

Overexpression and evaluation of an antimicrobial peptide from *Heuchera sanguinea* (Hs-AFP1) for inhibition of fungal pathogens in transgenic tobacco

by

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*Thesis presented in partial fulfillment of the requirements for the degree of
Masters in Science at Stellenbosch University*

March 2002

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis 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.

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SUMMARY

Seed germination is the most vulnerable time in a plant's life cycle, since the thick protective seed coat ruptures and the moist and humid soil environment not only favours seed germination, but also the growth and development of plant pathogens. Infection of plant seeds during germination, however, is the exception rather than the rule. Plant seeds have developed a complex preformed defense mechanism that includes antifungal agents that diffuse into the surrounding environment to form a protective layer around the seed. This protective layer prevents fungal and bacterial pathogens from infecting the young seedling.

Over the last decade, scientists have studied the defense mechanisms of different seeds in an effort to understand and ultimately to introduce and/or manipulate these mechanisms in plants as part of the plant's endogenous disease resistance to pathogens. Various chemical compounds, peptides and proteins that showed strong *in vitro* activities against various fungi were isolated in these efforts. The mere demonstration of *in vitro* activity alone, however, is not sufficient to assign a defense role to these antifungal agents. Typically, mutant plants that have lost the ability to produce the antifungal agent, or mutants that are overproducing the agent, have been used to correlate the mutant phenotype to either a decline or increase in disease resistance respectively. Genetic transformation and the subsequent development of transgenic plants have made an unprecedented impact in this regard, specifically in understanding the role of specific defense-related proteins and their interaction with plant pathogens.

In this study, the antifungal peptide, Hs-AFP1, from *Heuchera sanguinea*, a plant defensin, was evaluated in a heterologous *in planta* environment as a defense protein with potential for engineering disease resistant crops. The *in vitro* assays performed with Hs-AFP1 against *Botrytis cinerea* showed antifungal activities of 88% growth inhibition at a concentration of 8 µg/ml of the purified peptide, while inducing a characteristic hyperbranching effect on the *Botrytis* hyphae. Tobacco was subsequently transformed with a construct, pFAJ3068, expressing Hs-AFP1 under the strong constitutive 35S promoter. The peptide was targeted to the apoplastic region with the signal peptide from Mj-AMP2, an antimicrobial peptide from *Mirabilis jalapa*. Due to reports of peptide instability in transgenic plant systems, two additional constructs were prepared and transformed into tobacco to anticipate possible Hs-AFP1 instability in the heterologous tobacco environment. A putative peptide stabilization construct, pHs-EXG1, consisted of a fusion between Hs-AFP1 and the antifungal exo-glucanase (encoded by *EXG1*) from *Saccharomyces cerevisiae*. A control construct, pMj-EXG1, expressing *EXG1* targeted to the apoplastic region with the Mj-AMP2 signal peptide, was also prepared and transformed into tobacco to normalize the background antifungal activity as a result of the exoglucanase in the fusion construct lines.

Tobacco was successfully transformed with pFAJ3068, pHs-EXG1 and pMj-EXG1, resulting in transgenic tobacco lines designated THs, THE and TME respectively. Transgene expression was confirmed for the THs and THE transgenic lines. The translation of these transcripts into proteins was also confirmed with Western blot analysis. Moreover, the heterologous production of Hs-AFP1 in tobacco led to an increase in disease resistance to *B. cinerea* in the THs lines in comparison with the untransformed tobacco controls. An increase of up to 42% in disease resistance was observed in an *in planta* detached leaf assay. Crude protein extracts from the THs lines were also analyzed in an *in vitro* quantitative fungal growth assay. This assay confirmed the results obtained with the disease resistance assay, with crude protein extracts exhibiting up to 40% fungal growth inhibition. The incubation of *B. cinerea* in the presence of crude protein extracts from THs lines resulted in hyperbranching of the fungal hyphae, which is characteristic of Hs-AFP1 activity.

From these analyses it was clear that the heterologously expressed Hs-AFP1 was quite stable in the transgenic environment. The fusion between *Hs-AFP1* and *EXG1* did not increase the stability of Hs-AFP1, but rather led to a loss of the Hs-AFP1 activity. All the analyses performed showed the THE lines to be reduced in their ability to inhibit fungal infection in comparison to the THs line. Also, microscopic analysis of the effects of the crude THE extracts on *B. cinerea* growth showed no hyperbranching activity, again confirming the loss of peptide activity due to the fusion to *EXG1*. This is in agreement with previous work, in which sarcotoxin 1A was fused to a reporter gene and also lost activity.

Although integration of the Mj-EXG1 expression cassette was confirmed, no mRNA levels could be detected with Northern blot or RT-PCR analysis of the TME lines. These lines also did not show any *in vitro* antifungal activities, probably indicating post-transcriptional gene silencing. This silencing was overcome in the fusion constructs that were expressed in the THE plant lines. These lines also showed *EXG1* protein activity, as measured by β -glucosidase assays. Although the THE lines did not serve the functions originally envisaged, they fortuitously showed that a fusion strategy might stabilize glucanase expression in a transgenic environment. A variety of glucanases have been shown to be prone to gene silencing when overexpressed in a plant environment and the yeast glucanase can now be added to that list if it is not present as a fusion protein.

Overall, this study confirmed that Hs-AFP1 is involved in plant defense systems and provided valuable information on the stability of small peptides in a heterologous environment. The positive results obtained with overexpressed Hs-AFP1 on fungal inhibition in this study merits further investigations into the use of this peptide in the engineering of disease-resistant crops.

OPSOMMING

Saadontkieming is die mees vatbare tyd vir siekteontwikkeling gedurende 'n plant se lewenssiklus. Die saadhuid bars en die vogtige grondkondisies bevoordeel nie net saadontkieming nie, maar ook die groei en ontwikkeling van plantpatogene. Infeksie van plantsade tydens ontkieming is egter die uitsondering eerder as die reël. Plantsade besit komplekse verdedigingsmeganismes teen moontlike patogeënfeksies. Dié meganismes sluit die produksie van antifungiese agense, wat tydens saadontkieming na die omliggende omgewing diffundeer om 'n beskermende sone om die ontkiemende saad te vorm, in. Die gevolglike antifungiese sone beskerm die saad teen infeksie deur bakterieë en swamme.

Gedurende die laaste dekade het navorsers baie aandag aan die bestudering van plantsaadverdedigingsmeganismes gegee. Dié kennis word gebruik om die verdedigingsmeganismes beter te verstaan, asook om dié meganismes te manipuleer en/of oor te dra aan plantspesies met inherente swak weerstandsmeganismes wat gereeld aan plantpatogeënfeksies onderhewig is. Navorsing op plantsade het tot die isolasie van verskeie chemiese agense, peptiede en proteïene, wat sterk *in vitro* aktiwiteite teen 'n wye reeks swampatogene vertoon, gelei. Die vermoë van dié agense om swamme in 'n *in vitro* omgewing te inhibeer, is alleen egter nie 'n bewys dat hulle 'n rol in plantverdediging speel nie. Studies waar mutante gebruik word, is gewens om addisionele bewys te lewer dat die substansie 'n rol in plantverdediging vervul. Sodanige mutante sluit plantlyne, waarin die geen van belang gemuteer is of ooruitgedruk word om so die rol van die geen in 'n *in planta* omgewing te bepaal in. In hierdie toepassings het genetiese transformasie en die daarstelling van transgeniese plante 'n oëwenaarde bydrae gelewer.

In dié studie is die antifungiese peptied, Hs-AFP1, wat aan die peptiedgroep van plant-“defensins” behoort en van *Heuchera sanguinea* afkomstig is, in 'n heteroloë *in planta* omgewing geëvalueer as 'n verdedigingspeptied met die potensiaal om in die generering van transgeniese siektebestande gewasse gebruik te word. Die antifungiese aktiwiteit van Hs-AFP1 is teen *Botrytis cinerea* in 'n *in vitro* reaksie geëvalueer, waar die toediening van 8 µg/ml gesuiwerde Hs-AFP1 peptied aanleiding gegee het tot 'n 88% afname in hifegroei van *B. cinerea*. Hipervertakkings van swamhifes, 'n kenmerkende eienskap van Hs-AFP1 aktiwiteit, kon duidelik waargeneem word. Tabakplante is voorts getransformeer met 'n konstruk, pFAJ3068, wat die koderende geen van Hs-AFP1 onder die sterk konstitutiewe CaMV 35S promotor bevat het. Die peptied is met behulp van die seinpeptied wat afkomstig is van die *Mirabilis jalapa* antimikrobiële peptied, Mj-AMP2, na die apoplastiese omgewing geteiken. Voorheen is gerapporteer dat transgeniese peptiede in die heteroloë omgewing soms onstabiel is. Dit het gelei tot die generering van twee addisionele konstrukke om die moontlikheid van peptiedonstabieliteit te ondervang. 'n Stabiliseringskonstruk, pHs-EXG1,

bestaande uit 'n versmelting tussen Hs-AFP1 en 'n antifungiese eksoglukanase van *Saccharomyces cerevisiae*, gekodeer deur *EXG1*, is in tabakplante getransformeer. 'n Kontrolekonstruk, pMj-*EXG1*, met die *EXG1*-geen saam met die Mj-AMP2-seinpeptied, is ook voorberei en in tabakplante getransformeer. Dit is gebruik om die antifungiese aktiwiteit van die eksoglukanase in die antifungiese aktiwiteitstoetse van die stabiliseringskonstruk te kwantifiseer en te normaliseer.

Tabak is suksesvol met pFAJ3068, pHs-*EXG1* en pMj-*EXG1* getransformeer, wat onderskeidelik gelei het tot die sogenaamde THs, THE en TME transgeniese tabaklyne. Transgeentranskripsie en -translasie in die THs en THE tabaklyne is onderskeidelik deur Noordelike- en Westelike-kladanalises bevestig. Die aktiewe uitdrukking van Hs-AFP1 het die vermoë van tabakplante om *B. cinerea* infeksies te weerstaan, met tot 42% verhoog in vergelyking met ongetransformeerde kontrole tabakplante tydens 'n *in planta* siekteweerstandstoets. Totale proteïenekstrakte van THs tabaklyne is voorts ook in 'n *in vitro* inhibisietoets geëvalueer, wat gelei het tot resultate wat goed met dié van die *in planta* toetse ooreenstem. Die totale proteïenekstrakte het swamgroei met 40% geïnhibeer en die kenmerkende hipervertakking van Hs-AFP1-aktiwiteit is ook mikroskopies waargeneem.

Resultate wat verkry is vanaf al die analises wat op die transgeniese THs tabaklyne uitgevoer is, het aangedui dat Hs-AFP1 baie stabiel in die heteroloë tabakomgewing is en peptiedstabiliteit was dus nie 'n probleem, soos verwag is nie. Die fusie tussen *Hs-AFP1* en *EXG1* het dus nie die stabiliteit van die reeds stabiele Hs-AFP1 peptied verder verbeter nie, maar het wel tot die verlies van Hs-AFP1 aktiwiteit gelei. Die antifungiese analises van die THE tabaklyne het verder bevestig dat dié lyne selfs swakker inhibisie van *B. cinerea*-infeksies tot gevolg gehad het, as ongetransformeerde tabakplante. Mikroskopiese analises van totale THE proteïenekstrakte het voorts ook geen kenmerkende hipervertakkings in die swamhifes vertoon nie, wat alles daarop dui dat die Hs-AFP1-deel van die fusieproteïen as gevolg van die fusie met *EXG1* geïnaktiveer is. Dié resultaat is in lyn met vorige navorsing, wat getoon het dat 'n ander peptied, sarcotoxin 1A, sy antifungiese aktiwiteit verloor indien dit met 'n verklikkergeen versmelt word.

Alhoewel integrasie van die pMj-*EXG1*-konstruk in die TME-tabaklyne bevestig is, kon geen mRNA met Noordelike-klad- of trutranskriptase-PKR (RT-PKR)-analises waargeneem word nie. Die TME plant het ook geen antifungiese aktiwiteit in *in vitro* toetse getoon nie en dit het geblyk dat die pMj-*EXG1*-konstruk aan geenafskakeling in die heteroloë tabakomgewing onderworpe was. Dié afskakelingseffek is egter in die THE plante oorkom, aangesien laasgenoemde sterk *EXG1* proteïenaktiwiteit met β -glukosidase aktiwiteitstoetse vertoon het. Alhoewel die THE plante nie die stabiliteit van Hs-AFP1 verbeter het nie, het dit onwerwags tot die stabilisering van *EXG1* in 'n heteroloë omgewing gelei. Versmeltingstechnologie kan dus moontlik gebruik word as 'n strategie om ander glukanases, wat bekend is vir geenafskakeling in transgeniese omgewings, heteroloog uit te druk.

In die geheel gesien, het dié studie getoon dat Hs-AFP1 'n onbetwiste rol in plantverdedigingsmeganismes speel en daar is voorts ook meer kennis oor die stabiliteit van peptiede in 'n heteroloë plantomgewing ingewin. Die positiewe resultate t.o.v. die verhoogde siekteweerstand in die transgeniese THs plantlyne regverdig ook die verdere bestudering van dié peptied om transgeniese siekteweerstand in gewasse te bewerkstellig.

This thesis is dedicated to my parents

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. Abré enrolled at Stellenbosch University in 1995 and obtained the BSc degree in Microbiology and Biochemistry in 1997. In 1998 he received the BscHons degree in Wine Biotechnology through the Institute for Wine Biotechnology at the same University. In 1999 he enrolled at Stellenbosch University for his MSc degree in Wine Biotechnology through the Institute for Wine Biotechnology.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions for their invaluable contributions to the successful completion of this study:

Dr MA Vivier, Institute for Wine Biotechnology at Stellenbosch University, who acted as my supervisor, for accepting me as a student and for her enthusiasm and encouragement throughout this project;

Prof IS Pretorius, Institute for Wine Biotechnology at Stellenbosch University, who acted as my co-supervisor and for accepting me as student at the institute;

Prof WF Broekaert, who kindly supplied us with the antifungal peptides and plasmid constructs;

Dr BPA Cammue, who kindly supplied us with antibodies raised against Hs-AFP1 peptide and his advice regarding peptide and protein isolation and manipulation;

My colleagues in the laboratory for their support, encouragement and advice;

The staff at the Institute for Wine Biotechnology for their assistance;

The National Research Foundation (NRF), Stellenbosch University, Winetech and the **Harry Crossley Foundation** for financial assistance.

PREFACE

This thesis is represented as a compilation of four chapters. Each chapter is introduced separately. The chapters are written according to the style of *Plant Physiology* to which the manuscript will be submitted for publication.

- CHAPTER 1** **General Introduction and Project Aims**
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- CHAPTER 2** **Literature Review: preformed plant defense systems and the role of plant antimicrobial peptides**
- CHAPTER 3** **Research Results: Overexpression and evaluation of an antimicrobial peptide from *Heuchera sanguinea* (Hs-AFP1) for inhibition of fungal pathogens in transgenic tobacco**
- CHAPTER 4** **General Discussion and Conclusion**

I hereby declare that I was the primary contributor with respect to the experimental data presented on the multi-author manuscript presented in Chapter 3. My supervisors, Dr. MA Vivier and Prof. IS Pretorius were involved in the conceptual development and continuous critical evaluation of this study.

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Plants have always been subjected to pathogen attack, leading to the co-evolution of the two role players. The constant interaction between pathogens and plants has led to the development of complex defense mechanisms in the plant, whereby each individual plant cell can elicit its own defense (Terras et al., 1995; Broekaert et al., 1995, 1997; Heath 2000; García-Olmedo et al., 2001). Certain defense mechanisms, however, are specific for certain plant tissues and different plant organs. Of all the different plant organs, the plant seed boasts some of the most effective defense strategies.

Seed germination is the most vulnerable time in a plant's life cycle. The thick protective seed coat ruptures and the moist humid soil environment, which favors seed germination, is also the perfect breeding ground for plant pathogens (Terras et al., 1995; Liu et al., 2000). However, the infection of plant seeds during germination is the exception rather than the rule. The reason is that plant seeds have a multifaceted defense mechanism consisting of a preformed and an induced defense system (Bennett and Wallsgrave, 1994; Osbourn, 1996; Broekaert et al., 1997). Of these two defense systems, the preformed defense plays the most important role during seed germination. The preformed defense consists of biochemical and biological fungicides that are expressed constitutively. During germination and the subsequent rupture of the protective seed coat, these antifungal agents diffuse into the surrounding environment to form a protective layer around the seed (Terras et al., 1992, 1995; Osbourn, 1996; Broekaert et al., 1997; Osbourn, 2001). This protective layer prevents fungal and bacterial pathogens from infecting the young seedling.

Over the last decade, scientists have studied the defense mechanisms of different seeds in an effort to understand and manipulate these mechanisms for genetic engineering. This has led to the isolation and characterization of various seed defense proteins (Huynh et al., 1992; Regente and De la Canal, 2001) such as thionins (Bohlmann and Apel, 1991; Florack and Stiekema, 1994), defensins (Broekaert et al., 1997; Harrison et al., 1997; García-Olmedo et al., 1998), lipid transfer proteins (Arondel and Kader, 1991; Terras et al., 1992; Cammue et al., 1995; Kader, 1996, 1997; Regente and De la Canal, 2000; Wijaya et al., 2000), *Impatiens balsamina* antimicrobial peptides, Ib-AMPs, (Tailor et al., 1997), snakine (Segura et al., 1999) and chitin-binding peptides (Cammue et al., 1992; De Bolle et al., 1993, 1995; Nielsen et al., 1997; Liu et al., 2000). Peptides in all these families have been shown to have *in vitro* activity against fungal and/or bacterial pathogens.

The sole demonstration of *in vitro* activity alone, however, was not sufficient to assign a defense role to these peptides (Broekaert et al., 1997; García-Olmedo et al., 1998). To

confirm the role of these peptides in defense, it must be shown that a mutant plant that is unable to produce or over-produces a peptide leads to either a decline or increase in disease resistance, respectively. In this regard, genetic transformation and the subsequent development of transgenic plants have made an unprecedented impact on the understanding of the role of specific defense-related proteins and their interaction with plant pathogens. Transgenics have been implemented successfully to confirm the role of different proteins in the induced defense response system (Jach et al., 1995; Jongedijk et al., 1995; Shah, 1997; Oldroyd and Staskawicz, 1998; Salmeron and Vernooij, 1998), whereas the role of proteins present in the preformed defense mechanism has not yet been fully elucidated.

The study of defense mechanisms in seeds is important in two ways. Not only can the knowledge gained be applied to the mechanisms of disease resistance in the rest of the plant body, but the well-characterized defense proteins and their encoding genes act as a genetic resource in plant biotechnology.

The potential use of plant antimicrobial peptides in genetic engineering is well established, however (Carmona et al., 1993; Terras et al., 1995; Epple et al., 1997; Molina and García-Olmedo, 1997; Bi et al., 1999). The transformation of a plant defensin from *Medicago sativa*, alfAFP, into potato was the first reported case of a single defense gene conferring agronomically important disease resistance to potato (Gao et al., 2000).

Agricultural biotechnology is starting to play an increasingly important role in modern agriculture as more tissue culture systems and transformation protocols are developed for crop species. Agricultural biotechnology is seen as the answer to environmentally friendly agriculture (Van der Biezen, 2001). This makes the study of antimicrobial peptides and their physiology in heterologous plants systems very important. The expression, antifungal activity and spectrum, as well as the stability of these peptides in heterologous systems, must first be assessed in model systems, such as tobacco and *Arabidopsis*. Without the knowledge gained from model systems, the genetic engineering of crop species will be a costly affair of trial and error.

In this study, a similar approach has been taken to study the *in planta* activity of an antifungal peptide from *Heuchera sanguinea*, Hs-AFP1. This forms part of an initiative to upregulate and/or improve the plant host's defense mechanisms against fungal pathogens. Tobacco plant and the fungal pathogen *Botrytis cinerea* were used as model plant and pathogen systems, respectively, due to their ease of handling.

1.2 PROJECT AIMS

The specific aims of this project were as follows:

- i) The analysis of four different plant defense peptides obtained from Prof. Broekaert (Katholieke Universiteit Leuven), for their activity against the fungus *Botrytis cinerea*. This is an economically important pathogen and the causal agent of gray mold on several crops, including grapevine, which is our main concern. This destructive plant disease is responsible for huge crop losses each year and has a broad host range. In addition, this fungus has developed chemical resistant strains, rendering it immune to some chemical fungicides. The testing of these peptides for their activity is presented as an addendum to chapter 3 of this thesis.
- ii) The construction of plant expression vectors to introduce the Hs-AFP1-encoding gene into the tobacco genome via *Agrobacterium tumefaciens* transformation of tobacco leaf discs. The gene is under the control of the cauliflower mosaic virus promoter and the terminator of T-DNA gene 7 and targeted to the apoplastic region, with the signal peptide from Mj-AMP2, an antifungal peptide from *Mirabilis jalapa*.
- iii) The construction of a fusion protein by fusing the Hs-AFP1-encoding gene to the *EXG1* exoglucanase gene from *Saccharomyces cerevisiae* and its introduction into tobacco via *Agrobacterium* transformation. Previous transgenic work involving peptides reported instability at the protein level (De Bolle et al., 1996; Okamoto et al., 1998), due to foreign proteases acting on the small peptides. This problem could be overcome by a fusion to the GUS gene, but the peptide activity was subsequently lost due to probable errors in protein folding. Okamoto et al. (1998) suggested that another antifungal protein could be used to stabilize the small peptides, concurrently contributing to the antifungal activity of the fusion protein. Here the *EXG1* gene from yeast has been used, since the encoding exoglucanase protein has been shown to have strong antifungal activity.
- iv) The analysis of the transgenic lines for gene integration and copy number, gene expression, presence of the peptides and *in vitro* and *in planta* antifungal activity against *B. cinerea*. These experiments and their results are all presented in Chapter 3 of this thesis. This chapter was written in the style of the journal, *Plant Physiology*, to which this work will be submitted for publication.

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CHAPTER 2

LITERATURE REVIEW

**Preformed plant defense systems and
the role of plant antimicrobial peptides**

LITERATURE REVIEW

2.1 INTRODUCTION

Plants are constantly subjected to the threat of microbiological attack. A million years of evolution has led to the formation of plant defense systems, which can combat pathogen infection. Of these defense systems, the non-host plant defense system is the most important as it confers resistance to pathogens in non-domesticated plant species (Osbourn, 1996a; Heath, 2000). 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). The preformed defense system consists of 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 antifungal barriers, which prevent the spread of pathogen infection. These antimicrobial barriers are usually present in the outer cell layers of plant organs, which represent the first layers of tissue attacked by fungi and are in turn, the first plant cells to elicit a defense response (Osbourn, 1996b). The most noticeable preformed dense barriers are present in seeds, where they protect the plant material during seed germination (Terras et al., 1995).

Extensive research has been done on biochemical defense compounds such as phytoalexins (Fig. 1) that are induced during pathogen infection. The biochemical compounds present in the preformed defense have received less attention (Kùc 1990, 1992; Bennett and Wallsgrove, 1994; Kùc, 1995; Adrian et al., 1998).

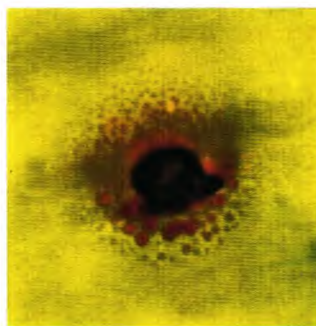


Figure 1. The formation and mobilization of phytoalexin-producing vesicles toward the site of fungal invasion by *Colletotrichum graminicola* in sorghum (Snyder and Nicholson, 1990). The dark structure in the centre of the photograph is a fungal appressorium. The red 3-deoxyanthocyanidin phytoalexins are visualized by light microscopy.

Collectively, the biochemical compounds involved in the preformed defense are named phytoanticipins (Van Etten et al., 1994). Phytoanticipins include cyanogenic glycosides, glucosinolates, cyclic hydroxamic acids and saponins (Osbourn, 1996a). Recent research conducted on the saponin group, avenacins, has shown that these chemicals are present at high levels in healthy plants, where they form anti-pathogenic barriers to prevent or inhibit the spread of pathogen infection (Fig. 2) (Morrissey and Osbourn, 1999; Papadopoulou et al., 1999).

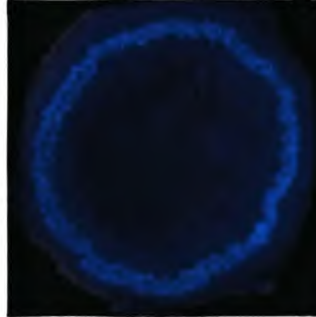


Figure 2. Preformed auto fluorescent avenacins, present in the epidermal cell layers of a young oat root, anticipating fungal attack (Osbourn et al., 1994).

Molecular biology has given an insight into the importance of these chemicals in the overall plant defense systems. Mutational analysis has shown that an absence or a decreased level of these compounds leads to a noticeable decline in disease resistance (Fig. 3) (Osbourn et al., 1994; Papadopoulou et al., 1999).

Although these phytoanticipins play an important role in the protection of plants against pathogen infection, it is questionable whether they will play a significant role in the engineering of disease-resistant crops. The biggest concern is that some fungal pathogens are able to detoxify phytoanticipins and thus overcome their antifungal effect (Morrissey and Osbourn, 1999). The carbohydrate residues in avenacins play a major role in their antifungal activity. The detoxification of avenacins by fungi usually involves the alteration of the carbohydrate composition, which leads to a loss in activity (Armah et al., 1999). The importance of saponin detoxification is best demonstrated for the oat root-infecting fungus, *Gaeumannomyces graminis* var. *avenae*. This plant pathogen produces an extracellular enzyme, avenacinase, which detoxifies avenacin through the removal of the terminal D-glucose molecules of the avenacin sugar chains (Fig. 4).

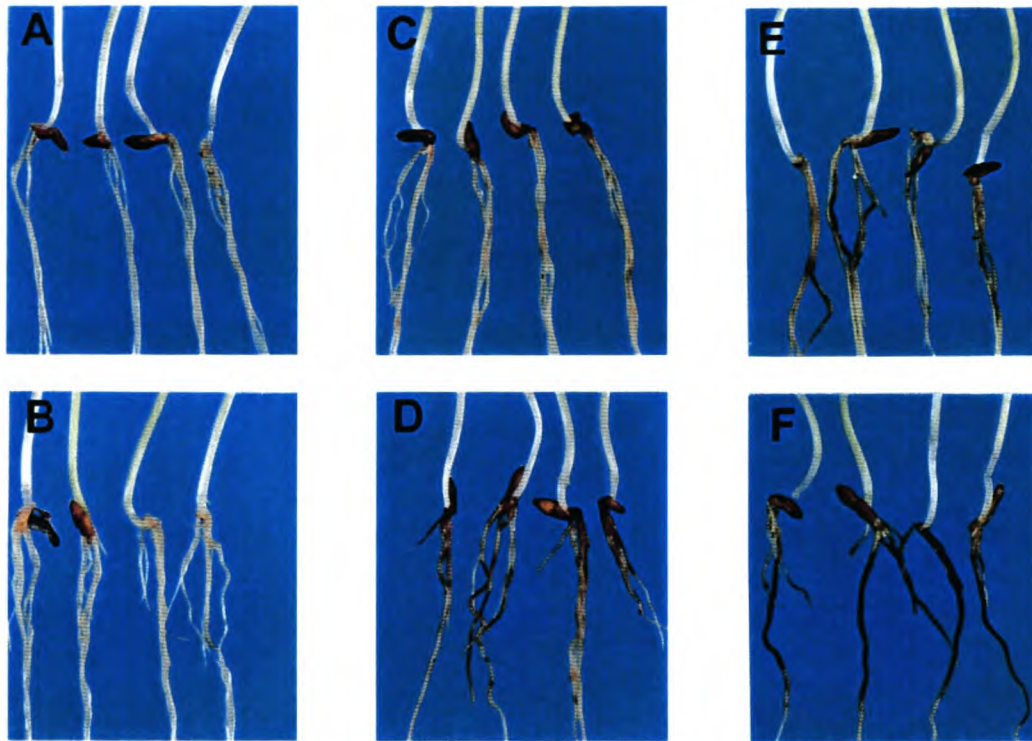


Figure 3. The disease severity caused by *Fusarium* spp. on wild type *Avena strigosa* and a saponin-deficient *A. strigosa* mutant. A, C and E represent the wild type seedlings, while B, D and F represent the *sad* mutant 610, lacking the ability to produce avenacin. A and B were mock inoculations, while C and D were inoculated with *Fusarium culmorum*. E and F were inoculated with *Fusarium avenaceum* (Papadopoulou et al., 1999).

G. graminis var. *avenae* mutants lacking avenacinase activity are unable to infect oats. This indicates that saponin, or in this case avenacin detoxification through avenacinase, is an essential factor in determining the host range for this specific plant pathogen (Osborn et al., 1991, 1996a, 1996b). It has been hypothesized that the hydrolysis of phytoanticipins is a prerequisite for pathogenicity in a wide range of plant pathogens (Bowyer et al., 1995; Osborn, 1999; Papadopoulou et al., 1999).

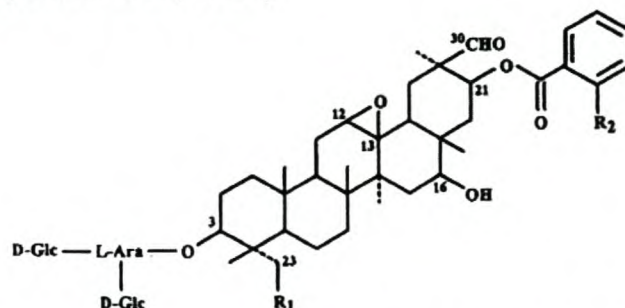


Figure 4. The chemical structure of the saponin, avenacin A-1, isolated from oat roots (Papadopoulou et al., 1999).

Although these compounds, especially saponins, have the potential to enhance disease resistance when expressed in a transgenic system, the risk of pathogen adaptation is too high to make it profitable in the design of commercial disease-resistant crop species. Pathogen adaptation is the reason why scientists look toward the proteinaceous molecules present in the preformed defense system. These peptides and proteins have unique mechanisms of action, which are not prone to pathogen adaptation (Van der Biezen, 2001). For the purpose of this study, the focus will fall on the peptides involved in the preformed and induced plant defense systems.

Plant antimicrobial peptides are proteins with antifungal and antibacterial properties that are less than 100 amino acids long (Ganz and Lehrer, 1995; Broekaert et al., 1997; Ganz and Lehrer, 1998, 1999). Most of the plant antimicrobial peptides characterized show antifungal activity, whereas only a few exhibit antibacterial activity (Broekaert et al., 1995, 1997; García-Olmedo et al., 1998). A wide range of plant antimicrobial peptides has been isolated from equally diverse plant species and it has been suggested that these peptides are present in all plant species (Broekaert et al., 1997). All the plant antimicrobial peptides have either 4, 6, 8 or 12 cysteine residues. These cysteine residues are connected by disulfide bonds that give rise to a very stable structure. The stabilization usually occurs due to the formation of a disulfide bond between the typical α -helical and β -sheet structures of the peptides.

These peptides have been isolated from various plant organs, i.e. leaves, flowers, tubers, roots and seeds. In normal, unstressed/uninfected tissue, they are present at a basal level in the outer cell layers, lining the plant organ. Localization of the peptides in the outermost tissue regions punctuates their role in the preformed defense mechanism of plants (Fig. 5) (Osborn, 1996b; Heath, 2000). The preformed defense is the main form of defense in nonhost-pathogen interactions and it is speculated that these preformed defense mechanisms are the determining factors when it comes to the host range of plant pathogens. Most of the characterized peptides have been isolated from seeds, in which they have been shown to play an important protective role before and during germination (Fig. 5).

Apart from their role in the preformed defense strategies, some of the genes expressing these peptides are transcriptionally up-regulated during pathogen infection, thus also emphasizing their role in the induced plant defense mechanisms (Fritig et al., 1998; Maleck and Lawton, 1998; Terras et al., 1998; Thomma and Broekaert, 1998). Plant defense mechanisms and the up-regulation thereof have been one of the main focus areas of plant biotechnology in the last 20 years. The ability to stably incorporate genes from diverse origins into economically important plant species has caused huge excitement and has led to innovative strategies to combat pathogen attack; these initiatives also included antimicrobial peptides. Before discussing the biotechnological applications of these peptides, the

classification and the modes of action of the most important groups of antimicrobial peptides will be discussed.

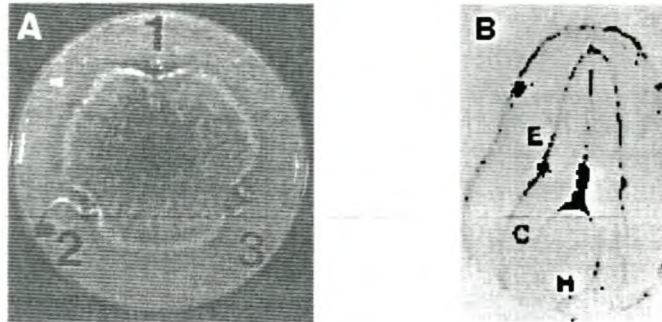


Figure 5. (A) The antifungal activity exhibited by germinating radish seeds through the secretion of antifungal agents. (B) A tissue print immunolocalization of radish seed antifungal peptides, Rs-AFPs. This picture clearly indicates the presence of the preformed defense barriers formed by Rs-AFPs between the different compartments of the seed tissue. C, cotyledon; E, endosperm; H, hypocotyls (from Terras et al., 1995).

2.2 PLANT ANTIMICROBIAL PEPTIDES: CLASSIFICATION AND MODES OF ACTION

Based on the primary structure and certain homologies between the characterized peptides, the plant antimicrobial peptides have been classified into eight families: thionins, defensins, lipid transfer proteins (LTPs), hevin and knottin-type peptides, MBP-1, Ib-AMPs and snakin-1 (García-Olmedo et al., 1998). Table 1 shows some characteristic features of these peptide families.

Table 1. Some features characteristic of the eight classified plant antimicrobial peptide families

Antimicrobial peptide family	Antifungal activity	Antibacterial activity	Cation sensitivity	Toxicity towards human cells
Thionins	High	High: against both Gram-(+) and Gram-(-)	High	Toxic
Defensins	High	Some: against Gram-(+)	Medium to High	None
LTPs	High to None	Very high: against both Gram-(+) and Gram-(-)	Low to High	None
Knottin- and Hevein-type peptides	High	Some: against Gram-(+)	High	None
MBP-1 and Ib-AMPs	High	Active against Gram-(+)	Low to High	None
Snakin-1	High	High	N/A	N/A

2.2.1 Thionins

Thionins are low molecular weight (M_r ca. 5000) plant peptides that have been isolated from seeds, roots, stems and flowers of various plant species (Bohlmann and Apel, 1991; Florack and Stiekema, 1994).

These peptides show a wide range of toxicity, including activities against fungi, yeast, and even mammalian cells. They are also the only known plant antimicrobial peptides that show high activity against both Gram-positive and Gram-negative bacteria (Bohlmann and Apel, 1991; Florack and Stiekema, 1994).

Thionins can be divided into five different types. This characterization is based on the number of amino acids present, the net charge of the peptide, the number of disulfide bonds present and the plant species and tissues in which they occur (Table 2) (Florack and Stiekema 1994; Broekaert et al., 1997; García-Olmedo et al., 1998).

Table 2. The classification of the known thionins and some feature characteristics determining the different types of thionins

Thionin type	Number of amino acids	Number of cysteine residues	Plant spp. isolated from	Disulfide bonds	Net charge of thionin type
I	45	8	<i>Poaceae</i> spp.	4	Highly basic
II	46-47	8	<i>Hordeum vulgare</i> <i>Pyrularia pubera</i>	4	Less basic
III	45-46	6-7	<i>Viscum album</i> <i>Dendrophthora clavata</i> <i>Phoradendron</i>	3-4	Less basic
IV	46	6	<i>Crambe abyssinica</i>	3	Neutral
V	37	6	<i>Aegilops</i> spp. <i>Triticum</i> spp.	2 + one presumably unique bond	Neutral

The type I to III thionins all share a basic net charge, whereas the type IV and V thionins have a neutral net charge (García-Olmedo et al., 1998). The non-toxic effect of crambin A and crambin B have been attributed to the neutral charge of the thionins. Type V thionins are more divergent than the rest of the thionin types. These thionins have two disulfide bonds, with the possibility of an unconfirmed unique third bond, unlike the four

disulfide bonds possessed by thionin types I and II. This phenomenon is due to the fact that type V thionins lack the cysteine at position four.

Twelve to 17% of the amino acids present in thionins consist of cysteine residues. There is also a high degree of conservation between the cysteine residues of the different thionin types (Fig. 6). Tertiary structure stabilization is also the direct result of disulfide bridge formation between these different cysteine residues (Broekaert et al., 1997; García-Olmedo et al., 1998). The first thionin structure to be analyzed was that of crambin, a type IV thionin from *Crambe abyssinica*. The crystal structure of crambin revealed that it contained three disulfide bonds.

Type I thionins	
Alpha-hordothionin	KSCCRSTLGRNCYNLCRVRG--AQKLCAGVCRCKLTSSGKCPDGFPPK
Beta-hordothionin	KSCCRSTLGRNCYNLCRVRG--AQKLCANACRCKLTSGLKCPSSFFPK
Alpha1-Purothionin	KSCCRSTLGRNCYNLCRSRG--AQKLCSTVCRCKLTSGLSCPFGFPPK
Alpha2-Purothionin	KSCCRSTLGRNCYNLCRARG--AQKLCAGVCRCKLTSSGLSCPFGFPPK
Beta-Purothionin	KSCCKSTLGRNCYNLCRARG--AQKLCANVCRCKLTSGLSCPDKFPPK
Alpha-Avenothionin	KSCCRDTLGRDCYDLCRSRG--APKLCATLRCRCKLTSSGLSCPDKFPPK
Beta-Avenothionin	KSCCKDTLGRDCYDLCRARG--APKLCSTLRCRCKLTSSGLSCPDKFPPK
Secale-thionin	KSCCKSTLGRDCYDLCRGRG--AEKLCDELRCRCKLTSSGLSCPDKFPPK
Type II thionins	
Leaf-thionin-DG3	KSCCKNTTGRNCYNACRFAGG-SRPVCCATACGCKIISGPTCPDYPK
Leaf-thionin-DB4	KSCCKDTLARNCYNTCRFAGG-SRPVCAGACRCKIISGPKCPDYPK
Leaf-thionin-BTH6	KSCCKDTLARNCYNTCRFAGG-SRPVCAGACRCKIISGPKCPDYPK
Pyricularia-thionin	KSCCRNTWARNCYNVCRLPGTISREICAKKCDCKIISGTCPSDYPK
Type III thionins	
Denclatoxin	KSCPTTAARNQYNIIRLPGT-PRPVALSGRCKIISGTPPPGYRH
Ligatoxin	KSCPTSTTARNIYNTIRLTGT-SRPTLSLGRCKIISGSTPSGWBH
Phoratoxin	KSCPTTTARNIYNTIRFGGG-SRPVCKLSGRCKIISGTPKDSGWNH
Viscotoxin-A2	KSCPNNTTGRNIYNTIRFGGG-SREVLSLGRCKIISASTPSYPDK
ViscotoxinA3	KSCPNNTTGRNIYNAIRLTGA-PRPTCKLSGRCKIISGSTPSYPDK
ViscotoxinB	KSCPNNTTGRNIYNTIRLGGG-SRERLSLGRCKIISASTPSYPDK
Viscotoxin-ThiVal	KIDRAPAGKKIYNLITALL--SSETNTVYRCKDVSGETPPADYPA
Viscotoxin-ThiVal2	KSCPNNTTGRNIYNAIRVPGT-PRPVLSLVDRCCKIISGKPPADYPR
Type IV thionins	
CrambinA	TTTSPSIVARSNFVIRLPGT-PEAIIATYTGIIIPGATPPGDYAN
CrambinB	TTTSPSIVARSNFVIRLPGT-SEAIATYTGIIIPAGTTPGDYAN
Type V thionins	
Ta-THV	VDGANPFKVAENSLLGPS-TVFQADFAIRLPAG
At-THV	VDGANPFKVAENSLLGPS-TVFQADFAIRLPAG

Figure 6. Sequence alignment of different thionins isolated from various plant species. The sequences are from hordothionins and leaf thionins from *Hordeum vulgare*, purothionins from *Triticum aestivum*, avenothionins from *Avena sativum*, secalet thionin from *Secale cereale*, viscotoxins from *Viscum album*, pyricularia thionin from *Pyricularia pubera*, ligatoxin from *Phoradendron liga*, phoratoxin from *Phoradendron tomentosum*, denclatoxin from *Dendrophthora clavata*, crambins from *Crambe abyssinica*, At-THV from *Aegilops tauschii* and Ta-THV from *Triticum aestivum* (Florack and Stiekema, 1994; García-Olmedo et al., 1998). The colour-coded cysteines represent the common cysteine pattern of the different types of thionins. The alignment was performed with the ClustalX programme (Thompson et al., 1997).

Proton Nuclear Magnetic Resonance (1 H NMR) studies conducted on α 1-purothionin (type I), viscotoxin (type III) and crambin (type IV) peptides showed that thionins of type I, III

and IV not only share a high level of homology at sequence level (Fig. 6), but are related structurally as well. Studies on the tertiary structure revealed that most thionins have a common L-shaped structure. The long arm is made up of two antiparallel α -helices, and the short arm of a β -sheet consisting of two antiparallel β -strands (Fig. 7).



Figure 7. The tertiary structure of viscotoxin revealing the common L-shape associated with all thionins (Romagnoli et al., 2000). The yellow arrows represent the two β -strands and the pink ribbons the α -helices forming the long arm of the L-shape. The bars (black) represent the disulfide bonds and the wire frame (red) the cysteines. The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).

Structural studies also revealed that thionins are amphipathic, with the hydrophobic residues on the outside of the long arm and the hydrophilic residues on the inside of the long arm. The eight-cysteine thionins, α 1-purothionin and β -purothionin, also appear to have a phospholipid-binding domain. This feature, together with the amphipathic nature of thionins, may be responsible for their toxic activity towards microorganisms (Florack and Stiekema, 1994).

The antimicrobial activity of thionins was first noticed in 1940, when scientists were searching for the substance reducing the baking quality of wheat flour. The substance they found was purothionin, a type 1 thionin. Purified purothionin showed *in vitro* activity against yeast, fungi and bacteria (Stuart and Harris, 1942), leading to the conclusion that thionins exhibit toxic activities against Gram-negative and Gram-positive bacteria, yeast, fungi and mammalian cells. It was suggested that the activity of thionin was the result of direct interaction with the membrane phospholipid bilayer and the subsequent formation of a membrane pore, resulting in the leakage of cations to and from the cell, ultimately leading to cell death (Fig. 8). Research showed that the interaction of α -hordothionin with *Neurospora crassa* hyphae led to a high Ca^{2+} uptake in the fungal cell, as well as a high K^+ efflux, resulting in medium alkalization (Thevissen et al., 1996). α -Hordothionin also caused permeabilization of the fungal membrane in the presence of non-metabolite α -aminoisobutyric acid, as well as an alteration of the electrical properties of artificial lipid bilayers, resulting in the rupturing of the lipid bilayer.

The mechanism of ion channel formation was recently confirmed for β -purothionin (Hughes et al., 2000). It was shown that β -purothionin forms cation-selective ion channels in artificial lipid bilayers and the plasmalemma of rat hippocampal neurons. This channel formation led to the dissipation of ion concentration gradients essential for the cellular homeostasis. It is now believed that all thionins, except the non-toxic type IV and V thionins, follow this mode of action.

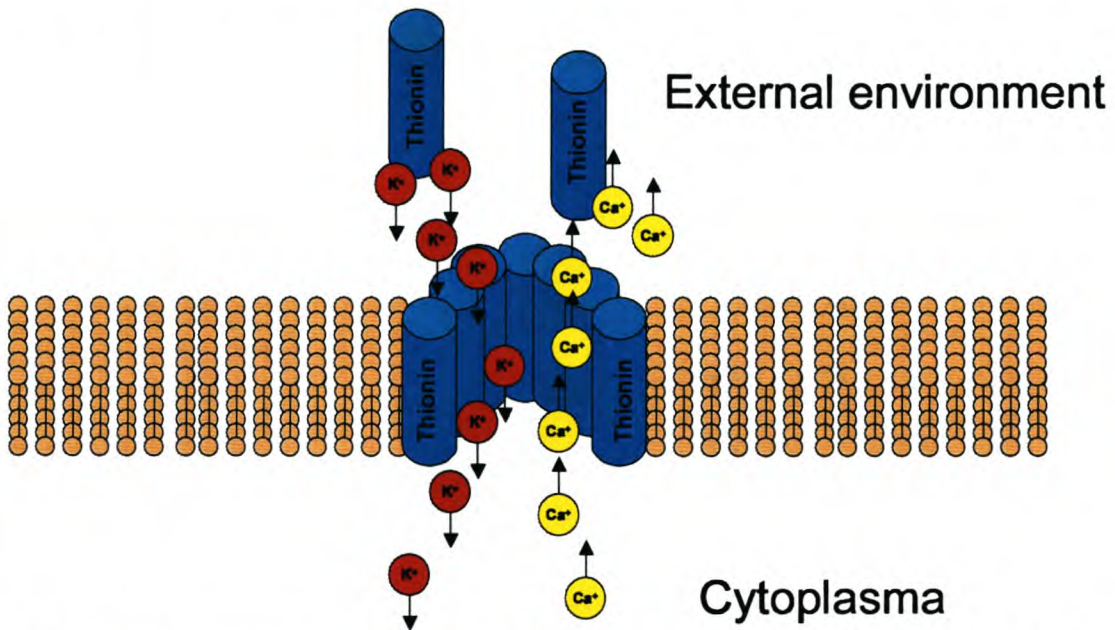


Figure 8. A schematic representation of thionin integration into the plasma membrane of cells and the subsequent pore formation, leading to cation leakage. The cations K^+ and Ca^{2+} are represented by red and yellow circles respectively.

2.2.2 Plant Defensins

Over the last decade, plant defensins have been researched actively, leading to the isolation of about 40 peptides from a range of plant species (Cammue et al., 1992; Terras et al., 1992a, 1992b, 1993; Broekaert et al., 1995; Osborn et al., 1995; Harrison et al., 1997; Taylor et al., 1997; Zhang and Lewis, 1997; Segura et al., 1998; Urdangarìn et al., 2000). These peptides are found not only in a wide variety of plant species, but also show a large degree of divergence when it comes to localization within the organs and tissues of the different source plants (Fig. 9). Localization studies conducted on defensins have shown them to be present in the intercellular spaces of the outermost cell layers of leaves, seeds, stems, flowers and guard cells surrounding the stomata, the latter being a favourite entry point of fungal pathogens (Kragh et al., 1995) (Fig. 10). It is in these locations that plant defensins play an active role in the preformed defense mechanism of plants.

When plant defensins were first isolated, they were thought to be another subgroup of thionins and were named γ -thionins (Colilla et al., 1990; Mendez et al., 1990; Karunanandaa et al., 1994). However, later studies revealed that plant defensins are completely different in structure and are in fact unrelated to thionins.

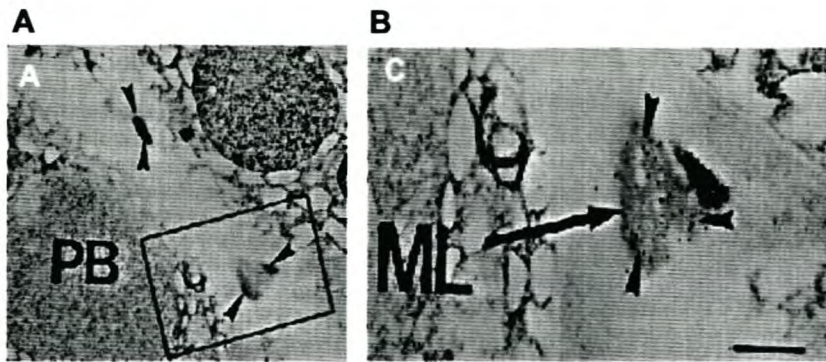


Figure 9. (A and B) Immunolocalization of Rs-AFP2 (a defensin from radish) in a radish seed tissue section. The arrowheads indicate the labelled peptides; ML, middle lamella; PB, protein body. Figure 9B is an enlargement of the boxed section in Figure 9A (Terras et al., 1995).

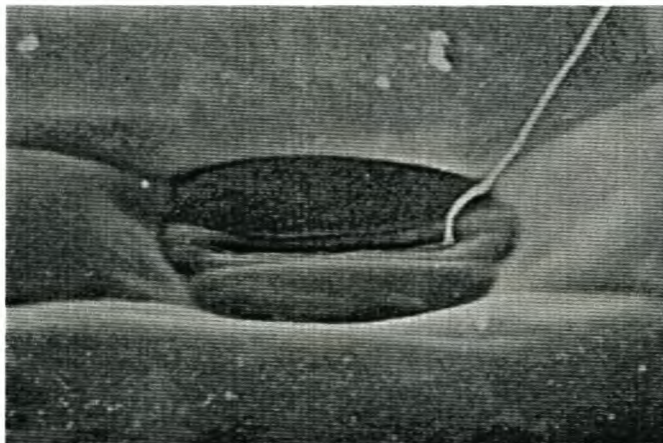


Figure 10. A fungal hypha, initiating infection by gaining access through the stomatal pore (Collinge, 2001).

Typically, plant defensins are polypeptides containing 45-54 amino acids. They also contain eight cysteine residues linked by four disulfide bonds. Plant defensins contain only one α -helix and a triple-stranded antiparallel β -sheet, which is a characteristic feature of all plant defensins. Also characteristic of defensins is a CXXXC segment in the α -helix that is connected by two disulfide bonds to a CXC segment of the C-terminal β -strand to form a structural motif known as the cysteine-stabilized α -helix motif (Kobayashi et al., 1991). The cysteine-stabilized α -helix motif also occurs in insect defensins, which show some homology to plant defensins on the tertiary level, but lack an amino-terminal β -strand present in all plant defensins (Fig. 11). Defensin-type peptides also have been isolated from various other

invertebrates, vertebrates and prokaryotes (Ganz and Lehrer, 1998, 1999; Lehrer and Ganz, 1999). This suggests that the mechanism of peptide defense has been conserved throughout evolution and thus can be described as the ancient defense of all living organisms (Charlet et al., 1996; Lemaitre et al., 1997; Cavallarin et al., 1998; Crouch, 1998; Medzhitov and Janeway Jr, 1998; De Lucca and Walsh, 1999; Lowenberger et al., 1999).

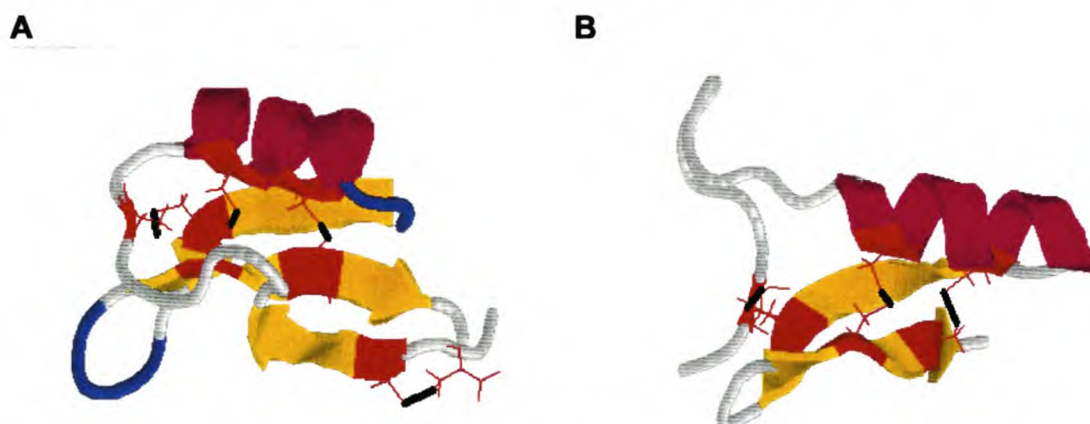


Figure 11. (A) The three-dimensional structure of Rs-AFP2, a plant defensin from radish, with its α -helix and three antiparallel β -strands characteristic of all plant defensins (Fant et al., 1998). (B) Insect defensin A with the same folding pattern as Rs-AFP2, but lacking the third amino-terminal β -strand (Cornet et al., 1995). The arrows (yellow) represent the β -sheet and the pink ribbons the α -helical turn. The bars (black) represent the disulfide bonds and the wire frame (red) the cysteines. The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).

There is very little homology on amino acid level between the different defensins (Terras et al., 1992b, 1993; Osborn et al., 1995; Zhang and Lewis, 1997; García-Olmedo et al., 1998; Broekaert et al., 1999) (Fig. 12). Apart from the eight common cysteines, only the two glycines at position 13 and 34, an aromatic residue at position 11 and a glutamic acid residue at position 29 are shared between the different plant defensins isolated to date (Numbering according to Rs-AFP1, Fig. 12). This variation in non-structural amino acids explains the divergence in biological activity of the different plant defensins.

Multi-alignment of different defensin amino acid sequences revealed the presence of five subgroups within the defensin family (Fig. 12). These subgroups were termed type I to type V. The aromatic residue at position 11 played an important role in the classification of the different defensin types. Type I and type II have tryptophan as aromatic residue, while type IV and type V have a phenylalanine residue. The defensins of type-III have a tyrosine as aromatic residue.

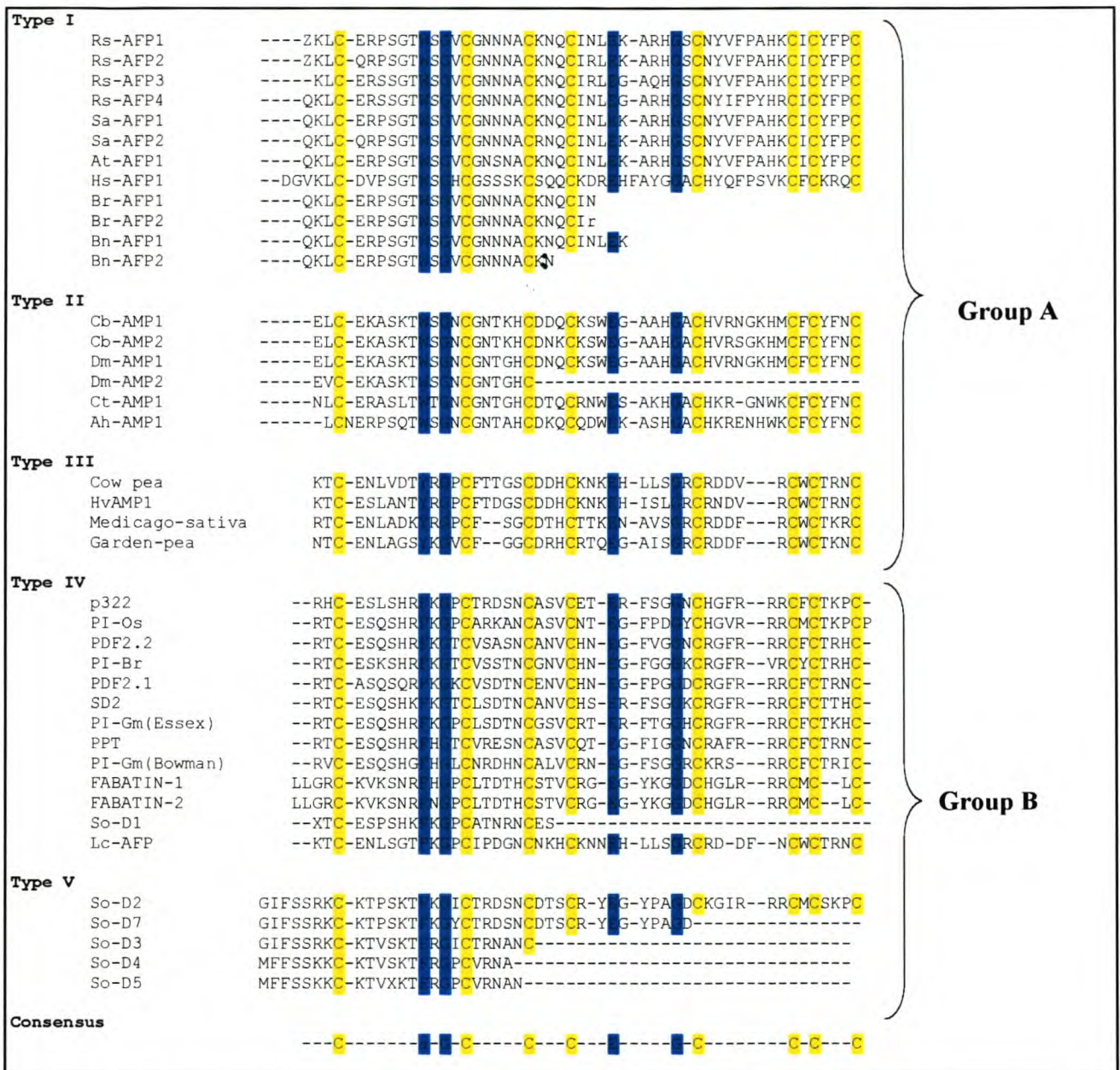


Figure 12. The sequence alignment of plant defensins isolated from various plant species. The sequences are Rs-AFPs from *Raphanus sativum*, Fabatins from *Vicia faba*, Sa-AFPs from *Sinapsis alba*, At-AFP1 from *Arabidopsis thaliana*, Cb-AMPs from *Cnicus benedictus*, Dm-AMPs from *Dahlia mercii*, Ct-AMP1 from *Clitorea ternatea*, Ah-AMP1 from *Amaranthus hypocondriacus*, Hs-AFP1 from *Heuchera sanguinea*, Bn-AFP1 from *Brassica naptus*, Lc-AFP1 from *Lathyrus cicera* and So-D1-7 from *Spinacia oleracea*, Br-AMPs from *Brassica rapa*, protease inhibitor PI-Os from *Oryza sativa*, Protease inhibitor PI-Br from *Brassica rapa*, PPT from *Petunia integrifolia*, SD2 from *Helianthus anuus*, P322 from *Solanum tuberosum*, Pi-Gm Essex and Bowman from *Glycine max* and PDF2.1 and PDF2.2 from *Arabidopsis thaliana*. The colour-coded cysteines represent the common cysteine pattern of plant defensins. The alignment was preformed with the ClustalX programme (Thompson et al., 1997).

The multi-alignment also revealed the presence of two distinct groups within the defensin family, namely group A and group B. Group A consisted of the defensins from type I to type III and group B of type IV and type V. The defensins in group A share 70% homology, but there is only 25% homology between group A and group B (Harrison et al., 1997; García-Olmedo et al., 1998; Thevissen et al., 1999, 2000b).

Plant defensins are active mostly against fungi and not bacteria. A few exceptions exist, such as Ct-AMP1 from *Clitoria ternatea* that is active against *Bacillus subtilis* (Osborn et al., 1995), and pseudothionin-St1 from potato shows activity against *Pseudomonas solanacearum* and *Clavibacter michiganensis* (Moreno et al., 1994). Defensins, unlike thionins, appear to have no detrimental effect on human cultured cells (Broekaert et al., 1997; García-Olmedo et al., 1998). The plant defensins can also be categorized into two distinct groups according to the morphological changes they induce on fungal hyphae. The morphogenic defensins cause a reduction in hyphae elongation, through the extensive increase of hyphae hyperbranching, whereas the non-morphogenic defensins reduce hyphae elongations without the induction of hyperbranching (Terras et al., 1992b, 1993). One common characteristic of both types of defensins is the decrease in activity when the ionic strength of the medium increases. This antagonistic effect is due to cations. Divalent cations are at least one order of magnitude more antagonistic than monovalent cations. This antagonistic effect, however, is also dependent on the fungal pathogen involved and the defensin type.

Research conducted by Thevissen et al. (1999, 2000b) suggests that defensins cause two different types of membrane permeabilization. Permeabilization is dependent on the cation concentration of the fungal growth medium and the defensin dose. At high defensin concentrations, between 10 and 40 μM , membrane permeabilization occurs due to a binding-site-independent interaction. Binding-site-independent permeabilization involves the direct interaction of defensins with the phospholipid components of the fungal plasma membrane and the insertion of the defensin into the plasma membrane, which results in membrane permeability. Binding-site-independent permeabilization is cation sensitive and can only be observed in low cationic strength media. High cation concentrations, especially of divalent cations, stabilize phospholipid structures in the plasma membranes, which counteracts the insertion of defensins into the plasma membrane. Binding-site-independent permeabilization does not appear to be the primary mode of action exhibited by defensins.

At low defensin concentrations (below 10 μM), weak, cation-resistant membrane permeabilization is observed. The doses required for cation resistant permeabilization correlate well with the defensin doses required for fungal inhibition. The spectrum of defensin activity also narrows in the presence of high cation concentrations and shows more variation among the different types of defensins. This mode of action involves binding-site-mediated permeabilization. The defensins bind to an as yet unknown receptor, followed by the

receptor-mediated insertion into the fungal plasma membrane (Fig. 13). This insertion leads to membrane disruption and permeabilization, resulting in ion fluxes across the membrane that ultimately lead to death. Binding-site-mediated resistance is seen as the primary mode of action of all defensins.

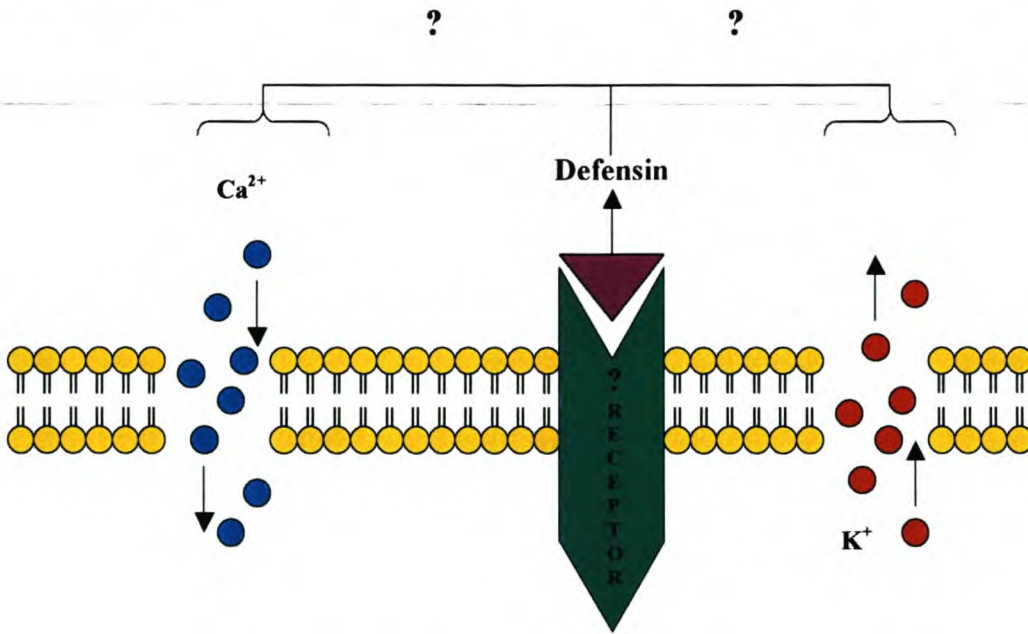


Figure 13. The proposed defensin-receptor interaction and subsequent cation channel formation as described by Thevissen et al. (1997, 2000a,b). The (?) represents an unknown interaction or mechanism initiated by defensins that leads to the leakage of Ca^{2+} (blue circles) and K^{+} (red circles).

Research also suggests that there are different receptors for the different types of defensins, since Dm-AMP2, a defensin from type II could not be out competed (for receptor binding) by other defensins belonging to type I, III and IV defensins (Thevissen et al., 2000b).

2.2.3 Lipid Transfer Proteins

Lipid transfer proteins (LTP) have been found in a wide range of plant species, such as *Arabidopsis*, barley, broccoli, carrot and maize. LTPs are expressed in a wide range of organs, including embryos, cotyledons, leaves, stems, siliques and various flower organs. LTPs accumulate in the epidermal or peripheral cells of these organs (Kader et al., 1984; Sterk et al., 1991; Molina et al., 1993; Segura et al., 1993; Kader, 1996; Dubreil, 1998).

LTPs from plants form a family of homologous peptides containing 90-93 amino acids, including eight disulfide-linked cysteines. The term LTP is given to these peptides because they have the ability to shuttle lipids from their point of synthesis, which is usually localized in organelles and vesicles, to membranes throughout the cell (Arondel and Kader, 1990). LTPs

not only have the ability to bind phospholipids (Fig. 14), but also fatty acids and glycolipids (Kader, 1990), and are henceforth termed non-specific LTPs (ns-LTPs). It has been suggested that a whole family of these peptides coexist in plant cells, with a wide range of biological functions besides lipid transfer (Wirtz and Gadella, 1990). It has been hypothesized that these peptides are also involved in β -oxidation (Breu et al., 1989), the formation of cutin layers through the transport of cutin monomers (Stern et al., 1991) and a role in defense against fungal and bacterial pathogens (García-Olmedo et al., 1995). The latter seems to be the most likely role of ns-LTPs in plants, since spatial expression studies conducted on a variety of ns-LTPs confirmed their presence at high levels in epidermal and peripheral cell layers (Sossountzov et al., 1991; Stern et al., 1991; Thoma et al., 1994). ns-LTPs were also found to be the most abundant protein present in the surface wax of broccoli leaves (Pyee et al., 1994). Moreover, the expression levels of these peptides rise upon fungal infection.

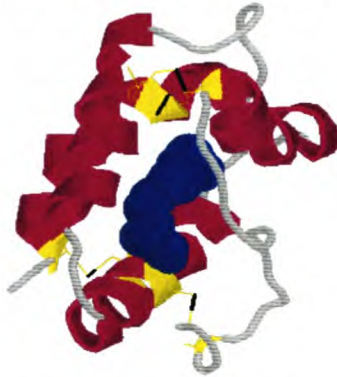


Figure 14. An ns-LTP isolated from wheat binding lyso-myristoyl-phosphatidylcholine (LMPC). The LMPC molecules are inserted head to tail in a hydrophobic cavity (Charvolin et al., 1999). The blue molecules represent LMPC6. The ns-LTP is represented by the α -helical turns (pink). The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).

The isolation of an ns-LTP-like peptide from *Allium cepa* (Ace-AMP1) that is unable to bind and transfer any phospholipids (Cammue et al., 1995) further punctuates the role of these types of peptides in plant defense responses. Although Ace-AMP1 only shows 76% homology to the other isolated ns-LTPs on amino acid sequence level (Fig. 15), it has the highest antimicrobial activity of ns-LTPs tested so far (Molina et al., 1993; Segura et al., 1993; Cammue et al., 1995).

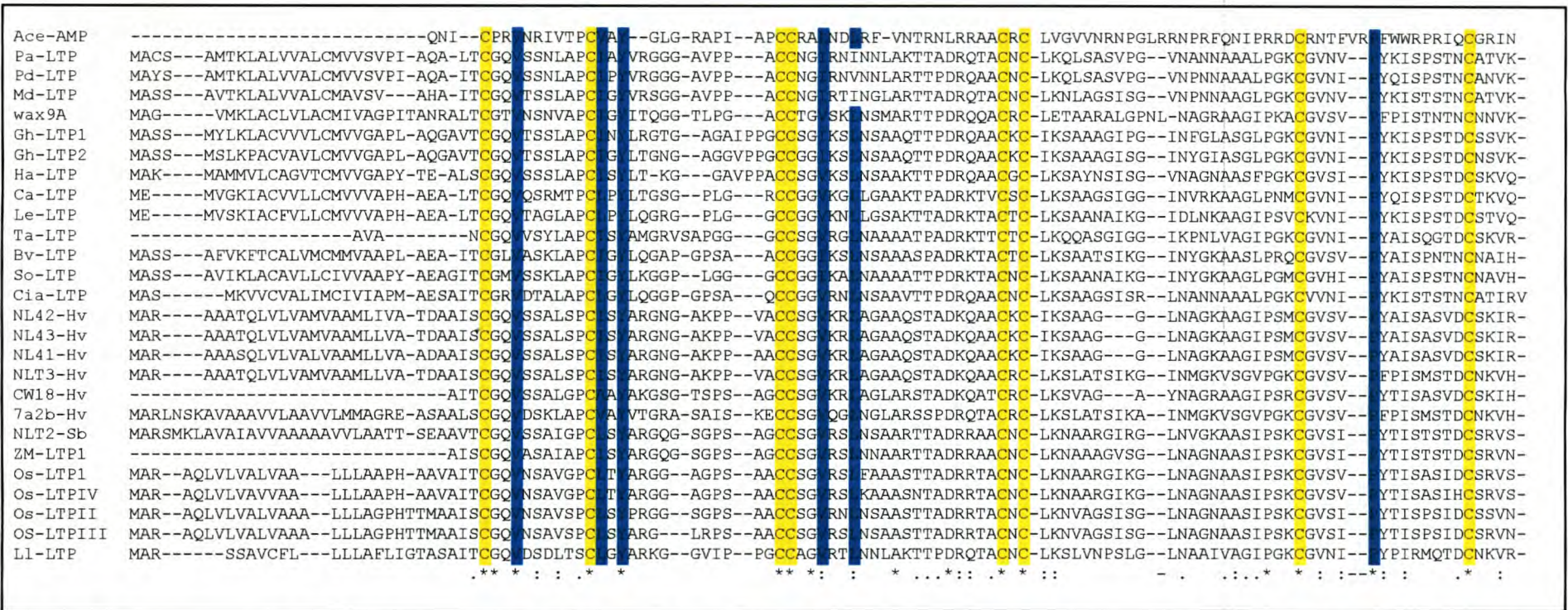


Figure 15. The preprotein sequence alignment of LTPs isolated from various plant species showing the conserved cysteine residues. The sequences are Ace-AMP1 from *Allium cepta*; Gh-LTP1 and 2 from *Gossypium hirsutum*; Ha-LTP from *Helianthus annuus*; Ca-LTP from *Capsicum annum*; Le-LTP from *Lycopersicon esculentum*; Bv-LTP from *Beta vulgaris*; So-LTP from *Spinacia oleracea*; Cia-LTP from *Cicer arietinum*; Pa-LTP from *Prunus avium*; Pd-LTP from *Prunus dulcis*; Md-LTP from *Malus x domestica*; Wax9A from *Brassica oleracea* var. botrytis; NL43,42, 41-Hv, NLT3-HV, CW18-Hv and 7a2b-Hv from *Hordeum vulgare*; NLT2-Sb from *Sorgum bicolour*; Zm-LTP from *Zea mays*; Os-LTP I-IV from *Oryza sativum*; Ta-LTP from *Triticum aestivum*; and LI-LTP from *Lilium longiflorum*. The colour-coded cysteines represent the common cysteine pattern of lipid transfer proteins. The * show homologous amino acids. The alignment was performed with the ClustalX programme (Thompson et al., 1997).

Exceptions to the general expression patterns and biological functions also exist in this group of peptides. An ns-LTP isolated from *Triticum aestivum*, ns-LTPe1, was not detected in the cell wall of wheat grains, but was present in the aleurone layer of the grain. Moreover, ns-LTPe1 had very low antifungal activities, but it enhanced the activities of thionins (Dubreil et al., 1998). It seems that there are different isoforms of this peptide group and that some play a role in defense and others in the depositing of extracellular lipids. These functions, however, might not be mutually exclusive, since some ns-LTPs may exert their antifungal activity after being deposited in the cell wall together with cutin precursors (Terras et al., 1992a).

When comparing the amino acid sequences of LTPs from different plant species, 30% conserved residues are found. These conserved areas include the eight cysteines and 12 other positions occupied by hydrophobic or aromatic residues (Fig. 15).

The folding pattern of ns-LTPs consists of a bundle of four α -helices linked by flexible loops (Gincel et al., 1994; Tassin et al., 1998) (Fig. 16). This structure also displays a hydrophobic cavity, which can accommodate a fatty acyl chain.

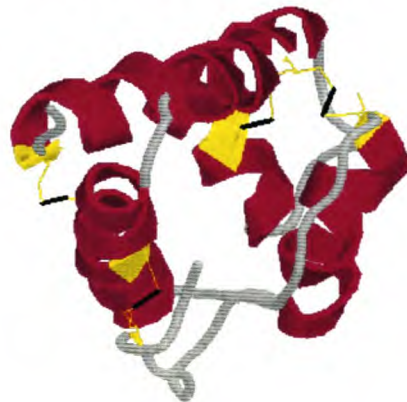


Figure 16. The three-dimensional structure of the wheat lipid transfer protein, as determined by HNMR studies, with four distinct α -helices. The helix (pink) represents the α -helical turns. The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).

From the studies conducted on the antimicrobial activities of ns-LTPs from different plant species, it is evident that significant variation exists in the inhibition potential against pathogens. An ns-LTP from onion seed, Ace-AMP1, is highly active against a broad range of fungi, whereas the ns-LTP from radish is approximately 10-fold less active. Peptides isolated from wheat and maize, however, show no antifungal activity. Some antibacterial activity has also been found for ns-LTPs from barley and spinach. These activities are 10-fold higher than that of wheat thionins against Gram-negative bacteria and three- to five-fold higher against Gram-negative bacteria (Molina et al., 1993; Segura et al., 1993). These peptides also exhibit

varying degrees of sensitivity to a high ionic environment. Onion ns-LTPs were nearly as potent in a low ionic synthetic medium than in the same medium supplemented with 1 mM Ca^{2+} and 50 mM K^{+} , whereas radish ns-LTP shows a reduced antifungal activity (Cammue et al., 1995).

ns-LTPs from onions, radish, maize or wheat did not show any detrimental effects on human erythrocytes or human fibroblasts, which suggests that LTPs have a more restricted cytotoxic effect than thionins.

2.2.4 Chitin-Binding Proteins and Knottin-Type Peptides

Chitin-binding proteins are able to reversibly bind to chitin and matrices composed of chitin. A common structural motif is observed when one compares the amino acid sequences of the chitin-binding proteins characterized to date. This motif is present between amino acids 30–43 and contains several cysteine and glycine residues at conserved positions. This chitin-binding domain is designated the hevein domain, since it is similar to hevein, the first chitin-binding protein isolated (Fig. 17). The hevein domain is a characteristic feature of all chitin-binding proteins, typically containing one or more of these domains in its structure (Raikhel et al., 1993). Although the term chitin-binding proteins is used, these proteins are also able to bind other complex glycoconjugates that contain N-acetylglucosamine (GlcNAc) or N-acetyl-D-neuraminic acid (NeuNAc) as primary building blocks. The natural ligands of these proteins have not yet been identified.

Chitin is not a substance found in higher plants, but is a rather important structural component of fungal cell walls and insect exoskeletons. This suggests that the chitin-binding proteins play a role in the host defense system. Other roles for these proteins cannot be excluded until further research establishes their exact physiological role *in planta*. For the purpose of this review, only the chitin-binding proteins containing less than 100 amino acids will be discussed.

Tomato Chitinase	-EQCGSQAGGARCAAGLCCSKFGWCGNTNDYCGPGN-CQSQCPCGG-
Bean Chitinase	-EQCGRQAGGALCPGGNCCSQFGWCGSTTDYCGPG--CQSQCPCGG--
Hevein	-EQCGRQAGGKLCNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDS-
Nettle lectin	-QRCGSQGGGGTCPALRCCSIWGWCGASSPYC-----
Wheat lectin	-QRCGEQGSNNECPNNLCCSQYGYCGMGGDYCGKG--CQDGACWTS

Figure 17. The sequence alignment of tobacco chitinase, bean chitinase, hevein, nettle lectin and wheat lectin, showing the cysteine/glycine-rich chitin-binding domain present in all chitin-binding proteins (in yellow). The alignment was performed with the ClustalX programme (Thompson et al., 1997).

Chitin-binding peptides are divided into two distinct groups, namely hevein- and knottin type peptides. These peptides, unlike most chitin-binding proteins, do not exhibit a multi-domain structure, but consist solely of the chitin-binding domain. The best-studied example of these peptides is hevein, which was isolated from the rubber tree (Archer et al., 1969;

Walujono et al., 1975; Broekaert et al., 1990). Hevein is present in the latex of the rubber tree, functioning to seal wounds sustained by the rubber tree and protecting it from potential pathogens. Hevein is located within luteoid bodies present in the latex sap. These luteoid bodies are vacuolar-derived organelles that contain a range of different proteins. Localization studies also revealed that hevein is present in the leaves and stems of the rubber tree, but not in the roots (Broekaert et al., 1990).

Hevein is a 5-kDa peptide containing 43 amino acids. The nucleotide sequence encoding this small peptide is much larger, however, and encodes a 204 amino acid preproprotein, consisting of a signal peptide, the mature hevein peptide and a hinged region followed by a C-terminal domain. Inefficient post-translational processing of this preproprotein results in incomplete cleavage, which generates mature hevein, a 20-kDa pro-hevein and a 15-kDa protein consisting of the C-terminal domain respectively (Broekaert et al., 1990).

Hevein has shown weak antifungal activity when compared to some other chitin-binding peptides and proteins. Nettle lectin, another chitin-binding peptide, is two- to five-fold more antifungal than hevein, whereas other hevein-like peptides isolated from *Amaranthus* (Ac-AMPs and Ar-AMP1), *Capsicum* (Ca-AMP1), *Beta* (IWF4) and *Briza* (Bm-AMP1) species also show high antifungal properties (Broekaert et al., 1992; De Bolle et al., 1993, 1995; Nielsen et al., 1997; Broekaert et al., 1999). Of these peptides, Ac-AMP1 and Ac-AMP2 are the smallest (29 and 30 amino acids respectively) and structurally the most simple chitin-binding peptides isolated thus far. When comparing the amino acid sequences from these peptides with that of hevein, it is evident that the *Capsicum*, *Briza*, *Beta* and *Amaranthus* peptides have truncated versions of the chitin-binding domain, although they are still able to bind chitin (Fig. 18).

Hevein	-EQCGRQAGGKLCPNLCCSQWGWCGSTDEYCS PDHNCQSNCKDS-
Bm-AMP1	---CS---SHNPCPRHQCCSKYGYCGLGSDYCGLG--CRGGPCDR-
Ac-AMP1	---VG-ECVRGRCPSGMCCSQFGYCGKGPKYCG-----
Ac-AMP2	---VG-ECVRGRCPSGMCCSQFGYCGKGPKYCGR-----
Ar-AMP1	---AG-ECVQGRCPSGMCCSQFGYCGKGPKYCGRR-----
IWF4	---SGECNMYGRCPGYCCSKFGYCGVGRAYCG-----
Ca-AMP1	QEQCQNQAGGRACANRLCCSQYGYCGSTRAYSG--VGCQSNCGR--

Figure 18. The sequence alignment of the hevein-type peptides from *Briza* (Bm-AMP1), *Amaranthus* (Ac-AMPs and Ar-AMP1); *Beta* (IWF4) and *Capsicum* (Ca-AMP1) compared with that of hevein. The yellow area indicates the truncated chitin-binding domain. The alignment was performed with the ClustalX programme (Thompson et al., 1997).

Another feature of these peptides is that they contain only six cysteines with three disulfide bonds, unlike the eight cysteines and four disulfide bonds of the other chitin-binding proteins. Three-dimensional studies conducted on Ac-AMP2 show that these peptides contain a small α -helix consisting of only one turn (Martins et al., 1996) (Fig. 19), whereas hevein contains an α -helix with one turn and a three-stranded β -sheet (Fig. 20).

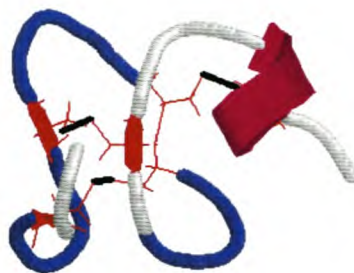


Figure 19. The three-dimensional structure of Ac-AMP2 (*Amaranthus cadautus*) with its small α -helix as determined by H NMR studies (Martins et al., 1996). The bars (black) represent the disulfide bonds and the wire frame (red) the cysteines. The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).



Figure 20. The three-dimensional structure of hevein as determined by H NMR studies showing the single α -helix and β -sheet made up of three antiparallel β -strands (Andersen et al., 1993). The bars (black) represent the disulfide bonds and the wire frame (red) the cysteines. The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).

The hevein-type peptides are highly active against a broad spectrum of fungi and some Gram-positive bacteria. Ac-AMPs and the IWF4 peptides from *Beta* have very low IC_{50} values against fungi. Ac-AMPs and the IWF4 peptides are also the most active of the chitin-binding peptides. Their activities can be contributed to their basic nature, which is more basic than that of hevein and other chitin-binding proteins. Research has shown that it is always the more basic peptides that have the highest activity (Cammue et al., 1992; Terras et al., 1992b).

The activities of these peptides result in morphological changes in the fungal hyphae, causing an inhibition of fungal growth. Although the mechanism involved in their antifungal activity has not been determined, it is speculated that they penetrate the fungal cell wall pores and interact with the fungal cell membrane. This hypothesis is supported by the fact that the fungal cell wall pores have a size exclusion of 15-20 kDa, which would allow the hevein-type

peptides of 5 to 8.5 kDa access to the cells. This might also be the reason why other chitin-binding proteins, such as *Gramineae* lectins (36 kDa), do not show any antifungal activity (Raikhel et al., 1993). Studies conducted on the antifungal activity of nettle lectin (8.5 kDa) showed that this peptide induced an increase in fungal hyphae chitin, which might result in enhanced cell wall plasticity. This interference in fungal cell wall composition results in abnormal apical growth, the latter being dependent on the mechanical strength of the hyphal cell wall. Whether this proposed mechanism of action applies to the other chitin-binding peptides, remains to be seen.

Over the last decade, chitin-binding peptides were isolated from *Mirabilis jalapa* (Mj-AMP1, Mj-AMP2) and, more recently, from *Phytolacca americana* (Pa-AMP1, Pa-AMP2) and *Mesembryanthemum crystallinum* (Mc-AMP1) seeds (Cammue et al., 1992; Shao et al., 1999; Liu et al., 2000). These peptides contain 36 - 38 amino acids, with six cysteine residues linked by three disulfide bonds. Although the cysteine motif present in these peptides and the disulfide bond linkage are identical to that of the first six cysteines of hevein, they are termed knottin-type peptides. The reason for this is that they show a high sequence homology to a group of peptides collectively known as knottins (Broekaert et al., 1997) (Fig. 21). This group is made up of proteinase inhibitors, the cellulose-binding domain of the cellobiohydrolases, calcium channel-binding toxins and a "sweet taste" modifying peptide from *Gymnema sylvestre* (Le-Nguyen et al., 1990; Chagolla-Lopez et al., 1994). All the knottin and knottin-type peptides isolated so far have a knot-like fold made up by a compact triple stranded β -sheet situated in a loop that connects the first and second β -strand (Lu et al., 1999) (Fig. 22).

Mj-AMP1	--QCIGN-GGRCNENVGP-PYCCSG--FCLR---QPGQGYGYCKNR-----
Mj-AMP2	--ACIGN-GGRCNENVGP-PYCCSG--FCLR---QPNQGYGVCKNR-----
Pa-AMP1	--AGCIKN-GGRCNASAGP-PYCCSS--YCFQ---IAGQSYGVCKNR-----
Pa-AMP2	--ACIKN-GGRCVASGGP-PYCCSN--YCLQ---IAGQSYGVCKKH-----
Mc-AMP1	--AKCIKN-GKGCREDPGP-PFCCSG--FCYR---QVGWARGYCKNR-----
OMN	---CKGK-GAPCRKTM---DCCSG--SCG-----RRGKC-----
OMG	---CKGK-GAKCSRLMY---DCCSG--SCG-----RSGKC-----
CELLOBIOH II	---CSSV-WGQCGQNWSGPTCCASGSTCVY---SN-DYYSQCLP-----
AXH	SPTCIPS-GQPCPYNEN---CCSQ--SCTFKENENGNTVKRCD-----
EIT	---ECVPE-NGHCRDWDYD---ECCGEG-FYCS---CRQPPKICRNN-----
GUR	---QCVKK-DELCIPYYL---DCCGEP-LECKK---VNWDHCKIG-----
AAI	---CIPK-WNRCGPKMDG-VPCCGEP-YTCTS-----DYYGNC-----
HWT	---ACKGV-FDACTPGKN---ECCPN-RVCS-----DKHKCKKWL-----
AGG	EDNCIAEDYGKCTWGGT---KCCRG-RPCRCRCS---MIGTNCECTPRLIMEGLSFA
	C-----C-----CC-----C-----C-----

Figure 21. The sequence alignment of the knottin-like peptides from *Mirabilis*, *Phytolacca* and *Mesembryanthemum* compared with that of the knottin group of peptides. The sequences are Mj-AMPs from *Mirabilis jalapa*; Pa-AMPs from *Phytolacca americana*; Mc-AMP1 from *Mesembryanthemum crystallinum*; OMN and OMG (ω -conotoxin-MVIlc and MVIlia) from *Conus magnus*; CELLOBIOH II from *Trichoderma reesei*; AXH (atractotoxin-HVI), EIT (μ -atactoxin) from a Blue Mountains funnel-web spider; GUR (gurmanin) from *Gymnema sylvestre*; AAI (α -amylase inhibitor I) from *Amaranthus hypocondriacus*; HWT (huwentoxin-I) from *Selenocosmia huwena*, a Chinese bird spider and AGG (ω -agatoxin-IVB), a funnel-web spider toxin. The colour-coded cysteines represent the common cysteine pattern of the different types of knottin-like peptides. The alignment was performed the ClustalX programme (Thompson et al., 1997).



Figure 22. The three-dimensional structure of α -Amylase inhibitor 1 determined by H NMR studies, showing the distinct Knot-like folding pattern (Lu et al., 1999). The arrows (yellow) represent the β -sheet and the helix (pink) indicates the α -helical turn. The bars (black) represent the disulfide bonds and the wire frame (red) the cysteines. The 3D structure was rendered with the RASMOL PDB viewer (Sayle, 1995).

Localization studies conducted on both the Mj-AMPs and Pa-AMPs confirmed their presence only in the seeds of *M. jalapa* and *P. americana*. The localization of the peptides in the seeds confirms their role in the plant defense system, since these peptides are released during germination to form an inhibitory zone around the seed to protect it from soil-borne pathogens (Cammue et al., 1992; Liu et al., 2000).

The knottin-type peptides show similar antimicrobial activities as those from the hevein-type peptides, including activity against Gram-positive bacteria and IC_{50} values as low as 5 $\mu\text{g/ml}$ against fungi. Although they show homology to certain toxins, they have no detrimental effect on human cultured cells. Their activities also depend on the cationic strength of the medium, since they are affected negatively by high cation concentrations. It is evident that the knottin and hevein-type peptides are inhibited more severely by cations than plant defensins and thionins. The exact mechanism of action of these peptides is not known, but it is suggested that they interact with a specific membrane-bound receptor rather than undergo random interactions with phospholipid membranes. The subsequent events following the binding of the receptor are unknown, but they typically lead to hyperbranching of the fungal hyphae, which results in growth inhibition.

2.2.5 Four Cysteine-Type Antimicrobial Peptides

This group of peptides has one unifying feature, namely that they contain four cysteines. One of the peptides in this group is MBP-1, a 33-residue peptide isolated from maize seed that contains four cysteine residues that are organised into two CXXXC segments. This peptide is inhibitory to several fungi and at least one Gram-positive (*Clavibacter michiganense*) and one Gram-negative (*Escherichia coli*) bacterium (Broekaert et al., 1997).

A novel class of antimicrobial peptides was also isolated from *Impatiens balsamina* seed and termed Ib-AMPs. This class of peptides includes four homologous, but non-identical, peptides, with a length of 20 amino acids (Fig. 23). All four peptides seem to be processed from a single multi-peptide precursor (Tailor et al., 1997). This multi-peptide precursor contains six repeated domains corresponding to one of the four mature Ib-AMPs. Multi-peptide precursors can be regarded as a means to diversify and broaden the activity spectrum of the defensive peptides. This makes them the smallest antimicrobial peptides isolated from plants. The peptides contain four cysteines that are clustered pair-wise in a CC and CXXC motif (Tailor et al., 1997). Proton Nuclear Magnetic Resonance studies conducted on Ib-AMP1 showed that this 20-amino acid peptide contained one β -turn, but no α -helices or β -sheet structures (Patel et al., 1998).

Ib-AMP1	QWGRRC ^{CC} GWGPGRRY ^C VRW ^C
Ib-AMP2	QYGRRC ^{CC} NWGPGRRY ^C KRW ^C
Ib-AMP3	QYRHR ^{CC} AWGPGRKY ^C KRW ^C
Ib-AMP4	QWGRRC ^{CC} GWGPGRRY ^C RRW ^C

Figure 23. The sequence alignment of the four mature Ib-AMPs from *Impatiens balsamina*. The colour-coded cysteines represent the common cysteine pattern of Ib-AMP peptides. The alignment was performed with the ClustalX programme (Thompson et al., 1997).

Ib-AMPs are inhibitory to a wide range of fungi and Gram-positive bacteria, but have no activity against Gram-negative bacteria. They also show no detrimental effect on human cultured cells (Table 1). The isoform with the highest isoelectrical point is least sensitive to cation inhibitions. This isoform still shows antifungal activity in a medium containing 1 mM CaCl_2 and 50 mM KCl. The exact mode of action of these peptides is not known, but since they do not contain any α -helices or β -sheets, it is unlikely that they interact directly with plasma membranes. Recent research has suggested that this peptide's activity is due to the inhibition of a distinct cellular process, rather than to ion channel or pore formation of cell membranes (Lee et al., 1999).

2.2.6 Snakin-1 (SN1)

A 63-amino acid peptide isolated from potato (*Solanum tuberosum* cv. *Desireé*) SN1 is a unique antimicrobial peptide with no homology to previously isolated plant antimicrobial peptides. SN1 contains 12 cysteine residues and has a molecular weight of 6.9 kDa. Although unrelated to other known plant antimicrobial peptides, it does show homology to cDNA-deduced clones encoding gibberlin-inducible mRNAs. SN1 also has some sequence motifs in common with the snake toxin kistrin (Fig. 24) (Segura et al., 1999). SN1 has high antimicrobial activity against pathogenic fungi and bacteria associated with potatoes, with IC_{50} values $<10 \mu\text{M}$ inhibiting these pathogens. The action of SN1 and that of PTH1 (a potato

defensin) are synergistic against *Clavibacter michiganensis* subsp. *sepedonicus* and additive against *Botrytis cinerea* (Segura et al., 1999).

SN1	G S N F C D S K C K L R C S K A G L A D R C L K Y C G I C C E E C K C V P S G T Y G N K H E C P C Y R D K K N S K G K S K --- C P
RC153	K S L F C A N K N D R C A R A G V K D R C I K Y C E I C C A E C K X V P S G T Y G N K H E C P C Y R D K K N S K G K S K --- C P
N37340	L E K W C G Q K C E G R C K A E G M K D R C L K Y C G I C C K D C C C V P S G T Y G N K H E C A C Y R D K L S S K G T P K --- C P
Os0951	G S D F C D G K C K V R C S K A S R H D D C L K Y C G V C C A S N C V P S G T T G N K D E C P C Y R D M D H R P W R S K E A Q V P
KIS	- S S P E N P C C D A A T C K L R P G A Q C G - - E G L C C E Q C K F S R A G - - - - - K I C R I P R G D M D D R C T G Q S A D C P

Figure 24. The sequence alignment of SN1 compared with those deduced from cDNA clones and that of kistrin, a snake venom toxin. The sequences are RC153 from *Ricinus communis*; N37340 from *Arabidopsis thaliana*; Os0951 from rice; and KIS from the Malayan pit viper. The colour-coded cysteines represent the common cysteine pattern of SN1. The alignment was performed with the ClustalX programme (Thompson et al., 1997).

SN1 also caused the aggregation of Gram-positive and Gram-negative bacteria. The induction of bacterial aggregation is not linked to the toxicity of SN1, however. It is rather hypothesized that this rapid aggregation of bacteria *in vitro* might play a role in the control of pathogen migration *in vivo* (Segura et al., 1999). The mechanism of action exhibited by SN1 has not been investigated, but initial studies showed that SN1 does not cause the aggregation and leakage of artificial liposomes, which are usually associated with the activity of other plant antimicrobial peptides.

2.3 PLANT ANTIMICROBIAL PEPTIDES: PRACTICAL APPLICATIONS

Plant antimicrobial peptides have the potential to play an important role in modern agriculture and medicine (Hancock and Lehrer, 1998; De Lucca and Walsh, 1999). In the pharmaceutical and medical fields, antimicrobial peptides might be highly useful to overcome or aid the problems of antibiotic resistance build-up in pathogens. Apart from the obvious medical applications, these peptides can also be used in agriculture (Shah, 1997; Rommens and Kishore, 2000). The ever-expanding human population is placing a heavy strain on water and soil resources and the agriculture industry in general to produce enough food without harm to the environment. Farmers are constantly challenged to increase yield and quality on the available resources, eliminate pests and pathogens from crops and use production practices that are environmentally friendly.

Farmers have always relied on chemical fungicides to protect crops from attack, but fungi are starting to develop resistance towards these chemicals at a rapid rate and consumers and environmentalist are demanding a reduction of fungicide use due to environmental issues (Hsiang, 1992). *B. cinerea*, a phytopathogenic fungus with a broad spectrum of hosts, is one of the fungal species showing resistance toward pesticides (Elad,

1992; Elad et al., 1992; Elad, 1996; Chapeland et al., 1999; Yourman and Jeffers, 1999). Even if this fungus does not reduce the yield of crops, it can interfere with the processing and production of secondary products from infected crops. This is especially true for the global wine industry, in which *B. cinerea* is a major problem in vineyards and the winemaking process (De Kock and Holz, 1991). Apart from the obvious loss in grape yield, the juice obtained from *B. cinerea*-infected grapes tends to be more viscous. This increase in viscosity causes problems in the pumping and filtering of juice. Wine made from *Botrytis*-infected grapes is more prone to oxidation, lowering the value of the wine.

One of the most important issues surrounding modern agriculture today is the effective and sustained control of fungal pathogens. Plant pathogen-related disease causes an estimated loss of 12% of potential crop yields throughout the world (Shah, 1997; Gao. et al., 2000). This figure is staggering, considering the fact that new fungicides and resistant crops are released each year. Much research has been conducted to find alternative methods to fight *B. cinerea* present in the vineyard and other crop fields. Some of these methods include: canopy management to manipulate the microclimate of the vines to prevent spore germination (Gubler et al., 1987; McLaughlin et al., 1992; English et al., 1993), the breeding of resistant cultivars, biological control (Tronsmo and Ystaats, 1980; Köhl et al., 1997, 1998; Paul et al., 1998; Moline et al., 1999; Paul, 1999) and the genetic improvement of plants through transformation technology.

2.3.1 Plant Antimicrobial Peptides as a Strategy for Engineering Disease-Resistant Crops

Genetic engineering of inherent disease resistance offers the potential for an environmentally and consumer-friendly alternative to manage agriculture in the 21st century. The potential of genetic engineering was first observed with the design of insect-resistant crops (Hilder and Boulter, 1999). To date, most of the work has been focused on the introduction of toxins from *Bacillus thuringiensis* (Bt) into commercial crops as a strategy against insect pests (Table 3).

Although genetic engineering of insect-resistant crops has been successful, the same cannot be said for the genetic engineering of resistance against fungal plant pathogens. Many plant antifungal proteins and peptides have been isolated over the last decade, and although they show great potential in *in vitro* studies, these antifungal agents disappoint *in planta*. The reasons for the loss of activity can range from incompatibility with the new host's internal protein modification pathways, stability in the transgenic host or the negative effect of the cationic makeup of the intercellular fluid (De Bolle et al., 1996).

Table 3. Transgenic plants expressing Bt toxins (Cry) from *Bacillus thuringiensis* (Schuler et al., 1998; Hilder and Boulter, 1999)

Crystal Prot.	Target insects	Transformed plants
Cry1Aa	<i>Lepidoptera</i>	Cranberry, poplar, rutabaga
Cry1Ab	<i>Lepidoptera heliothines</i> <i>Pectinophora gossypiella</i>	Apple, cotton, maize, poplar, potato, rice, tobacco, tomato, white clover, white spruce
Cry1Ac	<i>Lepidoptera heliothines</i> <i>Pectinophora gossypiella</i>	Apple, broccoli, cabbage, cotton, grapevine, oilseed rape, peanut, rice soybean, tobacco, tomato, walnut
Cry1Ba	<i>Lepidoptera</i>	White clover
Cry1Ca	<i>Lepidoptera</i>	Alfalfa, <i>Arabidopsis</i> , tobacco
Cry1H	<i>Lepidoptera</i>	Maize
Cry2Aa	<i>Lepidoptera</i>	Cotton
Cry3A	<i>Coleoptera</i>	Eggplant, potato, tobacco
Cry6A	<i>Coleoptera</i>	Alfalfa
Cry9C	<i>Lepidoptera</i>	Maize

There are several factors to consider before designing commercially-viable, disease-resistant crop species. This process involves trial and error before the right donor gene is obtained and usually involves *in vitro* studies with the protein concerned (Van der Biezen, 2001). In most cases, the desired disease resistance will not be achieved through the transformation of a single gene (Salmeron and Vernooij, 1998). The reason for this is that some genes only show antifungal activity in the presence of other substances that are up-regulated during pathogen infection. Another very important factor concerns pathogen adaptation. The products of the transgenes chosen must exhibit unique methods of activity, which cannot be overcome easily through random mutations of the fungal genome during evolution or enzyme degradation (Morrissey and Osbourn, 1999; Van der Biezen, 2001). The most important factor is the range of antifungal activity of the transgene product involved. The aim of genetic engineering is to reduce the costs associated with the production of food crops and if the transgene product does not have a broad range of antifungal activity, the crops will need to be sprayed with additional fungicides, which will cancel out the positive potential of the genetically-engineered crop.

Until recently, engineering disease resistance was achieved only through a strategy involving the introduction of more than one transgene. The best example thus far studied is

the strategy involving chitinase and glucanase genes (Jach et al., 1995; Dempsey et al., 1998; Fritig et al., 1998). Separately, these enzymes have little or no antifungal activity, but together they exhibit strong antifungal activity. This strategy has been applied to develop various disease-resistant crops. Co-expression of chitinase and glucanase genes in tomatoes, carrots and tobacco has led to enhanced resistance against *Fusarium* wilt disease, *Alternaria* and *Cerospora* species and *Rhizoctonia solani* respectively (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995). However, there are genes that, when transformed individually, will lead to enhanced disease resistance. These genes are becoming more important, since less work is required to design disease-resistant crops, which will reduce the costs involved.

For the purpose of this review, genetic engineering involving plant antimicrobial peptides will be discussed. Of the plant antifungal agents isolated, plant antimicrobial peptides show the best potential. These peptides have excellent characteristics that might make them suitable for engineering disease-resistant crops. Although more transgenic research needs to be done involving plant antimicrobial peptides, it is plant defensins and thionins that show the best results in transgenic lines thus far. These peptides also show a low risk of pathogen adaptation, because their antifungal mechanisms are targeted at the fungal membrane. Of all the plant antifungal agents isolated thus far, plant antimicrobial peptides have the lowest IC₅₀ values against a wide range of fungi (Table 4).

Table 4. The antifungal activities (IC₅₀ values µg/ml) in cation limited (-) and cation rich (+) medium of Type-I defensins isolated from *Raphanus sativa* (Rs-AFP1), *Heuchera sanguinae* (Hs-AFP1), *Brassica rapa* (Br-AFP1), *Brassica napus* (Bn-AFP1), *Sinapsis alba* (Sa-AFP1) and *Arabidopsis thaliana* (At-AFP1) (Terras et al., 1993; Osborn et al., 1995)

Fungi	Rs-AFP2		Br-AFP1		Bn-AFP1		Sa-AFP1		At-AFP1		Hs-AFP1	
	-	+	-	+	-	+	-	+	-	+	-	+
<i>B. cinerea</i>	10	100	1.5	100+	2	100+	1.8	100+	3.9	100+	6	25
<i>Fusarium culmorum</i>	1.5	6	2	19	2.8	33	4	40	3	35	1	3
<i>Verticillium albo-atrum</i>	12	100+	NA	NA	NA	NA	NA	NA	NA	NA	12	30
<i>Verticillium dahlia</i>	1.5	50	0.8	100+	1.2	100+	1.5	100+	1.5	100+	NA	NA

IC₅₀: Protein concentration required for 50% inhibition after 48 h at 25°C

Although the activities of the plant antimicrobial peptides are impressive in a cation-limited medium (Tables 4 and 5), the same cannot be said for their activity in media

containing cations representing an *in planta* environment (Tables 4 and 5). Although the activity of plant antimicrobial peptides is reduced *in planta*, this characteristic is dependent on the plant antimicrobial in question and the pathogen involved (Salmeron and Vernooij, 1998). This implies that a transgenic plant expressing plant antimicrobial peptides might show reduced activity against some pathogens, but will still exhibit enough activity to inhibit others effectively.

Table 5. The antifungal activities (IC₅₀ values µg/ml) in cation limited (-) and cation rich (+) media of type II defensins isolated from *Aesculus hippocastanum* (Ah-AMP1), *Clitoria tematea* (Ct-AMP1), *Dahlia merckii* (Dm-AMP1 and -2), *Hardenbergia violacea* (HvAMP1), a type III defensin, and So-D1, a type IV defensin from *Spinacia oleracea* (Osborn et al., 1995; Harrison et al., 1997; Segura et al., 1998)

Fungi	Ah-AMP1		Ct-AMP1		Dm-AMP1		Dm-AMP2		HvAMP1		So-D1	
	-	+	-	+	-	+	-	+	-	+	-	+
<i>B. cinerea</i>	25	100+	20	100+	12	100+	10	100+	15	100	NA	NA
<i>Fusarium culmorum</i>	12	100+	10	50	5	8	3	12	NA	NA	0.2	NA
<i>Verticillium albo-atrum</i>	6	100+	2	100+	4	100+	2	100+	NA	NA	NA	NA
<i>Verticillium dahlia</i>	NA	NA	NA	NA	NA	NA	NA	NA	100+	100+	NA	NA

IC₅₀: Protein concentration required for 50% inhibition after 48 h at 25°C

Disregarding the negative impact of cationic strength on the *in planta* environment, the transgenic expression of plant antimicrobial peptides has shown a lot of promise over the last 10 years. Various plant antimicrobial peptides have been introduced into crops species, resulting in a significant resistance against several plant pathogens (Table 6).

The transformation of potato with the *Medicago sativa* antifungal peptide (alfAMP) and the subsequent pathogen resistance is the first reported case, in which the transformation of a single defense-related gene resulted in agronomically-important disease resistance (Gao et al., 2000). These transgenic potatoes showed an increased fungal resistance to certain potato pathogens in greenhouse experiments. This increase in resistance could rival and even exceed the results achieved with fumigants. What is important is that this high level of antifungal activity, exhibited by the transgenic potatoes, could be maintained in field trial conditions. This single case of agronomically-important disease resistance achieved through defensin transformation confirms the important role that defensins and other antimicrobial peptides could play in the genetic engineering of disease-resistant crops.

Table 6. Crop plants genetically engineered with antimicrobial peptides for disease resistance

Crop	Plant antimicrobial peptide	Target pathogen	Reference
Tobacco	Rs-AFP2	<i>A. longipes</i>	Terras et al., 1995
Tobacco	Barley α -thionn	<i>P. syringae</i>	Carmona et al., 1993
Corn	AX1	<i>E. turcicum</i>	Shah, 1997
Corn	AX2	<i>E. turcicum</i>	Shah, 1997
Geranium	Ace-AMP1	<i>B. cinerea</i>	Bi et al., 1999
Tobacco	UDA	<i>B. cinerea</i>	Does et al., 1999
Potato	alfAMP	<i>V. dahliae</i>	Gao et al., 2000

Research already is being conducted to improve the activity of plant antimicrobial peptides (De Samblanx et al., 1997; Okamoto et al., 1998). These improvements include the reduction in sensitivity towards cation levels present in the *in planta* environment. The stability of these peptides in a foreign plant environment has also been achieved with the aid of peptide-protein fusion technology (Okamoto et al., 1998).

Synthetic peptides based on plant antimicrobial peptides are also being designed, although they have not rivalled the potential of the biological peptides to date (Ali and Reddy, 2000; Powell et al., 2000). Besides the obvious role played by these peptides in genetic engineering of disease resistance, they have the potential to act as bio-pesticides. When purified Mj-AMP1, isolated from *Mirabilis jalapa*, was sprayed on to sugar beet leaves to protect the plants against leaf spot disease (*Cercospora beticola*), it was as effective as the chemical fungicide hexaconazole at the same concentration (De Bolle et al., 1996). This makes the continuous isolation and improvement of plant antimicrobial peptides essential for the development of environmentally friendly agricultural industry. The potential impact of these small antimicrobial peptides on modern agriculture cannot be denied; they might just be the answer to the high demands of the modern health conscious consumer.

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CHAPTER 3

RESEARCH RESULTS

Overexpression and evaluation of an antimicrobial peptide from *Heuchera sanguinea* (Hs-AFP1) for inhibition of fungal pathogens in transgenic tobacco

**(To be submitted for publication in
Plant Physiology)**

RESEARCH RESULTS

Overexpression and Evaluation of an Antimicrobial Peptide from *Heuchera Sanguinea* (Hs-AFP1) for Inhibition of Fungal Pathogens in Transgenic Tobacco

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ABSTRACT

A plant defensin from *Heuchera sanguinea*, Hs-AFP1, shows high levels of *in vitro* activity against a broad range of fungi. In an effort to evaluate the possible application of Hs-AFP1 in the genetic transformation of crop plants to improve disease resistance, the Hs-AFP1 peptide was studied in transgenic tobacco. The gene encoding mature Hs-AFP1 was fused to a signal peptide from *Mirabilis jalapa* and was introduced into tobacco via *Agrobacterium* transformation. Southern blot analysis conducted on the resulting transgenic tobacco lines confirmed *Hs-AFP1* gene integration. *Hs-AFP1* was also transcribed actively and the presence of the transgenic protein was confirmed by Western blot analysis. Due to the well-documented instability of small peptides on the plant surface, a fusion between the Hs-AFP1 peptide and the antifungal exoglucanase gene, *EXG1*, from *Saccharomyces cerevisiae* was prepared and also introduced into tobacco. This fusion protein was also targeted to the apoplastic region with the signal peptide from *M. jalapa*. Northern blot analyses conducted on the resulting transgenic tobacco lines confirmed transcription of the fusion construct, whereas Western blot analyses confirmed the presence of the fusion protein. The exoglucanase activity of the fusion construct was evaluated with a β -glucosidase assay. All the transgenic lines that showed transcription of the various constructs were subjected to *Botrytis cinerea* inhibition and infection studies. Elevated levels of resistance towards *B. cinerea* were observed in several of the transgenic lines, reaching inhibition levels of 35-45%.

Key words: *Heuchera sanguinea* – defensin – antifungal – transgenic – disease resistance

3.1 INTRODUCTION

Plant diseases caused by bacterial and fungal pathogens result in huge crop losses every year (Baker et al., 1997; Gao et al., 2000). A rise in pesticide-resistant pathogens translates into an increase in pathogen damage to crops worldwide (Elad, 1992; Elad et al., 1992; Chapeland et al., 1999).

Strategies to improve the innate defense of plants against pathogens include the upregulation of plant defense pathways and improving the recognition between host and pathogen through the expression of genes encoding for defense elicitor molecules (Cao and Dong, 1998; Oldroyd and Staskawicz, 1998; Shen et al., 2000). The heterologous expression of hydrolytic enzymes isolated from various organisms, including fungi, has also showed some success in improving disease resistance (Lorito et al., 1998). Over the course of the last 15 years, scientists also have turned their attention to small, antimicrobial peptides in the manipulation of plant defense (Ganz and Lehrer, 1998; García-Olmedo et al., 1998; Ganz and Lehrer, 1999; Lehrer and Ganz, 1999; Powell et al., 2000).

One of the characteristic groups of small antimicrobial peptides is cysteine-rich peptides in which the high cysteine content favors the formation of disulphide bonds. These bonds are very strong and result in the formation of a stabilizing folding pattern, which involves antiparallel β -sheets (Broekaert et al., 1997).

Most of these peptides are present in the extracellular environment, where they play an important role in the innate immunity of various organisms. It has even been suggested that the cysteine-rich peptides represent the oldest form of defense that has stayed unchanged despite evolutionary pressure (Broekaert et al., 1995, 1997). *In vitro* research conducted on cysteine-rich peptides that were isolated from various organisms, has shown that these peptides are active against a broad range of human, as well as plant pathogens (Cavallarin et al., 1998; Mourgues et al., 1998; Lamberty et al., 1999). This broad spectrum of activity makes the cysteine-rich peptides excellent candidates for genetic engineering.

All the antimicrobial peptides thus far isolated from plants belong to the cysteine-rich peptide family and include thionins, plant defensins, lipid transfer proteins and chitin binding peptides (Broekaert et al., 1997; García-Olmedo et al., 1998). Although the *in vitro* activity of these peptides has been well documented, it is their performance in the *in planta* environment that will validate their role in plant defense mechanisms and their potential use for the engineering of disease-resistant crops.

This study evaluated four antifungal peptides from different plant sources for their *in vitro* activity against a pathogenic strain of *B. cinerea* that was isolated from grapevine. This led to the selection of a peptide from *Heuchera sanguinea* to be highly expressed and targeted to the apoplastic environment of transgenic tobacco lines. Due to the notorious instability of small peptides on the plant surface, a gene fusion strategy to potentially stabilize

the antifungal peptide was also evaluated. Successful integration and expression of the transgenes occurred in the various transgenic lines that were evaluated for their ability to resist infection by *B. cinerea*.

3.2 MATERIAL AND METHODS

3.2.1 Microbial strains and culture conditions

All microbial strains and plasmids used in the study are listed in Table 1. All percentages of media ingredients are indicated as weight per volume (w/v), unless otherwise stated. The *Agrobacterium tumefaciens* strain was cultured routinely in YEP medium (1% yeast extract, 1% peptone and 0.5% sodium chloride) at 28°C. *Escherichia coli* strain DH5 α was grown in Luria Bertani (LB) medium (0.6% yeast extract, 1.2% tryptone and 1.2% sodium chloride) at 37°C, or LB supplemented with the appropriate antibiotic for selection of transformants or to retain selective pressure.

A sporulating culture of *Botrytis cinerea* was obtained from the department of Plant Pathology at Stellenbosch University. The spores were germinated and maintained on apricot halves to sustain their high level of virulence. Apricot halves, canned in natural juice, were washed with sterile water, blotted dry on sterile paper towels and placed inside petri dishes (100 mm x 20 mm). Each apricot was inoculated with a few spores of *B. cinerea* and incubated in the dark at 25°C until sporulation. Spores were harvested with a bent glass rod and 3 ml of sterile water containing Tween 20 (90 μ L). The spore concentration was determined with a haemocytometer for subsequent applications.

3.2.2 Microscopic analyses of *Botrytis cinerea* inhibition by Hs-AFP1

To assess the antifungal activity of Hs-AFP1 peptides, 10 μ g/ml of purified peptide (kindly received from W. F. Broekaert at the F.A. Jansens laboratory, Katholieke Universiteit Leuven) was incubated with 2000 *B. cinerea* spores in 100 μ l half-strength potato dextrose broth (12 g/L)(DIFCO). The samples were incubated in the dark at 25°C for 3 days. Microscopical photographs were taken every 24 h of each sample, under 100x magnification, to assess their effect on *B. cinerea* hyphae morphology. A similar procedure was used to evaluate crude protein extracts from transgenic plant lines for their antifungal activities. These heterologous proteins were assayed at final concentrations of 5 mg/ml against *Botrytis* spores.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strain		
DH5 α	supE44 <i>lacU</i> 169[Φ 80 <i>lacZ</i> M15 <i>hsdR</i> 17 <i>recA</i> 1 <i>gyrA</i> 96 <i>thi</i> -1 <i>relA</i> 1]	Life Technologies (GIBCO/BRL)
<i>A. tumefaciens</i> strain		
EHA105	Disarmed, succinomopine-type strain	Hood et al., 1993
<i>Botrytis cinerea</i> strain	A highly virulent strain isolated in the Stellenbosch area	Department of Plant Pathology Stellenbosch University
Plasmids		
pGEM T-Easy	pGEM5Zf(+)-based PCR cloning vector	Promega
pRK2013	Helper plasmid used for tri-parental mating	
pEX191	pUC19-based plasmid; Ap ^R ; 3.95 kb <i>S. cerevisiae</i> genomic insert, comprising exoglucanase (<i>EXG1</i>) gene	Van Rensburg et al., 1997
pFAJ3068	A plant expression vector; Km ^R , driven by Nos promoter; <i>Hs-AFP1</i> , driven by the enhanced 35S promoter and fused to <i>M. jalapa</i> signal peptide encoding sequence	Prof. W.F. Broekaert, Jansens laboratory, Katholieke Universiteit Leuven
pFAJ-Hs-AFP1	pFAJ3068, <i>Hs-AFP1</i> was replaced with <i>Hs-AFP1</i> lacking a stop codon	This study
pFAJ-Mj	pFAJ3068, <i>Mj-Hs-AFP1</i> cassette deleted with <i>SacI/XhoI</i> ; <i>M. jalapa</i> signal peptide encoding sequence inserted	This study
pHS-EXG1	pFAJ-Hs-AFP1, <i>EXG1</i> cloned into <i>SacI</i> ; fusion between <i>HS-AFP1</i> and <i>EXG1</i>	This study
pMj-EXG1	pFAJ-Mj, <i>EXG1</i> cloned into <i>SacI</i> , fusion: <i>Mj-EXG1</i>	This study
pGEM-Hs-AFP1	pGEM5Zf(+)-based PCR cloning vector containing the <i>Hs-AFP1</i> insert lacking a stop codon	This study
pGEM-EXG1	pGEM5Zf(+); <i>EXG1</i> without start codon	This study
pGEM-MJ	pGEM5Zf(+); <i>M. jalapa</i> signal peptide	This study

3.2.3 Construction of plant expression cassettes

Standard DNA techniques were used for cloning, plasmid isolation and *E. coli* transformations (Ausubel et al., 1987; Sambrook et al., 1989). Restriction endonucleases and the PCR amplification reagents were purchased from Roche. T4 DNA ligase and the pGEM-T easy™ kit was purchased from Promega, whereas Shrimp alkaline phosphatase (SAP) was obtained from USB. All the plant expression vectors constructed relied on kanamycin selection (50 µg/ml) in *E. coli* and *Agrobacterium*.

The *Hs-AFP1* gene, lacking a stop codon, was PCR amplified with primers Hs-AFP1-5' and Hs-AFP1-Min-STOP-3' (see Tables 2 and 3 for primers and PCR program used respectively) and subcloned into the pGEM-T easy™ vector system (Promega), yielding pGEM-Hs-AFP1. The native *Hs-AFP1* sequence was replaced in pFAJ3068 with the *Hs-AFP1* fragment from pGEM-Hs-AFP1 at the *XhoI* and *SacI* sites to yield pFAJ-Hs-AFP1. An *EXG1* fragment was PCR amplified from pEX191 without its native start and stop codons (see Tables 2 and 3 for primers and PCR program used respectively) and was cloned into pGEM-T easy™ to yield pGEM-EXG1.

pFAJ-Hs-AFP1 was digested with *SacI* and dephosphorilated with SAP. The *EXG1* fragment was excised from pGEM-EXG1 using *SacI* and subcloned into the prepared pFAJ-Hs-AFP1 to yield the plant transformation vector pHs-EXG1 (Fig. 1).

The Mj-AMP2 signal peptide encoding sequence was PCR amplified from pFAJ3068 (see Tables 2 and 3 for primers and PCR program used respectively). The resulting fragment was cloned into the pGEM-T easy™ vector system to yield pGEM-Mj. pFAJ3068 was digested with *XhoI* and *SacI* to remove the native *Mj-Hs-AFP1* sequence. The Mj fragment was excised from pGEM-Mj with *XhoI* and *SacI* and subcloned into the corresponding sites of pFAJ3068 to yield pFAJ-Mj. pFAJ-Mj was digested with *SacI* and dephosphorilated with SAP. The *EXG1* fragment was excised from pGEM-EXG1 with *SacI* and subcloned into the prepared pFAJ-Mj plasmid to yield the plant transformation vector pMj-EXG1 (Fig. 1).

3.2.4 Tobacco transformation and plantlet regeneration

The three plant expression vectors, pFAJ3068, pHs-EXG1 and pMj-EXG1, were mobilized into *A. tumefaciens* EHA105 by triparental mating. Tobacco leaf discs were transformed by the standard leaf disc transformation method described by Horsh et al. (1985). Transgenic shoots were regenerated under kanamycin selection (120 µg/ml) (see Table 4 for designated transgenic lines). Root and shoot formation was induced by cultivation on rooting and shooting medium respectively (see Table 5).

Table 2. PCR primers used in this study

Primer name	5'-Sequence-3'	Template	Product
EXG1-MIN-ATG-5' (<i>SacI</i>)	5'-GGCC <u>GAGCTC</u> CTTTTCGCTTAAAC-3'	pEX191	EXG1 without start /stop codon
EXG1-MIN-STOP-3' (<i>SacI</i>)	5'-GGCC <u>GAGCTC</u> GTAGAAATTGTGCCACATTGG-3'	pEX191	EXG1 without start /stop codon
Hs-AFP1-5' (<i>XhoI</i>)	5'-GCCGCTCGAGTATTTTTACAATTACCAAC-3'	pFAJ3068	Hs-AFP1 without stop codon
Hs-AFP1-MIN-STOP-3' (<i>SacI</i>)	5'-GGCC <u>GAGCTC</u> GCATTGCCTCTTGCAGAAGC-3'	pFAJ3068	Hs-AFP1 without stop codon
Mj-AMP2SP-3'	5'-GGCCGAGTCTTGCTTCTAGCATGCCGGACATGG-3'	pFAJ3068	Mj-AMP signal peptide

The restriction sites are indicated by the underlined sequences.

Table 3. The PCR programs used in the construction of different plant expression vectors

PCR product	<i>Hs-AFP1</i> : without stop codon		<i>Mj-AMP</i> : signal peptide		<i>EXG1</i> : without start /stop codon	
	Temp	Time	Temp	Time	Temp	Time
1st Denaturation	94°C	2 min	94°C	2 min	94°C	2 min
Annealing	55°C	45 sec	55°C	45 sec	55°C	45 sec
Elongation	72°C	2 min	72°C	2 min	72°C	2 min
2nd Denaturation	94°C	45 sec	94°C	45 sec	94°C	45 sec

Table 4. The transgenic tobacco lines obtained after *A. tumefaciens* transformation

Plant Expression Vector	Designated Transgenic Line	Number of Transgenic Lines	F0/F1 generation
pFAJ3068	THs	15	F1
pHs-EXG1	THE	7	F1
pMj-EXG1	TME	14	F0

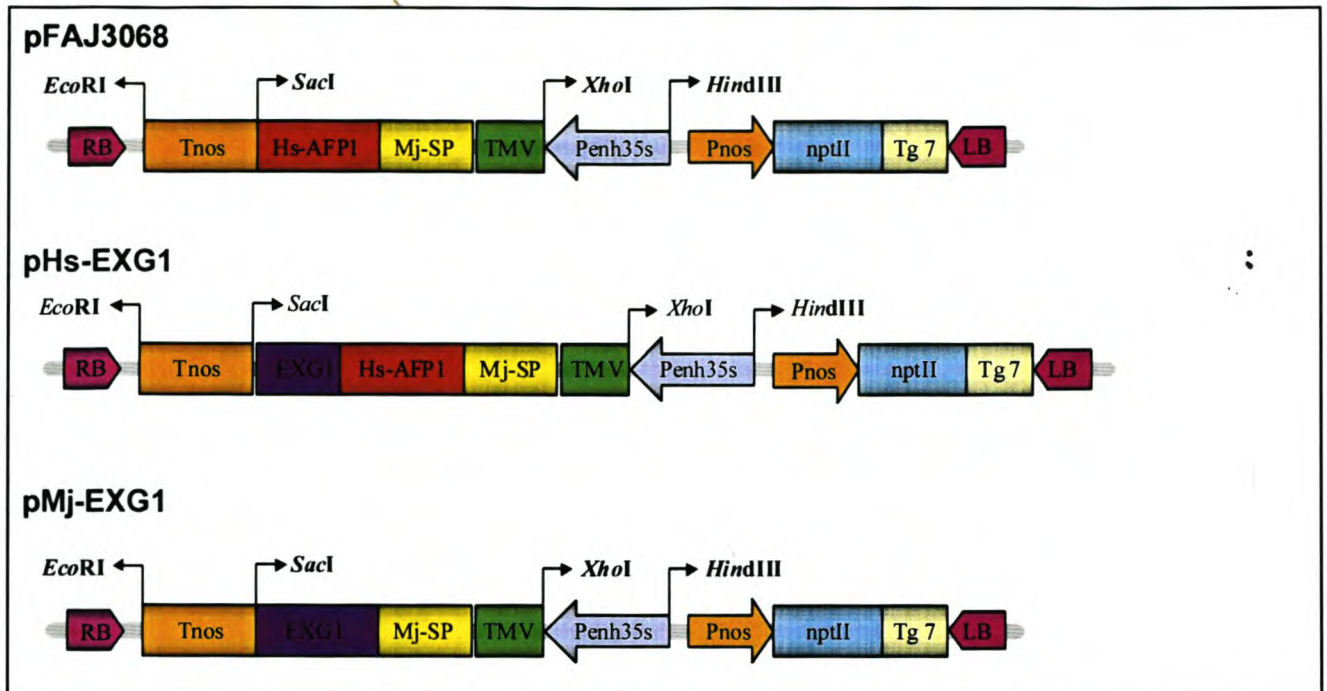


Figure 1. The plant expression cassettes used for tobacco transformation with an antifungal peptide from *Heuchera sanguinea* (*Hs-AFP1*). The abbreviations represent: **RB**, T-DNA right border; **LB**, T-DNA left border; **TMV**, Tobacco mosaic virus 5' leader sequence; **Penh35s**, Promoter of 35S 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; **Tg7**, Terminator of T-DNA gene 7; **Tnos**, Terminator of T-DNA nopaline synthase gene; **Hs-AFP1**, Mature protein encoding domain of *Hs-AFP1*; **EXG1**, Mature protein encoding domain of *EXG1*; **Mj-SP**, Signal peptide encoding domain of *Mj-AMP2* cDNA.

The resulting transgenic tobacco plantlets were sub-cultured to maintenance medium, and maintained at 25°C under a 16 h light cycle. Three-week-old plantlets were hardened-off in pot 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 THs and THE plant lines. Hardened-off primary transformants were used for the TME transgenic lines.

Table 5. Media used for the regeneration and cultivation of *in vitro* plant material

Organic constituent	Regeneration	Rooting	Shooting	Maintenance
Difco-agar (g.L ⁻¹)	8	8	8	8
Myo-inositol (mg.L ⁻¹)	100	100	100	100
Nicotinic acid (mg.L ⁻¹)	1	1	1	1
Pyridoxin HCl (mg.L ⁻¹)	1	1	1	1
Thiamin HCl (mg.L ⁻¹)	10	10	10	10
6-Benzylaminopurine (μM)	10	0	10	0
β-Naphthoxyacetic acid (μM)	0	0.5	0	0
Sucrose (g.L ⁻¹)	30	15	15	15
Cefotaxime (mg.L ⁻¹)	400	400	400	0
Kanamycin (mg.L ⁻¹)	120	120	120	0
pH (with 1 M KOH)	5.8	5.8	5.8	5.8

3.2.5 Southern blot analyses and PCR of transgenic tobacco lines

To confirm integration of the transgenes, Southern blot analyses were performed on the lines harboring pFAJ3068 and pHS-EXG1, whereas gene-integration was confirmed with PCR on the lines harboring pMj-EXG1.

For Southern blot analysis, leaf tissue (50 mg) from the F1 generation lines was ground to a powder in the presence of liquid nitrogen. The 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 pFAJ3068 and pHS-EXG1 expression cassettes were assessed through the digestion of the genomic DNA with *Hind*III. The digested DNA was separated on a 1% (w/v) agarose gel, followed by transfer to positively charged nylon membrane (Roche). The membrane was subsequently probed with a DIG-labeled, Hs-AFP1 probe. Pre-hybridization and hybridization were performed at 37°C. Detection was performed according to the DIG Manufacturer's protocol (Roche). Each hybridization signal represents a single gene copy. To confirm the positive integration of the pMj-EXG1 cassette, genomic DNA from the TME plant lines were used, together with primers Hs-AFP1-5' and EXG1-Min-Stop-3' in PCR analysis.

3.2.6 RNA manipulations to confirm gene expression in transgenic lines

All RNA isolations were performed with TRIZOL™ Reagent (GIBCO), according to the manufacturer's protocol. Leaf tissue (100 mg) was grounded to a fine powder in the presence of liquid nitrogen. RNA was extracted with 1 ml TRIZOL™ reagent, according to the manufacturer's protocol. Total RNA was separated on a 1.2% (w/v) formaldehyde gel and transferred to positively charged nylon membranes (Roche) as described by Maniatis et al. (1982). The THs and THE membranes were probed with a DIG-labeled, Hs-AFP1 probe. Pre-hybridization and hybridization was performed at 45°C. Detection was performed according to the manufacturer's protocol (Roche).

The TME lines were also subjected to RT-PCR analysis using the *EXG1* specific primers, EXG1-MIN-ATG-5' and EXG1-Min-Stop-3'. The RT-PCR reactions were performed with the *C. therm* one-step RT-PCR kit (Roche) according to the manufacturer's guidelines. cDNA synthesis was performed at 50°C, for 30 min, followed by a PCR reaction; denaturation occurred at 94°C for 2 min and at 94°C for 30 sec. The annealing temperature was 55°C for 30 sec with elongation at 72°C for 2 min. The program was run for 30 cycles. The PCR products were separated on an 1% (w/v) TAE gel. RT-PCR analysis was also performed on leaf RNA isolated from TME lines that were inoculated with 5000 *Botrytis* spores, 12 and 24 h after infection.

3.2.7 Protein isolation, gel electrophoresis and Western blot analysis

Crude protein extracts were prepared from the different transgenic F1 progeny lines. Five grams of leaf tissue were grounded to a fine powder in the presence of liquid nitrogen. The total crude protein fraction was extracted with 5 ml of ice-cold extraction buffer (50 mM phosphate buffer pH 7.0, 5 mM β-mercaptoethanol, 5 mM EDTA) as described by Jach et al. (1995). The protein extracts were dialyzed extensively against water and freeze-dried. The proteins were weighed and dissolved in dH₂O to a final concentration of 10 mg/ml.

Ten µg crude protein from the THs and THE tobacco lines was separated with Tricine-SDS-PAGE (Schagger and Von Jagow, 1987) and SDS-PAGE gel electrophoresis respectively. The separated proteins were transferred to a PVDF membrane (Millipore) using the semidry blotting method and a Semiphor™ transfer apparatus. For immunological detection of mature Hs-AFP1 and Hs-EXG1, an antibody raised against purified Hs-AFP1 was kindly obtained from Dr. B.P.A. Cammue. Goat-anti-rabbit peroxidase-linked antibody (IgG-horseradish peroxidase) was used as secondary antibody. Detection was performed with an enhanced chemiluminescence Western-blotting detection system (ECL, Amersham Life Science).

3.2.8 Microspectrophotometric assay of fungal inhibition in transgenic tobacco plants

The purified Hs-AFP1 peptides (obtained from W.F. Broekaert) as well as the F1 generations of the transgenic THs and THE tobacco lines were analyzed for their antifungal activities against *B. cinerea* by using a spectrophotometric assay. Purified proteins (6 and 8 μg) and total crude protein fractions, at a final concentration of 5 mg/ml, were analyzed in the presence of 2000 *Botrytis* spores according to the method of Broekaert et al. (1990). Measurements (A_{595}) were collected at time 0 and then every 24 h until 48 h past inoculation. All the values were corrected by subtracting the time zero value from the 24 and 48 h values. The activity of the different crude extracts was expressed in terms of percentage growth inhibition, which is defined as $100 \times$ 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. The percentage inhibition of the different crude extracts was plotted against each other. The microplate readings were performed on the PowerwaveX microplate reader (Bio-Tek instruments inc.).

3.2.9 β -Glucosidase activity assays

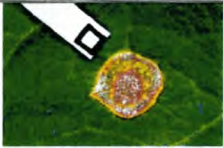
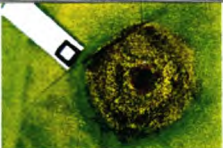


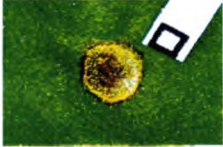
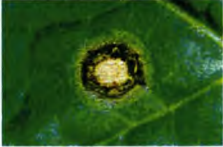

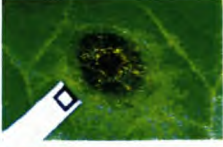


In an effort to determine if the EXG1 protein, as produced in the THE and TME lines, was still active, crude protein samples were subjected to a β -glucosidase assay. The total crude proteins were concentrated with 30 kDa cut-off Microcon™ columns (Millipore) and quantified using the Bradford method (Bio-Rad). Ten μl of crude protein extract, varying in protein concentration between the different transgenic lines, was analyzed in a 50 μl β -glucosidase assay, consisting of 4 mM p-Nitrophenyl- β -D-Glucopyranoside and 0.1 M NaOAc pH 5.5. The samples were incubated at 37°C for 1 h. The reaction was terminated with 100 μl 1M Na_2CO_3 . The A_{405} of all the samples was determined with a microplate reader. A reaction stopped at time zero served to normalize the samples. The corrected absorption values were converted to μM p-Nitrophenyl released per hour, using a p-Nitrophenyl standard curve. One unit of crude protein activity is defined as nM p-Nitrophenyl released per hour by 1 μg crude protein extract.

3.2.10 *Botrytis cinerea* infection studies

The TME, THs and THE plant lines were subjected to *B. cinerea* infection studies. The top three, fully expanded leaves from each plant were transferred to magenta pots containing water agar (8 g/L). Each leaf was inoculated with two spots of 5000 *B. cinerea* spores, suspended in 100% pure grape juice. The leaves were incubated at 22°C under a 16-h light/8-h dark cycle for 3 weeks. The disease symptoms were scored after 4 days and 7 days by measuring the lesion size. The lesion types were also observed for each transgenic

and control tobacco line. The appearance of the lesions was used to establish a lesion-type index with a ten-point scale that is presented in Table 6. The lesion size data were used to draw up a disease resistance index of all the transgenic tobacco lines. After three weeks, the infected leaves were inspected for appearance and the development of reproductive fungal organs.

Table 6. The lesion-type-index used in a *Botrytis* infection study

Lesion appearance*	Lesion index	Lesion appearance*	Lesion index
	Type 1 Dry; limited expansion; no hyphal growth; dark or light brown.		Type 6 Moist; expanding; no or minor hyphal growth; dark or light brown.
	Type 2 Moist; limited expansion; no hyphal growth; dark or light brown.		Type 7 Dry; expanding; hyphal growth; translucent.
	Type 3 Dry, minor expansion; no hyphal growth; dark or light brown.		Type 8 Dry; expanding; hyphal growth; dark or light brown.
	Type 4 Dry; limited expansion; hyphal growth; dark or light brown.		Type 9 Moist; expanding; hyphal growth; dark or light brown; physical damage.
	Type 5 Moist; minor expansion; hyphal growth; dark or light brown.		Type 10 Moist; expanding; hyphal growth; translucent; physical damage.

* The rectangular block on the white paper strip represents an area of 1 mm² and was used to standardize lesion magnification.

Transgenic and control tobacco leaves were inoculated with 5000 *B. cinerea* spores and incubated at 22°C under a 16-h light/8-h dark cycle.

To score lesion types, photographs were taken after 4 days and a lesion type index was drawn up. The scale proceeds from the most resistant phenotype (type 1) to the most susceptible (type 10)

3.3 RESULTS

3.3.1 *In vitro* activity of Hs-AFP1 against *Botrytis cinerea*

Data obtained from the microplate assay showed Hs-AFP1 to exhibit high levels of fungal growth inhibition at low peptide concentrations (Fig. 2). Hs-AFP1 at 6 µg/ml resulted in a decrease in fungal biomass of up to 65%, whereas peptide concentrations of 8 µg/ml almost totally inhibited fungal growth, showing a decline in fungal biomass of 88% over a 72-h period when compared to the control.

3.3.2 Tobacco transformation and regeneration

The plant expression cassettes, pFAJ3068, pHs-EXG1 and pMj-EXG1 (Fig. 1), were successfully mobilized into *N. tabacum* SR1, yielding transgenic lines designated THs, THE and TME respectively (Table 4). Typically, one to five copies of the expression cassettes integrated into the tobacco genome. The pFAJ3068 and pHs-EXG1 constructs were developed to study the *in planta* activity of Hs-AFP1 alone, or fused to a pathogenesis-related (PR) protein for the putative increased stability and/or activity of the transgenes respectively. The pMJ-EXG1 construct was intended as a control to assess the antifungal activity imparted by EXG1 in the fusion construct.

3.3.3 Southern blot analyses of transgenic tobacco lines

Southern blot analysis confirmed the stable integration of the pFAJ3068 (Fig. 3) and pHs-EXG1 (Fig. 4) expression cassettes into the tobacco genome. Typically, between one to five copies of the expression cassettes integrated into the tobacco genome. PCR analysis of genomic DNA from tobacco lines transformed with the Mj-EXG1 fusion cassette confirmed integration of the *EXG1* gene (Fig. 5).

3.3.4 Confirming gene expression in the transgenic tobacco lines

Northern blot analysis revealed the active transcription of the pFAJ3068 and pHs-EXG1 expression cassettes (Fig. 6). In the case of pFAJ3068, a transcript of 329 bp was visualized, which correlates with the *Hs-AFP1* DNA sequence. The THE lines revealed a signal at 1590 bp, which correlates with the size of the *Hs-AFP1-EXG1* fusion sequence.

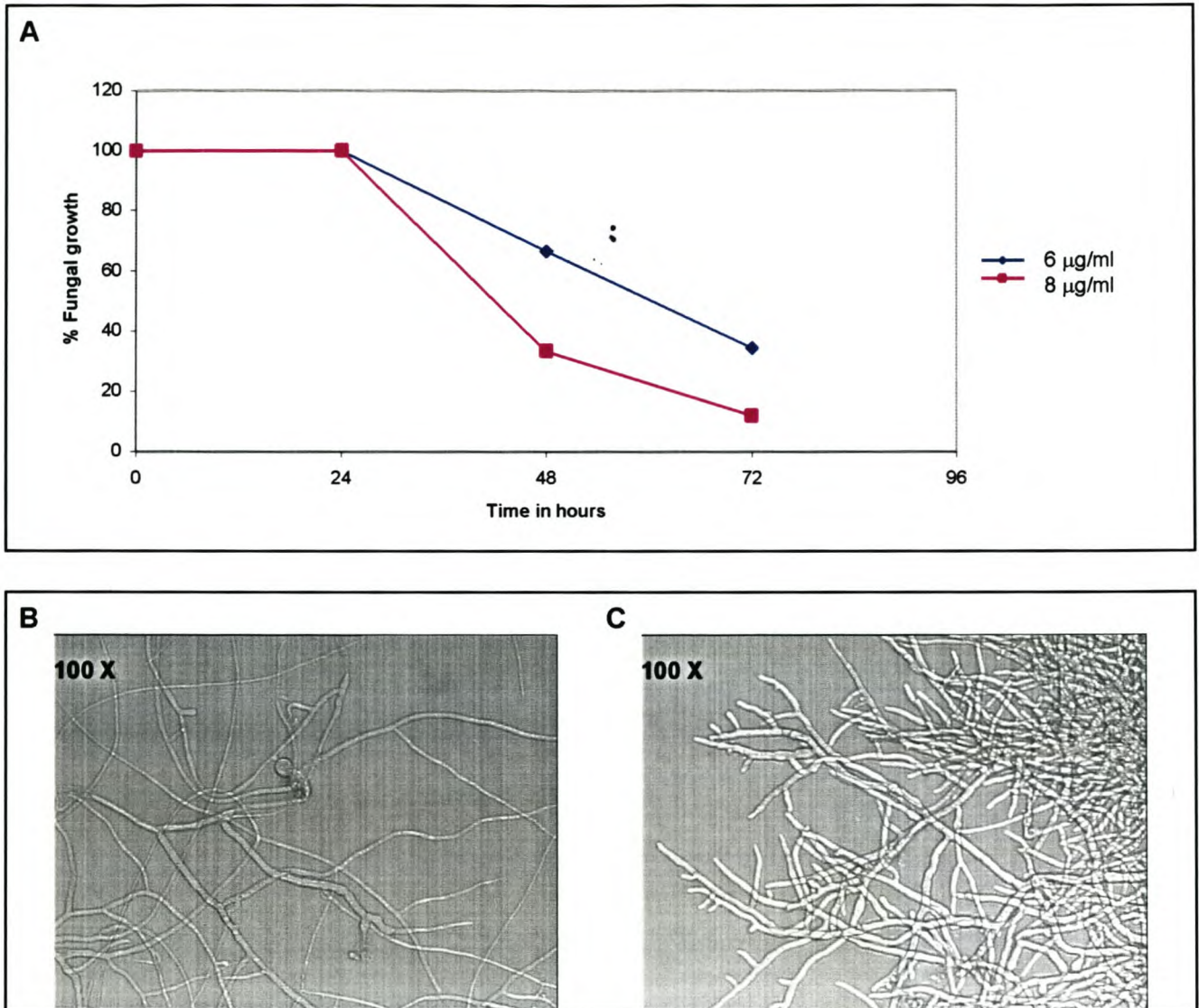


Figure 2. *In vitro* analyses of purified Hs-AFP1 from *Heuchera sanguinea* against *Botrytis cinerea*. (A) The decline in fungal biomass, as compared to the control, over a period of 72 h caused by the antifungal activity of Hs-AFP1 as determined by a quantitative fungal growth assay. The decline in biomass is the result of morphological changes induced by Hs-AFP1 on the normal growth of *B. cinerea*. (B) The natural growth pattern of *B. cinerea* in the control reaction lacking any antifungal agent. The rapid spreading, relatively unbranched hyphae are a sign of health. (C) The antifungal effect observed during the incubation of 10 µg/ml HS-AFP1 in the presence of *B. cinerea*. The hyperbranching effect induced by HS-AFP1 restricts the development of the hyphae, resulting in a loss of biomass over time. 100 X represents the microscopic magnification.

No mRNA signals could be detected for the TME tobacco lines by Northern blot analysis (results not shown). In a further attempt to confirm gene expression, RT-PCR analysis was performed, but still no product could be observed that indicated gene expression (Fig. 7).

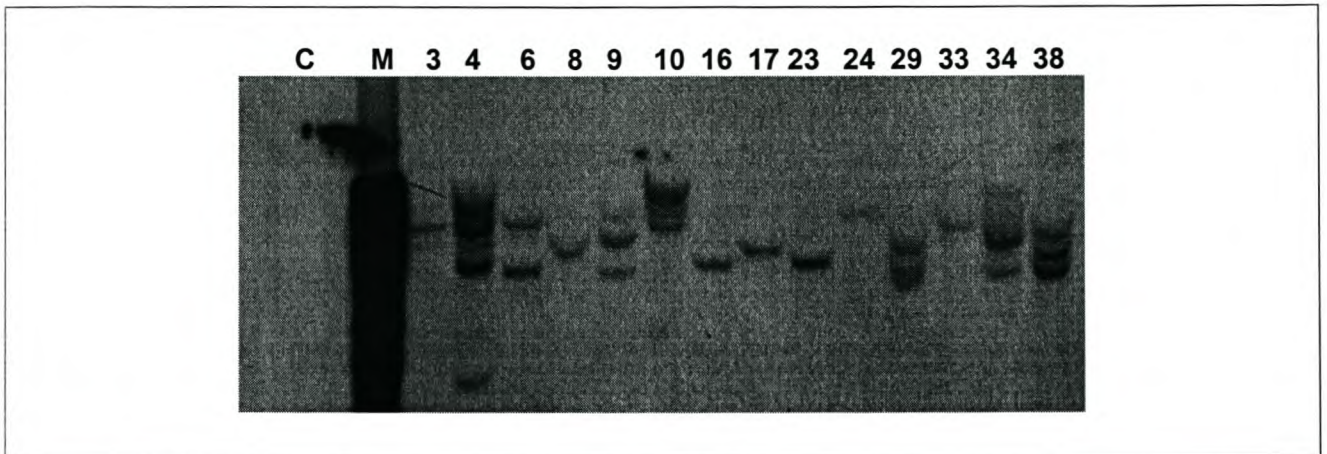


Figure 3. Southern Blot analysis of *HindIII*-digested genomic DNA from tobacco lines transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* and probed with a DIG-labeled *Hs-AFP1* sequence. Lane **C** represents a control tobacco plant line. Lane **M** represents the DNA marker, consisting of *BstEII*-digested Lambda DNA. The numbers in the subsequent lanes represent the different transgenic lines tested.

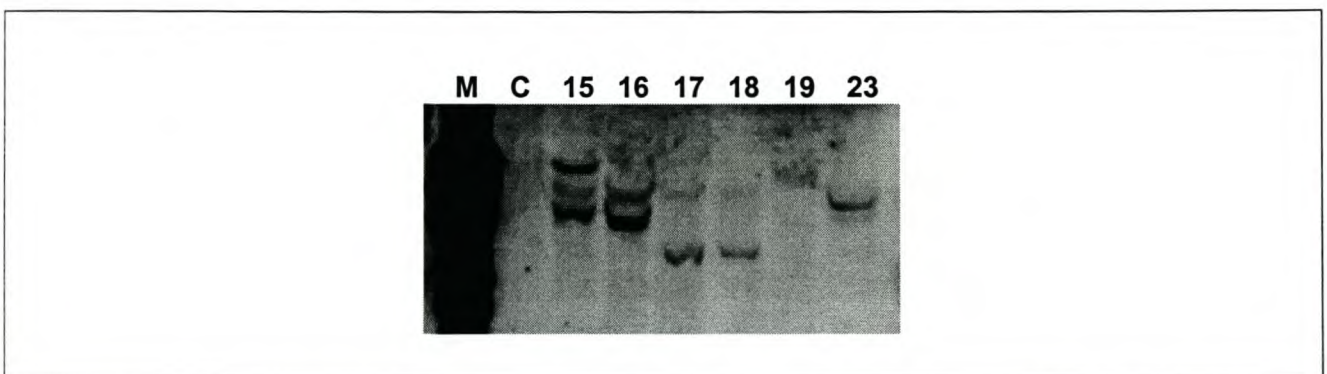


Figure 4. Southern Blot analysis of *HindIII*-digested genomic DNA from tobacco lines transformed with the *Hs-AFP1-EXG1* fusion cassette and probed with a DIG-labeled *Hs-AFP1* sequence. Lane **C** represents a control tobacco plant line. Lane **M** represents the DNA marker, consisting of *BstEII*-digested Lambda DNA. The numbers in the subsequent lanes represent the different transgenic lines tested.

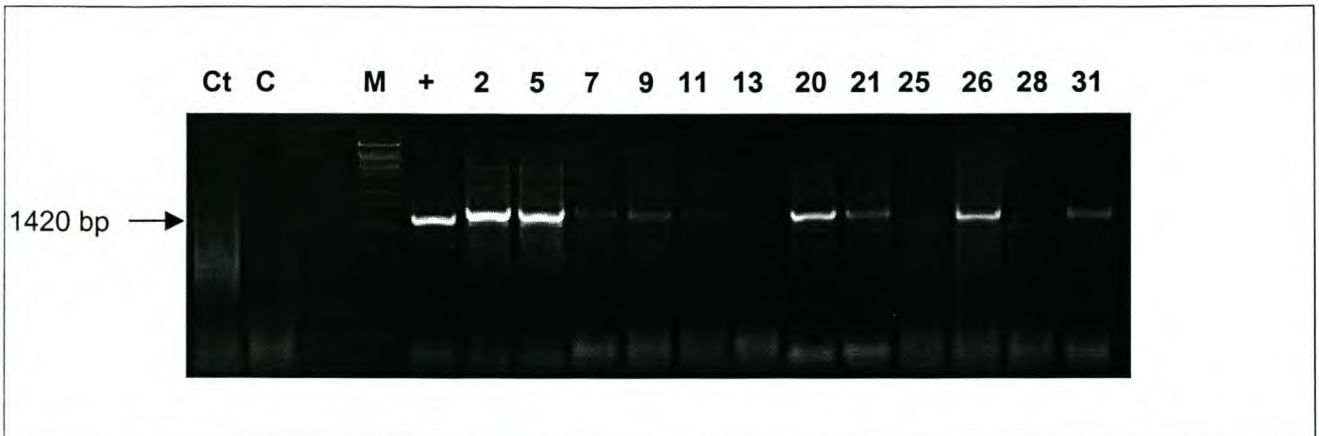


Figure 5. PCR analysis of genomic DNA from tobacco lines transformed with the Mj-EXG1 fusion cassette and PCR screened with Mj-EXG1 specific primers. Lane **Ct** and **C** represents a control tobacco plant line and a zero-DNA control respectively. Lane **M** represents the DNA marker consisting of *Bst*EII-digested Lamda DNA. Lane **+** represents a positive control, consisting of pMj-EXG1 plasmid DNA. The numbers in the subsequent lanes represent the different transgenic lines tested.

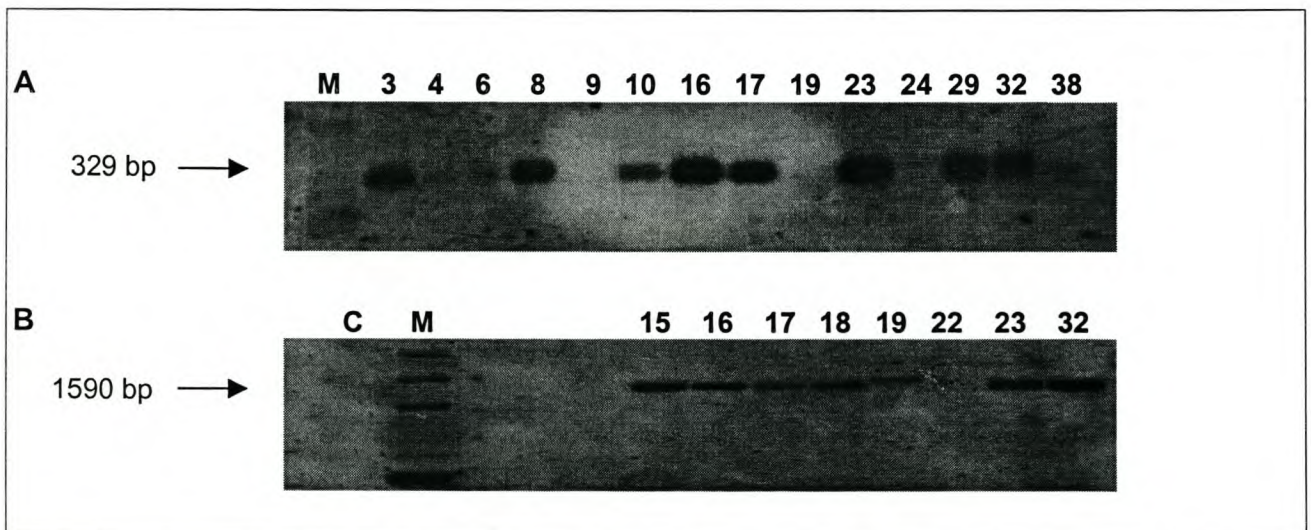


Figure 6. Northern blot analysis conducted on total RNA isolated from tobacco transformed with *Hs-AFP1* (transgenic lines designated THs) (A) and an *Hs-AFP1-EXG1* fusion cassette (transgenic lines designated THE) (B). Detection was achieved with a DIG-labeled *Hs-AFP1* sequence from pFAJ3068. Lane **C** represents an untransformed tobacco plant line. Lane **M** represents the RNA ladder from GIBCO. The numbers in the subsequent lanes represent the different transgenic tobacco lines.

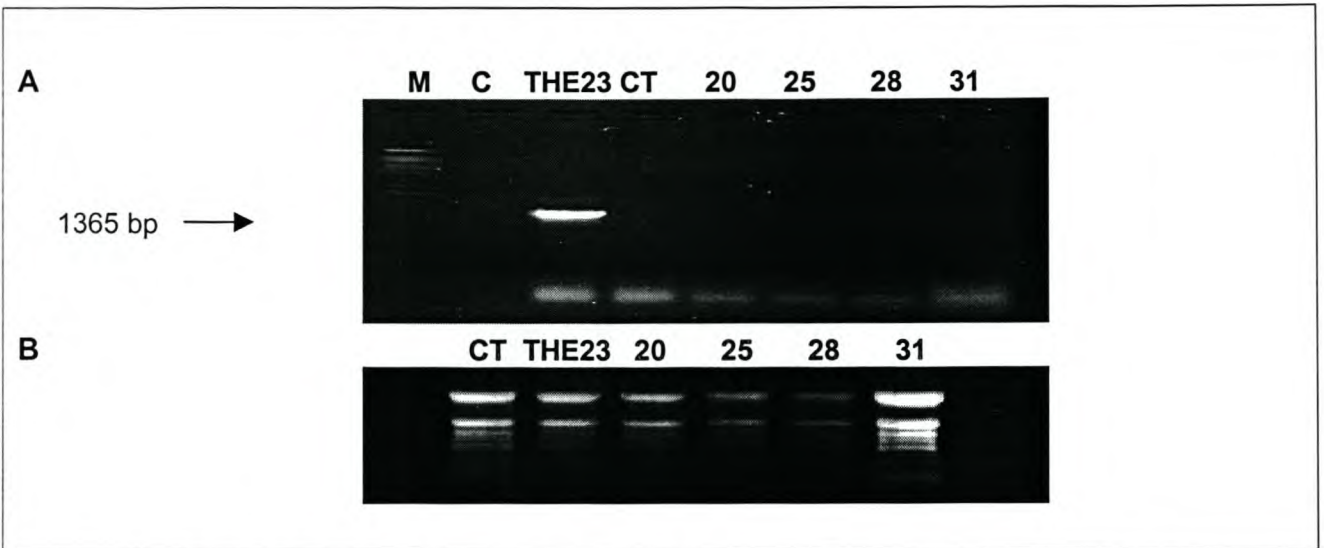


Figure 7. (A) The RT-PCR conducted on the total RNA isolated from tobacco transformed with the *Mj-EXG1* expression cassette (transgenic lines designated TME). RNA from a Northern positive *Hs-AFP1-EXG1* fusion (line THE23) was used as positive control. (B) The RNA samples used in the RT-PCR were separated on an 1% (w/v) agarose gel. Lane **C** and **CT** represent a zero-RNA control and RNA from an untransformed tobacco control respectively. Lane **M** represents the RNA ladder from GIBCO. The numbers in the subsequent lanes represent the different transgenic lines.

Since the incorporation of the *EXG1* gene has been confirmed by PCR-analysis on genomic DNA from the TME lines (Fig. 5), the possibility of post-transcriptional gene silencing due to the overexpression of the *EXG1* gene was suspected. In an attempt to evaluate this possibility, RT-PCR analysis of the total RNA isolated from *B. cinerea*-infected TME leaves were performed to see whether infection would derepress the suspected silencing phenomenon, but still no *EXG1* transcription levels could be detected (Fig. 8).

3.3.5 Western blot analysis of transgenic tobacco lines

Western blot analysis showed mature Hs-AFP1 of 5 kDa to be present in crude protein fractions of most of the transgenic THs tobacco lines (Fig. 9). The fusion protein, consisting of a fusion between Hs-AFP1 and *EXG1*-encoding genes could be detected readily in the crude protein fractions of the THE tobacco lines using Hs-AFP1 specific antibodies. The fusion protein was detected as a signal of 55 kDa corresponding to the expected molecular weight of the fusion protein (Fig. 10).

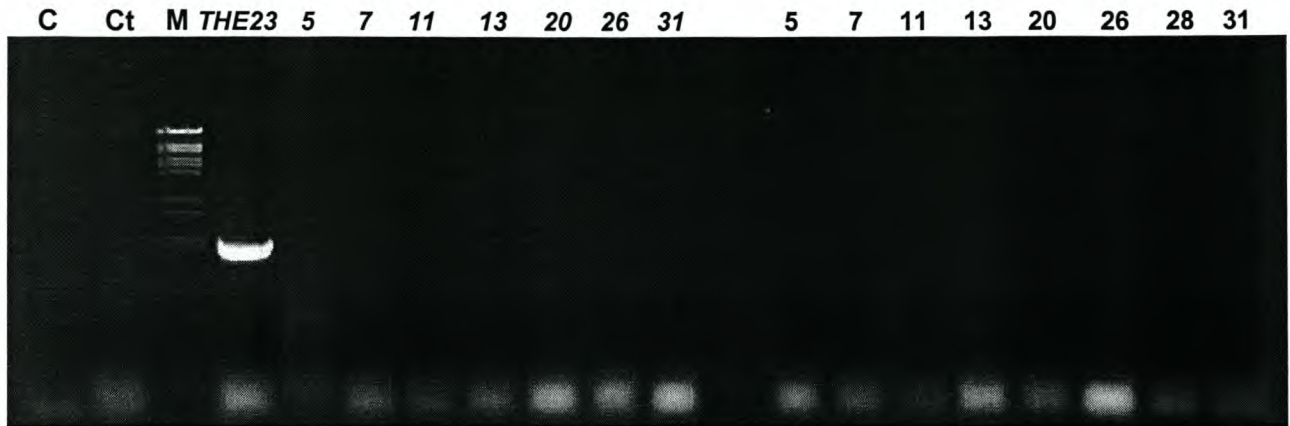


Figure 8. The RT-PCR analysis conducted on *B. cinerea*-infected leaves from tobacco transformed with the *Mj-EXG1* expression cassette. Leaves were inoculated with *B. cinerea* spores and total RNA was extracted 12 and 48 h after infection. RNA from a Northern positive *Hs-AFP1-EXG1* fusion (line *THE23*) was used as positive control. The total RNA was subjected to RT-PCR analysis using *EXG1*-specific primers. Lane **C** and **CT** represent a zero-RNA control and RNA from an untransformed tobacco control respectively. The numbers in the subsequent lanes represent the different tobacco lines. The italic numbers represent RT-PCR results after 12 h of infection and the non-italic numbers results after 24 h.

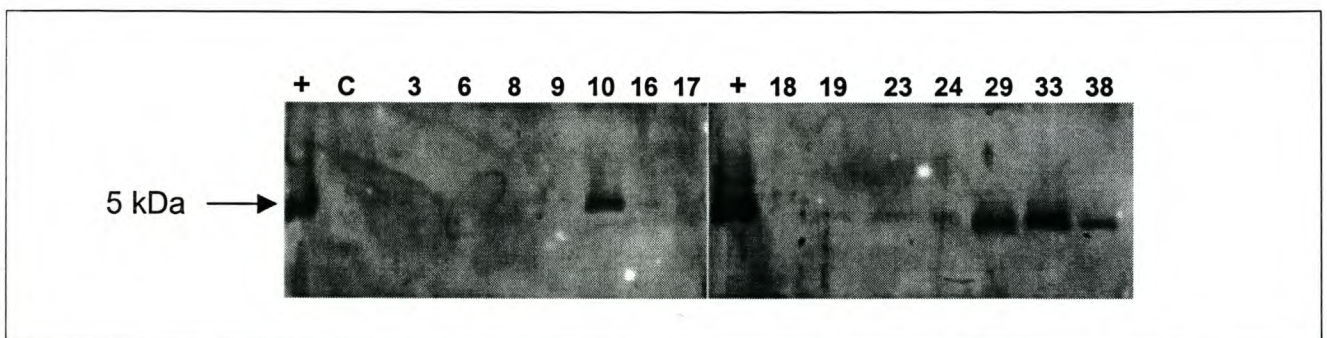


Figure 9. Western blot analysis conducted on the crude protein fraction from tobacco lines transformed with the *Hs-AFP1* gene from *Heuchera sanguinea*. Mature Hs-AFP1 was detected with antibodies raised against previously purified mature Hs-AFP1 and detected with the ECL chemiluminescent system. Lane **C** represents an untransformed tobacco plant line. Lane **+** represents the positive control, consisting of purified Hs-AFP1. The numbers in the subsequent lanes represent the different transgenic lines tested.

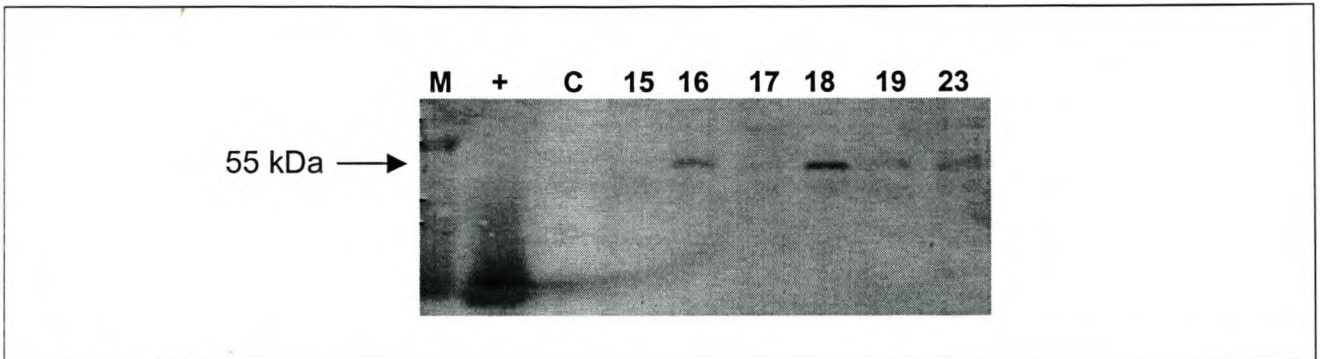


Figure 10. Western blot analysis conducted on crude protein extracts from tobacco lines transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* fused to the *EXG1* gene from *Saccharomyces cerevisiae*. Antibodies raised against mature Hs-AFP1 were used to detect the fusion protein. Lane **C** represents an untransformed tobacco plant line. Lane **+** represents the positive control, consisting of purified Hs-AFP1. Lane **M** represents the Rainbow marker from Promega. The numbers in the subsequent lanes represent the different transgenic lines tested.

3.3.6 β -Glucosidase activity assays

Untransformed tobacco lines showed endogenous β -glucosidase activity ranging from 3000-4000 units of activity. Compared to these values, crude protein extracts from the TME-lines mostly showed lower activity than that of the control. The same levels or slightly higher levels were detected only in a few lines. Only one line, TME25, showed significantly higher β -glucosidase activity with a three-fold increase in activity units (Fig. 11). The crude protein extracts from the THE tobacco lines, in contrast, showed higher β -glucosidase activities, ranging from a 1.5 to three-fold increase in activity units when compared to the untransformed control (Fig. 12).

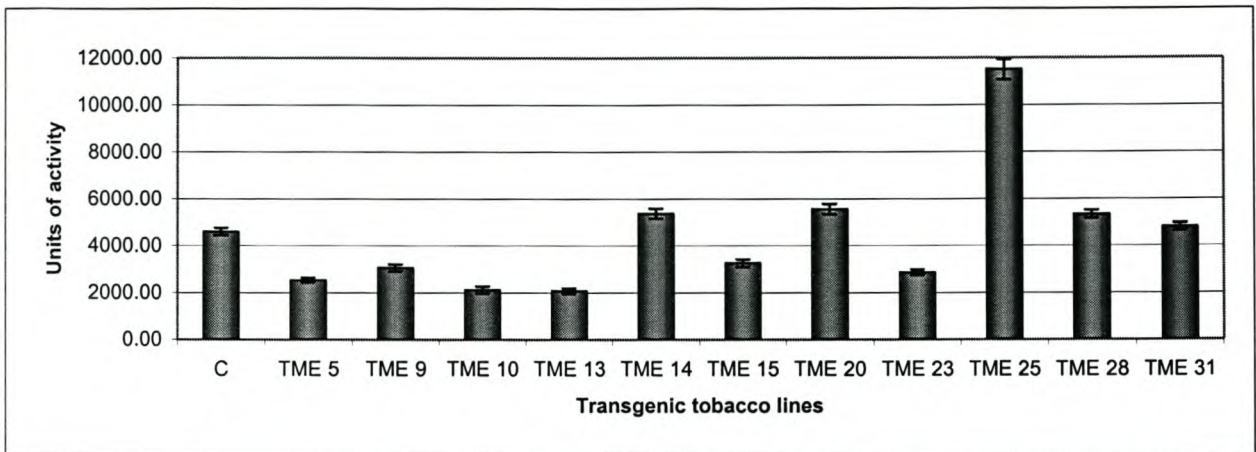


Figure 11. The β -glucosidase activity of crude proteins extracted from transgenic tobacco transformed with the *S. cerevisiae* exoglucanase (*EXG1*) gene under the Mj-AMP2 signal peptide (transgenic lines designated TME). One unit of activity is described as the amount of p -Nitrophenol (in nM) released per hour by 1 μ g crude protein at 37°C. Statistical analysis was performed with GraphPad Prism; standard deviation never exceeded 5%.

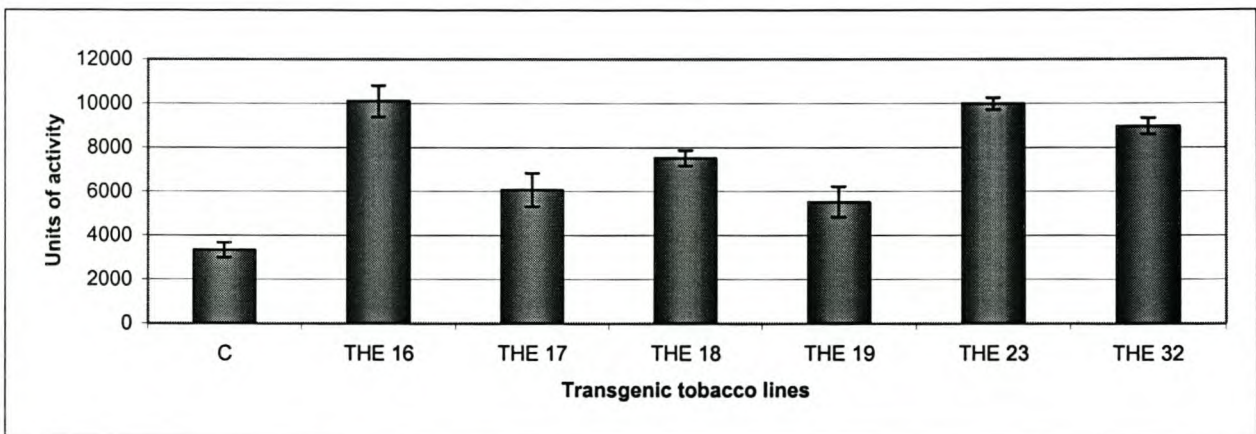


Figure 12. The β -glucosidase activity of crude proteins extracted from transgenic tobacco transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* fused to the *EXG1* gene from *Saccharomyces cerevisiae* (transgenic lines designated THE). One unit of activity is described as the amount of p -Nitrophenol (in nM) released per hour by 1 μ g crude protein at 37°C. Statistical analysis was performed with GraphPad Prism; standard deviation never exceeded 10%.

3.3.7 Fungal inhibition by transgenic Hs-AFP1

Microplate assays to quantitatively establish the fungal inhibition of THs and THE transgenic lines were conducted on crude protein extracts from the various lines that showed active transcription of *Hs-AFP1* and *Hs-AFP1-EXG1* respectively (Fig. 13). Five of the THs lines had very low percentages of growth inhibition (0-10%) against *B. cinerea*, whereas three lines exhibited significant levels of growth inhibition. The THs16 tobacco line resulted in 42% growth inhibition, whereas THs19 and THs23 resulted in growth inhibition activities of 32 and 30% respectively. The THE extracts all showed less activity than the control samples (Fig. 13).

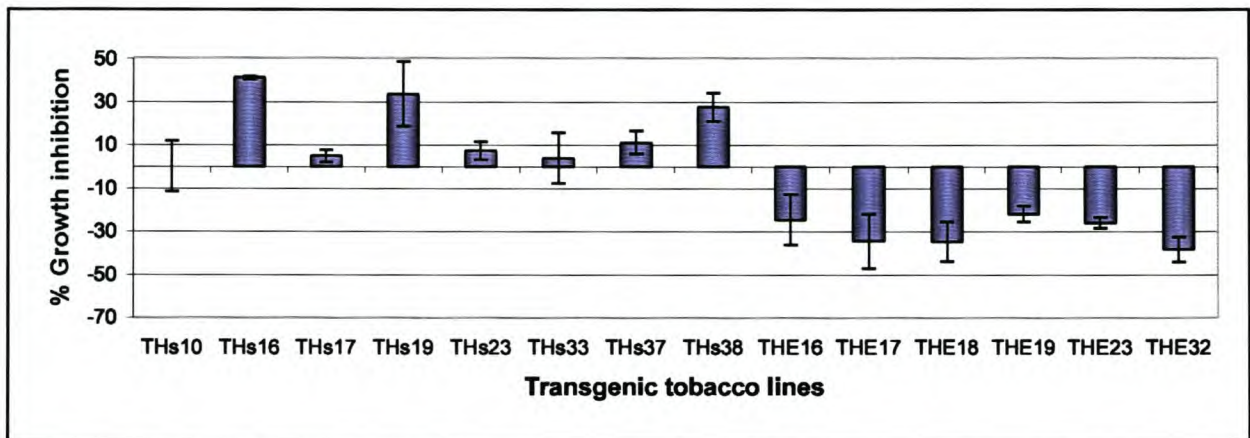


Figure 13. The antifungal activities against *B. cinerea* of crude protein extracts from tobacco lines transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* (transgenic lines designated THs) or *Hs-AFP1* fused to the *EXG1* gene from *Saccharomyces cerevisiae* (transgenic lines designated THE). Crude protein extracts (5 mg/ml) were assayed against 2000 *B. cinerea* spores in half-strength potato dextrose broth and incubated at 25°C for 48 h. The A_{595} (after 48 h) was used to determine the percentage growth inhibition normalized against the negative control (untransformed tobacco protein). The values were corrected by subtracting time zero measurements at A_{595} . Statistical analysis was performed with GraphPadPrism; standard deviation never exceeded 15%.

3.3.8 Microscopic analysis of transgenic Hs-AFP1 against *Botrytis cinerea*

Botrytis spores incubated for 48 h in the presence of 5 mg/ml crude protein extract from tobacco transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* showed a varied degree of health when compared to a control reaction lacking any antifungal agents (Fig. 14). Healthy fungal hyphae are long and relatively unbranched (Fig. 14A).

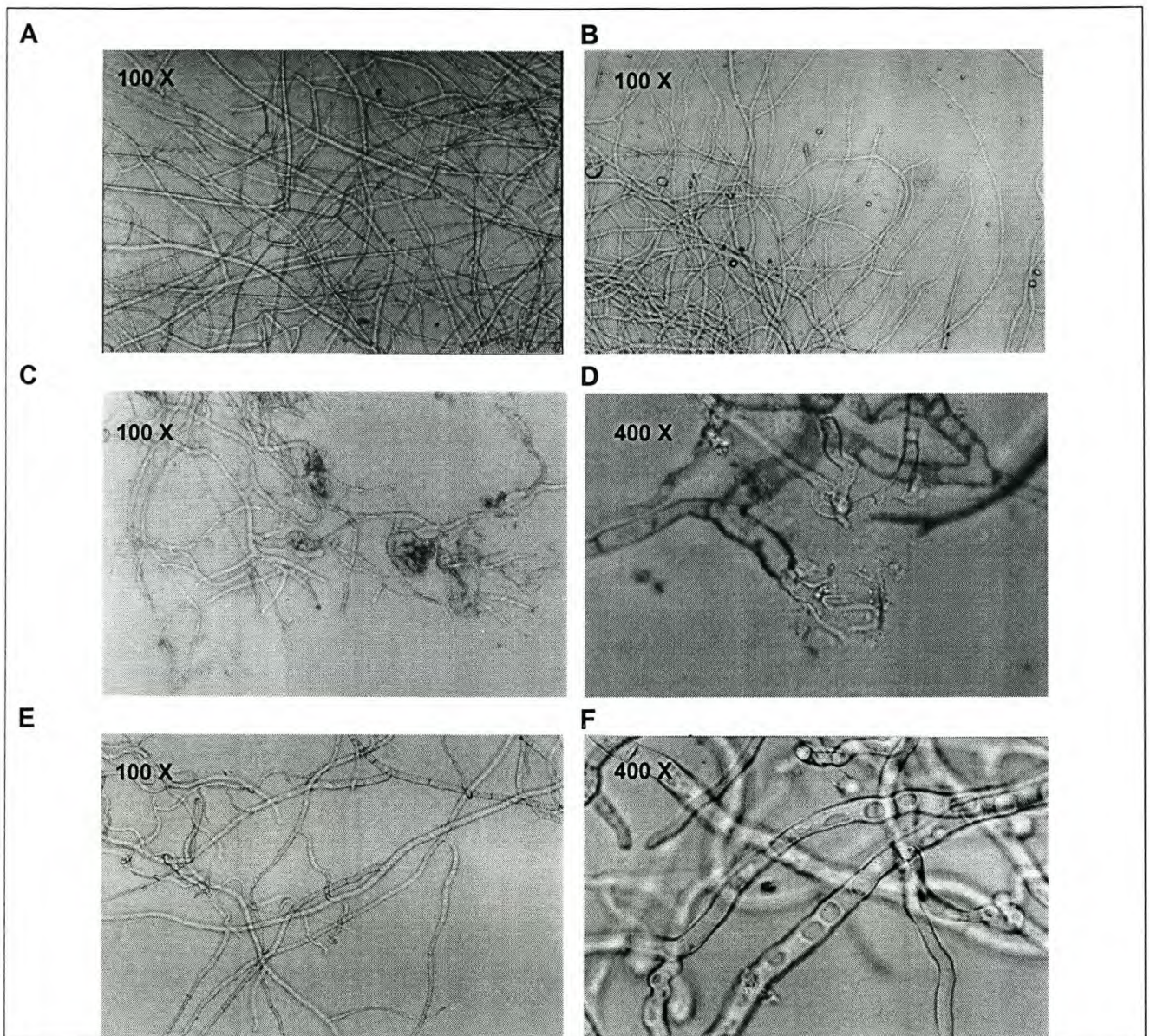


Figure 14. Microscopical analysis of *B. cinerea* hyphal changes induced by crude protein extracts isolated from tobacco transformed with Hs-AFP1, an antifungal peptide from *Heuchera sanguinea*. (A) An uninhibited *B. cinerea* culture in the control reaction lacking any antifungal agents. (B) The antifungal effect observed during the incubation of *B. cinerea* in the presence of 5mg/ml crude protein extracts, isolated from an untransformed tobacco control. (C) The severe morphological changes induced by 5 mg/ml crude protein extract isolated from THs16, clearly showing the hyperbranching effect characteristic of Hs-AFP1 activity. (D) The loss of hyphae integrity induced by the synergistic effect of HS-AFP1 and the native antifungal agents present in tobacco. (E and F) The morphological state of the *B. cinerea* hyphae incubated in the presence of 5 mg/ml crude protein extracts from THE transgenic lines. The lack of antifungal activity is clear considering the health of the hyphae. 100 X and 400 X represent the microscopic magnification.

The crude protein extract from THs16 had a severe effect on hyphal morphology, inducing hyperbranching (Fig. 14C) and even causing the hyphae to rupture, resulting in the leakage of fungal cytoplasm (Fig. 14D). The leaf extracts from the THE lines did not visibly influence the spore germination or hyphal growth of the *Botrytis* cultures (Fig. 14E,F).

3.3.9 *Botrytis cinerea* infection studies

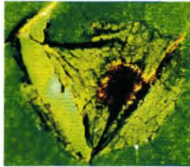
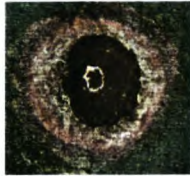


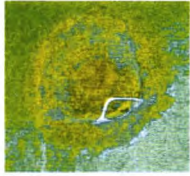

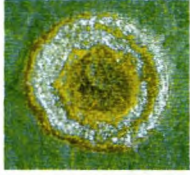

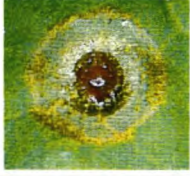

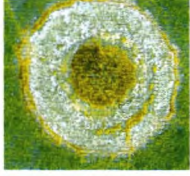

A detached leaf assay conducted on THs and THE lines to study the extent of disease resistance against *B. cinerea* fungal infection showed that varied resistance levels were obtained in the transgenic progeny. The detached leaves were inoculated with high spore concentrations and incubated in conditions favourable for disease development.

The lesions that developed were measured, as well as compared visually to a lesion-type index (Table 6). The tissue damage caused by *B. cinerea* to the untransformed control tobacco leaves was severe and spread actively (type 10 lesions) (Table 7). The average lesion sizes on the untransformed controls were 9 mm after 3 days, increasing to 9.8 mm after 7 days post-inoculation. In contrast, all the THs tobacco lines, except THs23, showed an approximate two-fold reduction in lesion sizes after 3 and 7 days (Fig. 15A), with observed lesion types ranging from type 3 to type 9 (Table 7).

The lesion sizes were normalized against those of the untransformed tobacco lines and expressed as percentage disease resistance (Figs. 15B and 16B for the THs and THE lines respectively). The highest percentage disease resistance was observed for lines THs16 and THs38 at 48% and 46% respectively, 7 days after inoculation (Fig. 15B). It was clear that the levels of resistance of several of the THs lines (THs4, 6, 8, 16 and 23) increased from day 4 to 7 post-inoculation, whereas the opposite occurred in lines THs18 and 19 (Fig. 15B). Line THs38 showed unchanged high levels of resistance over the 7-day incubation period. Good correlation with the observed lesion types could be drawn with the deduced disease resistances, i.e. lines THs16 and 38, which had the highest disease resistance, also exhibited the most resistant lesion types (Table 7). These lesions (type 3 and 4 respectively) appeared dry with little necrotic damage and a clear border between the infected and uninfected tissue.

When the inoculated leaves were kept for 3 weeks, extensive damage and complete destruction were visible on the leaves of the untransformed tobacco lines (Fig. 17). Again, a correlation was found between the observed disease resistance, the lesion type and the appearance of the leaves from transgenic lines three weeks after inoculations. The lesions on lines THs16 and THs38 were dry and confined after 7 days, as well as after 3 weeks. The leaves were still green and healthy and showed no signs of disease development or the formation of survival structures of *Botrytis* (Fig. 17).

Table 7. Lesion types scored 4 days after the inoculation of transgenic THs tobacco leaves with 5000 *B. cinerea* spores and incubation at 22°C under a 16-h light/8-h dark cycle

THs infection study		THE infection study	
Line and lesion type	Lesion appearance	Line and lesion type	Lesion appearance
Tobacco control Type 10		Tobacco control Type 9	
THs16 Type 3		THE16 Type 9	
THs17 Type 9		THE17 Type 9	
THs18 Type 5		THE18 Type 5	
THs23 Type 8		THE19 Type 9	
THs38 Type 4		THE23 Type 5	

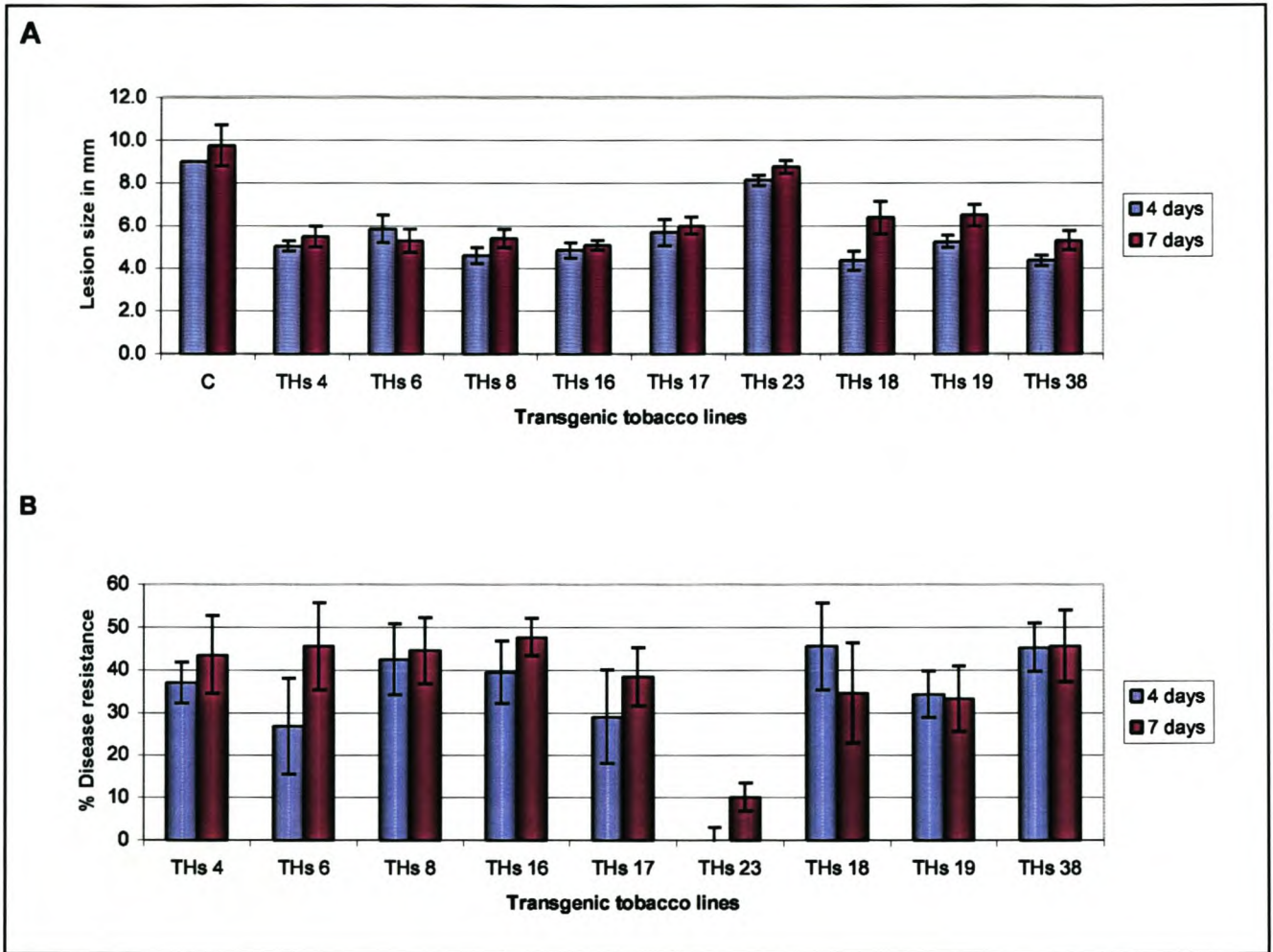


Figure 15. Results from a detached leaf assay of tobacco lines transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* (transgenic lines designated THs) and infected with *Botrytis cinerea*. Leaves were inoculated with 5000 *B. cinerea* spores and incubated at 22°C under a 16-h light/8-h dark cycle. (A) The lesion sizes (in mm) recorded 4 and 7 days after leaf inoculation. The lesion sizes shown are the mean of six repetitions. Lane C represents the untransformed tobacco lines. (B) Disease resistance expressed as a percentage of measured lesion sizes, normalized against those of the untransformed tobacco plant lines. Statistical analysis was performed with GraphPad Prism; standard deviation never exceeded 20%.

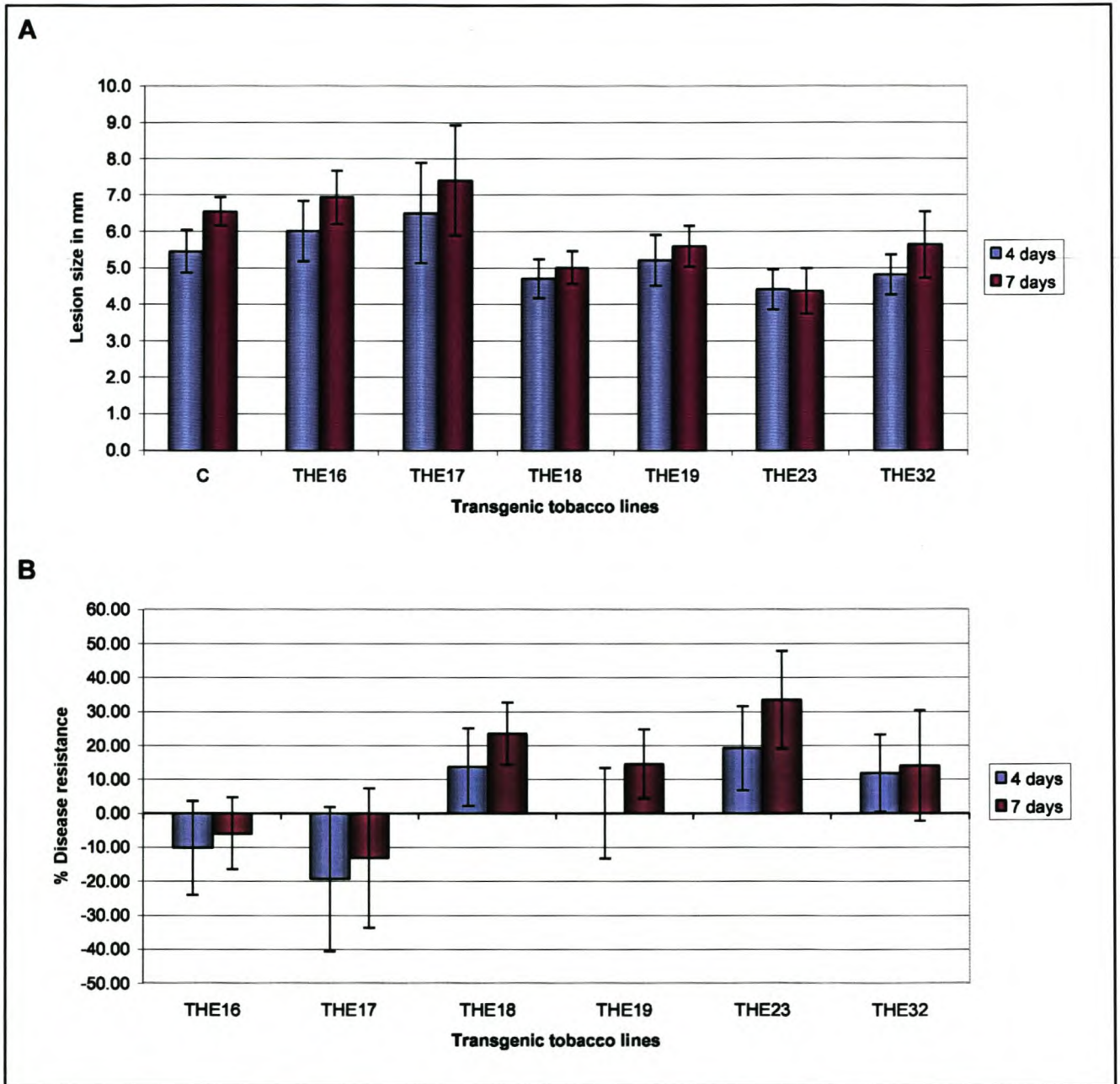


Figure 16. Results from a detached leaf assay of tobacco lines transformed with the *Hs-AFP1* gene from *Heuchera sanguinea* fused to the *EXG1* gene from *Saccharomyces cerevisiae* (transgenic lines designated THE) and infected with *Botrytis cinerea*. Leaves were inoculated with 5000 *B. cinerea* spores and incubated at 22°C under a 16-h light/8-h dark cycle. (A) The lesion sizes (in mm) recorded 4 and 7 days after leaf inoculation. The lesion sizes shown are the mean of six repetitions. Lane C represents the untransformed tobacco lines. (B) Disease resistance expressed as a percentage of measured lesion sizes, normalized against those of the untransformed tobacco plant lines. Statistical analysis was performed with GraphPadPrism; standard deviation never exceeded 20%.

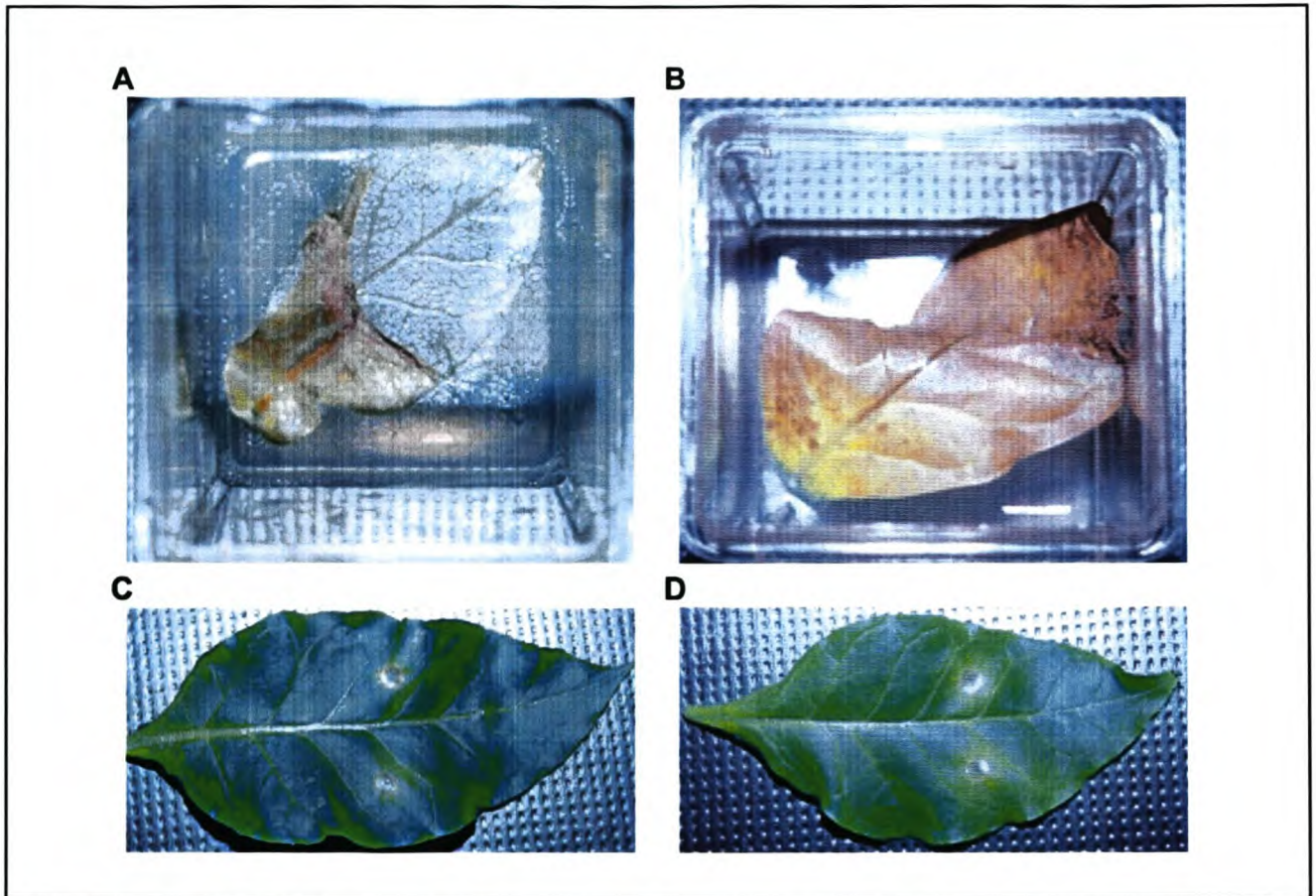


Figure 17. The resistance levels of the tobacco plants transformed with the Hs-AFP1 gene construct three weeks after inoculation with *Botrytis cinerea*. (A) The damage inflicted upon the untransformed tobacco control. (B) The destruction caused by *B. cinerea* on the weakest THs tobacco line, THs23. Although the leaf is dead, there are no signs of *Botrytis* reproductive structures. (C and D) The high levels of disease resistance in the transgenic THs16 and THs 38 tobacco lines. The spread of *Botrytis* infection halted seven days after inoculation.

3.4 DISCUSSION

3.4.1 *In vitro* activity of Hs-AFP1 against *Botrytis cinerea*

The Hs-AFP1 peptide, a defensin, is part of the cysteine-rich peptides well known for their antifungal activities as a result of membrane disruption by receptor-mediated insertion into membranes and/or pore formation (Thevissen et al., 1999, 2000). An *in vitro* assay revealed up to 88% fungal growth inhibition of *B. cinerea* by purified Hs-AFP1 (Fig. 2). Microscopic analysis of the inhibited *Botrytis* cultures showed severe hyperbranching (Fig. 2), a typical feature of the strong inhibitory action of the Hs-AFP1 peptide (Osborn et al., 1995).

This excellent inhibition of *Botrytis* hyphal growth merited the introduction of the Hs-AFP1-encoding gene into two genetic constructs (pFAJ3068 and pHs-EXG1) to analyze its activity in the resulting transgenic tobacco lines, THs and THE respectively. A construct containing the *EXG1* gene was used to quantify the contribution of the exoglucanase to the antifungal activity of line THE that contained *Hs-AFP1* fused to the *EXG1* gene.

3.4.2 Analysis of transgenic tobacco lines for gene integration, gene expression and peptide formation

The tobacco transformations yielded several transgenic lines that clearly contained copies of the transgenes in lines THs, THE and TME. Gene expression could also be confirmed in the THs and THE lines, but no gene expression was evident in the TME lines, not with Northern Blot or RT-PCR analysis. This led to the hypothesis that the *EXG1* gene expression was silenced. It was shown previously that glucanases are prone to tight regulation in the plant environment, often being gene-silenced at the post-transcriptional level when expression exceeds a certain tolerated maximum (Gutiérrez et al., 1999; Holtorf et al., 1999; Cogoni and Macino, 2000). Typically, regulation occurs by upregulation of mRNA degradation due to instability (Holtorf et al., 1999). In an attempt to alter this suspected silencing phenomenon, the physiological state of the plants was altered by infecting leaves with *Botrytis* spores and analyzing the glucanase expression in a time-course RT-PCR analysis, but the assay remained negative. The transgenic TME lines were also tested for β -glucosidase activity, but virtually no activity was observed when normalized against the untransformed tobacco control. Only line TME25 showed a significant increase in activity, while it lacked detectable *EXG1* transcripts in any of the analyses described. Although no conclusive explanation can be provided for this anomaly, one possibility is that it is the result of a transformation artifact linked to positional effects after gene integration into the genome. Another possibility is that gene expression does in fact occur in line TME25, but that the transcript levels are extremely low but highly translatable, leading to the observed β -glucosidase activity.

A surprising result was the fact that the *EXG1* gene-silencing phenomenon did not occur in the pHs-EXG1 fusion construct expressed in tobacco line THE. These lines exhibited transcripts representative of the size of the fusion construct. Moreover, the plant lines also showed a two- to three-fold increase in β -glucosidase activity compared to an untransformed control. These results indicated that the 5' extension (the Hs-AFP1-encoding sequence) protected the *EXG1* containing transcripts from the observed mRNA instability that is linked to gene silencing. A gene fusion strategy might prove beneficial in other circumstances in which high-level expression of glucanases is required.

Western blot analysis confirmed the presence of Hs-AFP1 in the foreign tobacco environment. Moreover, the peptide seemed to be stable and could be detected regularly with Hs-AFP1 antibodies. Several reports on peptide instability have shown that small antimicrobial peptides tend to be prone to protease attack and other mechanisms of protein degradation in a transgenic environment (Florack et al., 1995; De Bolle et al., 1996; Okamoto et al., 1998). This problem tends to be more severe if peptides are expressed in heterologous systems across kingdoms, i.e. an insect peptide in a plant environment (Florack et al., 1995). Sarcotoxin 1A from *Sarcofaga peregrina* was expressed in tobacco and although high-level expression was observed, no peptide could be detected, due to protein degradation. The problem of peptide stability was overcome by fusion of the sarcotoxin 1A-encoding gene to the β -glucuronidase (GUS)-encoding gene. The peptide stability increased dramatically, but it rendered the sarcotoxin 1A inactive (Okamoto et al., 1998). It was suggested that this might be due to properties inherent in the GUS protein (Okamoto et al., 1998). In anticipation of possible peptide stability problems, the Hs-AFP1 protein was fused to the *EXG1* gene from *S. cerevisiae*. This gene has been shown to inhibit fungal pathogens and is currently under evaluation as a possible PR protein in transgenic plants (personal communication with Ms E. Marais, Institute for Wine Biotechnology, Stellenbosch University). This fusion did not lead to a noticeable increase in the already stable Hs-AFP1 peptides, but, as mentioned previously, it led to the presence of stable and active *EXG1* protein activity, which could not be achieved with constructs harboring only the *EXG1* gene.

3.4.3 Antifungal activities of the transgenic proteins

Microscopical analysis, conducted on *B. cinerea* cultivated in the presence of transgenic crude protein extracts containing Hs-AFP1, confirmed that the heterologously expressed peptide was functional. The characteristic hyperbranching effect was elicited strongly by the transgenic Hs-AFP1. The negative effect of the transgenic peptides on the hyphal pathogen was further confirmed with a quantitative microplate assay that showed fungal inhibition of up to 40% with some of the transgenic peptides. The successful expression of Hs-AFP1 also resulted in a significant increase in disease resistance towards *B. cinerea in planta*. THs16, the transgenic line showing the strongest inhibition of *Botrytis*-induced lesion development, totally inhibited the spread of disease symptoms after 7 days. The pathogen was not able to overcome the inhibition effect of the Hs-AFP1 and hyphal development was arrested. The reduction in the primary lesion sizes is significant in curbing the infection and could be seen as the primary effect of Hs-AFP1 in the induced resistance strategy. The secondary mechanism of resistance involves the inhibition of normal fungal growth and the production of fungal reproductive organs. No fungal reproductive

organs were present on any of the transgenic lines, not even on those with lower observed disease resistances after a prolonged period of incubation following fungal infection. This is encouraging, especially since the conditions of the fungal infection assay were optimally suited to induce excessive infection and the formation of fungal reproductive organs, as were visible on various control plant lines. The phenomenon of reducing fungal inoculum over time is of great importance in the control of disease.

From microscopical analysis, as well as *in planta* infection studies, it was evident that the fusion between *Hs-AFP1* and *EXG1* most probably resulted in the loss of Hs-AFP1 activity. No hyperbranching effect on *B. cinerea* hyphae, indicative of Hs-AFP1 activity, could be detected in the crude protein extracts from the THE lines. Some fungal inhibition did occur in these lines, however, probably due to the actions of the exoglucanase portion of the fusion protein to which activity was ascribed with a β -glucosidase assay. Although unfortunate, the disruption of the peptide activity through fusion to the EXG1 protein is in accordance with the study of sarcotoxin 1A that had the same result (Okamoto et al., 1998). The receptor-mediated insertion of the peptide into the pathogen membranes is probably disrupted when the defensin forms part of a bigger protein with an abnormal conformation. This insertion event is critical to the physiological effects of the peptide on the membrane, namely the formation of pores and cell leakage. It has been shown that this insertion event proceeds from the 3'-portion of the mature peptide (Thevissen et al., 1999, 2000). It might be possible to overcome the disrupting effect of the fusion proteins if the peptide encoding sequence is fused to the 3' end of the partner gene to leave the 3' end of the defensin free to interact.

In conclusion, this study confirmed that antimicrobial peptides in general, and specifically the Hs-AFP1 defensin from *H. sanguinea*, could make an important contribution to the technology of the manipulated disease resistance of plant species. The observed stability of the Hs-AFP1 peptide in the heterologous environment is most encouraging, since one of the classic limitations of small antimicrobial peptides in transgenic expression is peptide instability. The observed disease resistance levels of up to 40%, the excellent control of fungal growth and the limitation of the formation of reproductive fungal structures in several of the lines validate further investigations into the use of this and similar peptides. The high-level expression that has been achieved recently by chloroplast-transformations of tobacco with antimicrobial peptides (DeGray et al., 2001) further widens the application possibilities of these peptides in enhanced disease resistance strategies of plants.

3.5 ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation and a Winetech grant. The authors would like to thank Prof. W.F. Broekaert and Dr. B.P.A. Cammue at the Katholieke Universiteit Leuven, Belgium, for supplying us with the pFAj3068 construct, purified peptide and antibodies raised against Hs-AFP1. We would also like to thank Prof. G. Holz at the Department of Plant Pathology for supplying the *Botrytis cinerea* culture.

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ADDENDUM A

A.1 *In vitro* antifungal activity assays on three additional antimicrobial peptides

Three purified antimicrobial peptides from *Dahlia mercii* (Dm-AMP1) (Osborn et al., 1995), *Raphanus sativa* (Rs-AFP2) (Terras et al., 1992) and *Allium cepa* (Ace-AMP1) (Cammue et al., 1995) were obtained from Prof. Broekaert at the Katholieke Universiteit Leuven. In an effort to determine their antifungal activity against *B. cinerea*, these three peptides (as well as Hs-AFP1 from *Heuchera sanguinea*; see chapter 3) were subjected to a microspectrophotometric assay at concentrations of 6 µg/ml and 8 µg/ml. The assay was performed according to Broekaert et al. (1990). Measurements (A_{595}) were collected at time zero and then every 24 h until 48 h past inoculation. All the values were corrected by subtracting the time zero value from the 24 and 48 h values. The activity of the different crude extracts was expressed in terms of percentage growth inhibition, which is defined as $100 \times$ 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. The percentage inhibition of the different crude extracts was plotted against each other. The microplate readings were performed on the PowerwaveX microplate reader (Bio-Tek instruments inc.).

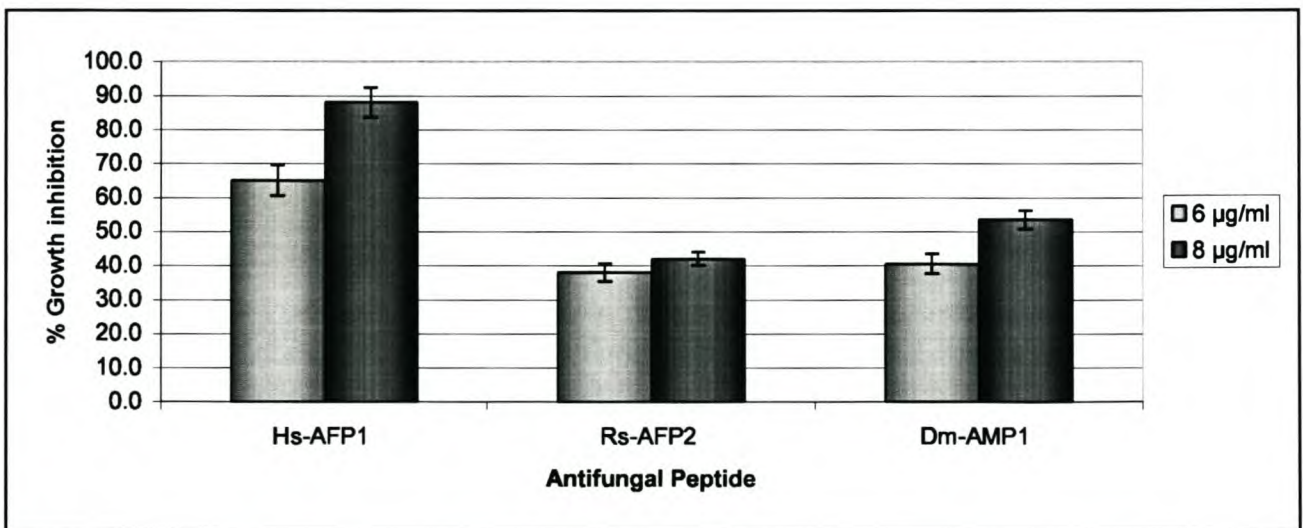


Figure A1. *In vitro* analyses of purified peptides from *Heuchera sanguinea*, *Raphanus sativa* and *Dahlia mercii* against *Botrytis cinerea*. The decline in fungal biomass, as compared to the control, over a period of 72 h was caused by the antifungal activity of these peptides as determined by a quantitative fungal growth assay. The decline in biomass is the result of morphological changes induced by these peptides on the normal growth of *B. cinerea*.

The data obtained from the microplate assay confirmed that Hs-AFP1 was the more active of the peptides tested (Fig. A1). Hs-AFP1 showed high levels of growth inhibition at very low concentrations, with IC_{50} values between 2 and 4 $\mu\text{g/ml}$. The microplate assay also showed that Rs-AFP2 and Dm-AMP1 had the same levels of activity after 72 h. Ace-AMP1, however, showed little or no activity against *B. cinerea* (data not shown).

A.2 Microscopic analyses of inhibition activity of the antifungal peptides

Hs-AFP1, Dm-AMP1, Rs-AFP2 and Ace-AMP1 were tested for antifungal activity against *B. cinerea* in a microscopic assay, at final concentrations of 10 $\mu\text{g/ml}$. The assay was conducted against 2000 *B. cinerea* spores in 100 μl half-strength (12 g/L) potato dextrose broth (PDB). The control consisted of 100 μl half-strength PDB with 2000 *B. cinerea* spores. The samples were incubated in the dark at 25°C for 3 days. Microscopical photographs were taken of each sample every 24 h.

The antifungal activity of the different peptides was clearly visible with the microscopical analysis. The three peptides, Rs-AFP2, Hs-AFP1 and Dm-AMP1, caused serious changes in the morphology of the *B. cinerea* hyphae (Fig. A2). These changes involved extensive hyperbranching of the fungal hyphae. This effect on hyphae morphology previously has been attributed to the antifungal activity typical of defensins. The peptide Ace-AMP1, in contrast, had no significant effect on fungal hyphae morphology and the sample containing this peptide showed no visible effect compared to that of the control (data not shown). Although the literature stated that the antifungal activity of Dm-AMP1 is due to the inhibition of fungal hyphae elongation, a strong hyperbranching effect also was observed (Fig. A2). It was also interesting to note that although Rs-AFP2 had a severe affect on the *B. cinerea* hyphae after 48 h (Fig. A2E), the fungus was able to overcome inhibition of the peptide after 72 h, as the severe hyperbranching effect caused by Rs-AFP2 disappeared.

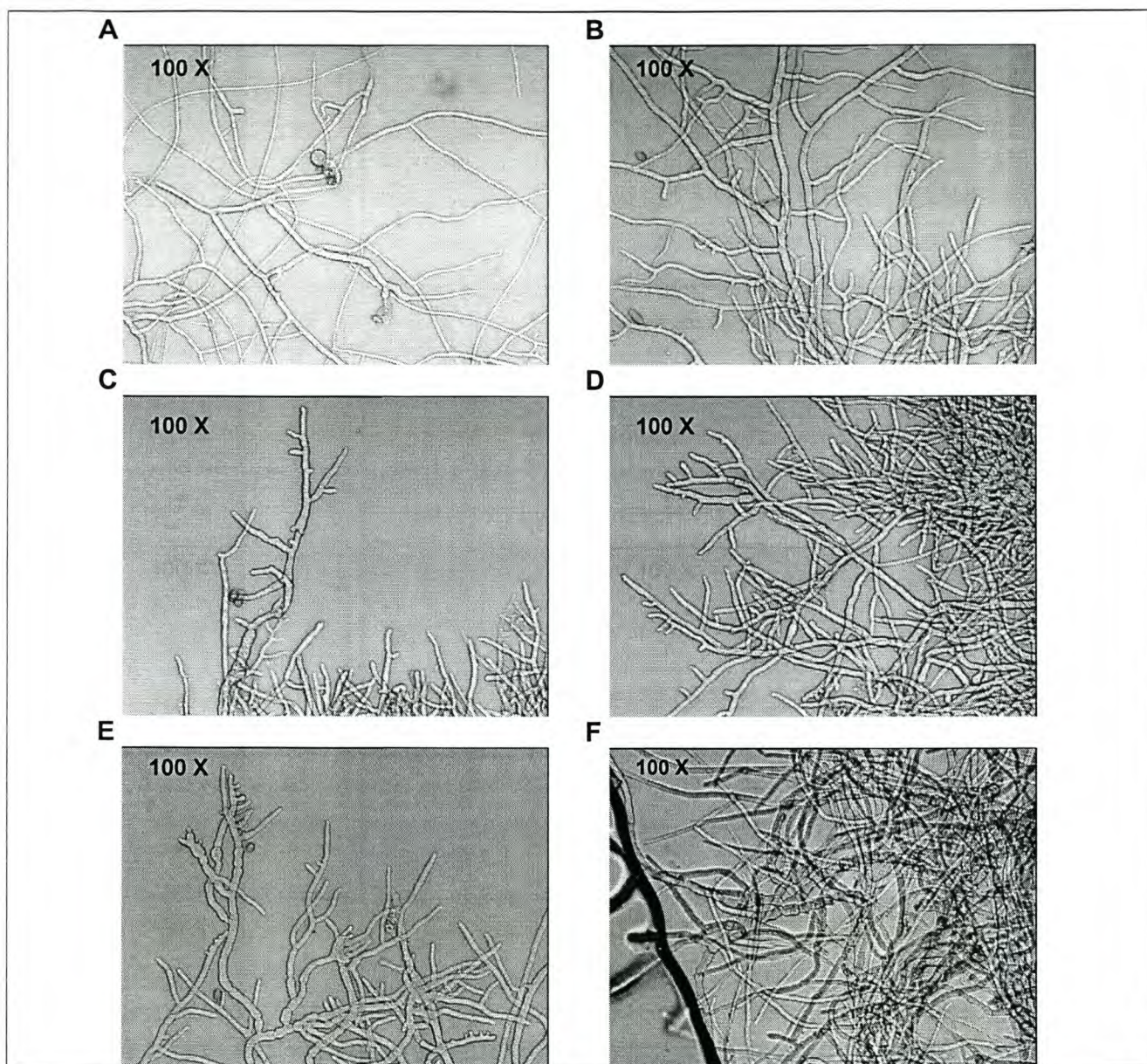


Figure A2. Microscopical analyses of purified peptides from *Heuchera sanguinea*, *Raphanus sativa* and *Dahlia mercii* against *Botrytis cinerea*. (A) The natural growth pattern of *B. cinerea* in the control reaction lacking any antifungal agent. The rapidly spreading, relatively unbranched hyphae are a sign of health. (B) The changes in hyphal morphology induced by 10 µg/ml Dm-AMP1 over a period of 72 h. (C and D) The antifungal effect observed during the incubation of 10 µg/ml HS-AFP1 in the presence of *B. cinerea*. The hyperbranching effect induced by HS-AFP1 restricts the development of the hyphae, resulting in a loss of biomass over time. (E) The changes in hyphal morphology induced by 10 µg/ml Rs-AFP2 over a period of 48 h. (F) The changes in hyphal morphology induced by 10 µg/ml Rs-AFP2 over a period of 72 h. *B. cinerea* was able to overcome the severe morphological changes induced by RS-AFP2 after 48 h. 100 X represents the microscopic magnification.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

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4.1 GENERAL DISCUSSION AND CONCLUSION

The ability to consistently harvest the necessary food and other resources obtained from plants is imperative to the optimal survival of the human race and its codependent communities of animals. In the dynamic interactions between plants and their pathogens lie a wealth of knowledge and resources that can be mined to eventually move towards a globally-sustainable agriculture, based on consistently high production, naturally high resistance of the plants against adverse biotic and abiotic factors, as well as integrated pest management with a low impact on the environment. Although this goal might seem overly optimistic at the current point in time, important initiatives have been launched to move in this direction.

The focus of this thesis falls broadly in this long-term vision, and the aim was to evaluate a defensin that is part of the innate disease resistance in several plant species for increased fungal resistance in a transgenic environment. Ultimately, this type of study is imperative to evaluate and validate the approach of over-expression of defense resistance genes in plant species. To this end, three plant expression vectors were designed and transformed into a model tobacco system. These constructs were firstly pFAJ3068, containing the Hs-AFP1 defensin from *Heuchera sanguinea* fused to the signal sequence of Mj-AMP2 from *Mirabilis jalapa* and under the control of the strong constitutive promoter. Secondly, pHs-EXG1 was constructed, in which the Hs-AFP1-encoding gene was fused to a yeast antifungal gene, the *EXG1* glucanase-encoding gene from *Saccharomyces cerevisiae*. A third construct, pMj-EXG1, was prepared to quantify the possible contribution of the *EXG1* gene alone to interpret the results from the fusion between *Hs-AFP1* and *EXG1* more optimally. The fusion protein was prepared to anticipate any peptide instability that might occur due to the small size of the peptides.

Tobacco was transformed successfully with these constructs. The expression and the resulting heterologous proteins could be detected readily in all the transgenic lines, except those transformed with the pMj-EXG1 cassette. The successful expression of pFAJ3068 led to a significant increase in disease resistance of the transgenic tobacco lines to *B. cinerea*, confirming the antifungal activities of the Hs-AFP1 peptide. These assays were performed both *in vitro*, with leaf extracts from transgenic lines, as well as in a detached leaf assay to assess the *in planta* activity. In both these assays, a good correlation could be drawn with the levels of resistance observed as well as with the morphological and physiological effects on the *Botrytis* hyphae in the presence of recombinant peptides. The characteristic

hyperbranching activity of Hs-AFP1 was observed when microscopical analysis was performed on the effect of leaf extracts from transgenic lines on the germination and growth of *Botrytis* spore cultures.

This study provided us with valuable information regarding the stability of heterologous peptides in a transgenic environment. Research done previously voiced fears of peptide instability in a transgenic environment, because small peptides are prone to rapid degradation by foreign proteases. The Hs-AFP1 defensin, however, showed high levels of stability in the heterologous tobacco system and could be detected readily with Western blot analysis. Previous transgenic work done using other plant defensins also showed no problems with instability. It seems that peptide instability, associated with incompatibility between the donor and the host, is more prominent when the donor peptide and the host are from different kingdoms. Although the defensin peptides were stable, the peptide levels were still low, but even these levels led to a significant increase in disease resistance.

With the advances made in designing high-level transgene expression systems, these levels can be greatly increased. Several high-level expression systems have been reported recently. Borisjuk et al. (2000) designed a vector capable of increasing the transgenic levels of the jellyfish green fluorescent protein (GFP) from 0.46 µg/mg to 2.28 mg/mg total soluble protein, constituting a 4500-fold increase in GFP levels. High levels of antimicrobial peptide expression in tobacco were also achieved using a chloroplast transformation system (DeGray et al., 2001). Other advantages of a chloroplast-transformation system include the targeting of integration as well as the control of the spread of the transgene due to the fact that transgenes would not be present in the pollen. Apart from high-level expression that is increasingly becoming possible, it is also feasible to utilize an inducible and/ or tissue-specific promoter to limit the expression to the tissues and developmental stages required.

Due the stability of transgenic Hs-AFP1 in our experiments, the Hs-AFP1-EXG1 fusion strategy did not enhance stability further. In fact, it was shown that the fusion, as it was prepared, led to the loss of peptide activity. The characteristic membrane pore-forming abilities of Hs-AFP1 through receptor-mediated insertion into the membrane, was probably disrupted due to the fusion of the *EXG1* gene to the 3' end of *Hs-AFP1*. It has been shown that the insertion into the membrane is initiated at the 3' end (Thevissen et al., 1999, 2000).

The fusion between *Hs-AFP1* and *EXG1*, however, did shed light on the expression of *EXG1* in a transgenic system. Transgenic *EXG1* gene expression was probably regulated at the post-transcriptional level, which resulted in total gene silencing. This silencing phenomenon was not present in the fusion of *Hs-AFP1* to the 5' region of the *EXG1* gene. This was a surprising result, but might be useful when glucanases need to be overexpressed on the plant surface. Frequent reports of gene silencing of glucanases in transgenic systems also have been documented previously (Holtorf et al., 1999). This fusion that overcame the silencing effect imparted on the glucanase might be a useful model system to study the

silencing phenomenon further. Moreover, it also emphasizes the dual role of transgenics; not only are they useful from a biotechnological viewpoint, but they also contribute significantly to the unraveling of fundamental processes.

In conclusion, this study has reiterated that a single gene introduced into a plant genome can have a significant effect on disease resistance mechanisms. This work will be expanded further, but has led to valuable information regarding the expression, stability and antifungal activities of the overexpressed plant defensin, Hs-AFP1.

4.2 LITERATURE CITED

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