Vitis* defensin sequences. The primer

set used was, forward primer Vitisdef-5′ (5′-GGCTCGAGATGGAAGGGCTCTCAA-CGTT-3′) together with the reverse primer Vitisdef-3′ (5′-CCGGATCCTTAACAA-TGCTTAGTGC-3′). PCR products obtained were cloned into the pGEM-T easy vector (Promega Corporation, Madison, USA) and sent for sequencing. Sequences obtained were analyzed using the BLAST algorithm and clones showing homology to the defensin family were termed *Vv-AMPs*.

4.3.3 Southern blot analyses of *Vv-AMP1*

Genomic DNA was isolated from *V. vinifera* cv. Pinotage as described above. Pinotage genomic DNA was digested with *Bam*HI, *Eco*RV, *Kpn*I, *Spe*I or *Xba*I and separated on a 0.8% [w/v] agarose TAE gel. After transfer to a nylon membrane (Sambrook J et al., 1989), the membrane was probed with a DIG-labeled cDNA probe of *Vv-AMP1*. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany). Each hybridization signal represents a single copy of *Vv-AMP1*.

4.3.4 Sequence analysis of *Vv-AMP1* within the *Vitis* genus

Germplasm of other *Vitis* species were obtained from the USDA-ARS National Clonal Germplasm Repository (Davis, CA 95616, USA). These included genomic DNA for *V. afganista, V. x andersonii, V. aestivallis, V. cinerea* var. floridana*, V. labrusca* and *Ampelopsis aconitifolia* var. galabra. Genomic copies of *Vv-AMP1* were isolated from the various germplasms using the same PCR based strategy to isolate the genomic copy of *Vv-AMP1* from Pinotage. Isolated genes were cloned into the pGEM-T easy vector and sequenced. Genomic sequences obtained for the different *Vitis* species were analyzed with the AlignX software from the VectroNTI suite (Invitrogen, Carlsbad, USA) and final alignments were created in ClustalX (Thompson et al., 1997).

4.3.5 Expression pattern of *Vv-AMP1*

Total RNA was isolated from *V. vinifera* cv. Pinotage leaves, flowers and the different developmental stages of berry ripening. Tissue was collected and ground in liquid nitrogen to a fine powder. Hundred mg tissue from leaf, flower and green berry tissue was extracted with 800 μl extraction buffer at 65°C (2% [w/v] CTAB, 2% [w/v] PVP-40, 100 mM Tris/HCl (pH 8.0), 25 mM EDTA, 2.0 M NaCl, 2% [v/v] β-mercaptoethanol and 0.5 mg ml^{-1} Spermidine) for 5 min (Chang et al., 1993). Total RNA from véraison to ripe berries was isolated with the sodium perchlorate method (Davies and Robinson, 1996).

Total RNA was separated on a 1.2% [w/v] agarose formaldehyde gel (QIAGEN RNA/DNA handbook) and transferred to positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) (Sambrook et al., 1989). Membranes were probed with a DIG-labeled *Vv-AMP1* cDNA probe and the Elongation factor1 alpha

(*EF-1α*) gene from *V. vinifera* (TC65250) as internal standard. Pre-hybridization and hybridization were performed at 50°C. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany).

For analysis of the chemical induction of *Vv-AMP1*, *V. vinifera* leaves were floated on 10 mM salicylic acid, 5 mM jasmonic acid, 300 mM NaCl or 5 mM abscisic acid. Material was collected after 1, 6, 16 and 24 hours after each induction experiment and frozen in liquid nitrogen. Induction of *Vv-AMP1* by wounding and *B. cinerea* infection was also assessed on leaf material. To evaluate the effect of wounding leaves were subjected to mechanical damage and floated on distilled water in Petri dishes. Leaf material was collected and frozen in liquid nitrogen 1, 6, 16 and 24 hours after the initiation of the wounding experiment. *B. cinerea* infection was achieved by submerging leaves in a spore suspension of 50% grape juice containing 2000 spores ml^{-1}. After inoculation leaves were placed in Petri dishes under conditions of 100% relative humidity and incubated at room temperature. Material was collected 6, 12, 24 and 48 hours after inoculation and frozen in liquid nitrogen. All induction experiments were repeated three times, the material pooled and subjected to two separate RNA extractions. RNA isolation and northern blot analysis were performed as described above.

4.3.6 Bioinformatical analysis of the deduced amino acids sequence of *Vv-AMP1*

The deduced amino acid sequence of *Vv-AMP1* were produced in VectorNTi and analyzed using the BLASTP algorithm (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Homologous sequences identified were further aligned using ClustalX (Thompson et al., 1997). The deduced Vv-AMP1 sequence was also subjected to disulfide bridge analyses using DIpro (http://contact.ics.uci.edu/bridge.html) and secondary structure analysis as well homology modeling were done using the software package LOOPP @ CBSU version 3.0 (http://cbsuapps.tc.cornell.edu/loopp.aspx). PDB files obtained were further analyzed using the software package VMD (Visual Molecular Dynamics) and final images were rendered using POV-Ray. Sub-cellular localization directed by the Vv-AMP1 signal peptide was predicted on the Proteome Analyst Specialized Sub-cellular Localization Server (PA-SUB, http://www.cs.ualberta.ca/%7Ebioinfo/PA/Sub/index.html). Peptide mass prediction was done with the Expasy tool, PEPTIDE-MASS (http://us.expasy.org/tools/peptide-mass.html).

4.3.7 Confirming the sub-cellular localization directed by the Vv-AMP1 signal peptide

In an effort to confirm the sub-cellular targeting predicted by PA-SUB, the signal peptide from Vv-AMP1 was fused to the Green Fluorescent Protein (GFP) reporter protein. The signal peptide from Vv-AMP1 was isolated by PCR using the primer set

Vitisdef-5′ and VitisSP-3′ (5′-AAGCTTAGCCTCAGCCACCATCGG-3′) and cloned into pGEM-T easy vector to yield pGEM-VvSP. The signal peptide was excised from pGEM-VvSP with *Xho*I and *Hin*dIII and cloned into the restriction enzyme prepared plant expression vector pART27 cassette, a pART27 vector (Gleave, 1992) containing the expression cassette from pART7 cloned into the *Not*I sites of pART27. The construct was termed pART27-VvSP.

A modified mGFP5 gene was prepared by PCR from the clone pLMNC92 obtained from the Arabidopsis Biological Resource Center (ABRC). The forward primer PIV2GFP5-5′ (5′-CCAAGCTTGTAAGTTTCTGCTTCTACCTTTGA-3′) and reverse primer PIV2GFP5-3′ (5′-GCCTCTAGATTATTTGTATAGTTCATCCATGC-3′) were used to PCR the GFP sequence from the plasmid pLMNC92. This GFP sequence lacked the N-terminal endoplasmic reticulum signal peptide as well as a C-terminal HDEL endoplasmic reticulum (ER) retention signal. The PCR product was cloned into pGEM-T easy vector to yield pGEM-GFP5. The GFP fragment was excised from pGEM-GFP5 with *Hin*dIII and *Xba*I and cloned into the restriction prepared pART27-VvSP vector to yield the plant expression vector pARTVvSP-GFP, which would allow for the expression of the Vv-AMP1 signal peptide fused to GFP, under control of the constitutive 35S cauliflower mosaic virus promoter.

The pARTVvSP-GFP construct was transformed into *Agrobacterium tumefaciens* strain EHA105 via electroporation (Mattanovich et al., 1989) and tobacco was transformed using a standard leaf disc method (Horsch et al., 1985). Plantlets were regenerated under kanamycin selection on MS medium and positive plantlets were identified by their fluorescent emissions after GFP excitation on a dark reader (Clare Chemical Research, CO, USA). Hand sections were prepared and mounted in MS salt solution containing 40% [v/v] glycerol. GFP localization was visualized under an Olympus IX 81 inverted microscope. Images were captured and processed with the CelliR® camera and software system (Olympus Soft Imaging Solutions GmbH).

4.3.8 Recombinant production of Vv-AMP1

The pGEX-2T system (Amersham Biosciences, NJ, USA) was used for the recombinant production of Vv-AMP1 in *E. coli*. This system would allow for the production of mature Vv-AMP1 peptide fused to a GST-tag. pGEM-Vv-AMP1 served as template to prepare the mature Vv-AMP1 sequence by PCR. This was achieved with the primer set Vv1-GST-5′ (5′-GGCCGGATCCAGGACCTGTGA-GAGTCAGAGCCACCG -3′) and Vitdef-3′. The resulting product was cloned into the pGEM-T easy vector and termed pGEM-GSTVv1 mature. The mature fragment was digested from pGEM-GSTVv1 with *Bam*HI and *Eco*RI and cloned into the *Bam*HI and *Eco*RI prepared pGEX-2T vector. Positive clones were sequenced and termed pGEX-Vv1.

pGEX-Vv1 was transformed into the BL21 (Rosetta-gami pLysS) DE3 (Novagen, Madison, WI, USA) and positive colonies were selected by plating onto LB

agar containing 50 μg ml^{-1} ampicillin, 12.5 μg ml^{-1} tetracyclin, 15 μg ml^{-1} kanamycin sulphate and 34 μg ml^{-1} chloramphenicol. A single colony was inoculated into 5 ml LB medium with antibiotics and grown overnight at 37°C. One ml preculture was inoculated into four 1 l Erlenmeyer flasks containing 400 ml LB medium with antibiotics and grown at 37°C with continuous shaking until an OD_{600} of 0.7. Expression of the GST-Vv1 fusion was induced with 0.4 mM IPTG for 5 hours at 22°C.

Bacterial pellets were collected from each flask by centrifugation and resuspended in 5 ml GST column binding buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM $NaCl_2$ pH7.6 and 4 mM PefaBloc from Roche Diagnostics GmbH, Mannheim, Germany) and frozen at -80°C. Cells were disrupted by repetitive freeze thaw cycles from liquid nitrogen to a 37°C water bath. Cell lysate was collected through centrifugation at 10 000 rpm and adjusted to 5 mM $MgCl_2$. Lysate was treated with 10 units of DNase I (Roche Diagnostics GmbH, Mannheim, Germany) for 15 min at room temperature to reduce viscosity. TritonX 100 was added to a final concentration of 1% (w/v) and the bacterial cell lysate was cleared through centrifugation at 10 000 rpm for 15 min and passed through a 0.22 μM filter.

Recombinant protein was batch purified with Glutathione agarose4B resin (Sigma, St Louis, USA). A 2 ml bed volume of Glutathione agarose was added to the filtered lysate and the recombinant peptide allowed to bind overnight on a rotor mixer. Unbound proteins were removed by washing twice with 10 ml GST binding buffer, followed by two washes of 10 ml of GST binding buffer containing 1% TritonX 100 to remove unspecific bound proteins. Bound recombinant GST-Vv-AMP1 peptide was eluted with 10 ml elution buffer (50 mM Tris-Cl pH 8.0, 10 mM reduced glutathione). The N-terminal GST-tag was removed by thrombin digestion overnight at room temperature with 20 units of enzyme (Amersham Biosciences, NJ, USA). SDS-PAGE analysis was used to confirm the purity of the recombinant fusion protein after affinity chromatography and the complete removal of the N-terminal GST-tag by thrombin digestion.

The GST-tag was separated from mature Vv-AMP1 peptide using cation exchange chromatography on a SP sepharose column (Amersham Pharmacia Biotech). Samples containing the mature Vv-AMP1 peptide were pooled and loaded onto a Strata C8 Solid Phase Extraction column (Phenomenex, Torrance, CA, USA). Bound peptide was desalted by washing with 5 column volumes of dH_2O containing 0.1% [v/v] Trifluoroacetic acid (TFA) and eluted with 5 ml of 60% [v/v] acetonitrile containing 0.1% [v/v] TFA. Eluted peptide was freeze-dried, dissolved in distilled water at a final concentration of 100 μg ml^{-1} and stored at -20°C.

4.3.9 Size determination and identification of heterologous Vv-AMP1

To confirm the purification of the Vv-AMP1-GST fusion protein and evaluate the cleavage of the GST-tag from Vv-AMP1, 2 μg purified protein was separated on a 15% [w/v] Tris-Tricine gel (Schagger H and G, 1987). After separation the gel was

microwave stained in staining solution (Coomassie R250 in 50% [v/v] ethanol, 10% [v/v] acetic acid). The gel was destained with 12.5% [v/v] isopropanol and 12% [v/v] acetic acid. The exact size of mature Vv-AMP1 peptide samples was determined by LC/MS analysis on a Waters API Q-TOF Ultima instrument.

Identification of the peptide was achieved by peptide mass fingerprinting. Forty five μg recombinant Vv-AMP1 peptide was digested with the ProteoExtract^{tm} all-in-one trypsin digest kit (Calbiochem, La Jolla, CA, USA) and subjected to LC/MSMS analysis on a Waters API Q-TOF Ultima instrument. The resulting peaks were analyzed with the Expasy program FindPep (http://www.expasy.org/tools/findpept.html).

4.3.10 Preparation of antibody and immunoblotting

Polyclonal antibodies against Vv-AMP1 were produced in mice by immunizing three mice with 300 μg of the GST-Vv-AMP1 fusion protein in Freund's complete conjugate. Two booster injections consisting of 100 μg protein in Freund's complete conjugate were given at 2 week intervals and a final injection with 100 μg purified Vv-AMP1 was given 2 weeks before the terminal bleed.

Western blot analysis was conducted on 400 ng purified Vv-AMP1. The peptide was separated on a 15% (w/v) Tris-tricine gel (Schagger and von Jagow, 1987) together with a low molecular weight marker (Sigma, St Louis, USA). One half of the gel was stained with Coomassie R250 and the other half electroblotted to a PVDF membrane (BioRad, Hercules, CA, USA).

The membrane was blocked for 3 hours in blocking buffer (phosphate buffered saline, 0.1% [w/v] Tween 20 and 5% [w/v] skim milk) before incubating overnight in a 1:500 dilution of primary antibody prepared in blocking buffer. Detection of Vv-AMP1 was achieved with anti-mouse IgG secondary antibody and the ECL chemiluminecent system according to Amersham Biosciences (NJ, USA).

4.3.11 Antimicrobial activity of recombinant Vv-AMP1

Quantitative antifungal activity of Vv-AMP1 was measured by microspectrophotometry as described by Broekaert et al. (1990). The assay was preformed in a 96-well microtiter plate (Bibby Sterilin Ltd, Stone, Staffs, UK), where each well contained 100 μl half strength Potato Dextrose Broth (PDB) with 2000 fungal spores and purified Vv-AMP1 peptide of 1-20 μg ml^{-1}, respectively. Control reactions contained no peptide. Plates were incubated in the dark at 25°C for 3 days. Microspectrophotometric readings were taken every 24 hours at A_{595}. All readings were corrected by subtracting the time zero readings from the time 24, 48 and 72 hour readings. Vv-AMP1 activity was scored after 48 hours and expressed in terms of percentage growth inhibition. Percentage growth inhibition is defined as 100× the ratio of the corrected A_{595} of the control minus the corrected A_{595} of the sample over the corrected A_{595} of the control. Each peptide concentration was assessed three times. Microscope images were also collected directly from the antifungal assays with

an Olympus IX70 inverted microscope. Images were captured with the Analysis® software (Olympus Soft Imaging Solutions GmbH).

The ability of Vv-AMP1 to cause fungal membrane permeabilization was assessed using a propidium iodide (PI) uptake assay (Gangwar et al., 2006), conducted on *F. oxysporum, F. solani* and *V. dahliae*. The permeabilization assay consisted of 200 μl half-strength PDB containing fungal spores (2 x 10^4 spores ml^{-1}) and Vv-AMP1 peptide at concentrations of 6 μg ml^{-1} for *F. solani*, 9.6 μg ml^{-1} for *F. oxysporum* and 1.8 μg ml^{-1} for the *V. dahliae* isolate. Fungal strains were incubated at 25°C in the presence of Vv-AMP1 for 40 hours. Control samples contained no Vv-AMP1. After incubation the samples were washed with 1x PBS and stained for 10 min in PI staining solution (25 μg ml^{-1} PI in PBS). Stained samples were washed twice with 1x PBS and viewed under an Olympus IX 81 inverted fluorescent microscope. Images were capture with the CelliR® digital camera and software system (Olympus Soft Imaging Solutions GmbH). The presence of fluorescence is indicative of a compromised fungal membrane.

4.3.12 Stability assessment of recombinant Vv-AMP1

The stability of the heterologous Vv-AMP1 peptide was assessed by an antifungal assay as described above. The heat stability of the peptide was assessed at a final peptide concentration of 20 μg ml^{-1} against *B. cinerea* spores, with the peptide being pretreated at 25°C, 50°C, 80°C and 100°C for 30 min, before commencing with the antifungal assay. The activity of Vv-AMP1 was scored against the control reaction conducted at 25°C. Vv-AMP1 peptide was also subjected to a proteinase stability assay. Vv-AMP1 was treated with 100 μg ml^{-1} proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for 16 hours. After digestion Vv-AMP1 was subjected to an antifungal assay against *V. dahliae* at a final concentration of 5 μg ml^{-1}. The activity of Vv-AMP1 was scored against a control reaction containing proteinase K (100 μg ml^{-1}), but without any Vv-AMP1 peptide added.

4.4 RESULTS

4.4.1 Isolation and genomic characterization of *Vv-AMP1*

Screening of the *Vitis vinifera* EST database at www.tigr.org with the BLAST algorithm yielded only one EST hit, TC69032. Primer design and subsequent PCR screening of cDNA batches made from root, leaf and berry material allowed for the isolation of a single complete coding sequence from grape berry cDNA. The sequence was termed *Vitis vinifera* antimicrobial peptide 1 (*Vv-AMP1*), because of its homology to the family of plant defensins. The complete coding sequence of *Vv-AMP1* is 234 bp in size and encodes for a predicted 77 amino acid peptide, comprising a 30 amino acid signal peptide and a 47 amino acid mature peptide (Figure 1A). The genomic copy isolated for *Vv-AMP1* is 742 bp in size and comparative analyses with the cDNA sequences revealed that a 508 bp intron

interrupts the predicted signal peptide (Figure 1B). Southern blot analysis conducted on genomic DNA from cultivar Pinotage revealed the presence of two hybridization signals for *Vv-AMP1* within the *V. vinifera* genome (Figure 2).

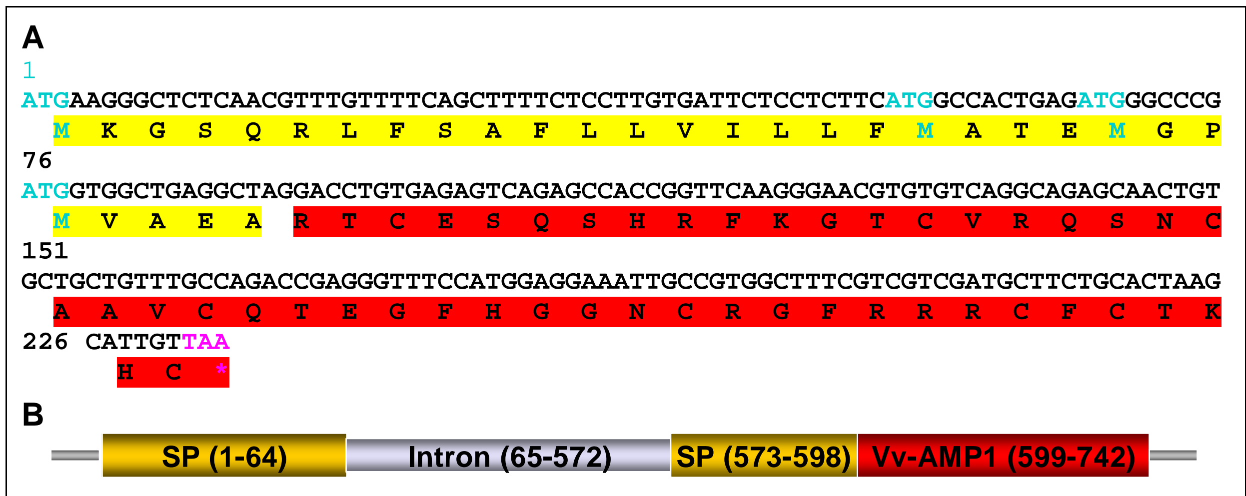

Figure 1. Gene structure of *Vv-AMP1* (A) The complete coding sequence of *Vv-AMP1* with its deduced amino acid sequence. The amino acids in yellow represent the signal peptide while red amino acids indicate the mature peptide. (B) The genomic structure of the *Vv-AMP1* gene. Yellow blocks represent the sequence encoding for the signal peptide of Vv-AMP1 and the red block the sequence encoding for the mature Vv-AMP1 peptide. The intron is indicated as a grey block. Numbering inside each block corresponds to the number of base pairs in each section.

BLAST analysis of the grapevine genome sequence at the National Centre for Biotechnological Information (NCBI) identified two possible contig sequences, VV78X055073.5 and VV78X034124.3. Alignment analysis of these two genomic sequences showed that the nucleotide areas upstream and downstream of the *Vv-AMP1* open reading were similar, suggesting that a single copy of *Vv-AMP1* is present in the *V. vinifera* genome.

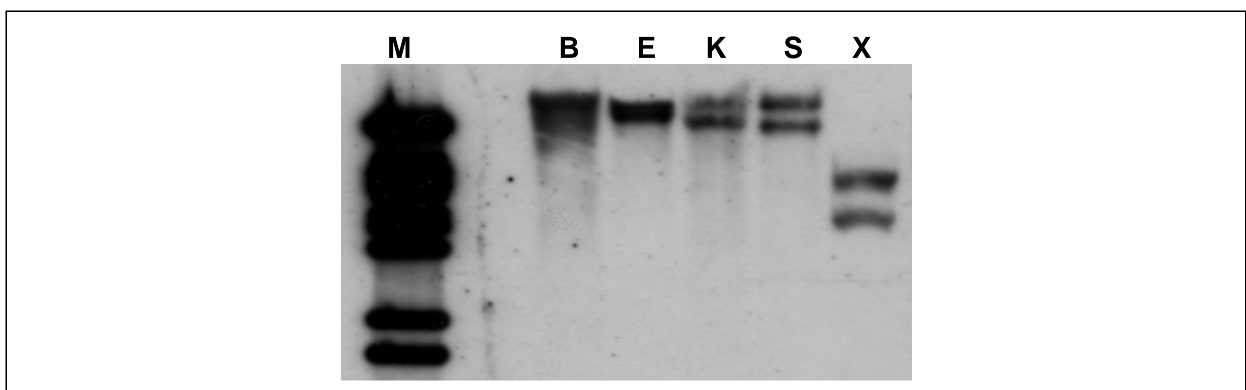

Figure 2. Southern blot analysis of *Vitis vinifera* genomic DNA to evaluate the genomic organization of *Vv-AMP1*. *Vitis vinifera* cv. Pinotage genomic DNA was digested with *Bam*HI (B), *Eco*RV (E), *Kpn*I (K), *Spe*I (S) or *Xba*I (X) and probed with a DIG labeled cDNA copy of *Vv-AMP1*. Lambda DNA digested with *Bst*EII was used as marker (M). Each signal indicates a single copy of *Vv-AMP1*.

Alignment analysis of the genomic sequences of *Vv-AMP1* isolated from non-vinifera *Vitis* species revealed a high level of homology (93%) at nucleotide level. When the deduced coding sequences for the different *Vv-AMP1* genes were compared, up to 95% homology was observed, translating into 93% identity at the deduced amino acid level (Figure 3). *Vv-AMP1* from *V. vinifera* showed the highest homology to the gene amplified from *V. afganista*, sharing 98.7% homology at amino acid level. The deduced amino acid sequences from *V. vinifera* and *V. afganista* differed from the rest by having one additional amino acid in their signal peptide region, with the introduction of isoleucine at position 15 (numbering according to Vv-AMP1 from *V. vinifera*).

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A.aconitifolia        MKGSQRLFTAFLLV-LLFMATEMGPMVAEARTCESQSHRFKGTCVRQSNCAAVCQTEGFN
V.cinerea_floridana   MKGSQRLFTAFLLV-LLFMATEMGPMVAEARTCESQSHRFKGTCVRQSNCAAVCQTEGFN
V.cinerea             MKGSQRLFTAFLLV-LLFMATEMGPMVAEARTCESQSHRFKGTCVRQSNCAAVCQTEGFN
V.aestivalis          MKGSQRLFSAFLLV-LLFMATEMGPMVAEARTCESQSHRFKGTCVRQSNCAAVCQTEGFN
V.afganista           MKGSQRLFSAFLLVILLFMATEMGPMVTEARTCESQSHRFKGTCVRQSNCAAVCQTEGFH
V.vinifera            MKGSQRLFSAFLLVILLFMATEMGPMVAEARTCESQSHRFKGTCVRQSNCAAVCQTEGFH
                      ********:***** ************:*******************************:

A.aconitifolia        GGNCRGFRRRCFCTKHC
V.cinerea_floridana   GGNCRGFRRRCFCTKHC
V.cinerea             GGNCRGFRRRCFCTKHC
V.aestivalis          GGNCRGFRRRCFCTKHC
V.afganista           GGNCRGFRRRCFCTKHC
V.vinifera            GGNCRGFRRRCFCTKHC
                      *****************
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Figure 3. Alignment analysis of the deduced amino acid sequences of the *Vv-AMP1* genes isolated from different *Vitis* species. The major differences are indicated in green, note the additional amino acid at position 15 of the sequences isolated from *V. vinifera and V. afganista* (numbering according to Vv-AMP1 from *V. vinifera*).

4.4.2 Bioinformatical analysis of the deduced Vv-AMP1 sequence

BLASTP results and further alignment analysis showed that the deduced amino acid sequence of *Vv-AMP1* shared high homology to the γ-thionins from *Castanea sativa* and PPT from petunia (Karunanandaa et al., 1994) (Figure 4). Vv-AMP1 also displays the following conserved amino acid residues: an aromatic residue at position 11, two glycine residues at positions 13 and 34 and a glutamate at position 29, as well as the eight cysteine residues at positions 4, 15, 21, 25, 36, 46, 48, 52 present in all plant defensins (numbering according to Rs-AFP1 Terras et al., 1995). Disulfide bridge analysis done with DIpro showed that the eight cysteine residues of Vv-AMP1 are connected by four disulfide bridges (Figure 4).

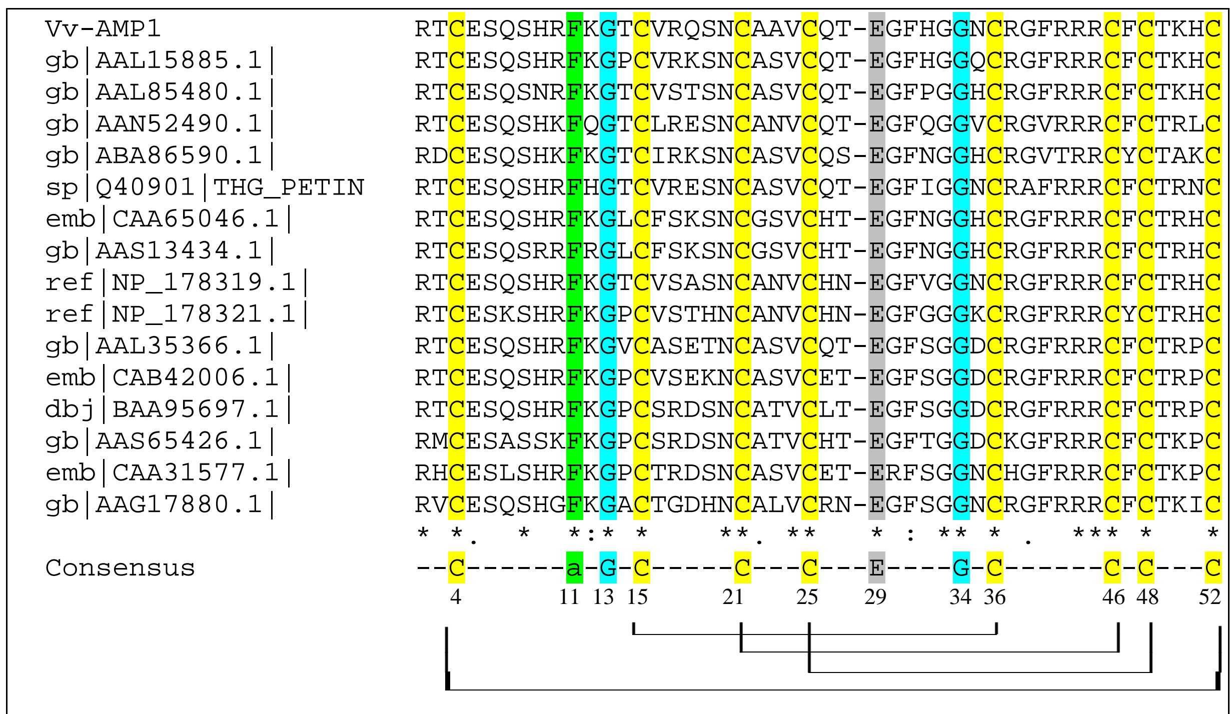

Figure 4. Amino acid alignment (ClustalX, Thompson et al., 1997) analyses of plant defensins belonging to subfamily B1 and Vv-AMP1 from *Vitis vinifera*: gb|AAL15885.1| putative γ-thionin [*Castanea sativa*]; gb|AAL85480.1| defensin protein 1 [*Prunus persica*]; gb|AAN52490.1| defensin EGAD1 [*Elaeis guineensis*]; gb|ABA86590.1| putative defensin 1 [*Aquilegia olympica*]; sp|Q40901|THG_PETIN γ-thionin homolog PPT precursor [*Petunia inflata*]; emb|CAA65046.1 unnamed protein product [*Capsicum annuum*]; gb|AAS13434.1| defensin [*Nicotiana attenuata*]; ref|NP_178319.1| LCR69/PDF2.2; protease inhibitor [*Arabidopsis thaliana*]; ref|NP_178321.1| LCR68/PDF2.3; protease inhibitor [*Arabidopsis thaliana*]; gb|AAL35366.1| defensin protein precursor [*Capsicum annuum*]; emb|CAB42006.1 γ-thionin [*Lycopersicon esculentum*]; dbj|BAA95697.1| thionin like protein [*Nicotiana tabacum*]; gb|AAS65426.1| Kunitz-type trypsin inhibitor [*Ipomoea batatas*]; emb|CAA31577.1 unnamed protein product [*Solanum tuberosum*]; gb|AAG17880.1| Kunitz trypsin inhibitor protein [*Phaseolus coccineus*]. The consensus sequence common to all defensins is indicated below with numbering according to Rs-AFP1 (Terras et al., 1995). The eight cysteines are indicated in yellow and the aromatic residue at position 11 in green. The conserved glycines are indicated in blue and glutamate at position 29 in grey. The gap was introduced to have number agreement with Rs-AFP1. The disulfide bridge organization within the Vv-AMP1 sequence is indicated below the consensus sequence.

Comparative homology modeling of the deduced amino acid sequence confirmed that the tertiary structure of Vv-AMP1 closely resembled that of hordothionin-γ (1GPT) from barley (Bruix et al., 1993) and had the typical defensin structure consisting of an α-helix and a triple-stranded antiparallel β-sheet, which are organized in a βαββ configuration (Figure 5). The structure is stabilized by intermolecular disulfide linkages between the eight cysteine residues.

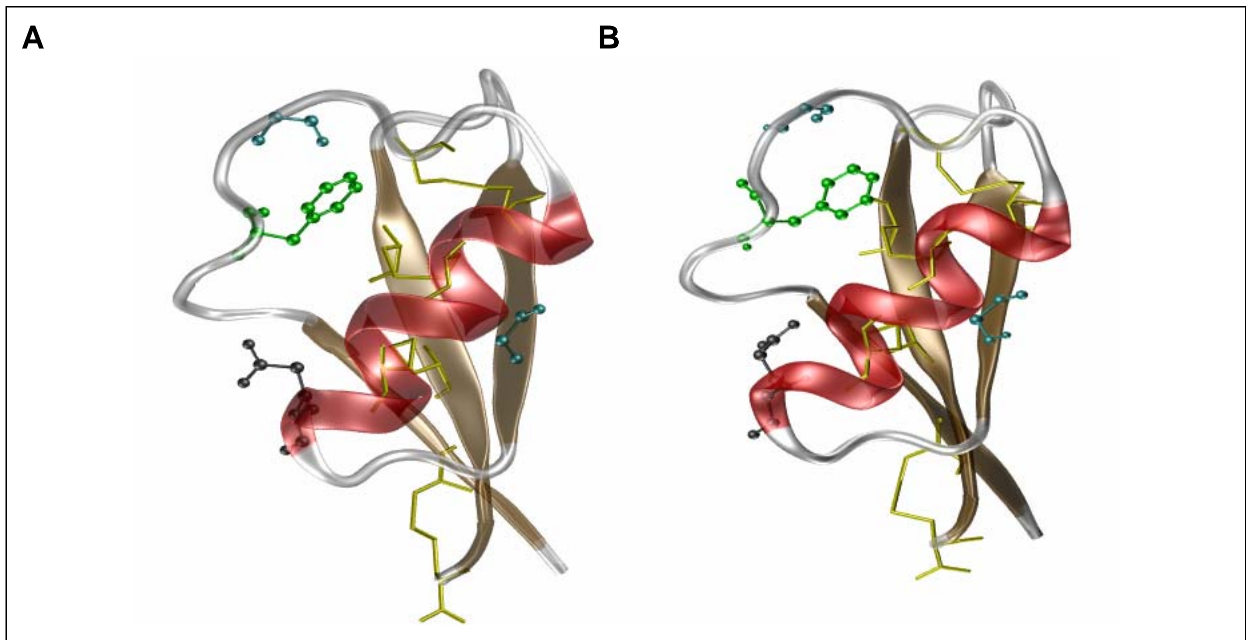

Figure 5. Comparison of the tertiary structure of Vv-AMP1 from grapevine (A) and hordothionin-γ from barley (Bruix et al., 1993) (B). The α-helix and β-sheet structures are represented in red and ochre respectively with the conserved amino acids represented in ball and stick models and coloured according to the conserved sequence in Figure 4.

4.4.3 Sub-cellular localization directed by the Vv-AMP1 signal peptide

PA-SUB predicted that the signal peptide of Vv-AMP1 directs its product to the apoplastic regions of plant cells. This was confirmed by fusing the Vv-AMP1 signal peptide to GFP under constitutive expression and transforming it into tobacco. Inverted fluorescent microscopy conducted on free-hand cross sections of the tobacco leaf petiole revealed that the GFP accumulated in the apoplastic regions (Figure 6A-C). High concentrations of GFP localization was observed in the guard cells of the stomata (Figure 6D and E), as well as the vascular tissues (Figure 6F).

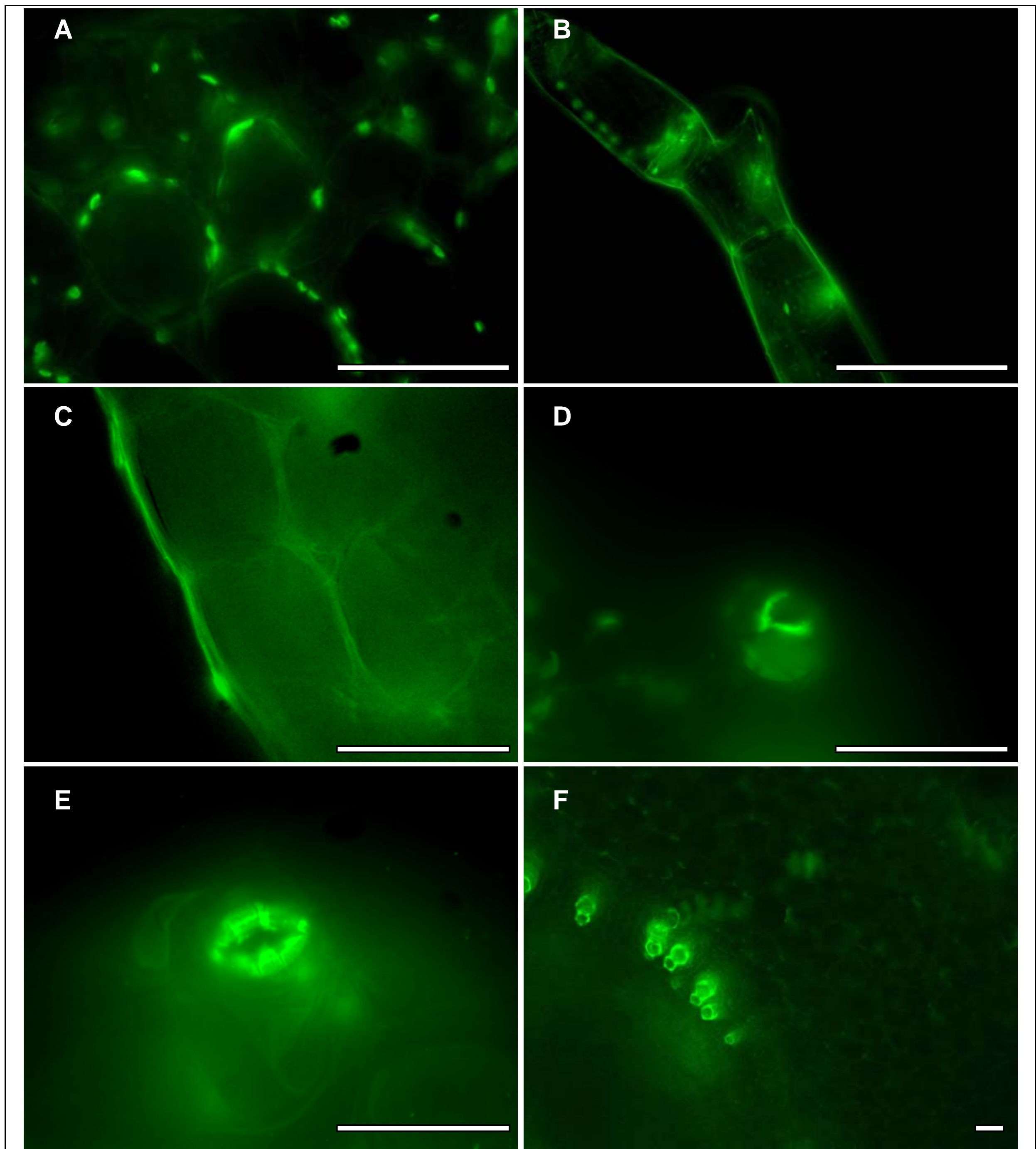

Figure 6. Localization of GFP in transgenic tobacco as directed by the signal peptide of Vv-AMP1 (A) Localization of GFP observed in the cortex of the tobacco leaf petiole. (B) GFP localization in the trichoma of the leaf petiole. (F) High levels of GFP localization directed to the vascular tissue of a tobacco leaf petiole. (D and E) localization of GFP to the guard cells of the stomata. (C) Localization in the epidermis layer of stem tissue. Bar = 50 μm.

4.4.4 Expression profile of *Vv-AMP1* in *V. vinifera*

Our investigation of the expression pattern of *Vv-AMP1* within grapevine revealed that this gene is expressed in a tissue-specific manner, being only expressed in berries (Figure 7A). Northern blot analysis on berries in different stages of development and ripening confirmed that the gene is developmentally regulated.

Vv-AMP1 expression was induced upon berry ripening, starting at véraison, 11 weeks post flowering (Figure 7B). Expression of *Vv-AMP1* remained high throughout the rest of the berry ripening stages.

Induction studies, conducted on grapevine leaf material, simulating osmotic stress, wounding, pathogen infection with *Botrytis cinerea* as well as treatment with ABA, were unable to induce *Vv-AMP1* expression (Figure 7C). Similar results were obtained when pre-véraison berries were induced (results not shown).

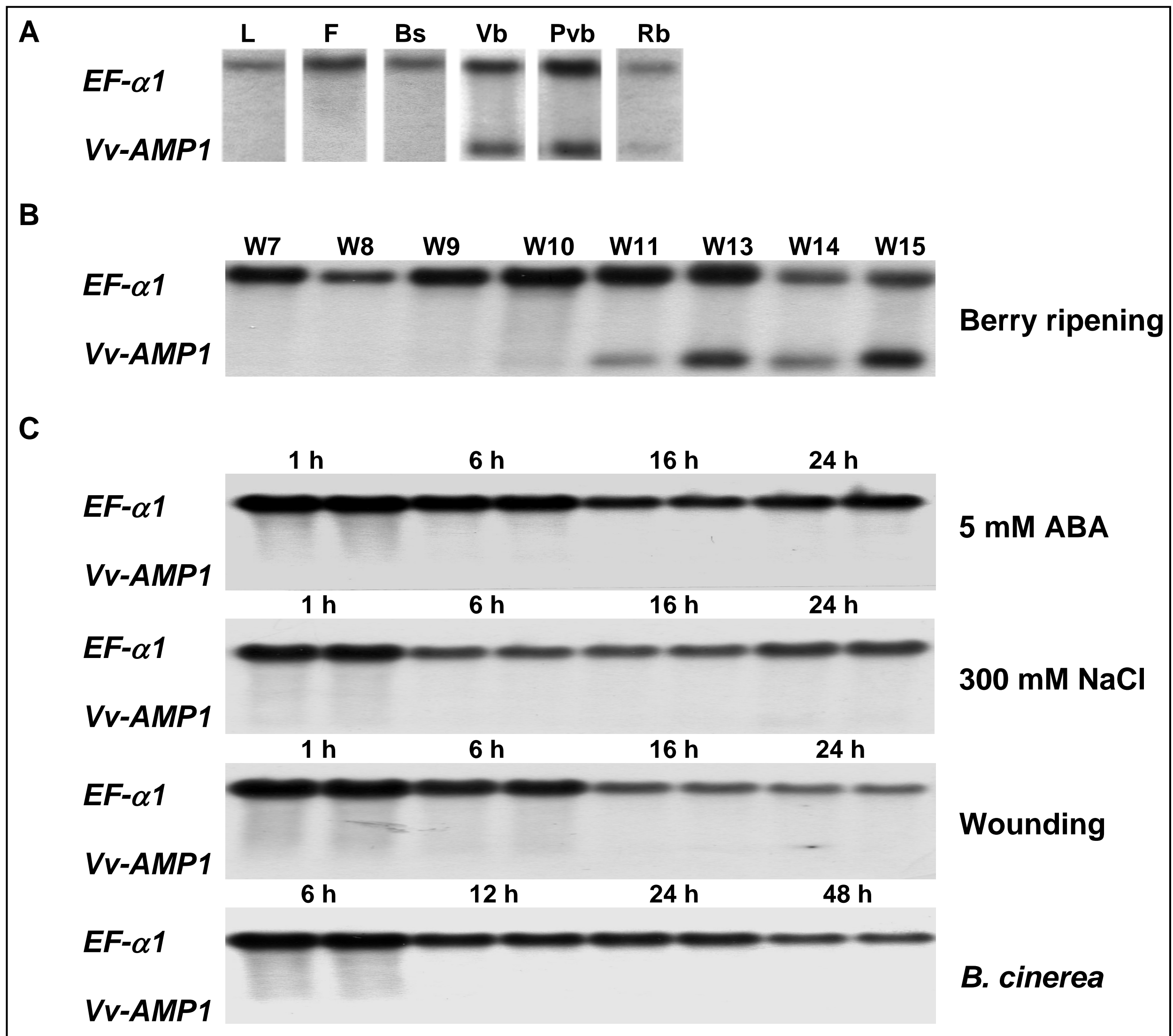

Figure 7. The expression profile of *Vv-AMP1* within the grapevine cultivar Pinotage. (A) Northern blot analysis of total RNA isolated from leaf (L) and flower tissue (F) as well as four berry developmental stages: Berry set (Bs), Véraison (Vb), Post véraison (Pvb), Ripe (Rb). (B) Induction of *Vv-AMP1* by developmental regulation (w=weeks post-flowering) (C) Biotic and abiotic induction studies were conducted on Pinotage leaf tissue. Time points indicate the time of tissue collection after the initiation of each induction experiment. Northern blot signals were detected by probing with a DIG labelled *Vv-AMP1* cDNA and a *V. vinifera* EF-α1 probe as internal standard. The *Vv-AMP1* signal hybridized at a molecular weight of 500 bp (size of Vv-AMP1 transcript with 5' and 3' UTR) and the internal standard at 2500 bp.

4.4.5 Heterologous expression and purification of Vv-AMP1

Recombinant Vv-AMP1, fused to the GST-tag, was successfully produced in *E. coli* using the Rosetta gami pLysS expression system. Purification of the recombinant peptide using a glutathione affinity chromatography system (Sigma, St Louis, USA) yielded 5 mg l^{-1} purified peptide. The recombinant fusion protein had a size of 31 kDa, consistent with the predicted size. Successful removal of the GST-tag was achieved by thrombin cleavage and confirmed with SDS-PAGE analyses (Figure 8A) and confirmed by western blot analysis (Figure 8A). Recombinant peptide was successfully separated from the cleaved tag, using ion exchange chromatography, and desalted on a C8 column. Mass-spectrometry revealed that the recombinant peptide had a size of 5.495 kDa, which matched the predicted mass (Figure 8B). Peptide mass fingerprinting confirmed that recombinant Vv-AMP1 resulted from the DNA sequence encoding for the mature Vv-AMP1 peptide.

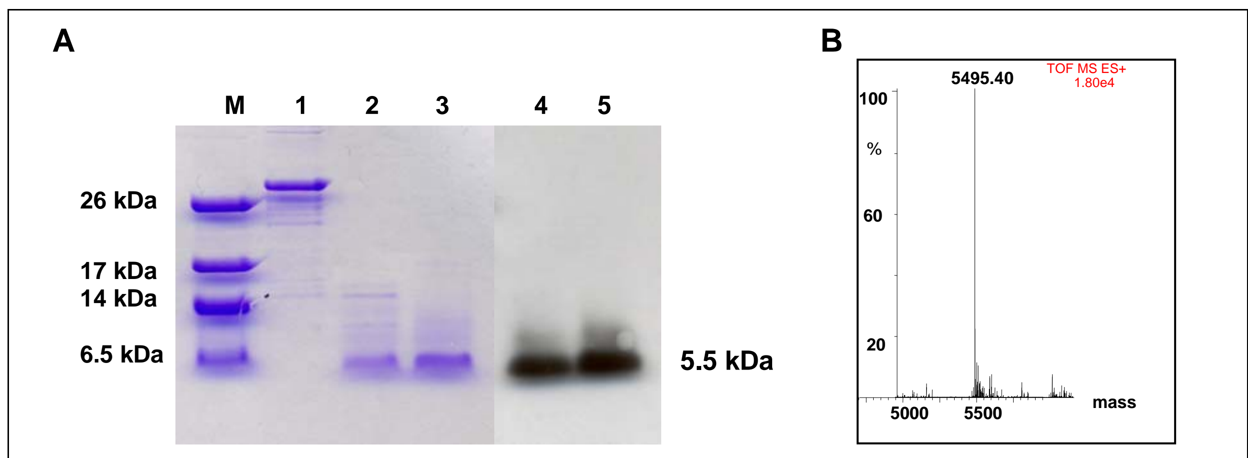

Figure 8. Size determination and western blot analysis of the purified recombinant Vv-AMP1 peptide (A) SDS-PAGE analysis of the GST-fusion protein before and after thrombin treatment; lane M, low molecular weight marker (Sigma, St Louis, USA); lane 1 GST-fusion protein, lane 2 and 3 purified Vv-AMP1 peptide after thrombin digestion and cation exchange chromatography; lanes 4 and 5, western blot analysis of Vv-AMP1. (B) Mass-spectrometric analysis of recombinant Vv-AMP1 after separation from the GST-tag using ion exchange chromatography.

4.4.6 Antimicrobial activity of recombinant Vv-AMP1

Recombinant Vv-AMP1 was tested against several plant pathogenic fungi using a dose-response growth inhibition assay. The activity of Vv-AMP1 on fungal hyphae was assessed by incubating fungal spores in the presence of various concentration of Vv-AMP1 over a 72 h period, with the IC_{50} value being determined after a 48 h of incubation (Figures 9 and 10).

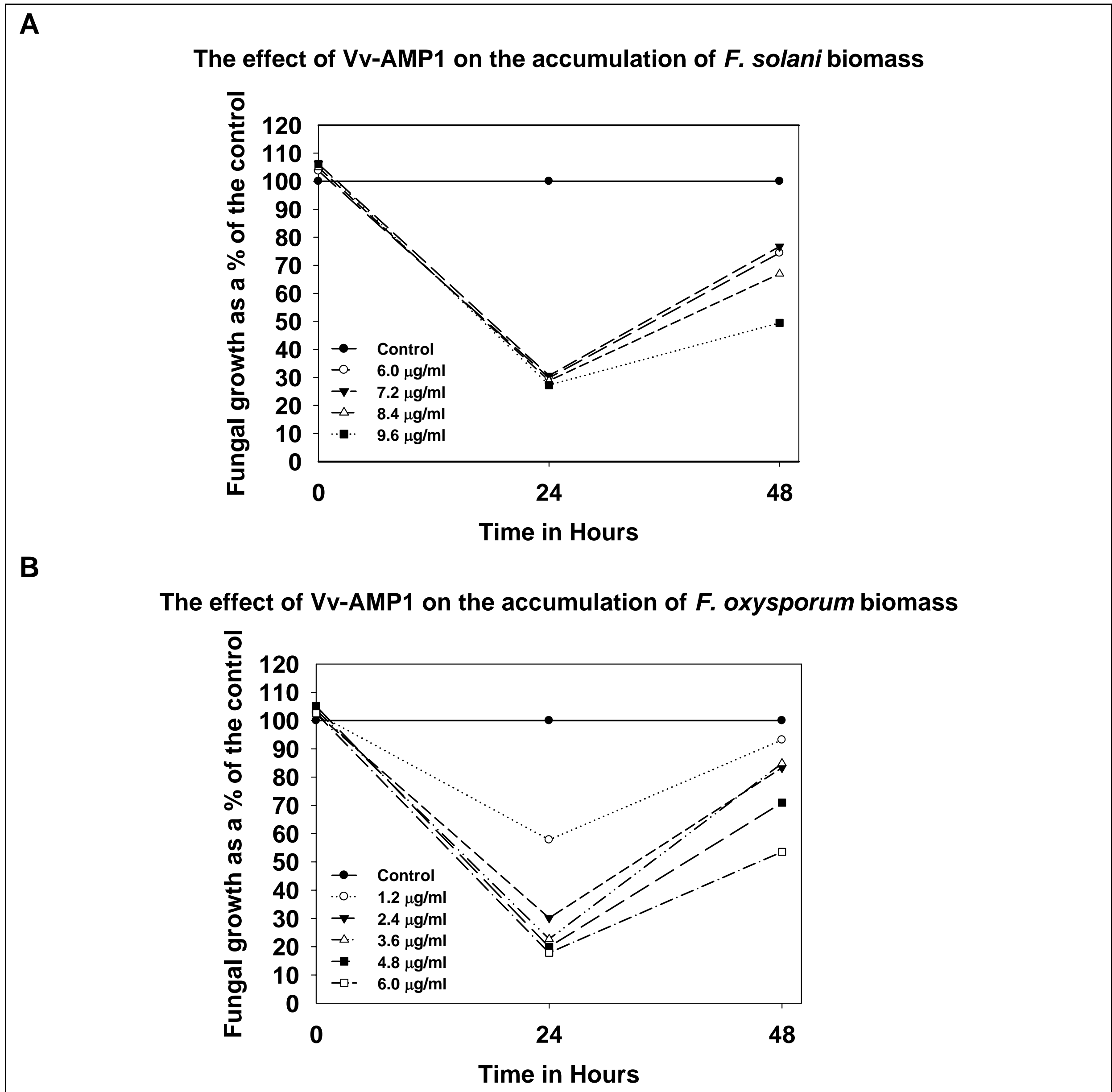

Figure 9. Antifungal activity of Vv-AMP1 on the growth of *F. oxysporum* and *F. solani*. Microspectrophotometric readings were recorded every 24 hours and compared to the untreated fungal controls. The data is represented as a percentage of fungal growth as compared to the untreated control reactions with no peptide. The standard deviation for each reaction was less than 5%. The effect of Vv-AMP1 on the germination and growth of *F. solani* (A) and *F. oxysporum* (B).

Vv-AMP1 had a severe effect on the accumulation of fungal biomass over time in all of the fungal isolates tested and was most active against *V. dahliae* (Figure 10B) and *F. oxysporum* (Figure 9B), the two causal agents of wilting disease, with IC_{50} values of 1.8 $\mu g\ ml^{-1}$ and 6 $\mu g\ ml^{-1}$, respectively. Vv-AMP1 was however less effective against *F. solani* with an IC_{50} value of 9.6 $\mu g\ ml^{-1}$ (Figure 9A).

The necrotrophic fungi *B. cinerea* (Figure 10A) was inhibited with an IC_{50} value of 13 μg ml^{-1}, but the peptide showed no inhibition of *A. longipes* even at peptide concentrations of 20 μg ml^{-1} (results not shown).

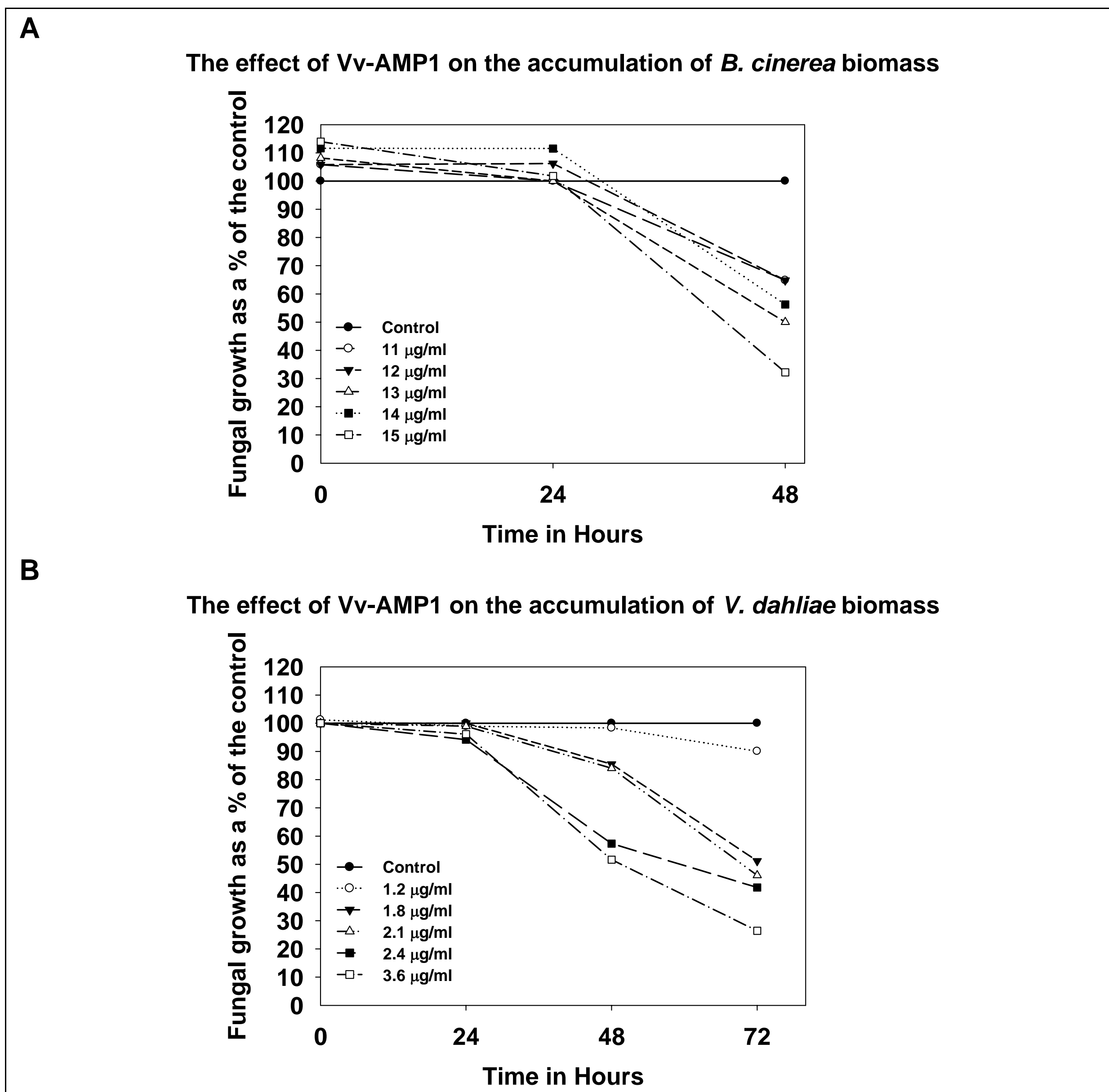

Figure 10. Antifungal activity of Vv-AMP1 on the growth of *B. cinerea* and *V. dahliae*. Microspectrophotometric readings were recorded every 24 hours and compared to the untreated fungal controls. The data is represented as a percentage of fungal growth as compared to the untreated control reactions with no peptide. The standard deviation for each reaction was less than 5%. The effect of Vv-AMP1 on the germination and growth of *B. cinerea* (A) and *V. dahliae* (B).

Treatment of *B. cinerea* spores with peptide concentrations above 15 μg ml^{-1} resulted in >95% growth inhibition, while a concentration of 30 μg ml^{-1} totally arrested spore germination of *B. cinerea* (Figure 11C).

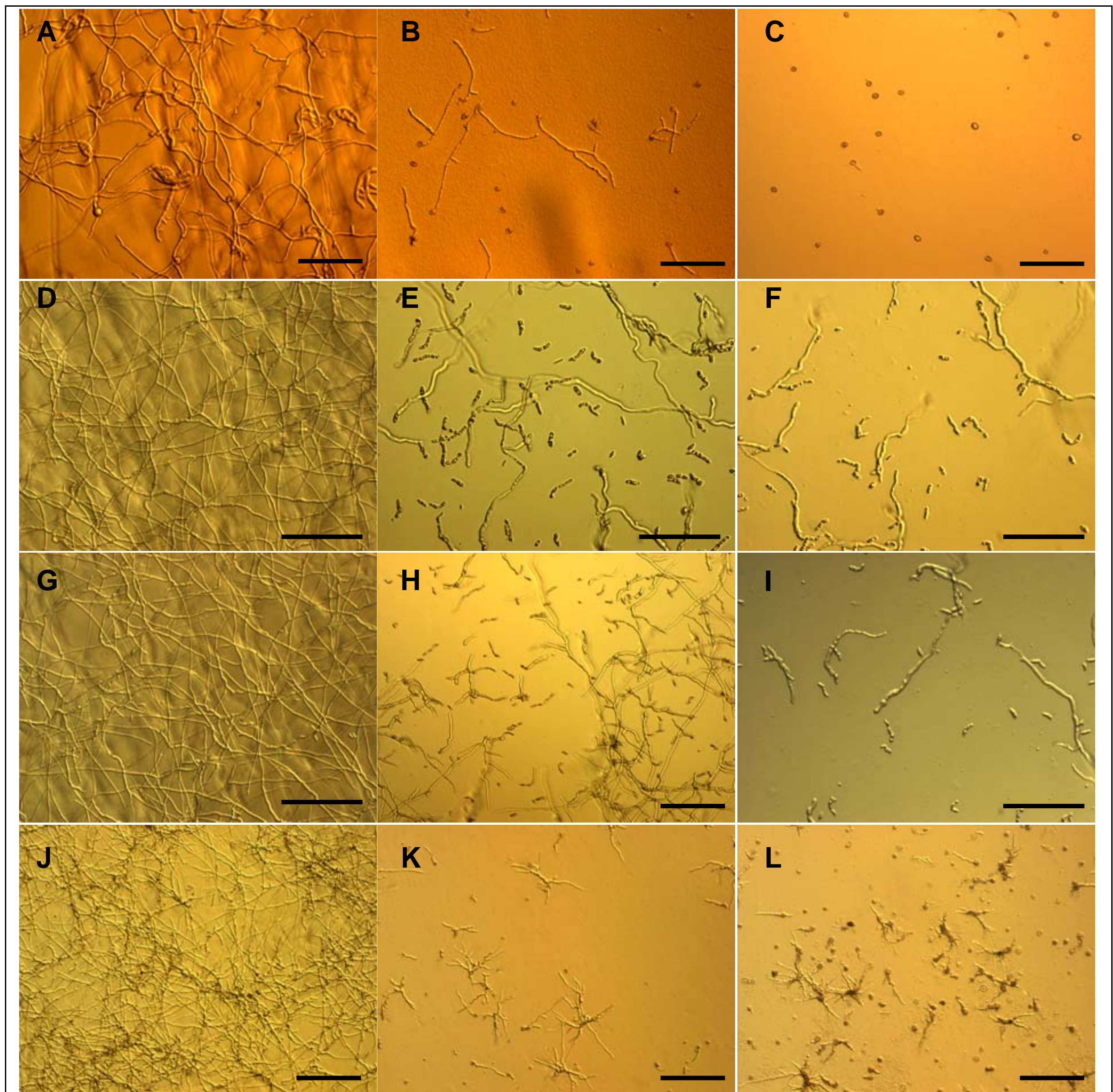

Figure 11. Microscopical analyses of the antifungal activity of Vv-AMP1 against a test panel of phytopathogenic fungi. (A, D, G, J) Control reactions of *B. cinerea, F. oxysporum, F. solani* and *V. dahliae*, without Vv-AMP1 peptide. (B, C) *B. cinerea* treated with 20 μg ml^{-1} and 30 μg ml^{-1} Vv-AMP1, respectively. (E, F) *F. oxysporum* treated with 6 μg ml^{-1} and 9.6 μg ml^{-1} Vv-AMP1, respectively. (H, I) *F. solani* treated with 6 μg ml^{-1} and 9.6 μg ml^{-1} Vv-AMP1. (K and L) *V. dahliae* treated with 4.8 μg ml^{-1} and 9.6 μg ml^{-1} Vv-AMP1. Photographs were taken after 48 h of incubation at 25°C. Bar = 100 μm.

Microscopical analyses of fungal hyphae treated with Vv-AMP1 showed no signs of the characteristic hyperbranching effect associated with some plant defensins. Vv-AMP1 did, however, severely alter the ability of fungal hyphae to elongate and most hyphal tips showed a swollen phenotype (Figure 11). Granulation of the hyphal cytoplasm was also observed in most fungi treated with Vv-AMP1.

Fluorescent microscopy done on propidium iodine stained fungal hyphae showed a high level of fluorescence present in the Vv-AMP1 treated samples

indicative of a permeabilized membrane, with the untreated fungi showing no fluorescence.

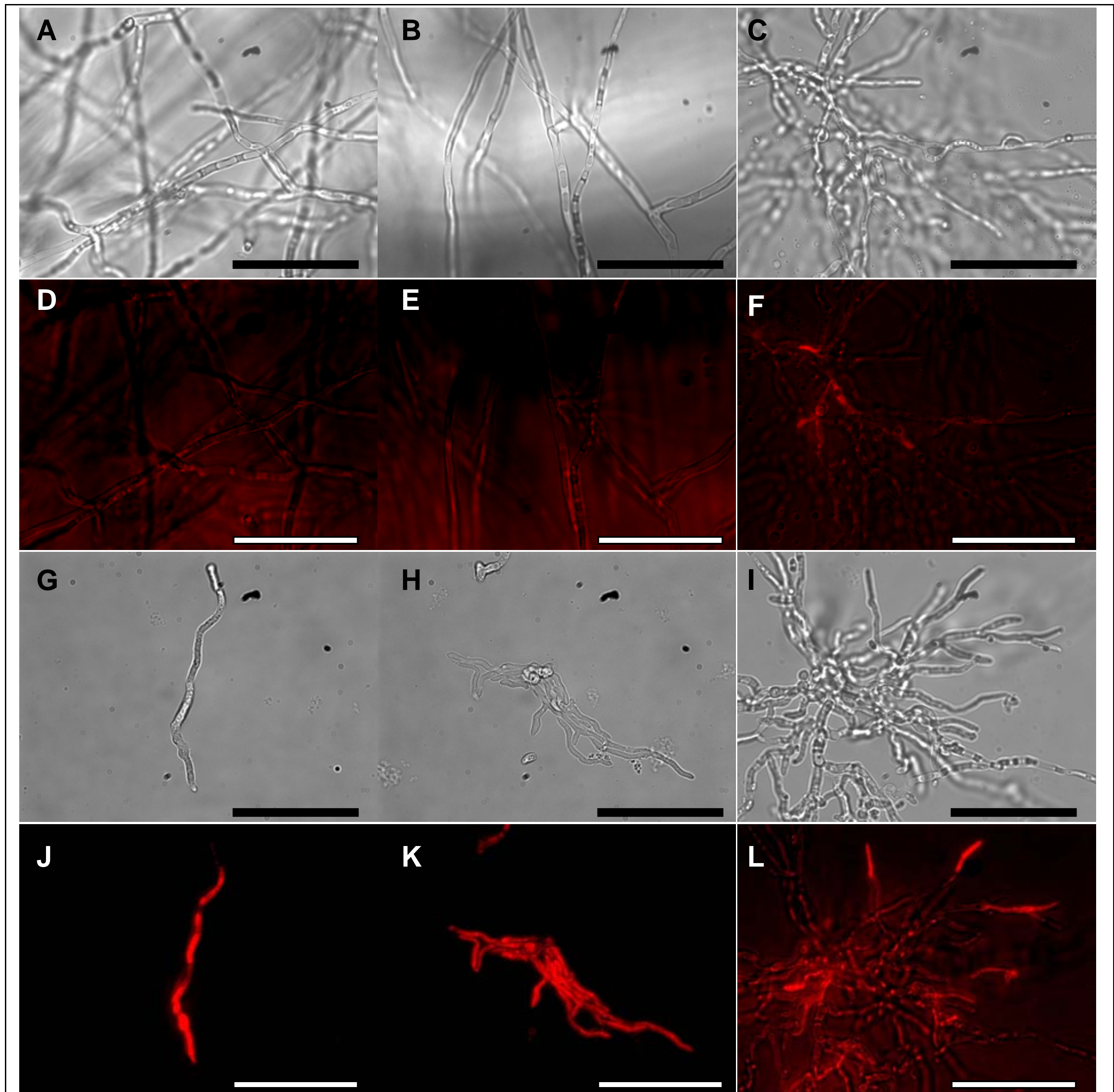

Figure 12. Fluorescent microscope analysis of propidium Iodide uptake during the membrane permeabilization assay. (A-C), light microscope images and (D-F) fluorescent images of untreated *F. oxysporum, F. solani and V. dahliae* hyphae respectively. (G-I), light microscope images and (J-L) of Vv-AMP1 treated *F. oxysporum, F. solani and V. dahliae* hyphae respectively. Fungi were grown for 40 hours in the presence of Vv-AMP1 at peptide concentrations of 6 μg ml^{-1} for *F. solani*, 9.6 μg ml^{-1} for *F. oxysporum* and 1.8 μg ml^{-1} for *V. dahliae*. Afterwards fungal hyphae were stained with propidium iodide for 10 min, washed with 1XPBS and subjected to fluorescent microscopic analysis. Bar = 50 μm.

4.4.7 Stability tests of recombinant Vv-AMP1

Vv-AMP1 was tested for its stability at different temperatures using an antifungal growth assay against *B. cinerea* (Figure 13A). Vv-AMP1 showed remarkable stability at temperatures up to 100°C. Ninety five percent of its antifungal activity was retained after 30 min of treatment at 80°C and 62% at 100°C for 30 min.

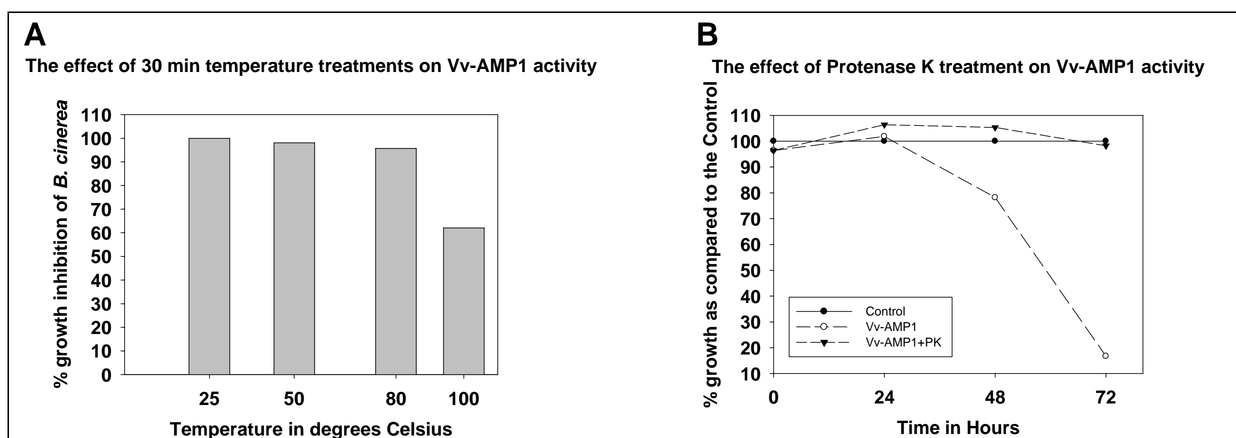

Figure 13. Stability assessment of Vv-AMP1. (A) Temperature stability of Vv-AMP1. After heat treatment the remaining antifungal activity of Vv-AMP1 was scored against the control with no heat treatment (25°C). (B) Stability of Vv-AMP1 against proteinase K (PK). The antifungal activity of Vv-AMP1 was scored against *V. dahliae* after treatment with 100 μg ml^{-1} proteinase K at 37°C for 16 h.

Vv-AMP1 showed no stability against proteinase K, confirming its proteinaceous nature. Enzyme treatment at 100 μg ml^{-1} totally abolished the activity of Vv-AMP1, as determined by an antifungal assay against *V. dahliae* (Figure 13B).

4.5 DISCUSSION

4.5.1 The isolation and characterization of a plant defensin encoding gene from grapevine

Plant defensins are small, cysteine-rich peptides with a basic nature that exhibit a broad spectrum of antimicrobial activity and have been implicated in the innate defense system of plants. Here we report the isolation and characterization of the first defensin peptide and its encoding gene from *Vitis vinifera*, the world's most important fruit crop. The 234 bp open reading frame isolated from *V. vinifera* berry cDNA (Figure 1) encoded Vv-AMP1, a peptide with significant consensus and structural homology to the peptide family of plant defensins. Analysis of the grapevine genome revealed that only one copy of *Vv-AMP1* is present in the *Vitis vinifera* genome and the two hybridization signals observed in the Southern blot analysis is due to heterogeneity in the *Vv-AMP1* locus. Moreover, the isolated gene is highly conserved within the *Vitis* genus (Figure 3).

Plant defensins exhibit an array of expression profiles and can be expressed constitutively, in a tissue specific pattern and also induced by various environmental stimuli (Manners et al., 1998; Oh et al., 1999; Moran and Thompson, 2001; van den Heuvel et al., 2001; Ahn et al., 2002; Koike et al., 2002; Hanks et al., 2005; Mirouze et al., 2006; de Zélicourt et al., 2007). The expression profiling of *Vv-AMP1* showed that this gene is highly regulated. *Vv-AMP1* showed a tissue specific and developmentally regulated expression pattern, being only expressed in grape berry material (Figure 7A-B). Under the conditions tested, no additional inducers were observed and none overcame the berry-specific expression pattern (Figure 7C). The lack of response to wounding and infection by the known grapevine pathogen *B. cinerea* suggest that induction of *Vv-AMP1* expression is independent of the plant defense signaling pathways directed by these external stimuli. The expression pattern of *Vv-AMP1* corresponds to berry ripening and it will be interesting to evaluate the recently identified inducers of berry ripening, such as brassinosteroids, on the expression of *Vv-AMP1* (Symons et al., 2006).

Plant defensins have a definitive preprotein structure consisting of a 29 to 30 amino acid N-terminal signal peptide followed by a mature defensin domain of between 45-54 amino acids. *Vv-AMP1* encodes for a deduced 77 amino acid peptide consisting of a 30 amino acid signal peptide and 47 amino acid mature defensin domain (Figure 1A). The signal peptide was shown to target GFP to the apoplastic regions when overexpressed in transgenic tobacco (Figure 6A-C). Interestingly, high levels of GFP accumulation was also observed around the xylem (Figure 6F) and in the guard cells of the stomata (Figure 6D), the latter a known entry point for grapevine pathogenic fungi.

4.5.2 Characteristic features of Vv-AMP1

Comparative analysis of mature Vv-AMP1 with other members of the defensin family revealed that Vv-AMP1 shared the conserved amino acids present in the majority of plant defensins and was closest related to the defensin PPT from petunia (Karunanandaa et al., 1994) (Figure 4). Classification studies of Vv-AMP1 grouped this peptide with subgroup B1 of the defensin family (Harrison et al., 1997), a subgroup well documented for its antifungal and antibacterial activity (Moreno et al., 1994; Meyer et al., 1996; Melo et al., 2002).

The Vv-AMP1 gene was successfully overexpressed in *E. coli* to yield purified peptide which could be subjected to accurate determination of size, as well as peptide fingerprinting. These results, as well as western blot analysis confirmed that the peptide was purified to homogeneity. The purified peptide was shown to be highly heat-stable, but readily deactivated by a proteinase enzyme.

4.5.3 Inhibition profile and antifungal characteristics of Vv-AMP1

Vv-AMP1 was especially effective against the causal agents of wilting disease, but also inhibited necrotrophic fungi such as *Botrytis*. Based on their antifungal activity plant defensins have been divided into three groups. The first group, termed morphogenic defensins, exhibit strong antifungal activity associated with induced morphological changes like hyperbranching upon treated hyphae. The second group exhibits strong antifungal activity, but without inducing morphological changes in treated hyphae, while the third group lacks antifungal activity. Vv-AMP1 limited the fungal biomass of the test organisms, but did not induce typical morphological changes in the treated cultures, classifying Vv-AMP1 as a non-morphogenic defensin peptide (group 2).

Another interesting observation was that two of the test organisms showed a dramatic reduction in optical density over the first 24 hours of the IC_{50} assay (Figure 9A, B). This initial reduction was then followed with a gradual increase in optical density to yield the final levels of inhibition at 48 hours. This observation might suggest that Vv-AMP1 either retards *Fusarium* spore germination, which usually occurs after 12 hours under the conditions tested, or that the peptide directly acts on the spores in a sporocidal effect, reducing the number of spores that can undergo hyphal development. In the treated *V. dahliae* and *B. cinerea* cultures, the initial decrease in optical density was not observed and the peptide probably acted on the germinated hyphae rather than on the spores, specifically since spore germination in these species only occurs between 16 and 20 hours of incubation.

The strong activity of Vv-AMP1 towards *V. dahliae* is of great interest in the engineering of disease resistant crops species. Vv-AMP1 seems to be more active against *V. dahliae* (IC_{50} values of 1.8 $\mu g\ ml^{-1}$) than alfAFP from *Medicago sativa* (published IC_{50} of 5.0 $\mu g\ ml^{-1}$), a peptide that has already showed great economical potential. When transformed into potato alfAFP was able to confer field trail resistance comparable to fumigation against the potato pathogen *V. dahlia* (Gao et al., 2000). The antifungal activities of the peptide are promising enough to merit further investigation of its potential in biotechnology approaches to increase fungal resistance in important crop species.

The observed activity of Vv-AMP1 was associated with alteration of fungal membrane permeability as indicated by the propidium iodide uptake assay and fluorescent microscopy (Figure 12). Previous studies have shown that plant defensin alter fungal membrane permeability which is associated with a rapid uptake and efflux of Ca^{2+} and K^{+} (Thevissen et al., 1996; Thevissen et al., 1999). It remains to be elucidated whether Vv-AMP1 used the same mechanism for the observed induction of membrane permeabilization.

Other questions that remain to be answered are the *in planta* stability of the peptide when overexpressed and the nature and degree of disease resistance that might be afforded by the peptide. The putative promoter of the Vv-AMP1 gene has

also been isolated from grapevine (results not shown) and might provide valuable insights into the regulation of this defensin.

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Chapter 5

RESEARCH RESULTS

In Planta Analysis of Vv-AMP1, a Ripening-Specific Defensin Isolated from *Vitis Vinifera* L. cv. Pinotage

Chapter 5 will be combined with data previously obtained and submitted for publication in Transgenic Research

RESEARCH RESULTS

In Planta Analysis of Vv-AMP1, a Ripening-Specific Defensin isolated from *Vitis Vinifera* L. cv. Pinotage

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5.1 ABSTRACT

The isolation and characterization of the first defensin from *Vitis vinifera*, *Vv-AMP1*, showed that the expression of the gene is strongly controlled. Expression was developmentally regulated and limited to the berries. The peptide had strong antifungal activity, inhibiting *Botrytis cinerea* and *Verticillium dahliae*, amongst others, with low doses in an *in vitro*-assay. This study is aimed at extending the functional analysis of the peptide, specifically in the *in planta* environment. The *Vv-AMP1* gene was successfully overexpressed in *Nicotiana tabacum* cv. Havana Petite transgenic lines. Genetic characterization confirmed the integration and expression of the gene in the heterologous tobacco environment. The peptide was under control of its native signal sequence which has been shown to direct its product to the apoplastic regions of cells. The transgenic lines were analyzed to determine the presence and activity of the grapevine defensin peptide. The transgenic strategy ensured that the strict regulation in terms of tissue-specificity and developmental regulation that is imparted on the gene in the native grapevine hosts was lifted since the expression was directed by the constitutive CaMV35Sp. Western blot analyses of partially purified plant extracts detected a signal of the expected size in both the untransformed control and the transgenic lines. Comprehensive analysis of EST databases identified three highly homologous sequences from tobacco that probably caused the background signal in the control. These crude protein extracts were able to inhibit the growth of *V. dahliae in vitro* when tested in a microtiter plate assay, but the inhibition could not be conclusively linked to the presence of the transgenic peptide, since non-expressing transgenic lines, included as controls, also showed inhibition. Similar results were obtained with infection studies, clearly showing that despite successful integration and expression of the transgene, the peptides was either not functional in the heterologous environment, or perhaps unstable under the particular regulatory conditions.

5.2 INTRODUCTION

Plants have developed various strategies to combat their bacterial and fungal pathogens. These defense mechanisms can be biochemical in nature (Kuc, 1990; Bennett and Wallsgrove, 1994; Kuc, 1995; Osbourn, 1996a; Osbourn, 1996b; Osbourn, 1999) or consist of peptides and proteins that interact with the bacterial and fungal pathogen to inhibit its growth and cause its death (Bohlmann and Apel, 1991; Broekaert et al., 1995; Broekaert et al., 1997; Fritig et al., 1998; Garcia-Olmedo et al., 1998; Lay and Anderson, 2005). The defense mechanisms in plants can be divided into the preformed or induced defense systems. The preformed defense is seen as an important factor in determining the host range of a specific plant pathogen (Morrissey and Osbourn, 1999). The most noticeable preformed defense mechanisms are the physical barriers and the preformed substances present in plant seeds. During seed germination the components of the preformed defense diffuse into the surrounding soil to form a protective barrier around the emerging seedling, thereby protecting it from soil-borne pathogens (Almeida et al., 2000; Wijaya et al., 2000; Wong and Ng, 2003; Balandin et al., 2005; Chen et al., 2005; Song et al., 2005). A major component of this defense mechanism is small peptides known as plant defensins.

Plant defensins are small, basic, cysteine rich peptides, consisting of 45-54 amino acids. The cysteine residues are connected by disulfide bridges to give these peptides a specific conformational peptide fold known as the cysteine stabilizing motif (Fant et al., 1998; Janssen et al., 2003; Lay et al., 2003b). Structurally these peptides consist of one α-helix and one β-sheet consisting of three antiparallel β-strands. These peptides show a broad spectrum of activity against oomycetes, fungi as well as some Gram positive bacteria and play an important role in plant defense (Harrison et al., 1997; Dahot, 1999; Garcia-Olmedo et al., 2001; Thomma et al., 2002; Pelegrini and Franco, 2005; Franco et al., 2006; Langen et al., 2006). Although the majority of these peptides have been isolated from seeds, they have also been isolated from leaves, tubers, flowers and fruits, were they are components of both the preformed and induced defense systems (Meyer et al., 1996; Segura et al., 1999; Berrocal-Lobo et al., 2002; Park et al., 2002; Janssen et al., 2003; Lay et al., 2003a). Most of the plant defensins isolated to date are induced upon pathogen attack and by other environmental stress factors. There are however a few of these defensins that show a strict tissue specific and developmental pattern of regulation (Meyer et al., 1996; Park et al., 2002).

Plant defensins have made a significant contribution to the engineering of disease resistant crops and have proven very successful in the engineering of disease resistant potatoes against the economical important pathogen *Verticillium dahliae* (Gao et al., 2000).

Here we report on the *in planta* analysis of Vv-AMP1, a developmentally regulated plant defensin from *Vitis vinifera*, by overexpressing the grapevine peptide in tobacco. Analysis of the transgenic population confirmed the presence and

expression of the transgene, but this could not be conclusively correlated to Vv-AMP1 levels and activity. These results present interesting possibilities that should be considered when overexpressing peptides from this subclass of plant defensins.

5.3 MATERIALS AND METHODS

5.3.1 Plant material and microbial strains

Tobacco seeds (*Nicotiana tabacum* cv. Havana petite SR1) were purchased from Lehle Seeds, Round Rock TX 78681 USA, and tobacco plants were maintained in growth rooms with a 16h/8h day/night cycle and a temperature of 25°C. *Escherichia coli* strain DH5α was used for all cloning strategies. *Agrobacterium tumefaciens* strain EHA105 was used for the transformation of tobacco. The fungal pathogen *Botrytis cinerea* was obtained from the Department of Plant Pathology, Stellenbosch University, while *Verticillium dahlia* was obtained from the American Type Culture Collection. *B. cinerea* cultures were maintained on apricot halves and cultivated in corning tissue culture plates until sporulation. *V. dahliae* was maintained on potato dextrose agar until sporulation.

5.3.2 Construction of the Vv-AMP1 expression vector and tobacco transformation

The complete coding sequence for *Vv-AMP1* has been isolated previously (see chapter three) and cloned into pGEM-T vector to create pGEM-Vv-AMP1. The coding sequences of *Vv-AMP1*, consisting of the native signal peptide and mature peptide, were excised from pGEM-Vv-AMP1 with *Xho*I and *Spe*I cloned into the *Xho*I and *Xba*I sites of the plant expression vector pART27cassette, a pART27 vector containing the expression cassette from pART7 cloned into the *Not*I sites of pART27, to be expressed under the constitutive control of the CaMV35S promoter (Gleave, 1992). The plant expression vector was termed pART27-Vv1 (Figure 1).

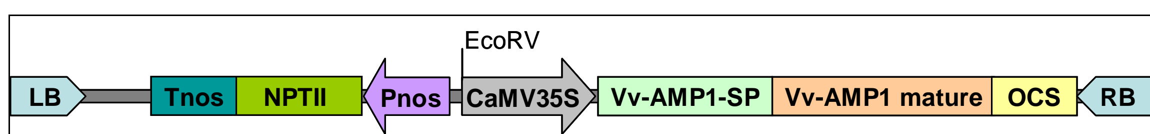

Figure 1. The plant expression cassettes used for tobacco transformation with an antifungal peptide from *Vitis vinifera* (Vv-AMP1). The abbreviations represent: **RB**, T-DNA right border; **LB**, T-DNA left border; **CaMV35S**, promoter of 35S RNA of cauliflower mosaic virus with duplicated enhancer region; **Pnos**, promoter of T-DNA nopaline synthase gene; **NPTII**, coding region of neomycin phosphotransferase II gene; **Tnos**, terminator of T-DNA nopaline synthase gene; **Vv-AMP1-SP**, signal peptide encoding domain of Vv-AMP1; **Vv-AMP1 mature**, mature protein encoding domain of Vv-AMP1; **OCS**, terminator of the octopine synthase gene.

The plant expression vector was mobilized into *A. tumefaciens* EHA105 via electroporation. Tobacco leaf discs were transformed by the standard leaf disc transformation method (Horsch et al., 1985). Transgenic plants were regenerated under kanamycin selection (120 $\mu g\ ml^{-1}$) and termed Tvv. The resulting transgenic tobacco plantlets were sub-cultured to maintenance medium, Murashige Skoog (MS) medium (Murashige and Skoog, 1962) without hormones and maintained at 25°C under a 16 h light cycle. Three-week-old plantlets were hardened-off in potting soil under glasshouse conditions. The primary, hardened-off transformants were allowed to self-pollinate. The resulting seeds were germinated under kanamycin selection to yield the F1-generations that were used for all subsequent analyses of the transgenic plant lines.

5.3.3 Southern blot analyses of transgenic tobacco lines

Southern blot analyses were conducted on all plantlets regenerated under kanamycin selection to confirm integration of the *Vv-AMP1* expression cassette as, well as copy number and integration patterns.

Fifty mg leaf tissue from each line of the F1 generation was ground to a powder in the presence of liquid nitrogen. Genomic DNA was extracted with 800 μl of extraction buffer (3% [w/v] CTAB, 1.4 M NaCl, 0.02 M EDTA, 1 M Tris-HCl pH 8.0), according to McGarvey and Kaper (1991). The copy numbers of the expression cassettes were assessed through the digestion of 10 μg genomic DNA with *Eco*RV, where each hybridization signal represents a single copy of the integrated expression cassette. The digested genomic DNA was separated on a 1% (w/v) agarose gel, followed by transfer to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). The membrane was subsequently probed with a DIG-labeled probe representing the coding sequence of *Vv-AMP1*. Pre-hybridization and hybridization were performed at 37°C. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany).

5.3.4 RNA manipulations to confirm gene expression in transgenic lines

RNA isolations were performed according to the protocol of Chang et al. (1993). Leaf tissue (100 mg) was grounded to a fine powder in the presence of liquid nitrogen and extracted with 800 μl extraction buffer (2% [w/v] CTAB, 2% [w/v] PVP-40, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 $g\ L^{-1}$ spermidine). Five microgram total RNA was separated on a 1.2% (w/v) formaldehyde agarose gel and transferred to positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) as described by Sambrook et al. (1989). The membranes were probed with a DIG-labeled probe representing the coding sequence of *Vv-AMP1*. Pre-hybridization and hybridization was performed at 50°C. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany).

5.3.5 Western blot analysis of Vv-AMP1 expressed in the transgenic tobacco lines

Tobacco leaf material was frozen in liquid nitrogen and ground to a fine powder. Five grams of ground tissue was extracted with 10 ml extraction buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM KCl, 1.5 % [w/v] PVPP, pH 7.5 and complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) for three hours. The supernatant was collected through centrifugation and the basic protein fraction separated from the acidic fraction, using cation exchange chromatography. This was achieved by passing the crude extract over a SP-Sepharose column (Amersham Biosciences, NJ, USA) equilibrated with 50 mM phosphate buffer pH 7.3. The crude basic protein fraction was eluted from the column with 50 mM phosphate buffer pH 7.3 containing 700 mM NaCl. Eluted protein fractions were bound to a C8 reverse phase column (Isolute) and desalted by washing with 5 ml of ddH2O + 0.1% (v/v) Trifluoroacetic acid (TFA). The crude protein fractions were eluted with 50% (v/v) acetonitrile + 0.1% (v/v) TFA, freeze dried and dissolved in 200 μl distilled water.

Protein concentrations were determined using the BCA assay (Pierce) and 5.6 μg of the semi purified crude basic protein was separated on a 16.5% (w/v) Tris-Tricine gel (Schagger and von Jagow, 1987). Separated proteins were transferred to PVDF membrane (BioRad, Hercules, CA, USA) using a semi-dry transfer apparatus (Hoeffer), the membranes were blocked for three hours with 5% (w/v) skim milk in phosphate buffer saline with 0.1% [w/v] Tween 20 and incubated overnight in a 1/200 dilution of primary antibody, raised against Vv-AMP1 in mice (see chapter 4 of this dissertation). Vv-AMP1 was detected with secondary IgG mouse antibody and the ECL plus detection system (Amersham Biosciences, NJ, USA). Purified Vv-AMP1 produced in *E. coli* (see chapter 4 of this dissertation) was used as positive control and to confirm the correct size of the peptide produced in tobacco.

5.3.6 Microspectrophotometric assay of crude protein extracts on fungal growth

Crude protein extracts were prepared from the F1 generation tobacco by centrifugation. Five hundred mg of powdered tobacco leaf tissue was placed in a 2 ml centricon unit where the membrane filter has been replaced by 3MM Whatman paper. The tissue was centrifuged for 20 min at 8000 rpm to collect all the liquid present in the tobacco tissue. Crude protein samples were filter sterilized and analyzed for their antifungal activities against *V. dahlia* by using a spectrophotometric assay. *V dahlia* was chosen to evaluate the antifungal activity of the crude plant extracts due to its sensitivity towards Vv-AMP1 at low concentrations (see chapter 4 of this dissertation). The microtiterplate readings were performed on the PowerwaveX microplate reader (Bio-Tek instruments Inc).

Crude protein fractions, at a final concentration of 50% (v/v) in a 100 μl reaction, were analyzed in the presence of 2000 fungal spores according to the method of

Broekaert et al. (1990). Measurements (A_{595}) were collected at time 0, 24 h and 48 h past inoculation. All the values were corrected by subtracting the time 0 values from the 24 h and 48 h values. The growth was recorded after 48 h of incubation and each reaction was repeated three times.

5.3.7 *Botrytis cinerea* infection studies

The transgenic plant lines were subjected to *B. cinerea* infection studies. Leaves 3 to 5 (numbered from the apex of each plant) were transferred to plastic containers containing water agar (8 g L^{-1}). Each leaf was inoculated with four 2 μl spots containing 2000 *B. cinerea* spores, suspended in 50% pure grape juice. The leaves were incubated at 22°C under a 16 h light/8 h dark cycle for 2 weeks. The disease symptoms were scored after 4 and 6 days (for the hardened off plants) and 3 days (for the *in vitro* plants) by measuring the lesion size.

5.3.8 Statistical analysis of data and bioinformatics

All data obtained in the *in vitro* and *in vivo* assays were subjected to ANOVA analysis using the software package STATISTICA (StatSoft, Inc. (2004)). Evaluation of the *Nicotiana tabacum* transcriptome for the presence of peptides homologous to Vv-AMP1 was performed *in silico* by analyzing available data present in nucleotide databases and combining it with bioinformatical evaluation in the software packages ClustalX (Thompson et al., 1997) and AlignX of the VectorNTi 9.0 package (Invitrogen, Carlsbad, USA).

5.4 RESULTS

5.4.1 Molecular characterization of the transgenic Tvv tobacco lines

Transformation of tobacco leaf discs yielded 30 positive transgenic tobacco lines with the *Vv-AMP1* expression cassette as confirmed by Southern blot analysis (Figure 2). The Southern blot integration patterns revealed that 20 individual transgenic lines were regenerated and they were termed Tvv transgenic tobacco. Northern blot analysis of the 20 Southern positive lines revealed that *Vv-AMP1* was successfully expressed in 18 of the 20 transgenic Tvv-lines. Lines Tvv18 and Tvv32 showed a silencing phenotype, being Southern blot positive, but lacking expression of the *Vv-AM*P1 expression cassette (Figure 2). These lines were subsequently used as non-expressing controls in the *in vitro* antifungal assays and infection studies conducted with *V. dahliae* and *B. cinerea*, respectively.

Western blot analysis revealed the presence of a 5 kDa signal present in the semi-purified protein extracts of the transgenic lines that correlated with the positive control consisting of Vv-AMP1 peptide heterologously produced in *E. coli*.

Plant line	WT1	Tvv 4	Tvv 5	Tvv 7	Tvv 8	Tvv 9	Tvv 10	Tvv 12	Tvv 13	Tvv 15	Tvv 17
Southern Blot											
Integration	0	2	2	5	5	3	4	4	5	>5	3
Northern Blot											
RNA											
Western Blot											

Plant line	WT2	Tvv 18	Tvv 19	Tvv 20	Tvv 23	Tvv 24	Tvv 27	Tvv 29	Tvv 32	Tvv 33	Tvv 36
Southern Blot											
Integrations	0	3	5	4	>5	>5	>5	>5	4	4	>5
Northern Blot											
RNA											
Western Blot											

Figure 2. Molecular characterization of the untransformed WT tobacco lines and the transgenic Tvv-lines regenerated to overexpress the Vv-AMP1 plant defensin from grapevine under control of the constitutive CaMV35S promoter.

A signal was also detected in the untransformed WT control plants lacking the *Vv-AMP1* expression cassette. Screening of the *Nicotiana tabacum* EST database at The Institute for Genomic Research (www.tigr.org), revealed the presence of three ESTs showing homology to *Vv-AMP1*. The homology between these genes and *Vv-AMP1* were 70%, not high enough at nucleotide level to result in a signal during Southern blot or northern blot analysis (Figure 3) with the high stringency wash steps employed during the post hybridization processing of the membranes.

```
TC13691         ATGGCTGGCTTTCCCAAAGTGCTTGCAATTGTTTTCCTTATGCTGATGCTGGTTTTTGCT
TC8608          ATGGCTGGCTTTCCCAAAGTGCTTGCAATTGTTTTCCTTATGCTGATGCTGGTTTTTGCT
TC10253         ATGGCTGGCTATCCCAAAGTTGTTGCAACTGTTTTCCTTATGATGATGCTGGTTTTTGCT
Vv-AMP1         ATGAAGGGCTCTCAACGTTTGTTTTCAGCTTTTCTCCTTGTGATTCTCCTCTTCATGGCC
                ***   **** **      *  ** **  * ** ***** ** *  * **  *  * **

TC13691         AATGAGATGGGACCAATGGTGGCTGAGGCGAGGACCTGCGAGTCGCAGAGTCACCGATTC
TC8608          AATGAGATGGGACCAATGGTGGCTGAGGCGAGGACCTGCGAGTCGCAGAGTCACCGATTC
TC10253         ACTGAGATGGGACCGATGGTGGCTGAGGCGAGGACCTGTGAGTCGCAGAGTCACCGATTC
Vv-AMP1         ACTGAGATGGGCCCGATGGTGGCTGAGGCTAGGACCTGTGAGAGTCAGAGCCACCGGTTC
                * ********* ** ************** ******** ***   ***** ***** ***

TC13691         AAGGGGCTGTGCATTAGTAAGAGCAACTGTGCGTCAGTTTGCCATACTGAGGGCTTTAAC
TC8608          AAGGGGCTGTGCATTAGTAAGAGCAACTGTGCGTCAGTTTGCCATACTGAGGGCTTTAAC
TC10253         AAAGGACTGTGTTTCAGTAAAAGCAACTGTGCTTCTGTTTGCCATACAGAGGGCTTTTAC
Vv-AMP1         AAGGGAACGTGTGTCAGGCAGAGCAACTGTGCTGCTGTTTGCCAGACCGAGGGTTTCCAT
                ** **   ***  * **  * ***********  * ******** ** ***** **  *

TC13691         GGTGGCCATTGCCGTGGATTCCGTCGCCGTTGCTTCTGCACCAGACATTGTTAA
TC8608          GGTGGCCATTGCCGTGGATTCCGTCGCCGTTGCTTCTGCACCAGACATTGTTAA
TC10253         GGTGGCCACTGCCGTGGATTCCGTCGCCGTTGCTTCTGCACCAGACACTGTTAA
Vv-AMP1         GGAGGAAATTGCCGTGGCTTTCGTCGTCGATGCTTCTGCACTAAGCATTGTTAA
                ** **  * ******** ** ***** ** *********** *  ** ******
```

Figure 3. Alignment of *Vv-AMP1* coding sequence with the nucleotide sequences of the EST identified in the tobacco EST database at TIGR. ***** indicates homology. Alignments were generated in ClustalX (Thompson et al., 1997).

Comparison between the amino acid sequence of mature Vv-AMP1 and the deduced amino acids of the ESTs present in tobacco did show a high level of homology (80.9%, determined with AlignX) between the amino acid sequence of Vv-AMP1 and the tobacco defensins (Figure 4).

```
TC13691         RTCESQSHRFKGLCISKSNCASVCHTEGFNGGHCRGFRRRCFCTRHC
TC8608          RTCESQSHRFKGLCISKSNCASVCHTEGFNGGHCRGFRRRCFCTRHC
TC10253         RTCESQSHRFKGLCFSKSNCASVCHTEGFYGGHCRGFRRRCFCTRHC
Vv-AMP1         RTCESQSHRFKGTCVRQSNCAAVCQTEGFHGGNCRGFRRRCFCTKHC
                ************ *. :****:**:**** **:***********:**
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Figure 4. Amino acid alignment of Vv-AMP1 with the translated sequences of the ESTs identified in the tobacco EST database at TIGR. (*) indicates areas of homology and (**:**) synonymous changes. Alignments were generated in ClustalX (Thompson et al., 1997).

Transgenic lines Tvv15, 17, 18, 19, 20, 27, 29, 32 and 33 had a lower western blot signal intensity than that of the untransformed WT plants. There appeared to be no correlation between the levels of *Vv-AMP1* expression, as detected by northern blot analysis, and the lack of protein production in these transgenic lines, specifically since the two non-expressing lines are amongst these lines with lower levels.

The rest of the transgenic lines showed a western blot signal intensity equal or greater than the WT plant lines, with transgenic lines Tvv4, 7, 8, 9, 12, 13, 24 and 36 showing higher signal intensities than the WT control plants. In these lines there appeared to be a positive correlation between the amount of Vv-AMP1 peptide produced and the observed northern blot signal.

5.4.2 Antifungal activity of crude protein extracts

The crude protein extracts of the WT and transgenic lines showed varied activity against the wilting disease pathogen *V. dahliae* (Figure 5). Growth of *V. dahliae* was greatly reduced in the transgenic lines Tvv7, 8, 13, 23, 24, 33, but also in the non-expressing control line, Tvv18.

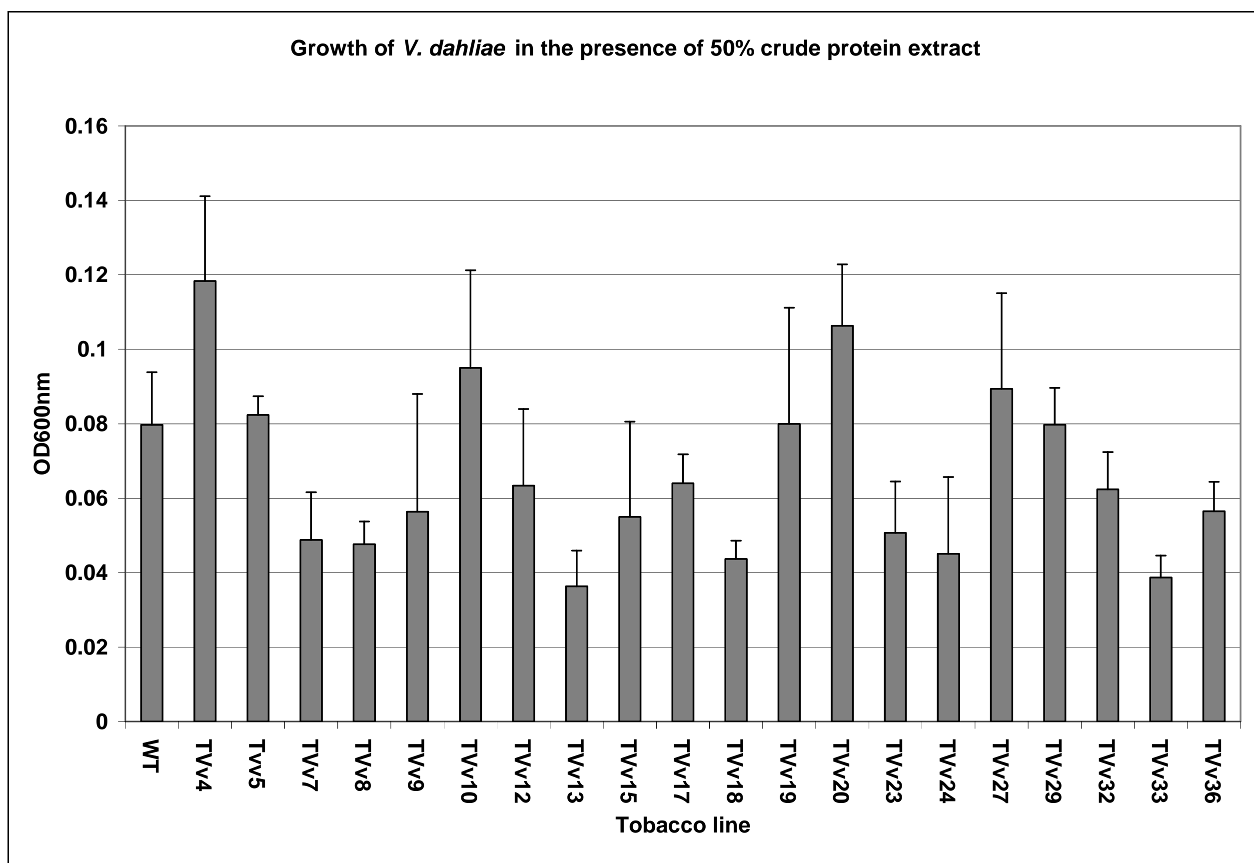

Figure 5. The average growth of *V. dahliae* recorded as optical density in the presence of crude protein extracts from WT tobacco and transgenic lines overexpressing *Vv-AMP1*. Crude extracts was prepared by collecting the liquid present in 500 mg of plant tissue through centrifugation. Error bars denote standard deviation.

Statistical analysis of *V. dahliae* growth in the presence of crude protein extracts from WT and transgenic lines revealed that no statistical significant ($P<0.05$) difference in growth existed between the WT and non-expressing controls and the transgenic lines (Table I).

Table I. Statistical analysis of *V. dahliae* growth in the presence of crude protein extracts from WT tobacco plants and transgenic Tvv-lines overexpressing *Vv-AMP1*.

Transgenic line	Mean of OD_{600}	Homogenous group (Tukey test[a])
Tvv13	0.031250	****
Tvv18[b]	0.038500	****
Tvv8	0.041750	****
Tvv33	0.047250	****
Tvv7	0.048750	****
Tvv32[b]	0.055250	****
Tvv36	0.056500	****
Tvv17	0.057500	****
Tvv23	0.062250	****
Tvv24	0.067750	****
Tvv15	0.069250	****
Tvv9	0.075500	****
Tvv27	0.076000	****
Tvv29	0.079750	****
Tvv10	0.082000	****
Tvv12	0.085500	****
WT[b]	0.092250	****
Tvv20	0.093000	****
Tvv19	0.096750	****
Tvv4	0.097250	****
Tvv5	0.100500	****

[a] ANOVA analysis was performed with the Statistica package using the Tukey HSD test.

[b] The WT and non-expressing controls are indicated in red.

5.4.3 Infection studies conducted on transgenic tobacco lines overexpressing *Vv-AMP1*

Due to the difficulty in establishing successful infections conditions for *V. dahliae* using a soil based infection assay it was decided to evaluate the transgenic lines against the necrotrophic plant pathogen *B. cinerea* using a detached leaf assay.

B. cinerea were able to infect the *in vitro* leaves of the wild type (WT) control and transgenic Tvv-lines at a rapid rate. Lesions were visible after 3 days of incubation at 22°C and 100% relative humidity. Statistical analysis of the recorded lesion size data (Figure 6) using the ANOVA method of the Statistica software revealed that there were no statistical significant differences ($p < 0.05$) between the transgenic lines and the control lines, including the non-expressing transgenic lines Tvv18 and 32 (Table II).

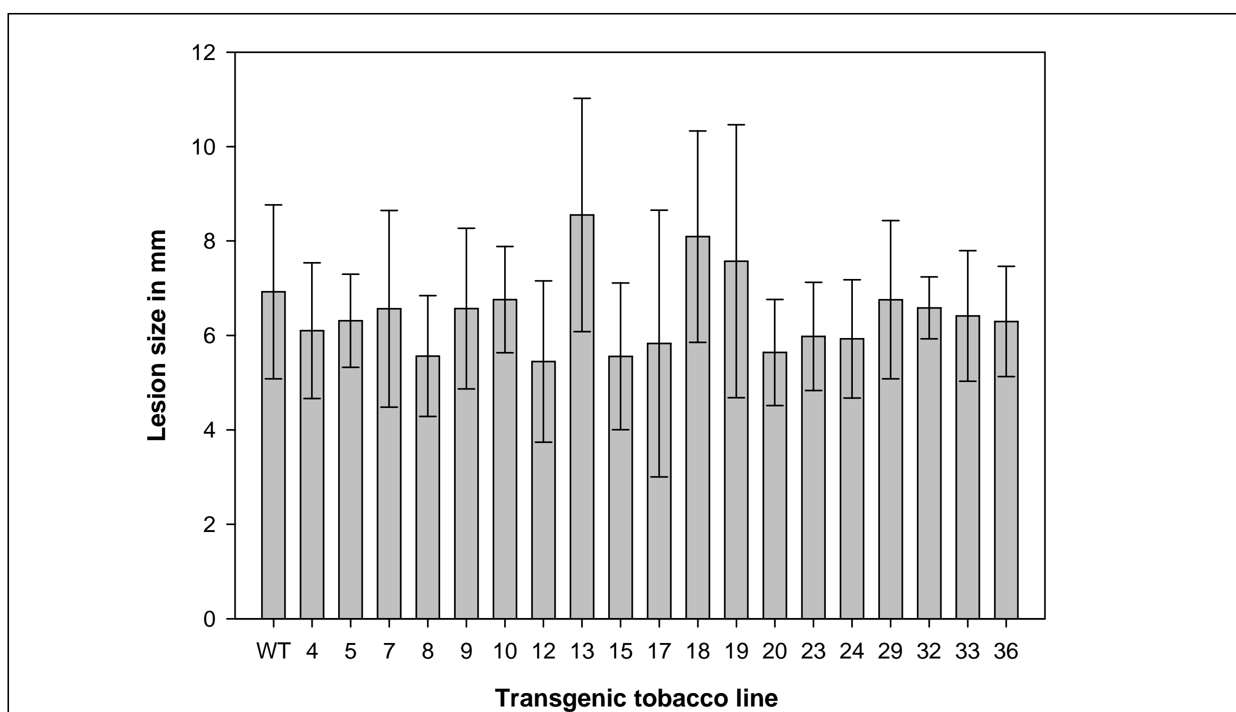

Figure 6. *B. cinerea* infection study conducted on *in vitro* propagated untransformed (WT) and Tvv transgenic lines. Lesion sizes were recorded 3 days after the day of inoculation. Error bars denote standard deviation of the data analyzed.

Table II. Statistical analysis of infection data obtained during the detached leaf assay conducted on *in vitro* transgenic plant material.

Tobacco line	Mean of lesion size	Homogenous group (Tukey test)[a]		
		1[c]	2[c]	3[c]
Tvv15	6.24783	****		
Tvv24	7.16400	****	****	
Tvv8	7.18125	****	****	
Tvv33	7.50952	****	****	
Tvv4	7.63654	****	****	
Tvv29	7.81304	****	****	
Tvv12	7.96786	****	****	
Tvv36	8.09444	****	****	
Tvv20	8.10882	****	****	
Tvv23	8.43824	****	****	
Tvv10	8.47895	****	****	
Tvv17	8.52353	****	****	
Tvv5	8.97045	****	****	
Tvv18[b]	8.98000	****	****	****
Tvv9	9.29375	****	****	
WT[b]	9.60091		****	
Tvv7	9.63500	****	****	
Tvv32[b]	9.73250	****	****	
Tvv13	13.72321			****

[a] ANOVA analysis was performed with the Statistica package using the Tukey HSD test.

[b] The WT and non-expressing controls are indicated in red.

[c] Homogenous group number assigned by Statistica

Infection studies conducted on randomly selected hardened-off leaves of the different transgenic tobacco lines were scored after 6 days of infection with *B. cinerea*. Comparison of the average lesion sizes that developed over the 6 day period and subsequent statistical analysis revealed that no transgenic plant line performed better than the untransformed and non-expressing control lines (Table III).

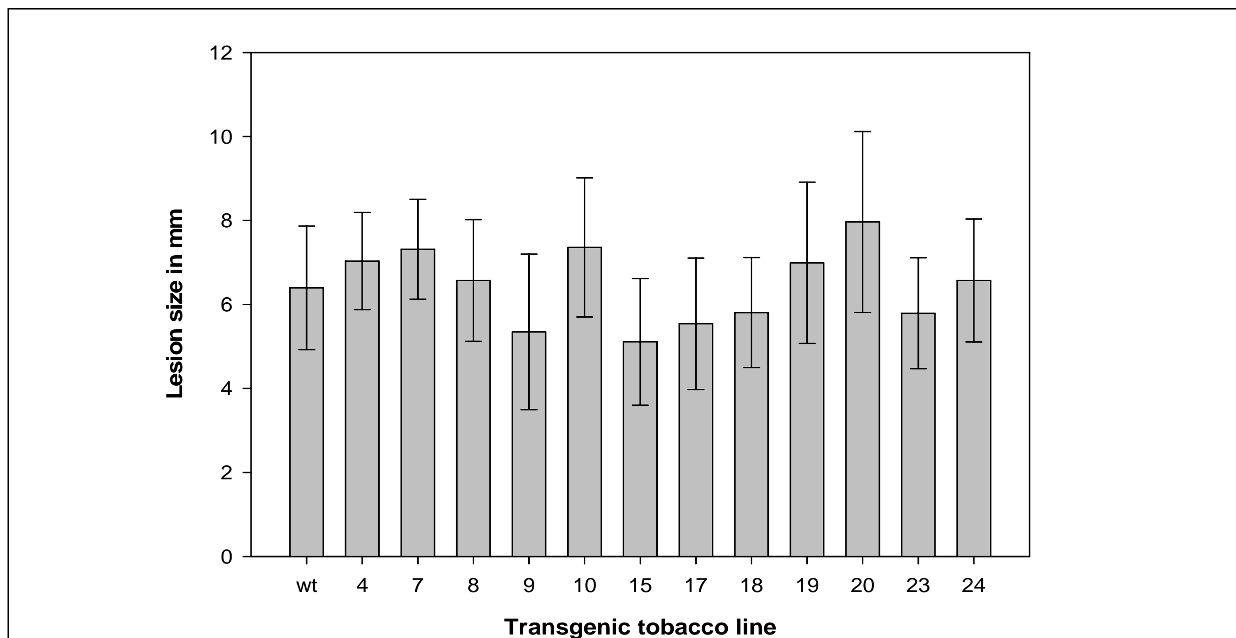

Figure 7. *B. cinerea* infection study conducted on randomly selected hardened off transgenic Tvv-lines and untransformed WT tobacco plants. Data was recorded 6 days after inoculation and growth at 22°C in 100% relative humidity. Error bars denote standard deviation within the data analyzed.

Table III. Statistical analysis of infection data obtained during the detached leaf assay conducted on hardened off transgenic leaf material.

Transgenic line	Mean lesion size	Homogenous group (Tukey test)[a]				
		1[c]	2[c]	3[c]	4[c]	5[c]
Tvv15	5.110526	****				
Tvv 9	5.347931	****				
Tvv 17	5.541250	****	****			
Tvv 23	5.790789	****	****	****		
Tvv 18[b]	5.805556	****	****	****	****	****
WT[b]	6.395444		****	****	****	
Tvv 8	6.572034			****	****	
Tvv 24	6.572321			****	****	
Tvv 19	6.991525			****	****	
Tvv 4	7.035000				****	****
Tvv 7	7.315000				****	****
Tvv 10	7.358929				****	****
Tvv 20	7.965254					****

[a] ANOVA analysis was performed with the Statistica package using the Tukey HSD test.

[b] The WT and non-expressing controls are indicated in red.

[c] Homogenous group number assigned by Statistica

5.5 DISCUSSION

Vv-AMP1 was successfully expressed in tobacco with 20 individual transgenic lines (confirmed with Southern blot) regenerated under kanamycin selection. Southern blot and northern blot analysis revealed that two lines (Tvv18 and 32) were silenced (Figure 2). Although previous reports have suggested that high copy numbers might lead to silencing of expression cassettes (Cogoni and Macino, 1999; Rocha et al., 2005), the silencing in this case was not associated with an excessive number of integration events. Since expression was silenced in only two of the 20 lines, the silencing is probably linked to the transformation event in those specific lines, rather than a phenomenon linked to the presence of the specific transgene. These lines were used as additional negative controls in the phenotypical analyses of the expressing transgenic lines.

The overriding outcome of all subsequent analyses (western blots, as well as *in vitro* and *in planta* antifungal activity and inhibition assays) was that we could not conclusively confirm the presence (western data) and activity (activity and infection tests) of the heterologously overexpressed peptide. The non-expressing controls specifically were instrumental to lead us to this conclusion, since all analyses, also after statistical evaluation could not discriminate any of the transgenic lines from the untransformed controls and the non-expressing lines.

Firstly, our western blot analysis and subsequent bioinformatical analysis of expressed sequences of tobacco confirmed that this genotype contains peptides with putative overlapping activities. When the project was initially designed and the transformations performed, two of the three EST sequences were not present in the databases. These EST sequences, although mainly present in tobacco flowers have also been detected in tobacco leaf material and share enough homology with Vv-AMP1 at deduced amino acids level to allow detection by the mouse polyclonal antibody raised against Vv-AMP1. In retrospect, the tobacco host does not provide a null background like we initially anticipated and our analysis clearly shows that we cannot conclude that the western blot signals obtained are linked to the translation of the grapevine defensin.

Furthermore, it was interesting to note that some of the Tvv-lines showed no, or a lower signal intensity at western blot level than the untransformed WT control, despite having a regularly detected Vv-AMP1 signal at northern blot level (Figure 2). There have been reports of instability of apoplastically overexpressed small peptides, typically leading to problems with western blot detection (De Bolle et al., 1996; Okamoto et al., 1998). Our results might indicate that the heterologously overexpressed peptide was subject to protein degradation strategies that could have been triggered by the plant's mechanism of controlling abundant "foreign" peptides, specifically since the peptides might share similar activities to the products of the highly homologous EST's present in tobacco.

If the grapevine peptides are prone to accelerated degradation, it might also be influenced by the overexpression of the peptide, and specifically the lifting of the

native regulatory mechanisms that exist in grapevine. Expression of *Vv-AMP1* under the CaMV35Sp abolishes tissue specific expression and allows for the expression of the peptide in al tissue types of the transgenic tobacco lines, including areas where instability might occur. *Vv-AMP1* is only expressed in grape berries and only at the onset of berry ripening (see chapter 4 of this dissertation). This regulation might be linked to favorable physiological and/or metabolic conditions important for Vv-AMP1 activity, or perhaps stability of the peptide. Supporting data for this possibility is that we could never detect *Vv-AMP1* expression in the grapevine leaves and expression was also never induced in leaves (or in berries) with the induction stimuli, including *B. cinerea* infection, that were tested (see chapter 4 of this dissertation).

Current work involving the overexpression of *Vv-AMP1* in grapevine might shed more light on the stability of this peptide in the leaf environment (personal communication M. Tredoux). Another possibility that exists is that Vv-AMP1 is toxic when overexpressed in certain plant tissues. Most transgenic work involving defensin genes have focused on the overexpression of defensin peptides belonging to subgroup A of the defensin superfamily, which has been conclusively linked to strong antifungal activities. Vv-AMP1, however, belongs to subgroup B of the plant defensins (see chapter 4 of this dissertation). The peptides in this group have other activities, such as the inhibition of *in vitro* translation systems and protease inhibitory activity as well, which might interfere with the natural biochemistry of the transgenic host and thus activating some regulatory control over peptide levels and/or activity.

The western blot analysis showed that within the transgenic Tvv-lines, an overall reduction of defensin peptide concentrations could be observed, including those of the native (contaminating) tobacco defensins. Again, it might infer that the peptides were subject to post-translational control or increased sensitivity towards proteases present in leaf apoplasts. This theory can also explain why some of the transgenic lines performed worse than the WT and non-expressing plant lines in the *in vitro* and infection studies against *V. dahliae* and *B. cinerea.*

In combination these results indicate that the observed phenotypes could not be conclusively linked to the presence of the Vv-AMP1 peptide. These results are significantly different that those obtained when we overexpressed a plant defensin from *Heuchera sanguinea* (subclass A defensin) in tobacco (De Beer and Vivier, 2002). In that and several other studies it was shown that targeted overexpression of defensin peptides could lead to stable and high levels of heterologous peptides in the apoplasts, as well as increased resistance against fungal pathogens (Broekaert et al., 1999; Saitoh et al., 2001; Banzet et al., 2002; Francois et al., 2002; Kanzaki et al., 2002; Langen et al., 2006).

Further research will need to be conducted on the overproduction of Vv-AMP1 in other hosts and different tissues to clarify the issue surrounding the stability and/or activity of this peptide in heterologous plant environments. It will also be of great interest to isolate and study the promoter of this strictly regulated gene and to identify its regulatory elements and the mode of action of this peptide.

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ADDENDUM A

A 1. PREPARATION FOR THE PRODCUTION, ISOLATION AND BIOCEHMICAL CHARACTERIZATION OF *HELIOPHILA CORONOPIFOLIA* DEFENSINS

Due to the comprehensive chromatographic requirements to separate different defensins peptides isolated from native host tissues it was decided to produce the proteins in a heterologous bacterial expression system by using the defensin genes isolated in chapter three of this dissertation. The nucleotide sequences encoding for the mature peptide regions (mCDS) of Hc-AFP1, 3, 4 and 5 were fused to the 6 X histidine tag of the bacterial expression vector pET14b (Novagen, Madison, WI, USA), or the GST tag from pGEX-2T (Amersham Biosciences, NJ, USA) (Figure 1). Primers were designed to PCR-amplify the mCDSs from their respective pGEM-T clones generated during the isolation of the respective genes (Table I).

Table I. Primers used in the design of all the expression vectors used in this study.

Primer	Primer sequence	Enzyme	Template
pETHc1-5’	5’- CTCGAGAGGTACTGTGAGAGATCGAG -3’	*Xho*I	pGEM-Hc-AFP1
pETHc4-5’	5’- CTCGAGAAGTTGTGCCAGAGACAGAG -3’	*Xho*I	pGEM-Hc-AFP4
pETHc5-5’	5’- CTCGAGCAGAAGTTGTGTGAGAGACC -3’	*Xho*I	pGEM-Hc-AFP5
Hc1-GST-5’	5’-CCGGATCCAGGTACTGTGAGAGATCGAG-3’	*Bam*HI	pGEM-Hc-AFP1
Hc4-GST-5’	5’-CCGGATCCAAGTTGTGCCAGAGACAGAGTG-3’	*Bam*HI	pGEM-Hc-AFP4
Hc5-GST-5’	5’-CCGGATCCCAGAAGTTGTGTGAGAGACCAA-3’	*Bam*HI	pGEM-Hc-AFP5
Hc-AFP1-3’	5’-CGCGGGATCCTCAACATGGGTAGTAACAGA-3’	*Bam*HI	pGEM-Hc-AFP1
Hc-AFP4-3’	5’-CGCCGGATCCTTAACATGTGAAGTAACAGATAC-3’	*Bam*HI	pGEM-Hc-AFP4
Hc-AFP5-3’	5’-CGGCGGATCCTTAACATGGGAAGTAACAGA-3’	*Bam*HI	pGEM-Hc-AFP5

pETHc1-5’ together with Hc-AFP1-3’ amplified both the mCDSs of *Hc-AFP1* and *Hc-AFP3*, as did the corresponding Hc1-GST-5’ primer. PCR reactions were performed on 10 ng template DNA with the Expand high fidelity PCR enzyme (Roche Diagnostics GmbH, Mannheim, Germany) in 50 μl reactions (1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 μM forward primer, 2.5 μM reverse primer). PCR reactions were performed on a Biometric thermocycler with the following program: 94°C for 5 min; 94°C for 1 min, 53°C for 30 sec, 72°C for 1 min, 35 cycles; 72°C for 5 min. PCR products obtained were cloned into the pGEM-T easy vector (Promega Corporation, Madison, USA) and termed: pGEM-Hc1-14b, pGEM-Hc3-14b, pGEM-Hc4-14b and pGEM-HC5-14b. Clones from the PCR products obtained using the Hc-GST-5’ primers were termed pGEM-Hc1GST, pGEM-Hc3GST, pGEM-Hc4GST and pGEM-Hc5GST. The mCDSs were excised with *Xho*I and *Bam*HI (Roche Diagnostics GmbH, Mannheim, Germany) and cloned into the prepared pET14b vector (see Table II) The mCDSs of the pGEM-HcGST clones were excised with *Bam*HI and *Eco*RI (Roche Diagnostics GmbH, Mannheim, Germany) and cloned into the

prepared pGEX-2T vector (see Table II). The integrity of the affinity tag and the mCDSs were confirmed by sequencing.

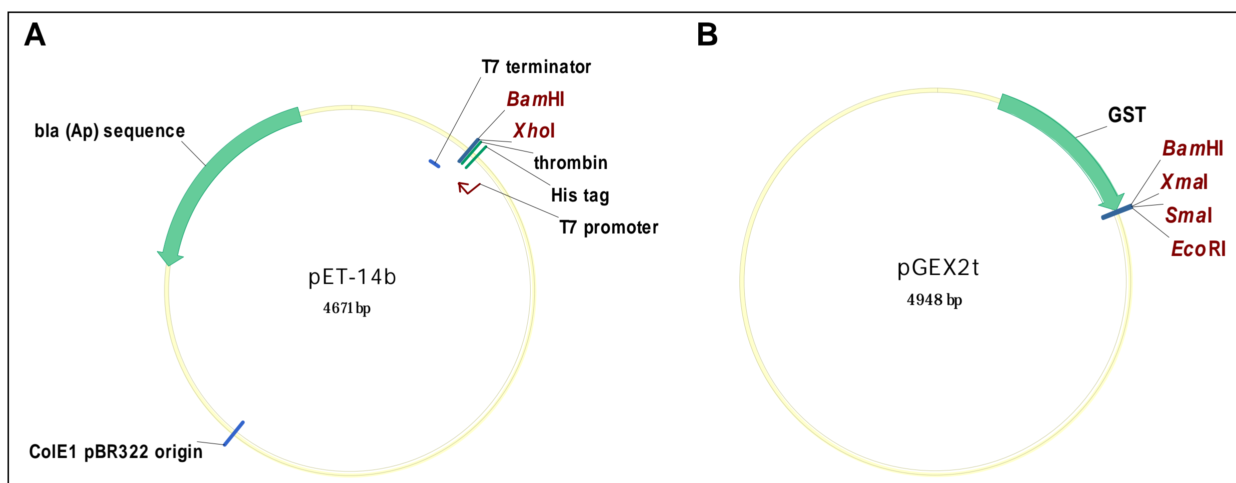

Figure 1. Vector maps of the bacterial expression vectors pET14b from Novagen (A) and pGEX-2T from Amersham (B) used in this study to produce recombinant plant defensin peptide in *E. coli.*

Table II. Expression vectors successfully created for the heterologous defensin production in bacteria.

Defensin gene	Vector	Affinity tag	Expression vector
Hc-AFP1	pET14b	6 x histidine	pET14b-Hc1
	pGEX-2T	GST tag	pGEX-Hc1
Hc-AFP3	pET14b	6 x histidine	pET14b-Hc3
	pGEX-2T	GST tag	pGEX-Hc3
Hc-AFP4	pET14b	6 x histidine	Nc[a]
	pGEX-2T	GST tag	pGEX-Hc4
Hc-AFP5	pET14b	6 x histidine	pET14b-Hc5
	pGEX-2T	GST tag	Nc[a]

[a] NC = Not created

The bacterial expression cassettes were transformed into the bacterial expression host BL21(DE3) (Novagen, Madison, WI, USA) via electroporation and positive transformants were selected on LB agar containing 50 μg ml^{-1} ampicillin, 12.5 μg ml^{-1} tetracyclin, 15 μg ml^{-1} kanamycin sulphate and 34 μg ml^{-1} chloramphenicol (Table III).

Table III. Bacterial expression systems used for the production of *H. coronopifilia* defensins.

Expression vector	*E. coli* host	Expression system
pE14b-Hc1	BL21(DE3)	BL21-pET14b-Hc1
	BL21(DE3)Rosetta-gami pLysS	Rosetta-gami-pET14b-Hc1
pGEX-Hc1	BL21(DE3)Rosetta-gami pLysS	Rosetta-gami-pGEX-Hc1
pET14b-Hc3	BL21(DE3)	BL21-pET14b-Hc3
	BL21(DE3)Rosetta-gami pLysS	Rosetta-gami-pET14b-Hc3
pGEX-Hc3	BL21(DE3)Rosetta-gami pLysS	Rosetta-gami-pGEX-Hc3
pGEX-Hc4	BL21(DE3)Rosetta-gami pLysS	Rosetta-gami-pGEX-Hc4
pET14b-Hc5	BL21(DE3)	BL21-pET14b-Hc5
	BL21(DE3)Rosetta-gami pLysS	Rosetta-gami-pET14b-Hc5

A single colony of the confirmed recombinants was inoculated into 5 ml LB medium with antibiotics and grown overnight at 37°C. One ml preculture was inoculated into four 1 L Erlenmeyer flasks containing 400 ml LB medium with antibiotics and grown at 37°C with continuous shaking until an OD_{600} of 0.7 was reached. Expression of the pET-Hc and pGEX-Hc fusion proteins were induced with 0.4 mM IPTG (Roche Diagnostics GmbH, Mannheim, Germany) for 5 hours at various temperatures (37°C, 30°C and 22°C) as an optimization step.

The Hc-AFP defensins were initially expressed in *E. coli* strain BL21(DE3), but no detectable levels of the 6 x his-Hc-AFP fusion proteins were observed after induction with 0.4 mM IPTG for 5 hours at 37°C. It was decided to analyze the deduced Hc-AFP amino acid sequences with the Vector NTI suite (Invitrogen, Carlsbad, USA) to determine the level of codon bias that existed between the plant peptides and the BL21(DE3) *E. coli* host. Significant levels of bias was observed and it was decided to change the recombinant *E. coli* host to BL21(DE3) Rosetta-gami pLysS (Novagen, Madison, WI, USA) that contains a plasmid encoding for the 10 most rare codons of *E. coli*.

When recombinant production was initiated with 0.4 mM IPTG for 5 hours at 37°C in the BL21(DE3) Rosetta-gami pLysS host, containing the pET-Hc1, 3 and 5 expression vectors, a rapid decline in OD_{600} was observed from 0.7 to 0.1 within the first hour of induction. The experiment was repeated at different temperatures including 30°C and 22°C, but a decline in OD_{600}, indicative of lyses of the *E. coli* host was observed in all the optimizations and repeat experiments.

The plant defensins were then fused to the large (26 kDa) GST tag in the pGEX-2T vector in the hope of abolishing the antimicrobial activity of the peptides by inhibiting the folding into the correct conformation, which would allow for the production of these peptides in *E. coli.* Initiation of the BL21(DE3) Rosetta-gami pLysS host, containing the pGEX-Hc1, 3 and 4 expression vectors with 0.4 mM IPTG for 5 hours at 37°C still resulted in a slight decline in OD_{600} from 0.7 to 0.6, and only a negligible amount (<100 μg) of fusion peptide could be purified from 1.6 L of

culture. The other induction temperatures of 30°C and 22°C have not been tested with this system.

Although the production of the four defensin peptides from *H. coronopifolia* in *E. coli* has proven to be problematic, it was however expected, since recombinant defensin production in *E. coli* is reported to be difficult (Park et al., 2002; Chen et al., 2004). Lyses of the bacterial cells, for all four defensin genes, upon induction with IPTG were of real interest, since these peptides belong to subgroup A3 of the defensin family, which is characterized as not having antibacterial activity. The recombinant production of the peptides for further characterization is currently being further optimized.

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ADDENDUM B

B 1. PREPARATION OF NOVEL DEFENSIN GENES INTO PLANT EXPRESSION CASETTES, TRANSFORMATION VECTORS AND PROGRESS WITH TRANSFORMATION TO PLANT HOSTS FOR COMPLETE *IN PLANTA* FUNCTIONAL ANALYSIS

The aim of the grapevine biotechnology group at the Institute for Wine Biotechnology is to develop novel technologies and resources for the genetic improvement of grapevine through the use of genetic engineering. This involves both the improvement of resistance towards biotic and abiotic factors and improving aspects of quality. Genes with favorable characteristics identified through *in vitro* experiments must be evaluated *in vivo*, before entering the final stages of our biotechnology program. To this end we set out to evaluate the defensin genes from *H. coronopifolia* isolated in chapter 3 and the *Vitis vinifera* defensin isolated in chapter 4 of this dissertation.

The complete coding sequences for the *H. coronopifolia* defensins were expressed in tobacco under control of the 35S cauliflower mosaic virus promoter (CaMV35Sp) and their native signal peptides. The complete coding sequence (CDS) for Hc-AFP1, 3, 4 and 5 were obtained from their respected pGEM-T clones generated during the isolation of these genes (see chapter 3 of this dissertation). The CDS were excised with *Xho*I and *Bam*HI respectively and clone into the *Xho*I/*Bam*HI sites of the expression vector pART27casette, a pART27 vector containing the expression cassette from pART7, cloned into the *Not*I sites of pART27 (Gleave, 1992) (Figure 1A). The expression vector for the expression of *Vv-AMP1* under its native signal peptide was created in chapter 5 of this dissertation.

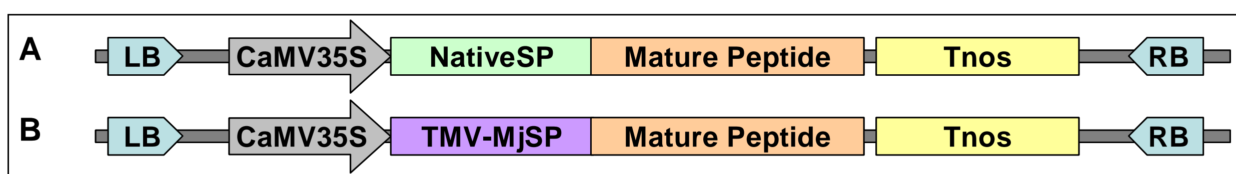

Figure 1. The plant expression cassettes used for tobacco transformation with the antifungal defensins from *H. coronopifolia* (Hc-AFPs) and *Vitis vinifera*. (A) The expression cassette for the over expression of plant defensins under their native signal peptides. (B) The expression cassette for the overexpression of plant defensins under the *M. jalapa* signal peptide. The abbreviations represent: **RB**, T-DNA right border; **LB**, **T-DNA** left border; **CaMV35S**, promoter of 35S RNA of cauliflower mosaic virus with duplicated enhancer region; **Pnos**, promoter of T-DNA nopaline synthase gene; **NPTII**, coding region of neomycin phosphotransferase II gene; **Tnos**, terminator of T-DNA nopaline synthase gene; **NativeSP**, signal peptide encoding domain of native *Hc-AFP* and *Vv-AMP1* genes; **TMV-MjSP**, TMV leader sequence and *Mirabilis jalapa* signal peptide; **Mature Peptide**, mature protein encoding domain of plant defensin genes; **OCS**, terminator of the octopine synthase gene.

Sub-cellular targeting of the defensin peptides were also placed under direction of the proven *Mirabilis jalapa* signal peptide from the chitin binding peptide Mj-AMP2 (Figure 1B) (De Bolle et al., 1996). The nucleotide sequences encoding for the mature peptide regions (mCDS) of the defensin peptides were isolated by PCR (see Table I for primers used) and cloned in to pGEM-T easy to yield pGEM-matureHcs and pGEM-matureVv1. The mCDSs were excised using *Hin*dIII and *Bam*HI and cloned into the prepared pART-TMV-Mj SP vector (Figure 2) to yield pART-TMV-HCs and pART-TMV-Vv1. The expression cassettes were excised with *Not*I and cloned into *Not*I prepared pART27. All plant expression vectors are listed in (Table II).

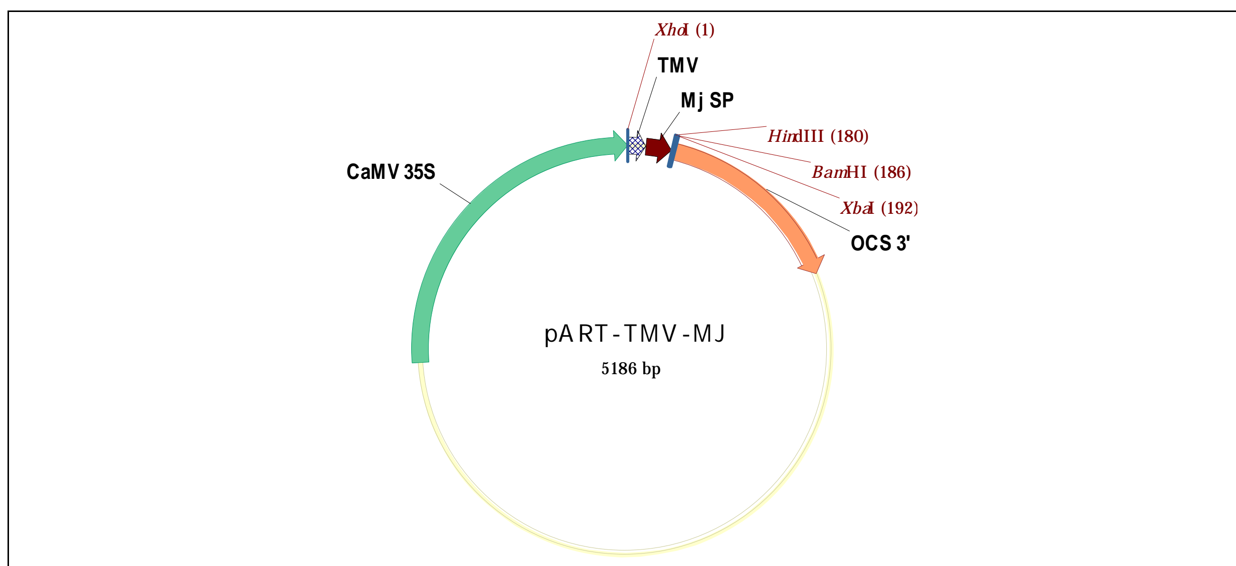

Figure 2. The pART-TMV-Mj SP vector that allows for the expression of the plant defensin genes under control of the CaMV35S promoter and localization directed by the signal peptide of Mj-AMP2 from *Mirabilis jalapa* (De Bolle et al., 1996).

Table I. Primer sets used in the construction of the expression vectors where the localization of the plant defensins is directed by the Mj-AMP2 signal peptide.

Primer name	Primer sequence	Restriction enzyme	Primer partner
Hc-AFP1-5’	5’-CGCGAAGCTTAGGTACTGTGAGAGATCGAG-3’	*Hin*dIII	Hc-AFP1-3’
Hc-AFP4-5’	5’-CGCGAAGCTTAAGTTGTGCCAGAGACAGAGTGG-3’	*Hin*dIII	Hc-AFP4-3’
Hc-AFP5-5’	5’-CGCGAAGCTTCAGAAGTTGTGTGAGAGACC-3’	*Hin*dIII	Hc-AFP5-3’
Vitis mature def5'	5’-CCA AGC TTA GGA CCT GTG AGA GTC A-3’	*Hin*dIII	Vitisdef-3’
Hc-AFP1-3’	5’-CGCGGGATCCTCAACATGGGTAGTAACAGA-3’	*Bam*HI	Hc-AFP1-5’
Hc-AFP4-3’	5’-CGCCGGATCCTTAACATGTGAAGTAACAGATAC-3’	*Bam*HI	Hc-AFP4-5’
Hc-AFP5-3’	5’-CGGCGGATCCTTAACATGGGAAGTAACAGA-3’	*Bam*HI	Hc-AFP5-5’
Vitisdef-3’	5’-CCGGATCCTTAACAATGCTTAGTGC-3’	*Bam*HI	Vitis mature def5'

Table II. Expression vectors created for the *in planta* analysis of the defensin genes isolated from *H. coronopifolia* and *V. vinifera*.

Defensin gene	Signal peptide	Promoter	Expression vector
Hc-AFP1	Native	CaMV35S	pART27-Hc1
	Mirabilis jalapa	CaMV35S	pART27Mj-Hc1
Hc-AFP3	Native	CaMV35S	pART27-Hc3
	Mirabilis jalapa	CaMV35S	pART27Mj-Hc3
Hc-AFP4	Native	CaMV35S	pART27-Hc4
	Mirabilis jalapa	CaMV35S	Nc
Hc-AFP5	Native	CaMV35S	pART27-Hc5
	Mirabilis jalapa	CaMV35S	pART27Mj-Hc5
Vv-AMP1	Native	CaMV35S	pART27-Vv1
	Mirabilis jalapa	CaMV35S	pART27Mj-Vv1

The plant expression vectors were mobilized into *A. tumefaciens* EHA105 via electroporation (Mattanovich et al., 1989). Tobacco leaf discs were transformed by the standard leaf disc transformation method (Horsch et al., 1985). Transgenic plants were regenerated under kanamycin selection (120 μg ml^{-1}). The resulting transgenic tobacco plantlets were sub-cultured on MS medium (Murashige and Skoog, 1962) without hormones and maintained at 25°C under a 16 h/8 h light cycle (Table III). Three-week-old plantlets were hardened-off in soil under greenhouse conditions. The primary, hardened-off transformants were allowed to self-pollinate. The resulting seeds were germinated under kanamycin selection to yield F1-generations.

Grapevine cultivar Sultana was transformed with pART27-Hc1 and pART27-Hc4 and pART27MjSP-Vv1 via *Agrobacterium* transformation of embryogenic callus (done by Dr Krishnan Vasant at the Institute for Wine Biotechnology). Transgenic embryos were generated under kanamycin selection (100 μg ml^{-1}) and the transgenic grapevine plantlets regenerated on MS medium without hormones at 25°C under a 16 h/8 h light cycle (Table III). Transgenic grapevines were maintained on MS medium without hormones at 25°C under a 16 h/8 h light cycle until enough material could be collected for clonal propagation of each transgenic line.

The transformations of all the constructs mentioned in Table III are currently being completed. Once transgenic populations of a suitable size have been established they will be characterized genetically and their expression profiles determined.

Table III. Transgenic populations created with expression vectors overexpressing the *H. coronopifolia* and *V. vinifera* defensin genes.

Expression vector	Transgenic host	Number of plants	Seeds for F1
pART27-Hc1	Tobacco	Not transformed	
pART27Mj-Hc1	Grapevine	>50	Not applicable
pART27-Hc3	Tobacco	Not transformed	
pART27Mj-Hc3	Tobacco	11	Yes
	Grapevine	>50	Not applicable
pART27-Hc4	Tobacco	14	Yes
pART27Mj-Hc4	Grapevine	>50	Not applicable
pART27-Hc5	Tobacco	Not transformed	
pART27Mj-Hc5	Grapevine	Not transformed	Not applicable
pART27-Vv1	Tobacco	20	Yes
pART27Mj-Vv1	Grapevine	>50	Not applicable

Molecular and biochemical analyses of the populations will include evaluation of transgene stability as well as the stability of the heterologous peptides in a foreign environment. All transgenic plants will be analyzed in fungal infection studies to determine the contribution of the various defensin transgenes in establishing a resistance phenotype towards fungal disease. The available transgenic populations will be very useful in studying the effects of the various peptides and to characterize the various phenotypes that might be linked to the various transgenes and the activities of their encoded products.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

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6.1 GENERAL DISCUSSION AND CONCLUSIONS

Modern agriculture will only be successful if it manages to produce enough food of good nutritional quality to satisfy world needs under the prevailing environmental conditions. Efficient management of crop pests is essential to ensure high yields of commercial crops, thus ensuring a constant food supply for an ever growing world population. The agricultural sector is in a constant battle to develop new strategies as new and resistant species of plant pathogens and pests arise. Pest management has traditionally relied upon the use of chemical pesticides and fungicides to protect crops against insect pest and microbial spoilage, but a rise in chemical resistance among plant pathogens have greatly reduced their efficiency in protecting economical important crop species (Chapeland et al., 1999; Yourman and Jeffers, 1999; Hayashi et al., 2002). The effect of chemical pesticides on the environment and human health (Barr et al., 2004) is paramount among the concerns of consumers and has led to a public drive for a reduction in the use of chemical fungicides.

Over the last fifteen years industry and researchers have been investigating alternative measures to protect crops against pests (McLaughlin et al., 1992; Elad et al., 1993; Shah, 1997; Montesinos et al., 2002). The traditional methods of breeding have always proven successful, but are time consuming and can not keep pace with the development of new resistant variants of pathogens. Another drawback of plant breeding is that quality traits are not always associated with disease resistance traits, resulting in a crop where either one of the traits is compromised. The potential of biological control has not yet been proven on a commercial scale and the effectiveness of these strategies is always subject to environmental factors. Genetic engineering has made a huge impact on the protection of commercial crop species without altering the nutritional quality and yield of the crops. Genetic engineering has however been more successful in protecting crops against insects, where many engineered crops have been commercialized (Dempsey et al., 1998; Schuler et al., 1998; Hilder and Boulter, 1999). The engineering of microbial resistant crop species have however proved less successful.

Currently scientists are evaluating the potential of the natural defense systems in plants to protect susceptible crop species through genetic engineering (van der Biezen, 2001; Montesinos et al., 2002). Many natural defense proteins and chemical compounds show potential, but it was a small plant defensive peptide belonging to the family known as plant defensins that yielded the first disease resistant engineered crop, showing resistance at field trial level (Gao et al., 2000). For this ground-breaking result, the defensin from alfalfa was introduced into potatoes, yielding transgenic potato lines with high levels of resistance to the wilting disease causing fungus *Verticillium dahliae*. Plant defensins have also been expressed in various crop species, resulting in enhanced defense to some major economically important fungal

pathogens (Terras et al., 1995; Shah, 1997; Garcia-Olmedo et al., 1998; Broekaert et al., 1999; Kanzaki et al., 2002; Sjahril et al., 2006).

Plant defensins are small basic peptides with a broad spectrum of antifungal activity and have been shown to play an important role in the innate immunity of plants (Lay and Anderson, 2005; da Cunha et al., 2006; Kiraly et al., 2007). With the isolation and characterization of more members of this peptide family, it has become apparent that these peptides have a broad spectrum of biological activities, ranging from anti-insecticidal, metal tolerance and the inhibition of plant parasite growth (Bloch Jr and Richardson, 1991; Mirouze et al., 2006; de Zélicourt et al., 2007). Some peptides have also been linked to activities important for medical application, including activities such as the inhibition of HIV reverse transcriptase and anti-tumor properties (Wong and Ng, 2003 and 2005). What is important, is that these activities were correlated with single amino acid changes within the 45-54 amino acid peptides (Lay and Anderson, 2005; Zhu et al., 2005). This makes the continued isolation of new and novel defensin peptides all the more important in the identification of activities that could be of agricultural and medical importance.

The work presented in this dissertation contributes information regarding 15 new antifungal peptides. These peptides have been isolated and characterized to provide novel genetic resources that may be used in biotechnological applications. Equally important however, is the insights they provide into the activities, regulation and possible mode of actions for some of the peptides that were studied more in depth. Only one of the peptides, a defensin from grapevine was comprehensively studied, but all the other novel peptides provide significant scope for additional work to characterize these putatively valuable peptides.

Nucleotide databases and PCR-amplification strategies provide valuable tools in the isolation of new plant defensin genes

Plant defensin peptides isolated from the *Brassicaceae* family have made the biggest contribution to the successful engineering of disease resistant crop species (Gao et al., 2000). Screening of the basic, heat-stable peptide fractions from three local *Brassicaceae* spp against the fungal pathogen *Botrytis cinerea*, revealed the presence of small antifungal peptides inducing characteristic morphological changes associated with antifungal plant defensin activity (Chapter 3 of this dissertation). A strategy was devised that would allow for the quick and easy isolation of genes encoding for these putative plant defensin genes.

Extensive database analysis and homology profiling of plant defensin genes belonging to the *Brassicaceae* family have allowed us to implement a homology-based amplification strategy to isolate new and novel plant defensin encoding genes. The strategy was based on the design of PCR primers that would recognize nucleotides encoding for the signal peptide, as well as the C-terminal regions of the *Brassicaceae* defensins.

These primers allowed us to isolate 14 new defensin genes from *Brassicaceae* genera present in South Africa, including the native species *Heliophila coronopifolia* or sun flax (Chapter 3 of this dissertation). Even though this strategy relies on nucleotide homology to existing members of the *Brassicaceae* defensins, newly isolated defensin genes shared less than 56% homology at the nucleotide level and 42% homology at the deduced amino acid level. This variance in amino acid sequence is of great importance in the search for new biological activities of plant defensins, where single amino acids can give rise to new biological activities. The phylogenetic relationship of the isolated defensins with other members of the defensin superfamily was also assessed, but no relation was observed with the defensins isolated from the *Solanaceae*, *Poaceae* and *Fabaceae* families. This would suggest that the isolation strategy is selective for defensins from the *Brassicaceae* species and that each plant family will require its own set of primers to isolate their respective defensin genes. Some of these defensin genes are also being prepared to enter our biotechnology program (Addendum A and B of this dissertation).

With the improvement of bioinformatical algorithms to search nucleotide databases it is apparent that genes encoding for these small cysteine-rich antifungal peptides contribute significantly to the genetic material of plants, with more than 300 defensin genes identified in the *Brassicaceae* model plant *Arabidopsis thaliana* (Silverstein et al., 2005; Silverstein et al., 2007). With more sequences being isolated, homology based isolation methods can be improved in sensitivity and selectivity and with more than 3000 plant species present in the *Brassicaceae* genera, this isolation strategy might allow for the isolation of peptides with unique activities important for the agricultural and medical biotechnology sectors.

A ripening induced defensin from *Vitis vinifera* shows strong antifungal activity

Through our extensive analysis of sequence databases for novel plant defensin encoding genes we identified a putative plant defensin encoding gene within the fruit crop *Vitis vinifera* and termed it *Vitis vinifera* antimicrobial peptide 1 (*Vv-AMP1*) (Chapter 4 of this dissertation). Genetic characterization and subsequent analysis of the newly released *V. vinifera* genome, showed that *Vv-AMP1* was present as a single copy gene on chromosome 1. Analysis of the deduced amino acid sequence and subsequent phylogenetic analysis grouped Vv-AMP1 with subgroup B of the plant defensin superfamily, a group known for both antifungal and antibacterial activity (Harrison et al., 1997).

Vv-AMP1 showed a well regulated pattern of expression, being highly tissue specific and developmentally regulated. *Vv-AMP1* expression was only present in grape berries and only at the onset of véraison. This form of developmental regulation has also been reported for other members of the defensin superfamily (Lay et al., 2003; Nielsen et al., 2006). What makes the regulation of *Vv-AMP1* unique, however, is the lack of response to the plant hormones salicylic-, jasmonic- and abscisic acid. *Vv-AMP1* gene expression was strictly developmental and did not

respond to pathogen attack by the well known grape pathogen *B. cinerea* or to wounding and osmotic stress. This interesting expression pattern, lack of induction and strong antifungal characteristics makes this peptide an interesting defensin to study, specifically to gain more understanding of its biological role, mode of action and relevance *in planta*.

With the completion of the *V. vinifera* genome the putative promoter sequence of *Vv-AMP1* has become available and will facilitate the isolation and characterization of the *Vv-AMP1* promoter region. Characterization of the promoter region might shed light on the factors contributing to the strict level of control on the *Vv-AMP1* gene.

Localization of plant defensin peptides to the apoplastic regions of plant tissues are always predicted, but rarely proven for the individual defensin peptides. Localization of Vv-AMP1 directed by its signal peptide was evaluated with the green fluorescent protein (GFP) reporter gene. Fluorescent microscopy confirmed the predicted apolastic targeting directed by the Vv-AMP1 signal peptide. What were interesting though were the high levels of GFP accumulation in the vascular tissue and guard cells of the stomata. Localization to these plant cell types by the defensin signal peptides have never been observed before. The stomata are favorite entry points for fungal pathogens and targeting of the Vv-AMP1 defensin peptide to these areas supports a proposed role in plant defense.

Recombinant Vv-AMP1 produced in *E. coli* confirmed that the peptide has properties characteristic of plant defensin peptides, being highly basic and having a molecular weight below 5.5 kDa. Plant defensins are well documented for their strong antifungal activity and Vv-AMP1 was no exception, showing strong antifungal activity at low concentrations against a broad spectrum of fungal pathogens (Terras et al., 1993; Osborn et al., 1995; Terras et al., 1995; Thomma et al., 2002; Song et al., 2004; Lay and Anderson, 2005; Da-Hui et al., 2007; Odintsova et al., 2007). Vv-AMP1 activity was associated with induced membrane permeabilization, as indicated by the propidium iodide uptake assay. In the relative few conclusive mode-of-action studies on defensins, the induced membrane permeabilization was a phenomenon thus far only reported for members of subgroup A of the plant defensin family (Thevissen et al., 1996; Thevissen et al., 1999; Thevissen et al., 2003a; Thevissen et al., 2003b). Whether membrane permeabilization of this subclass B defensin is linked to changes in fungal membrane ion fluxes, as is the case for subgroup A defensins, remain to be seen and present very exciting prospects to study the mode of action of this peptide further.

Overexpresssion of *Vv-AMP1* in tobacco does not reduce disease susceptibility towards *B. cinerea*

Vv-AMP1 was successfully overexpressed in transgenic tobacco under control of the constitutive CaMV35S promoter and localization directed by its native signal peptide (see Chapter 5 of this dissertation). Expression of Vv-AMP1 was confirmed for 18 of the 20 individual transgenic tobacco lines, with two lines showing a silencing

phenotype. The silencing was not correlated to excessive integration of the expression cassette (Cogoni and Macino, 1999; Rocha et al., 2005).

Western blot analysis revealed production of Vv-AMP1 as well as correct post translational processing resulting in removal of the signal peptide. Only 14 of the 18 northern positive transgenic tobacco lines gave western blot signals. More disconcerting was the presence of a western blot signal in the untransformed WT tobacco lines despite no Southern or northern blot hybridization signal. BLASTN analysis of the *Nicotiana tabacum* EST database with the cDNA sequence of Vv-AMP1 showed the presence of three EST sequences in tobacco that could be responsible for the background signal in the untransformed control lines of the western blot analysis.

Of the 20 individual transgenic lines obtained nine showed no western blot signal or a signal less intense than the untransformed control lines, not withstanding detectable levels of expression. This suggest that Vv-AMP1 is either post translationally regulated, resulting in the degradation of not only Vv-AMP1, but the native tobacco defensins as well, or Vv-AMP1 is unstable in the physiological conditions of tobacco leaves. This could be due to sensitivity towards proteases present in the apolastic regions of the leaf tissue.

The possibility of Vv-AMP1 instability in the leaf environment might explain why Vv-AMP1 is so tightly regulated in its native host *Vitis vinifera*. *Vv-AMP1* expression has never been detected in grapevine leaf material and is only present in grape berries and only at the onset of berry ripening. This tissue specific expression pattern could never be overcome with treatment by hormones, osmotic stress, wounding or even infection by *B. cinerea*. This might suggest that the stability of Vv-AMP1 is dependant on certain physiological and/or metabolic conditions only present in grape berries and only at the onset of berry ripening.

In vitro antifungal assays conducted against *Verticillium dahliae* and infection studies conducted with *B. cinerea* did showed that some of the transgenic lines, with the stronger western blot signals, did perform slightly better than the untransformed WT tobacco lines, but when compared with the non-expressing Southern positive transgenic lines no statistical significant reduction in disease susceptibility was observed. This was the case in the *in vitro* assays and the infection studies conducted with *in vitro* and hardened-off transgenic tobacco lines. No clear link could thus be established between the presence of Vv-AMP1 peptide and the resistance phenotypes observed. The question thus remains; what is the biological role of Vv-AMP1 within grape berry development?

Currently, we are investigating the role of Vv-AMP1 within its native host. This includes analysis of the Vv-AMP1 promoter to identify the elements responsible for its strict developmental control. Other opportunities are explored by studying the effect of the peptide on economically important grapevine pathogens, specifically since preliminary *in vitro* analysis indicated that the peptide is highly active against a range of pathogens (current MSc study of M. Tredoux, personal communication). Moreover,

the transgenic tobacco and grapevine populations (see Addendum B) that resulted from this study could also be analyzed on the -omics level (transcriptomic, proteomic and metabolomic analyses) to gain more insight into the mode of actions, molecular control mechanisms, as well as cross-talk within the complex plant-pathogen interaction.

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