Mode of action studies of defensin peptides from native South African *Brassicaceae* species

by

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Summary

Plant defensin peptides have become promising and attractive candidates to be used as antifungal agents in agricultural biotechnology. These peptides have a broad spectrum antifungal activity and play a vital role in the innate immune system of plants. Plant diseases caused by fungi are a major contributor to the decrease in the quality and safety of agricultural products. Due to the dangerous effects and negative environmental impact of pesticides, an effective, safe, natural and durable method to control crop pathogens has therefore become one of the major concerns in modern agriculture. Although these peptides are promising and attractive candidates, their precise mechanism of action is to date still unknown. Several common observations have been made. These include the antagonistic effect of cations on the activity of plant defensins. It is of vital importance to understand the underlying mechanism of the cation-antagonistic effect on the antifungal potency of defensin peptides in order to evaluate the possible contribution to defence reactions against microorganisms *in planta*.

To this end we set out to characterize the effect of cations in the form of biological salts, NaCl, KCl, $MgCl_2$ and $CaCl_2$ on the structural stability and activity in terms of growth inhibition, morphological effects and permeabilization. In order to perform these characterization experiments, a production method resulting in a greater yield and involving simple and rapid purification methods was required. *Heliophila coronopifolia* peptides have previously been produced in a bacterial system, however the purification methods were tedious resulting in poor yields. *Pichia pastoris* was selected as production system as several other plant defensins have been successfully produced in this eukaryotic system. Hc-AFP1 and Hc-AFP3 was successfully produced using the *Pichia* production system and rendered active peptides. Hc-AFP2 and Hc-AFP4 was, however, not produced correctly, due to a post-translational modification event leading to the cyclization of the N-terminal glutamine to generate pyroglutamic acid. This modification negatively influenced the activity of these peptides. An active Hc-AFP2 could be produced by replacing the production buffer with a reduced ionic buffer.

The effect of divalent and monovalent cations on the secondary structure of Hc-AFP1 was evaluated by circular dichroism spectroscopy. These cations induced a conformational change in the secondary structure of Hc-AFP1, with NaCl and $MgCl_2$ inducing a more defined secondary structure and KCl and $CaCl_2$ inducing a less defined secondary structure. Monovalent cations caused a slight reduction in the growth inhibition activity of Hc-AFP1 on *Botrytis cinerea*, however, characteristic hyperbranching and other morphogentic effects were still visible. Divalent cations had a greater antagonistic effect on the activity of Hc-AFP1, completely abolishing the growth inhibitory activity of the peptide, but the induced morphological effects on hyphae remained present. The activity of Hc-AFP1 to permeabilize *B. cinerea* hyphae was not influenced by the addition of cations, however it was in fact increased to up to 10-fold. However, since the growth inhibition activity of Hc-AFP1 was reduced in the presence of the biological salts indicates that permeabilization is not the sole activity responsible for growth inhibition caused by Hc-AFP1. This peptide probably has an alternative/primary target and more complex MOA. This is the first known report of the investigation of the influence of cations on the structure of plant defensin peptides. It is clear that cations induce a secondary structural conformational change in Hc-AFP1. This may be linked to the antagonism on the activity of this peptide. This study provides significant progress towards the structure-function analysis of plant defensins.

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Opsomming

Plantdefensinpeptiede word beskou as belowende en aantreklike kandidate vir gebruik as swammiddles in agribiotegnologie. Hierdie peptiede beskik oor breë spektrum antifungiese aktiwiteit en speel ‘n essensiële rol in die ingebore immuunsisteem van plante. Plant siektes wat deur swamme veroorsaak word dra betekenisvol by tot die afname in die kwaliteit en veiligheid van landbouprodukte. As gevolg van die skadelike effekte en negatiewe omgewingsimpak van plaagdoders, het effektiewe, veilige, natuurlike en duursame metodes om gewaspatogene te beheer, van die belangrikste vraagstukke van moderne landbou geword. Alhoewel hierdie peptiede belowende en aantreklike kandidate is vir die toepassing, is hulle presiese meganisme van aksie tot vandag toe steeds onbekend. Verskeie algemene waarnemings is egter al gemaak. Dit sluit die antagonistiese effek van katione op die aktiwiteit van plantdefensinpeptiede in. Dit is kernbelangrik om die onderliggende meganisme van die katioon-antagonistiese effek op die antifungiese effektiwiteit te verstaan om die moontlike bydrae van die peptiede tot die verdedigingsreaksies teen mikro-organismes *in planta* te evalueer.

Met die doel voor oë het ons gemik om die effek van katione, spesifiek in die vorm van die biologiese soute NaCl, KCl, $MgCl_2$ en $CaCl_2$, op die strukturele stabiliteit en aktiwiteit in terme van groei inhibisie, morfologiese effekte en permeabilisasie te karakteriseer. Om uiteindelik hierdie karakterisasie eksperimente uit te voer was dit nodig om ‘n metode met ‘n groter opbrengs en wat vinnige suiwering van die peptied ondersteun, te optimiseer. *Heliophila coronopifolia* peptiede was voorheen in ‘n bakteriese sisteem geproduseer, maar die suiweringsmetodes was tydsaam en het gelei tot ‘n swak opbrengs. *Pichia pastoris* is dus geselekteer as die produksie sisteem aangesien verskeie ander plantdefensinpeptiede al suksesvol geproduseer is in hierdie eukariotiese sisteem. Hc-AFP1 and Hc-AFP3 is suksesvol vervaardig in die *Pichia* sisteem en het aktiewiteit getoon. Hc-AFP2 and Hc-AFP4 kon egter nie korrek vervaardig word nie as gevolg van ‘n na-vertalingsverandering wat gelei het tot die siklisering van die N-terminale glutamien, om piroglutamiensuur te lewer. Hierdie verandering het die aktiwiteit van die peptied negatief beinvloed. ‘n Aktiewe Hc-AFP2 kon wel vervaardig word deur die produksiebuffer te vervang met ‘n lae-ionise buffer.

Die effek van divalente en monovalente katione op die sekondêre struktuur van Hc-AFP1 is ge-evalueer deur van sirkulêre dikroisme spektroskopie gebruik te maak. Hierdie katione het ‘n vouingsverandering in die sekondêre struktuur van Hc-AFP1 geïnduseer, NaCl and $MgCl_2$ het ‘n meer gedefinieërde sekondêre struktuur induseer, terwyl KCl and $CaCl_2$ ‘n minder gedefinieërde sekondêre struktuur geinduseer het. Monovalente katione het ‘n effense vermindering in die groei-inhibisie aktiwiteit van Hc-AFP1 op *Botrytis cinerea* veroorsaak, alhoewel kenmerkende hife-oorvertakking en ander morfologiese effekte nogsteeds sigbaar was. Divalente katione het ‘n sterker antagonistiese effek gehad op die aktiwiteit van Hc-AFP1, waar dit totaal en al die groei-inhibisie aktiwiteit van die peptied vernietig het, alhoewel die geïnduseerde morfologiese effekte op die hiffes steeds sigbaar was . Die aktiwiteit van Hc-AFP1 om *B. cinerea* hyphae te permeabiliseer is nie negatief beinvloed deur die byvoeging van katione nie, tewens dit het die aktiwiteit tot 10-voudig verhoog. Aangesien die groei-inhibisie aktiwiteit van Hc-AFP1 nie verminder is in die teenwoordigheid van die biologiese soute nie, dui dit aan dat permeabilisasie nie die enigste aktiwiteit is wat die groei inhibisie veroorsaak het nie. Die peptied het dus moontlik ‘n alternatiewe of primêre teiken en ‘n meer komplekse meganisme van aksie. Dit is die eerste verslag wat die invloed van katione op die struktuur van plantdefensinpeptiede ondersoek het. Dit is duidelik dat katione ‘n sekondêre strukturele vouingsverandering in Hc-

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AFP1 induseer. Hierdie verandering mag dalk bydra tot die antagonistiese uitwerking op die aktiwiteit van hierdie peptied. Hierdie studie het betekensisvolle vordering gemaak met die analise van die struktuur-funksie interaksie van plantdefensinpeptiede.

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This thesis is dedicated to
My brother and parents

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Biographical sketch

Helmien Barkhuizen was born in Bellville, South Africa, on 2 June 1988. She matriculated from Bellville High in 2006. In 2007 Helmien enrolled for a BSc-degree in Molecular Biology and Biotechnology at Stellenbosch University, which she obtained in 2009. In 2010 she received a HonsBSc-degree in Biochemistry. She enrolled for her MSc in Wine Biotechnology in 2011.

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Preface

This thesis is presented as a compilation of chapters. Each chapter is introduced separately and is written according to the style of the journal of Plant Physiology.

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Plant defensins

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The recombinant production and activity analysis of four *Heliophila coronopifolia* defensin peptides

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Chapter 1

Introduction and project aims

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GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Plant diseases caused by fungi are a major contributor to the decrease in the quality and safety of agricultural products (Montesinos, 2007). The effective and durable control of pathogens of crops has therefore become one of the major concerns in modern agriculture (Gao *et al.*, 2000; Kaur *et al.*, 2011). Due to the dangerous effects and negative environmental impact of pesticides, antimicrobial peptides like plant defensins are considered attractive and promising candidates to be used in agricultural biotechnology due to their broad antifungal activity (Van der Biezen, 2001; Lay and Anderson, 2005; Stotz *et al.*, 2009). Plant defensins form part of a large family of cationic host defence peptides that are widely distributed throughout the plant kingdom. These peptides form part of the innate immune system and play a vital role in the protection of plants against invading fungal pathogens (Broekaert *et al.*, 1995; Osborn *et al.*, 1995; Terras *et al.*, 1995; Thevissen *et al.*, 1997; Thomma *et al.*, 2003; Lay and Anderson, 2005).

Plant defensins have a broad spectrum antifungal activity and these peptides are not only active against the phytopathogenic fungi but also against baker's yeast, *Saccharomyces cerevisiae* and the human pathogenic fungi *Candida albicans* (Broekaert *et al.*, 1995; Osborn *et al.*, 1995; Thevissen *et al.*, 1997; Almeida *et al.*, 2000; Carvalho and Gomes, 2009; Stotz *et al.*, 2009). Defensin peptides are expressed during normal plant growth and development and are present in the peripheral cells of different plant organs which are the first barriers to pathogen invasion (Broekaert *et al.*, 1995; Terras *et al.*, 1995). Some plant defensins can be induced in response to fungal infection and mechanical wounding, whereas others are constitutively expressed (Broekaert *et al.*, 1995; Osborn *et al.*, 1995; Terras *et al.*, 1995; Thevissen *et al.*, 1997; Thomma *et al.*, 2003; Lay and Anderson, 2005).

Several defensin genes have been successfully transformed into various plant hosts (Gao *et al.*, 2000; Lay and Anderson 2005). Although the constitutive overexpression of several plant defensins have shown to significantly enhance disease resistance of hosts under greenhouse-conditions, the effectiveness of defensins to maintain this resistance under field-conditions have only been demonstrated in a few cases (Terras *et al.*, 1995; Koike *et al.*, 2002; Kaur *et al.*, 2011). The plant defensin AlfAFP, isolated from the seeds of *Medicago sativa,* was transformed into potato and provided increased resistance against *Verticillium dahliae* that is responsible for the "early dying" disease of potato under greenhouse-conditions and field-conditions. Moreover, the level of resistance obtained

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against *V. dahlia* was equal or exceeded the level of resistance obtained by the conventional fumigants used (Gao *et al.*, 2000). Another plant defensin, NmDef02, a novel defensin isolated form *Nicotiana megalosiphon,* was also over expressed in transgenic potato plants. These transgenic potato plants demonstrated enhanced resistance against *Phytophthora infestans*, an economically important pathogen that causes potato late blight, under greenhouse and field conditions (Portieles *et al.*, 2010). These results indicated that the expression of a single defensin gene contains the ability and potential to increase the field disease resistance of certain crops and provide protection against several fungal diseases (Gao *et al.*, 2000; Portieles *et al.*, 2010).

It is essential to have insight and detailed knowledge of the mode of action of plant defensin peptides in order to rationally design their potential application. The exact mechanism of antifungal action to date is unknown; however, several common observations regarding the possible mode of action have been made. Plant defensins bind to the plasma membranes of sensitive fungi with high affinity and this result in permeabilization and cell growth arrest (Thevissen *et al.*, 1997; Thevissen *et al.*, 1999). The ability of plant defensins to permeabilize plasma membranes is not a direct indication of the antifungal activity and potency of these peptides (Sagaram *et al.*, 2011). This membrane permeabilization induced by plant defensins seems to be a secondary effect (Thevissen *et al.*, 1996). The presence of specific high affinity binding sites for some plant defensins has been established. The explicit binding sites of defensins are specific sphingolipids present in the plasma membrane of the sensitive fungi. The plant defensin DmAMP1 from *Dahlia merckii* binds to the sphingolipid mannose(inositol-P)$_2$-ceramide (M(IP)$_2$C), whereas the plant defensin RsAFP2 from *R. sativus* binds to the plasma membrane sphingolipid glucosylceramide (GlcCer) (Terras *et al.*, 1992; Thevissen *et al.*, 2000; Thevissen *et al.*, 2003a; Thevissen *et al.*, 2003b; Thevissen *et al.*, 2004). However, it is still unclear how this defensin/sphingolipid interaction results in the growth arrest of the fungus. It remains to be determined if this interaction leads to the activation of specific signalling events essential for the antifungal action (Kaur *et al.*, 2011). Recently it has been established that some plant defensins are internalized by a fungal cell and bind to intracellular targets (Van der Weerden *et al.*, 2008; Van der Weerden *et al.*, 2010; Lobo *et al.*, 2007). Whether or not these plant defensins inhibit cell wall synthesis or rather nucleic acid or protein synthesis of enzymatic activities remains to be determined (Kaur *et al.*, 2011). It has been recognized that some plant defensins induce the production of reactive oxygen species (ROS), involving the MAP kinase signalling pathway. Furthermore, some plant defensins have been shown to induce apoptosis. These ROS play a critical role in the antifungal activity of defensin peptides (Aerts *et al.*, 2007). However, whether or not this ROS accumulation and induction of apoptosis is associated with defensin-induced fungal growth arrest still remains to be determined (Kaur *et al.*, 2011).

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The antimicrobial activity of plant defensins is strongly antagonized by the addition of monovalent and divalent cations to the medium (Ameilda *et al.*, 2000; Osborn *et al.*, 1995, Terras *et al.*, 1992 and Terras *et al.*, 1993). This antagonistic effect of cations on the antifungal activity appears to depend strongly on the fungal pathogens tested (Terras *et al.*, 1992, Osborn *et al.*, 1995). The precise mechanism of this antagonistic effect on the antifungal activity is still unknown. It is proposed that the antagonistic effect is the result of an electrostatic interaction between the fungus and the cations rather than a conformational change in the peptide due to the direct interaction between the peptide and the cations (Terras *et al.*, 1992). However, this still remains to be determined. It is well-known that the defence reactions mounted by plants typically involve changes in cations. One of the earliest known reactions following pathogen infection is a series of ion fluxes at the plasma membrane (de Bruxelles and Roberts, 2001; Yeaman and Yount, 2003). It has been demonstrated that pathogen elicitor treatment stimulates rapid and transient increase in Ca^{2+} influx and increase in the extracellular Cl^- and K^+ concentrations in cultured parsley cells. Furthermore, these ion fluxes results in the alkalinisation of the culture medium (Jabs *et al.*, 1997). The influx of Ca^{2+} in the cytoplasmic environment is associated with the activation of the Ca^{2+} binding messenger protein, calmodulin, that has been demonstrated to modulate elicitor-induced gene expression, suggesting a role in signalling (de Bruxelles and Roberts, 2001). Ca^{2+} plays a vital role in the signal transduction events of plants (White and Broadley, 2003). All these initial ion fluxes are prerequisites for further signalling transduction events possibly involving a complex signalling network that activates the overall defence response (Somssich and Hahlbrock, 1998). It is therefore important to understand the underlying mechanism of the cation-antagonistic effect on the antifungal potency of defensin peptides in order to evaluate the possible contribution to defence reactions against microorganisms *in planta*.

In the Institute for Wine Biotechnology, four plant defensin peptides have recently been isolated from *Heliophila coronopifolia,* a native South African *Brassicaceae* species. Analysis of the deduced amino acid sequences of Hc-AFP1-4 showed that the peptides were 72% similar and grouped closest to defensins isolated from other *Brassicaceae* species. The Hc-AFP1 and Hc-AFP3 peptides shared high sequence homology (94%) and formed a unique grouping amongst the Brassicaceae defensins, whereas Hc-AFP2 and Hc-AFP4 formed a second homology grouping with defensins from *Arabidopsis* and *Raphanus*. Furthermore, homology modelling showed that the few amino acids that differed between the four peptides had an effect on the surface properties of the defensins, specifically in the alpha helix and the loop connecting the second and third beta strands ($L\beta_2\beta_3$) (De Deer and Vivier, 2011) (Figure 1). These areas, and specifically $L\beta_2\beta_3$ (also termed the γ-core), are implicated in determining differential activities of defensins. The major determinants of the antifungal

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activity and induced morphogenic effects of plant defensins reside in this structurally conserved motif (Yount and Yeaman, 2004). These four defensin peptides were found to exhibit high inhibitory activity against *Botrytis cinerea* and *Fusarium solani*. The antifungal activities and morphogenic effects induced by the four peptides against the two pathogens were extremely diverse, despite their high sequence similarity. Hc-AFP2 and Hc-AFP4 was found to be the most active against the pathogens tested. All the defensin peptides induced changes in hyphal morphology of *B. cinerea*, including hyper-branching, fungal tip swelling, increased granulation of hyphae and spores, as well as hyphal and spore disruption. Moreover, the activity of all four of these plant defenins was associated with membrane permeabilization on both, or at least one of the pathogens (De Beer and Vivier, 2011).

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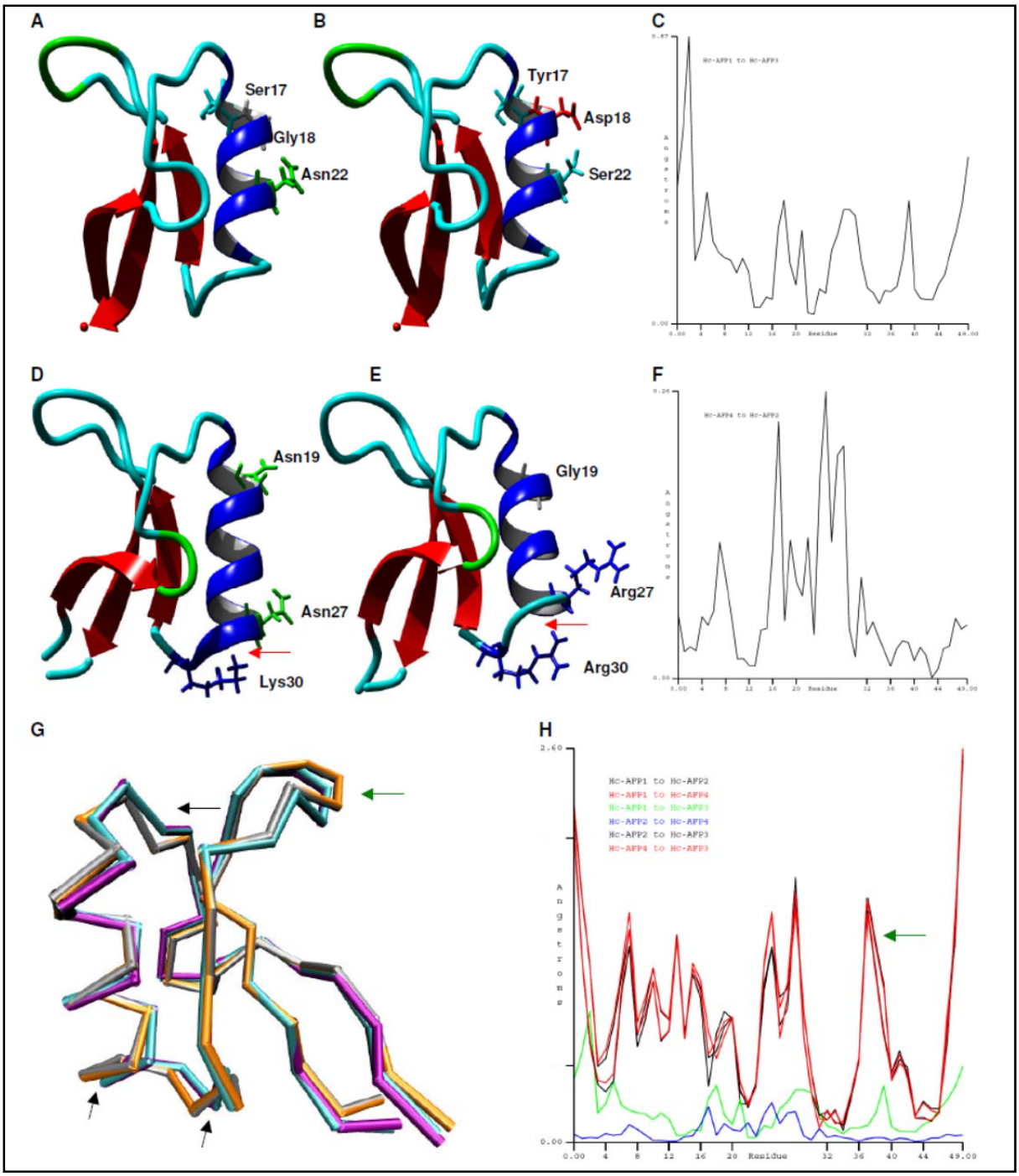

Figure 1.1 Homology models of *H. coronopifolia* defensins depicting the amino acid differences observed in the alignment of the deduced amino acid sequences. (A) Hc-AFP1, (B) Hc-AFP3, (D) Hc-AFP2, (E) Hc-AFP4, (C and F) RMSD analysis of Hc-AFP1 and 3 and Hc-AFP2 and 4 respectively. (G) Structural alignment of the backbones of Hc-AFP1-4. (H) RMSD analysis of the structural alignment of Hc-AFP1-4. Models were created using Rs-AFP1 (Protien DataBank: 1AYJ) as template. Acidic residues are indicated in red, basic in blue and polar residues in green. The black arrows indicate significant difference ins in the structure between Hc-AFP1 and 3 (Group 1) and Hc-AFP2 and 4 (Group 2). The green arrow indicates the difference in the presence of the loop connecting the β-strand 2 and 3 (Taken from De Beer and Vivier, 2011).

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1.2 RATIONALE AND PROJECT AIMS

This project was initiated to continue the characterization of the four *H. coronopifolia* defensin peptides in terms of stability and activity, as well as their possible mode of action (MOA). Moreover, the effect of monovalent and divalent cations on the structure and activity of the Hc-AMP plant defensins are not known and therefore formed part of the proposed study. These aspects formed the basis of the project aims; the project also relied on a few pertinent methods as introduced below:

We set out to characterize the effects of monovalent and divalent cations on the secondary structure of plant defensins by using circular dichroism spectroscopy. Circular dichroism spectroscopy is a very useful technique to study changes in the secondary structure conformation of antimicrobial peptides (Gopal 2012). This technique can be used to differentiate between unordered (random coil) and ordered (α-helix or β-sheet) secondary structures (Kelly *et al.*, 2005; Gopal *et al.*, 2012). Moreover, the effect of the cations on the MOA of the peptides would be monitored with *in vivo* permeabilization studies. These studies make use of propidium iodide treatment combined with fluorescent microscopy. Propidium iodide is a membrane-impermeable intercalating fluorochrome that is restricted to enter membranes of stressed, injured or dead cells. This fluorochrome binds to DNA and RNA where it fluoresces red (Nussbaum *et al.*, 1997; Ganwar *et al.*, 2006). To further assess the effect of the cations on the ability of these peptides to cause growth inhibition of the pathogen(s) a well-established microspectrophotometric assay would be used (Broekaert *et al.,* 1990), as well as following the effects on the hyphae microscopically.

The *H. coronopifolia* defensin peptides were previously produced in a bacterial system. This resulted in a very low peptide yield of 0.2 mg mL^{-1}. In order to perform all the above mentioned characterization experiments a method resulting in a greater yield was required. Furthermore the previously used bacterial production system involved purification methods that were extremely tedious (De Beer and Vivier, 2011). *Pichia pastoris* provides a promising alternative for the recombinant production of peptides and has been used to produce large quantities of other plant defensin peptides (Almeida *et al.*, 2001; Cabral *et al.*, 2003; De-Paula *et al.*, 2008; Marqués *et al.*, 2008). In this study we set out to express the four *H. coronopifolia* peptides in *P. pastoris* and optimize the production and purification of the defensins since yields of 1-3 mg would be required in order to perform characterization experiments on these peptides.

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The specific aims of this project were as follows:

1. The recombinant production and purification of *H. coronopifolia* peptides Hc-AMP1-4, from *P. pastoris* and;

2. In-depth characterization of *H. coronopifolia* peptides in terms of activity and stability, as well as to make progress in our understanding of the MOA;
    a. Circular dichroism spectroscopy of the *H. coronopifolia* peptides;
    b. *In vivo* permeabilization studies of *H. coronopifolia* peptides.

The results linked to these aims are presented in the research Chapters 3 and 4, following a concise literature review in Chapter 2. The outcomes of this study are concluded upon in Chapter 5.

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Chapter 2

Literature review

Plant Defensins

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LITERATURE REVIEW

2.1 Introduction

Throughout a plant's lifespan, it is constantly threatened by various invading pathogens and pests. For protection against these pathogens, plants rely on their dynamic defence mechanisms. Plants have the ability to produce a wide variety of antimicrobial molecules, including several antimicrobial peptides (AMPs) (Broekaert *et al*., 1995, Broekaert *et al*., 1997, Lay and Anderson, 2005, Thevissen *et al*., 2007 and De Oliceria Carvalho and Gomes, 2000). These antimicrobial peptides are single gene products and plants produce these peptides effortlessly and rapidly, without excessive energy and biomass input (Bowman and Hultman, 1981 and Broekaert *et al*., 1995). Among the numerous types of antimicrobial peptides produced by plants for protection against invading pathogens and pests is a class of peptides called plant defensins. Defensin peptides are present in vertebrates, invertebrates and plants and they all share structural and functional homology (Broekaert *et al*., 1995).

Plant defensins are described as small (45 to 54 amino acids long), basic, positively charged, cysteine-rich antimicrobial peptides that possess a diverse array of antimicrobial activities (Broekaert *et al*., 1955, Lay and Anderson, 2005, Thevissen *et al*., 2007 and De Oliceria Carvalho and Gomes, 2000). The first plant defensins were isolated form wheat and barley endosperm (Colilla *et al*., 1990 and Mendes *et al*., 1990). These plant defensins were originally classified as new members of the thionin family, namely γ-thionins, due to the fact that their amino acid sequences (45-54 amino acids), size (~5 kDa) and number of cysteine residues (8), closely resembled that of thionins (Colilla *et al*., 1990). However, structural studies by Bruix *et al* (1993) revealed differences between thionins and defensins, including the distribution of basic amino acids and the organizational pattern of disulphide bridges. This indicated that defensins and thionins should be separated (Bruix *et al*., 1993) and when Terras *et al.* (1995) isolated two novel antifungal peptides, RsAFP1 and RsAFP2 from *Raphanus sativus,* that resembled those of insect and mammalian defensins in terms of their structure and function, this family of peptides were renamed as "plant defensins" (Terras *et al*., 1995).

Plant defensins form an important component of the innate defence system of plants where they act as protective antimicrobial barriers between tissue types of plant organs as well as around seeds (Terras *et al*., 1995). Furthermore, plant defensins are crucial components of the plant's defence system and display an array of biological activities including protein translation inhibition activities and enzyme inhibitors of α-amylases and proteases (Bloch and Richardson, 1991; Broekaert *et al*., 1995; Carvalho and Gomes, 2000; Liu *et al*., 2006; Lin *et al*., 2007; Pelegrini *et al*., 2008). These peptides play an important role in the protection of seeds where they are released upon mechanical wounding of seeds as well as during germination. During the latter critical phase

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of plant growth, peptides are released to form an inhibitory zone around the seed to protect the germinating seedling from soil-borne or seed-borne fungal pathogens (Terras *et al*., 1995, Broekaert *et al*., 1995 and Lay and Anderson, 2005). Plant defensins are mostly located in the periphery of a range of organs. These locations are consistent with a role for these peptides as "first line of defence" against pathogens (Terras *et al*., 1995 and Lay and Anderson, 2005). These peptides can mostly be induced under pathogenic stress conditions or mechanical wounding and they are able to confer increased protection against pathogens in vegetative tissues (Broekaert *et al*., 1995 and Lay and Anderson, 2005)

Due to their broad antifungal activity, plant defensins have become promising candidates to be used in agricultural biotechnological approaches as well as in the pharmaceutical industry (Lay and Anderson, 2005; Thevissen *et al*., 2007; Stotz *et al*., 2009). These peptides have the potential to generate transgenic crops with improved pathogen resistance and are likely to reduce dependence on environmentally unfriendly chemical pesticides used in the agricultural industry (Stotz *et al*., 2009). Furthermore these peptides also have the potential to serve as templates to develop novel antifungal therapeutics for humans and animals. This is of great importance to the pharmaceutical industry due to increasing drug resistance of pathogens (Thevissen *et al*., 2007). Although plant defensins have potential to be used in the agricultural and pharmaceutical industries, it is essential to have insight and detailed knowledge of their modes of action in order to rationalize/sustain the potential application of these peptides. This crucial aspect is still unclear and for most plant defensins, the underlying molecular components involved in signalling pathways and the intracellular targets are also still unknown. Several common observations regarding the possible mode of action have been made. Furthermore, only for the best studied plant defensins namely, DmAMP1, a plant defensin isolated from *Dahlia merckii,* and RsAFP2, a plant defensin isolated form *Raphanus sativus,* the molecular basis for their growth inhibitory activity have been established (Thomma *et al*., 2003; Aerts *et al*., 2008; Wilmes *et al*., 2011).

This review focuses on the structure, localization, biological activities, the current understanding of the mode of action, as well as the structure-function relationship of plant defensin peptides.

2.2. Plant defensin structure

Plant defensin peptides can be divided into two major groups based on their precursor protein structure. In the first and largest group, an amino acid secretion signal peptide, comprising 29 to 30 amino acids, and the mature defensin domain, consisting of 45-54 amino acids occur (Broekaert *et al*., 1995; Thomma *et al*., 2003; Lay and Anderson, 2005). The second group of defensins has only been observed in solanaceous species and has an additional C-terminal pro-domain of approximately 33 amino acids (Figure 2.1) (Lay and Anderson 2005).

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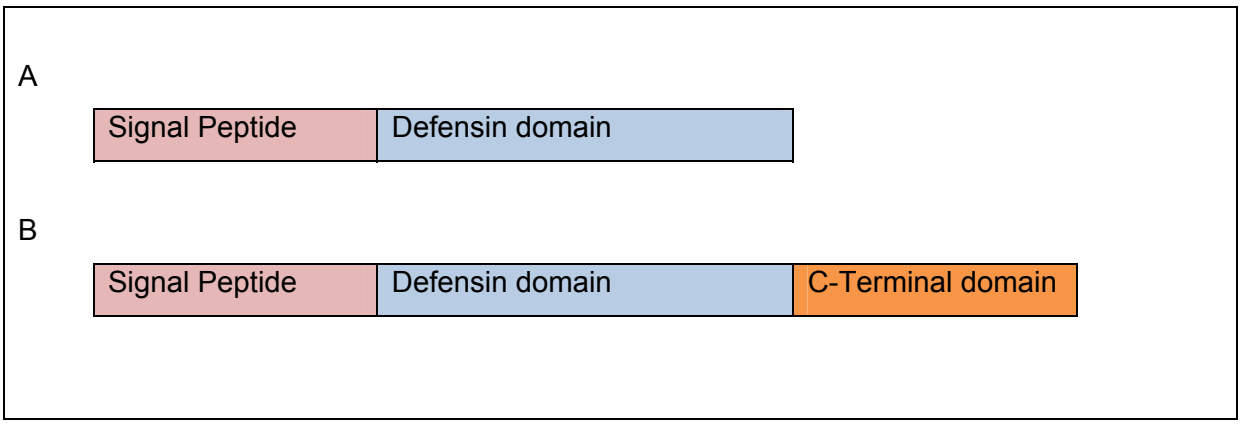

Figure 2.1 The two major classes of plant defensins. (A) The general precursor structure observed in the majority of plant defensins consists of a signal peptide followed by a mature defensin domain. (B) The precursor structure observed in plant defensins isolated from *Solanacea* species, containing an additional C-terminal pro-domain (Lay *et al*., 2005).

The C-terminal pro-domain observed in solanaceous defensins has been characterized and is acidic in nature with a high content of acidic and hydrophobic amino acids. This acid net charge of the pro-domain is able to counteract the basic nature of the defensin domain leading to an overall neutrally charged defensin peptide (Lay *et al*., 2003b; Lay and Anderson, 2005). In contrast, the sunflower defensin HaDEF1, contains a positively charged C-terminal pro-domain which is of an unusual length (30 amino acids) and contains a high number of proline residues (De Zélicourt *et al*., 2007). The role of the C-terminal pro-domain in floral defensins is unclear, but it has been suggested to act as a targeting sequence for sub-cellular sorting and post-translational proteolytic processing. The high content of acidic and hydrophobic amino acids in the C-terminal pro-domain are indicative of vacuolar sorting signals (Lay *et al*., 2003b; Lay and Anderson, 2005). This hypothesis was further supported by the discovery of the presence of high levels of NaD1 defensin in the tobacco vacuole (Lay *et al*., 2003b). Several other roles, including the assistance in the maturation of the defensin, have been suggested. This role is consistent with that of mammalian, α-defensins, insect defensins and the C-terminal pro-domain of thionins. Another suggested function is that the C-terminal pro-domain acts as an intermolecular steric chaperone (Lay and Anderson 2005).

The mature peptide domain consists out of 45-54 amino acids and is basic with a pI of around 9. The processed domain constructs a small cationic molecule with a molecular mass of 5-7 kDa (Broekaert *et al*., 1995; Lay and Anderson, 2005; Aerts *et al*., 2008; Carvalho *et al*., 2009). Defensins display very limited sequence conservation that is usually limited to the presence of eight cysteine and two glycine residues at positions 13 and 34, an aromatic residue at position 11 and a glutamic acid at position 29 (numbering relative to the radish seed-isolated plant defensin, RsAFP1) (Broekaert *et al*., 1995). The significant variation in the remaining amino acid residues might explain the wide range of biological activities that has been attributed to this group of antimicrobial peptides (Lay and Anderson, 2003a). The eight conserved cysteine residues are responsible for the characteristic four disulphide bridges that play a vital role in the stabilization of

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the three dimensional structure of these peptides (Broekaert *et al*., 1995; Lay and Anderson, 2005; Aerts *et al*., 2008; Carvalho *et al*., 2009).

Some defenins have been successfully crystallised. The solution structure of the RsAFP1 was determined by nuclear magnetic resonance spectroscopy. RsAFP1 was confirmed to adopt a globular structure that consists of one α-helix (from Asn^{18} to Leu^{28}), with three helical turns and three anti-parallel β-sheets (β1 consists of the amino acid residues from Lys^{2} to Arg^{6}, β2 from His^{33} to Tyr^{28} and β3 from His^{43} to Pro^{50}). This globular structure is stabilized by four disulphide bridges. Two of the four disulphide bridges (Cys^{21}-Cys^{45} and Cys^{25}-Cys^{47}) connect the central strand of the triple-stranded β-sheet to the α-helix. Furthermore, the third disulphide bridge (Cys^{15} and Cys^{36}) connects the loop with β-strand 2, whereas the fourth disulphide bridge (Cys^{4}-Cys^{51}) connects the N and C termini (Bruix *et al*., 1993 and Fant *et al*., 1998).

The solution structure of the first floral defensin, isolated from *Nicotiana alata*, NaD1 was also determined and characterized structurally. As previously mentioned, floral defensins differ from the seed defensins in that they are produced from precursor proteins that not only contain the secretion signal peptide and mature defensin domain, but they also contain a C-terminal pro-domain of up to 33 amino acid residues. NaD1 adopted a structure that consisted of triple stranded anti-parallel β-sheets (β1 consisted of amino acid residues from Cys^{3} to Glu^{6}, β2 from Asp^{31} to Cys^{34} and β3 from Cys^{41} to Lys^{45}), and a single α-helix (amino acid residues from Lys^{71} to Ser^{26}) and the structure was stabilized by four disulphide bridges. The α-helix was cross-linked to the central strand (β3) of the β-sheet by two disulphide bridges (Cys^{20}-Cys^{41} and Cys^{24}-Cys^{43}). The third disulphide bond was located between Cys^{14} and Cys^{34} and linked the loop to strand β2 of the β –sheet. The fourth disulphide bridge was located between Cys^{3}-Cys^{47} and this accommodated the N and C termini of the molecule (Lay *et al*., 2003a). The three-dimensional structure of NaD1 is shown in Figure 2.2.

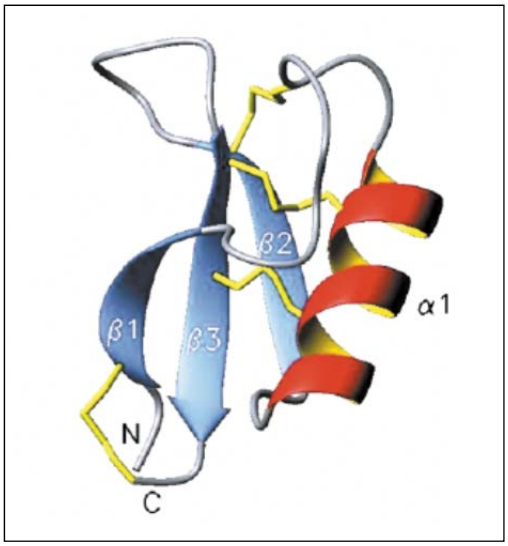

Figure 2.2. Three dimensional representation of NaD1 showing the characteristic structure of plant defensins (Lay *et al*., 2003a). The α-helix is indicated in red and the three β-sheets in blue. The four disulphide bonds responsible for stabilization are indicated in yellow.

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A comparison of the seed-isolated RsAFP2 and the flower-isolated NaD1 showed that these two peptides only share 34% sequence identity based on amino acid comparison, whereas the tertiary structures of these two defensin peptides were found to be very similar. The topologies of the disulphide bridges of both peptides are identical, but they differ largely in their loop regions. There is a difference in the conformations of the loop between strands β2 and β3. This can be attributed to the difference in the number of amino acid residues, i.e. the loop of NaD1 is two residues shorter than that of RsAFP2. Furthermore, there is a significant difference in the long loop between strand β1 and the α-helix. This loop consists of amino acids Pro^{7} to Asn^{17} in RsAFP1 and Ser^{7} to Thr^{16} in NaD1. The difference in conformation in this loop can thus be attributed to the difference in loop length as well as the difference in amino acid sequences (Lay *et al*., 2003).

Several other plant defensin structures have been determined including Psd1, a plant defensin isolated from the seed of *Pisum sativum*. Most plant defensins, including Psd1, presented a significantly similar pattern that contains all the conserved structural characteristics (Almeida *et al*., 2002; Liu *et al*., 2006). Although most plant defensins contain four disulphide bridges, some peptides, such as PhD1 and PhD2 from *Petunia hybrid,* contain five disulphide bridges instead. The fifth disulphide bridge connects the α-helix and the β1-strand, providing further stabilization of the defensin peptide structure (Lay *et al*., 2003b).

Plant defensins also contain a distinctive, conserved structural feature, namely the cysteine-stabilized α-helix motif (CSαβ) (Broekaert *et al*., 1995, Cornet *et al*., 1995, Fant *et al*., 1998 and Lay *et al*., 2003). This motif was named by Cornet *et al.* (1995) after solving the solution structure of insect defensin A (Cornet *et al*., 1995). This motif consists of a pair of cysteines separated by a tri-peptide (Cys-X-X-X-Cys) in the α-helix and is cross-linked to two disulphide bridges to a second pair of cysteines, separated by a single amino acid residue in (Cys-X-Cys) in the C-terminal β-strand. This characteristic structural motif is known as the cysteine-stabilized α-helix motif (CSαβ) (Kobayashi *et al*., 1991 and Broekaert *et al*., 1995) and is generally found in peptides that posses antimicrobial activity. These include insect defensins, although their structure differs slightly from that of plant defensins in that they do not contain the domain corresponding to the amino-terminal β-strand (Bruix *et al*., 1993; Broekaert *et al*., 1995, Carvalho *et al*., 2009).

A study by Yang *et al* (2009) investigated the amino acid tolerance of the CSαβ motif of *V. radiate* defensin 1 (VrD1) in order to further understand the structural stability, biochemical function as well as the target interaction of this evolutionary conserved motif. This was achieved by performing a systematic Ala substitution of non-cysteine residues on VrD1. Interestingly, the Ala substitution of each position did not have a significant effect on the structure, but these substitutions caused the inhibitory effect of the peptide to either be altered or abolished (Yang *et al*., 2009).

Another highly conserved sequence found throughout disulphide-containing antimicrobial peptides is the γ-core motif (Figure 1.3). This motif comprises two antiparallel β-sheets with an

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interposed turn region. The consensus sequence of the γ-core motif, also called the $\beta_2\beta_3$ loop, is Gly-X-Cys(X_{3-9})Cys. This conserved sequence is also orientated in reverse in some peptides and is then referred to as levomeric isoforms, as outlined below and depicted in Figure 2.3:

NH_2...[X_{1-3}]-[GXC]-[X_{3-9}]-[C]...COOH (dextromeric)

NH_2...[C]-[X_{3-9}]-[CXG]-[X_{1-3}]...COOH (levomeric isoform 1)

NH_2...[C]-[X_{3-9}]-[GXC]-[X_{1-3}]...COOH (levomeric isoform 2)

This motif can represent the entire peptide or form part of other structural domains. The γ-core transcends previously identified defensin-like consensus motifs like the cysteine-stabilized α-helix motif (CSαβ). The γ-core motif is further characterized by a net cationic charge, periodic charge and hydrophobicity, that provide an amphipathic stereogeometry to this motif. Furthermore, the γ-core motif takes part in one to four disulphide bonds (Yount and Yeaman, 2004). Interestingly, this motif contains specific, highly conserved amino acid residues contributing to the secondary structure (these include residues proline and cysteine), and also contributing to amphipathicity that includes residues that are hydrophobic and charged (Yount *et al*., 2006). This motif plays an important role in the antimicrobial activity of these peptides (Yount and Yeaman, 2004).

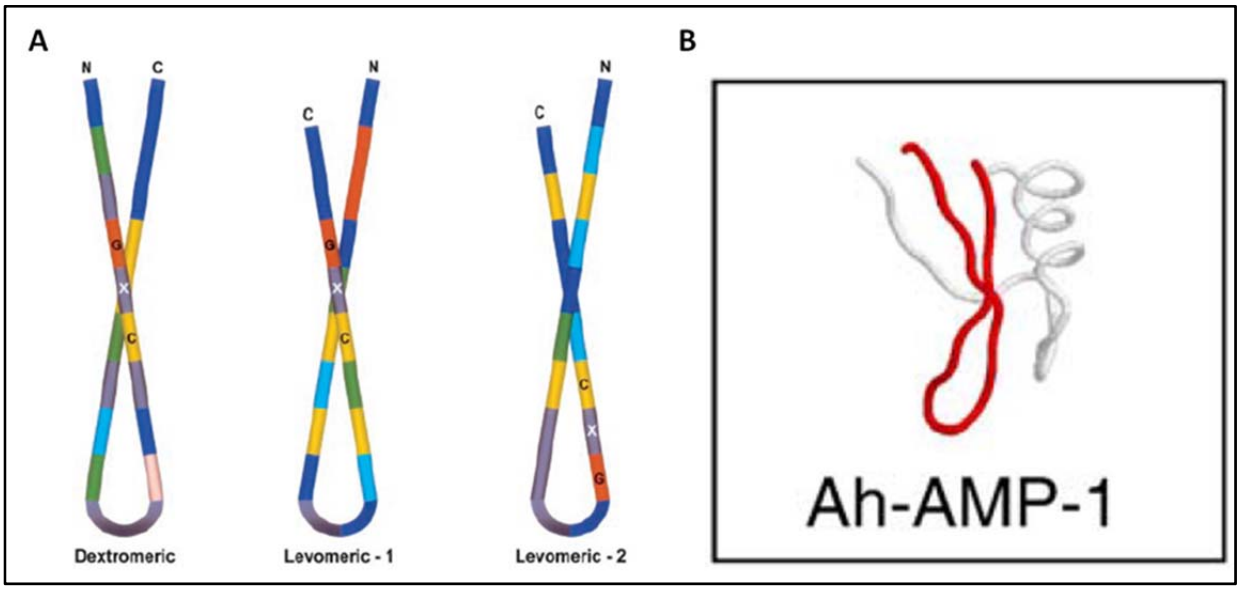

Figure 2.3. (A) Three dimensional representation of the three γ-core motif sequence isoforms. The conserved cysteine (C) residues are indicated in yellow and glycine (G) in orange (Yount and Yeaman, 2004). (B) The three-dimensional conformation of the chestnut antifungal peptide, Ah-AMP1 with the conserved γ-core motif indicated in red (Adapted from Yount and Yeaman, 2006).

Interestingly, Schaaper *et al.* (2001) investigated the optimal length of the loop sequence, $\beta_2\beta_3$/γ-core of RsAFP2 by synthesizing different linear and cyclic peptides with increasing rigidity. This

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was achieved by the introduction of one or two cysteine bridges and then evaluated their antifungal activities. The vital role of N-terminal positively charged amino acids and C-terminus hydrophobic amino acids in the antifungal activity of the loop peptides were confirmed in this study. The addition of His^{43} and Tyr^{48} in the C-terminus and the addition of His^{33} and Arg^{32} amino acid residues at the N-terminus both lead to an increase in the antifungal activity of the loop peptides. More importantly this study emphasises the important role of the $\beta_2\beta_3$loop in the antifungal activity of the 51-residue RsAFP2 as this peptide, activity could be mimicked by a 19-residue peptide derived from this loop (Schaaper *et al*., 2001).

Sagaram *et al.* (2011) characterized the role of the γ-core motif in the antifungal activity of MsDef1, a plant defensin isolated from the seed of *M. sativa* and MtDef4, a plant defensin isolated from the model legume *M. truncatula*. The three-dimensional structures of these two plant defensins are depicted in Figure 2.4. Both these peptides contain the γ-core motif encompassing the β2-β3 loops of their homology predicted three-dimensional structures. During the amino acid sequence analysis of MsDef1 and MtDef4 another motif was discovered also containing the GXC sequence. Sagaram *et al*. (2011) named this sequence as the α-core motif and it contains a consensus sequence of Gly-X-Cys(X_{3-5})Cys. The three dimensional structures of MsDef1 and MtDef4 are depicted in Figure 2.4 and the positions of the γ-core and α-core motifs are indicated. Sagaram *et al.* (2011) replaced the γ-core motif of MsDef1 with that of MtDef4 in order to determine what the effect of this substitution would be on the biological activity of MsDef1 on *F. graminearum* and in effect the role of the γ-core motif in the antimicrobial activity of plant defensin peptides. This newly synthesized peptide was named MsDef1-γ4 and contained a net positive charge of +7 that was significantly higher than that of the original MsDef1 with a net charge of +3. MsDef1-γ4 was found to have a high growth inhibitory activity against *F. graminearum* at concentrations as low as 0.375 μM. Compared to MsDef1, MsDef1-γ4 showed significantly higher antifungal activity, partially comparable to that of MtDef4. The native MsDef1 causes a morphological effect on the hyphae of *F. graminearum*, namely hyper-branching, however this effect was not observed in the presence of the MsDef-γ4 variant. Therefore, the substitution of the γ-core leads to the enhancement of the antifungal activity of MsDef1. Furthermore, this substitution also lead to the transition of the morphogenicity of the antifungal activity of MsDef1 from morphogenic to non-morphogenic and highlighted the vital role of the γ-core motif in antifungal activity. Furthermore, MsDef1 was shown to be inactive against a mutant strain of *F. graminearum*, ΔFggcs1. This strain does not contain the sphingolipid, glugosylceramide (GlcCer) and was sensitive to MtDef4 and MsDef1-γ4, indicating the important role of the γ-core motif of MsDef1 during its interaction with GlcCer. Moreover, the γ-core motif of MsDef1 is directly or indirectly involved in the interaction with GlcCer. To further investigate the contribution of the γ-core motif to the antifungal activity of MsDef1 and MtDef4, Sagaram *et al.* (2011) synthesized and tested the antifungal activity of two peptides, GMA1-C and GMA4-C, which only consisted of the γ-core motifs

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of MsDef1 and MtDef4 and six carboxy-terminal amino acids of the respective peptides. Both these synthesized peptides exhibited strong antifungal activity; but it was less potent than the native defensin peptides. Furthermore, GMA1 failed to cause hyper-branching of the fungal hyphae, which is characteristic to the antifungal activity of MsDef1. The γ-core motifs, GMA1 and GMA4 of MsDef1 and MtDef4 respectively, were subsequently synthesized and tested for antifungal activity. Although GMA4 was found to have antifungal activity, it was less potent compared to that of the native MtDef4, whereas GMA1 showed no antifungal activity. Together these results indicate that the γ-core motif plays in vital role in the antifungal activity of defensin peptides. However, this motif alone is not sufficient to support the antifungal activity. The determinants of the antifungal activity therefore reside both inside and outside the γ-core motif (Sagaram *et al*., 2011).

The four plant defensin peptides that have been isolated from *Heliophila coronopifolia,* a native South African *Brassicaceae* species; differ specifically with regards to their $L\beta_2\beta_3$ areas. Homology modelling showed that these changes had an effect on the surface properties of the defensins which might explain, at least partly, their differential activities (De Beer and Vivier, 2011).

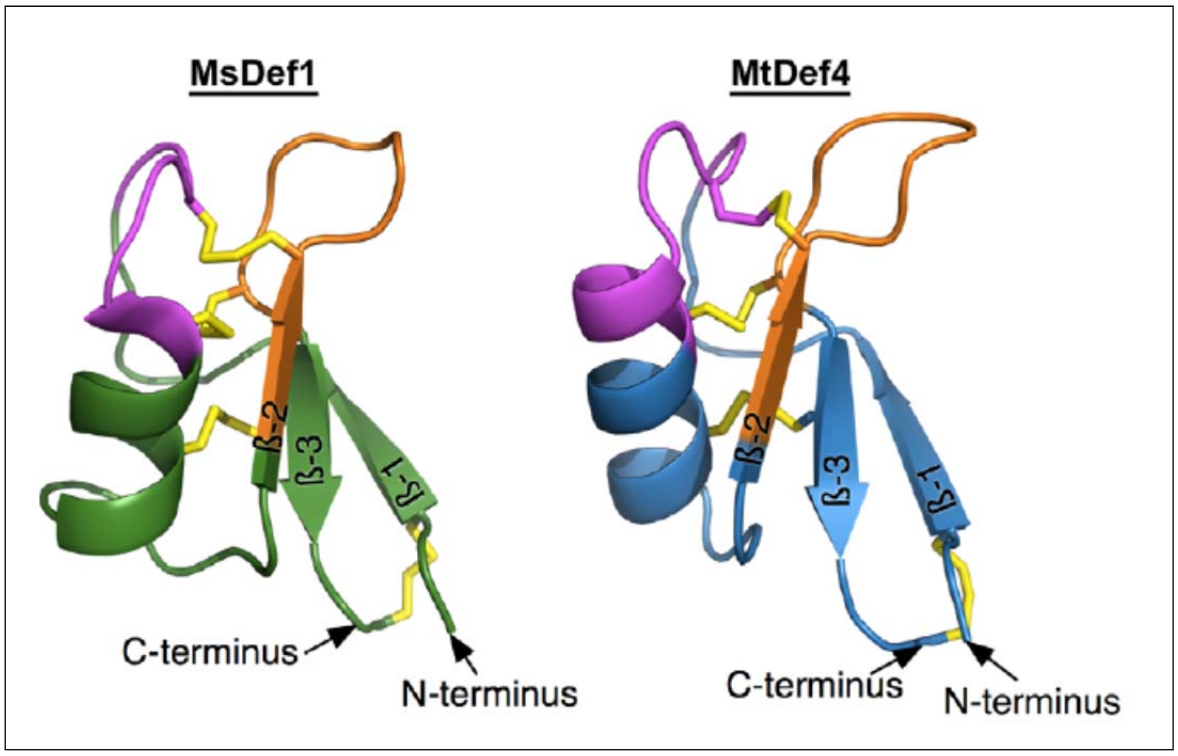

Figure 2.4. Three-dimensional homology-based models of MsDef1 and MtDef4. The γ-core motif is indicated in orange; the α-core motif is indicated in purple and the four disulphide bridges are indicated in yellow (Lay *et al*., 2011).

Several other structure-function analyses have been performed in order to investigate the roles of the highly conserved motifs in the function of plant defensin peptides.

Spelbrink *et al.* (2004), investigated the importance of certain regions in a plant defensin isolated from *M. sativa* (MsDef1) for antifungal activity as well as the hyper-branching morphogenic effect, characteristic to the antifungal activity of MsDef1. This was done by dividing MsDef1 and

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MtDef2 (isolated from *M. truncatula*) into three regions of similar length and combining these regions in all six possible combinations to create chimeric proteins named Def1-2C1 to Def1-2C6 (Figure 2.5). The antifungal activities of these chimeric proteins were assessed against *F. graminearum* in terms of growth inhibition as well as the degree of hyper-branching induced (Spelbrink *et al*., 2004). This analysis revealed that the C-terminal sequence (amino acid residues 31-45) of MsDef1 contains vital determinants for the antifungal activity of this peptide. It was observed that the presence of the C-terminal determinants were required for the antifungal activity of MsDef1, since the chimeric proteins lacking the MsDef1 C-terminal region (Def1-2C1 and Def1-2C6) failed to cause growth inhibition as well as hyper-branching of the test fungus. Furthermore, the chimeric protein Def1-2C2, containing the MsDef1 C-terminal sequence, displayed antifungal activity comparable to that of MsDef1 whereas Def1-2C3, containing the C-terminal of MtDef2, was inactive against the test pathogen. Interestingly, the N-terminal sequence (amino acid residues 1-15) of MsDef1 seems to play a role in the antifungal activity of this peptide. In addition, the presence of both N- and C-termini were required to reach the same degree of antifungal activity as the original peptide.

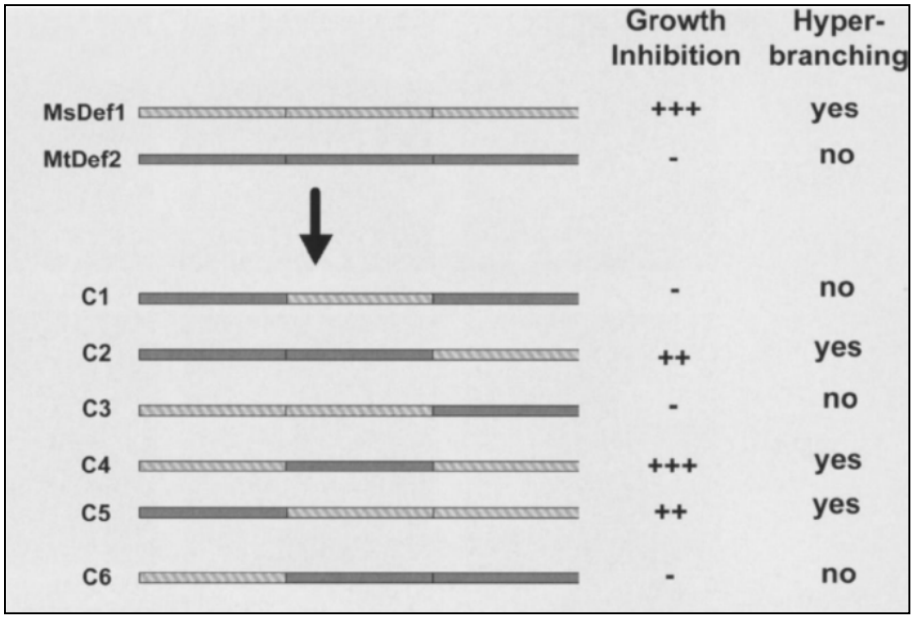

Figure 2.5 Six chimeric protein created from, MsDef1 and MtDef2 and their corresponding antifungal activity against *F. graminearum* in terms of growth inhibition and hyper-branching are shown (Spelbrink et al., 2004). The six chimeric proteins were created by dividing MsDef1 and MtDef2 into three regions of similar length and combining these regions in all six possible combinations, termed Def1-2C1 to Def1-2C6 (Spelbrink *et al*., 2004).

Spelbrink *et al.* (2004) showed that Arg^{38} plays a major role in the antifungal activity of MsDef1. This was revealed by site-directed mutagenesis of Arg^{38}. This specific residue was chosen for site-directed mutagenesis for the following reasons: This residue is located in a motif of MsDef1 that shows sequence homology to the active site of KP4, a known ion channel blocker, as well as the scorpion toxin AaHII. These proteins both have a basic residue at the base of the loop between β2

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and β3 that has been reported to play a critical role in antifungal activity. Moreover, Arg^{38} is replaced by a Gln in MtDef2, which might explain the difference in their antifungal activities. A single amino acid substitution was performed in both MsDef1 (R38Q) and MtDef2 (Q39R) and the antifungal activities of these mutated peptides revealed that Def2-Q39R was significantly more potent compared to the MtDef2 and had activity almost identical to that of MsDef1. In contrast the substitution of an Arg^{38} with Gln^{38} resulted in a significant decrease in the antifungal activity compared to MsDef1. This antifungal activity of Def1-R38Q was comparable to that of MtDef2. This emphasises the important role of this specific amino acid residue (Arg^{38}) in the antifungal activity of MsDef1 and this residue might play a role in the target recognition of this peptide (Spelbrink *et al*., 2004).

A structure-activity analysis of RsAFP2 was performed in order to identify the possible amino acid residues involved in the antifungal activity of this peptide. Two series of RsAFP2 analogues were produced by mutational analysis. The first series was produced mutating specific amino acids corresponding to amino acid residues of plant defensin SIα2 that does not present antifungal activity. These mutations revealed the importance of several amino acids that play a role in antifungal activity, including Gly^{9}, Thr^{10}, Ser^{12}, Leu^{28}, Ala^{31}, Tyr^{38}, Val^{39}, Phe^{40}, Pro^{41}, Ala^{42}, Lys^{44}, Ile^{46} and Phe^{49}. The second series was produced by substituting specific amino acid residues by an Arg at several non conserved positions. This was done since RsAFP2 has a higher net positive charge than RsAFP1, but also a 2-30-fold higher activity compared to RsAFP1. These results revealed that the increasing of the overall charge, especially at positions 9 and 39, resulted in the increase of the antifungal activity of Rs-AFP2. Furthermore, De Samblanx *et al.* (1997) discovered two adjacent sites important for antifungal activity. The first one is the region around the type VI β-turn that connects β-strands 2 and 3. This consists of amino acids 38, 40-42, and 46. The second site is the region in the loop that connects β-strand 1 with the α-helix (amino acids 10 and 12). Furthermore, this also includes residues on the α-helix and β-strand 3 (amino acids 28 and 49 respectively) (De Samblanx *et al*., 1997). These two sites have proven to be important for antimicrobial activity of RsAFP2 and might represent two sites important in the binding of the single binding site located on the pathogen (De Samblanx *et al*., 1997, Thomma *et al*., 2003).

These examples highlight the proven importance of the conserved structures of defensins and also highlight the variable activities of closely related peptides that are often explained by specific amino acid changes in the sequences.

2.3 Expression and localization pattern of plant defensins

Plant defensins have been isolated from a wide range of plant species and plant organs, including seeds, leaves, pods, flowers and tubers. The preferential location of plant defensins reveal the vital role they play in the protection of the plant. These peptides are expressed constitutively or can be induced upon fungal infection or mechanical wounding (Broekaert *et al*., 1997)

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The special expression patterns of the plant defensin NaD1 revealed that this defensin was detected in anthers, pistils, ovaries and petals from ornamental tobacco flowers. NaD1 expression was highest in young floral buds and decreased significantly as the flower matures. However, the expression level of this defensin peptide was barely detectable in the roots and was not detectable in the leaves. It is noteworthy that this peptide was expressed in the outermost layers of the sepals and petals and in tissues that surround the pollen or pollen tubes. The location of NaD1 is consistent with its defence role as it protects the germ cells against possible damage by invading pathogens (Lay *et al*., 2003b). Similarly, defensin peptides isolated from the seed of pea (*P. sativum*), was shown to be localized primarily in vascular bundles and epidermal tissues of pea pods, which are the first barriers to pathogen invasion (Almeida *et al*., 2000).

Terras *et al.* (1998) analyzed the effect of fungal infection, wounding and the treatment with various chemicals on the accumulation of plant defensin transcripts and peptides. The chemicals tested included ethylene, mercuric chloride, methyl jasmonic acid (MeJA), salicylic acid, 2,6-dichloroisonicotinic acid, γ-resorcylic acid, aminobutryric acid isomers, the herbicide paraquat and nicotinamide. Of all these chemicals tested, only mercuric chloride, MeJA, ethylene and paraquat led to the induction of defensin gene expression. The effect of fungal infection on the accumulation of defensin transcripts and peptide concentration was investigated by infecting radish plants with *Alternaria brassicicola.* This revealed that defensins accumulated in the areas immediately around the inoculation sites of infected leaves. Furthermore, the defensin levels of the rest of the infected plant, as well as the control leaves of the same infected plant, were significantly higher than that of the mock-inoculated leaves. Furthermore, the plant defensin distribution in infected leaves changed over time. The defensins were detected at the site of infection for the first three days after inoculation, but after four days peptides started to accumulate in the surrounding areas. These observations further highlighted the vital role these plant defensins play in the defence of plants against invading pathogens. Terras *et al.* (1998) explained these results by suggesting that the plant defensins are produced in higher concentrations at the site of infection where they are most needed to arrest the fungal infection. Moreover, in the surrounding tissue and rest of the plant they are produced in more moderate levels to prevent further infections (Terras *et al*., 1998).

Plant defensins play a very important role in the protection of seed and seedlings from soil borne pathogens. This was demonstrated by Terras *et al*. (1995) where radish seeds, germinating on a medium supporting fungal growth, formed an inhibition halo around the seed. This effect could be mimicked by the application of 1 μg mL^{-1} of RsAFP1 or RsAFP2 in a plate assay. Interestingly, the growth inhibition halo was produced only after the seed coat was disrupted by germination or mechanical wounding. It was subsequently showed that 30% of the proteins released from seeds where the seed coats were mechanically damaged, were RsAFPs. Investigation of the location of these plant defensins within the seed revealed that they are located in high levels in the outer cell layers and in the spaces between different seed organs (Figure 2.6). More specifically, these

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peptides were located in the middle lamellae of the cell walls of the different seed tissues. Like the other defensins mentioned previously, the location of RsAFPs in seeds are at positions where the first contacts with invading fungal pathogens occur.

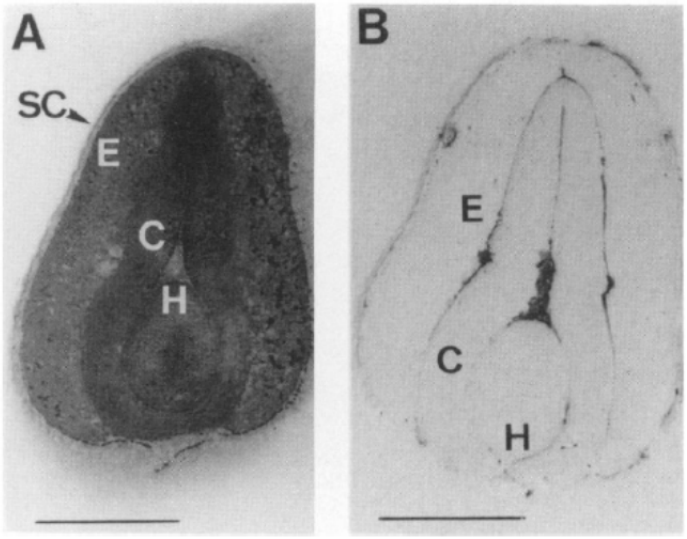

Figure 2.6. Tissue print immunolocalization microscopy of seed RsAFPs. (A) Amido black protein stain for the detection of the total protein. (B) Immunolocalization section performed with anti-RsAFPs (C, Cotyledon; E, endosperm; H, hypocotyls; SC, seed coat (taken from Terras *et al*., 1995).

2.4 Biological activities of plant defensins

Plant defensins are a very diverse group of peptides and possess a wide range of biological activities in addition to their widely documented antimicrobial role (Refer to Table 2.1 for a summary).

Table 2.1 Pertinent examples of different biological activities attributed to plant defensins.

Biological activity	Examples	Plant source	Reference
Antifungal	RsAFP1-4	Raphanus sativus	Terras *et al*., 1992; Terras *et al*., 1995
	HcAFP1	Heuchera sanguine	Osborn *et al*., 1995; Terras *et al*., 1992
	DmAMP1	Dahlia merckii	Osborn *et al*., 1995; Terras *et al*., 1992
	AhAMP1	Asculus hippocastanum	Osborn *et al*., 1995; Terras *et al*., 1992
	CtAMP1	Clitoria ternatea	Osborn *et al*., 1995; Terras *et al*., 1992
	HsAMP1	Heuchera sanguine	Osborn *et al*., 1995;

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Table 2.1 (cont.)

Biological activity	Examples	Plant source	Reference
Antibacterial	DmAMP1	Dahlia merckii	Osborn *et al*., 1995; Terras *et al*., 1992
	AhAMP1	Asculus hippocastanum	Osborn *et al*., 1995; Terras *et al*., 1992
	CtAMP1	Clitoria ternatea	Osborn *et al*., 1995; Terras *et al*., 1992
Insecticidal	VrCRP	Vigna radiata	Cheng *et al*., 2002
α-Amylase inhibitor	VuD1	Vigna unguiculata	Pelegrini *et al*., 2008
	SIα1-3	Sorghum bicolour	Bloch and Richardson, 1991
	VrD1	Vigna radiata	Lin *et al*., 2007
Protease inhibitor	5495 Da plant defensin	Crassia fistula	Wijaya *et al*., 2000
Ion channel blockers	γ1- and γ2-zeothionins	Zea mays	Kushmerick *et al*., 1998
	MsDef1	Medicago sativa	Ramamoorthy *et al*., 2007
Protein translation inhibitor	γ-hordothionin	Hordem vulgare	Mendes *et al*., 1990, Mendes *et al*., 1996 and Carvalho and Gomes, 2009
	ω-hordothionin		

2.4.1 Plant defensins as enzyme inhibitors

Some plant defensins has the ability to inhibit α-amylase enzymes of the insect gut. This α-amylases-inhibitor activity protects the plant against some seed and plant feeding insects (Broekaert *et al*., 1995). This is supported by the observation that VrCRP, a mung bean defensin, was lethal to the larvae of the bruchid, *Callosobruchus chinensis* (Chen *et al*., 2002), Similarly, three peptides from sorghum (*Sorghum bicolour*), SIα1, SIα2 and SIα3, which share high sequence similarities with the γ-purothionins isolated form wheat endosperm (Bloch and Richardson, 1991), have strong inhibitory action against insect α-amylases, but weak inhibition of α-amylases from fungal and human saliva origin and no activity against α-amylases form porcine pancreas, barley and bacteria. Another example is *Vigna radiata* plant defensin 1 (VrD1), that has also been shown to inhibit *Tenebrio molitor* α-amylase (TMA). This is thought to explain the previously reported *in vitro* insect-resistant activity against bruchids (Chen *et al*., 2002; Chen *et al*., 2004; Chen *et al*., 2005; Liu *et al*., 2006).

When the possible binding of plant defensins with insect α-amylases was investigated, it was shown that the loop between β2 and β3 was the probable docking area to the active site of the α-amylase enzyme. By comparing the structures of VrD1 with defensins that lack α-amylase inhibition activity, it was demonstrated that VrD1 contains a longer loop between the β2 and β3-

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sheets, facilitating the binding. Peptides with shorter loops are thus incapable to bind the active site of the α-amylase enzyme (Liu *et al*., 2006). Moreover, by comparing the three-dimensional structures of VrD1 and VrD2 (*V. radiate* plant defensin 2), the latter lacking the α-amylase inhibitory activity, it was obvious that although these two peptides share little sequence identity, they have similar global structures. It was observed that the three-dimensional structures of VrD1 and VrD2 differ in the loop connecting the β1-sheet to the α-helix and the loop that connects the β2 and β3-sheets. VrD2 does not contain the 3^{10} helix. Furthermore, the loop of VrD2 that connects the β2 and β3-sheets is one residue shorter than that of VrD1. The conserved positively charged Arg residue resides at position 40 in VrD2, whereas this residue is at position 38 in VrD1. To investigate whether these structural differences might explain the lack of α-amylase inhibitory activity in VrD2, the structural loop of VrD1 was used to replace the corresponding loop of VrD2. Interestingly, this domain swapping led to the recombinant VrD2 to have TMA inhibitory activity higher than that of VrD1. This study once again revealed the importance of structural features such as the length of the loop that connects the β2 and β3-sheets, as well as the electrostatic distribution in the α-amylase inhibitory activity of plant defensins (Lin *et al*., 2007).

Another defensin with α-amylase inhibitory activity is the defensin from cowpea (*Vigna unguiculata*), *Vigna unguiculata* defensin 1 (VuD1). This plant defensin was found to inhibit the α-amylases form gut extracts of the pest insects *Acanthoscelides obtectus* (been weevil) and *Zabrotes subfasciatus* (Mexican bean weevil). VuD1, however, showed low inhibitory activity towards the mammalian α-amylases from porcine pancreas and human saliva. Molecular modelling revealed that the N-terminal end of VuD1 plays a vital role in the binding of the active site of *Z. subfasciatus* α-amylase (ZAR) enzyme. It was demonstrated that the cationic Lys^{1} from VuD1 interacts with the Asp^{204} located in the active site of ZAR (Pelegrini *et al*., 2008).

Some plant defensins have also been shown to contain protease inhibitor activity. One such plant defensin is the 5495 Da plant defensin isolated from the seeds of *Crassia fistula.* This defensin peptide was shown to inhibit trypsin with an IC_{50} of 2 μM. This was the first report of a plant defensin to posses protease inhibitor activity. Another defensin peptide (5144 Da) was isolated from *C. fistula* but this defensin did not contain any trypsin inhibitor activity. When the sequences of these two *C. fistula* isolated defensins were compared, the 5144 Da defensin was found to contain only 44% sequence identity to the 5459 Da defensin. The investigation of the three-dimensional structures revealed that the Lys at position 25 of the 5459 Da defensin is located within the α-helical domain. The change of the Thr^{25} to Lys^{25} in the 5495 Da defensin is believed to be the critical for trypsin inhibitory activity. *C. fistula* is known for its insecticide properties and it is suggested that this trypsin-inhibitory plant defensin plays a role in the defence of the plant against insects (Wijaya *et al*., 2000).

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These enzyme inhibitory activities of plant defensins form part of their defence mechanism and resistance against insects and this further highlighted the important role plant defensins play in the protection of plants against invading pests and pathogens (Liu *et al*., 2006).

2.4.2. Plant defensins as ion channel blockers

The first evidence of a plant protein that blocks the sodium channel in animal cells was obtained from a study by Kushmerick *et al.* (1998). They investigated the effect of γ1 and γ2-zeothionins, plant defensin peptides isolated form maize (*Zea mays*), on voltage-gated ion channels in the GH3 cell line for American Type Culture Collection (ATCC). A dose response curve of the inhibition of sodium currents by γ1 and γ2-zeothionins revealed IC_{50} values of 62 and 33 μM, respectively. The authors suggested that these γ-zeathionins block the conductance of the sodium channel since no effects were observed on the voltage dependence of inactivation or steady-state inactivation that might explain the inhibition. Furthermore, they block the conductance of the sodium channel by reducing the open channel conductance or by decreasing the amount of channels that open during a voltage step (Kushmerick *et al*., 1998).

Spelbrink *et al.* (2004) showed that MsDef1 blocks the mammalian L-type Ca^{2+} channel. This peptide was able to block up to 90% of the Ca^{2+} channel. Maximum inhibition of the Ca^{2+} channel occurred after 13 minutes of exposure of the cells to the defensin. RsAFP2 that shares a three-dimensional structure with MsDef1, did not present any ion-channel blocking activity. Furthermore, MtDef2, which is closely related to MtDef1, also did not present any ion-channel blocking activity. It is suggested that the Ca^{2+} ion-channel blocking activity of MsDef1 may be attributed to the structural homology it shares with the known ion-channel blocker KP4. It is suggested that MsDef1 acts via the disruption of a Ca^{2+} gradient, as Ca^{2+} is one of the major signalling molecules that plays an important role in the life cycle of fungi. Furthermore, the disruption of the Ca^{2+} gradients is known to cause hyper-branching in fungal hyphae. This is consistent with the biological activity of MsDef1 (Spelbrink *et al*., 2004).

2.4.3. Plant defensins as protein translation inhibitors

Two peptides isolated for the endosperm of barley, namely γ-hordothionin and ω-hordothionin, were demonstrated to inhibit protein synthesis in a variety of translation systems (Mendes *et al*., 1990, Mendes *et al*., 1996 and Carvalho and Gomes, 2009). Mendes and colleagues assayed the effect of ω-hordothionin on protein synthesis in several cell-free systems derived from mammals (rat liver), bacteria (*E. coli*) monocotyledonous and dicotyledonous plants and compared it to that of γ–hordothionin. The latter inhibit protein synthesis in rat liver, and in *Triticum aestivum*, *Cucumis sativus*, *Vicea sativa* and *Hordeum vulgare.* Compared to γ-hordothionin, ω-hordothionin did not cause any inhibition of protein translation in plant systems. However, it was found to strongly inhibit the protein translation in rat liver, rabbit reticulocytes and bacteria (Mendes *et al*., 1996). The exact

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mechanism of how these plant defensins inhibit the protein translation activity in cell-free systems has not been fully examined. However, Mendez *et al* (1990) has reported that γ-hordothionin inhibits the primary polypeptide chain initiation. Furthermore, this activity is partially dependent on the presence of the disulphide bonds in γ-hordothionin. When disulphide bonds were disrupted, a decrease in translation inhibition of the native γ-hordothionin was observed. Moreover, the authors reported that at high concentrations, this defensin peptide induces an increased phosphorylation of the α-subunit of eukaryotic polypeptide chain initiation factor 2 (eIF-2α). This most likely occurs via the activation of eIF-2α kinase in the hemin-supplemented reticulocyte lysates. It was further suggested that this activation may be caused by the oxidation of sulphydryl groups involved in the activation of the heme-controlled translation inhibitor (HCI). These results suggest that these peptides are exerting their action at numerous steps in the translation process (Mendez *et al*., 1990). VaD1, (*V. radiate* plant defensin 1) has been demonstrated to inhibit protein translation in a cell-free system derived from wheat germ (Chen *et al*., 2004). The biological significance of this translation-inhibiting activity still remains to be determined.

2.4.4 Activity of plant defensins against mammalian cells

To date, none of the plant defensins have been shown to cause any harmful effects on cultured human cells (Broekaert *et al*., 1995). All the peptides isolated from radish were tested against human umbilical vein endothelial cells and skin-muscle fibroblasts. None of these cell types showed a decrease in cell viability in the presence of the peptides, even up to a concentration of 500 μg mL^{-1} (Terras *et al*., 1992). Furthermore, Tavares *et al* (2008) tested the ability of RsAFP2 to release lactate dehydrogenase (LDH) from the human brain endothelial cells strain U87 and in effect its toxicity. RsAFP2 was unable to induce the release of LDH, in other words cause cell damage. Similarly, defensin peptide NaD1was found to be non-toxic to mammalian HeLa cells (Van der Weerden *et al*., 2008).

2.4.5 Plant defensins as microbial inhibitors

The antimicrobial activity of plant defensins is mostly linked to activity against fungi (Osborn *et al*, 1995, Terras *et al*, 1992, Terras *et al*, 1993; Broekaert *et al*., 1995), however, some has been reported to be active against some bacteria, especially Gram positive bacteria (Osborn *et al*., 1995; Carvalho and Gomes, 2000; Lay and Anderson, 2005, and Stotz *et al*., 2009). DmAMP1, isolated form *D. merckii,* CtAMP1, isolated form *C. ternatea* and AhAMP1, isolated form *A. hippocastanum* were found to inhibit the growth of *Bacillus subtilus* at peptide concentrations of 150, 15 and 100 μg/ml, respectively (Osborn *et al*., 1995). RsAFP2, isolated form *R. sativus*, inhibited the growth of *B. megaterium* at a peptide concentration of 200 μg mL^{-1} (Terras *et al*., 1992). The antibacterial activity of plant defensins is, however, very modest compared to the antifungal activity of these peptides.

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Plant defensin peptides have also shown to be active against *Saccharomyces cerevisiae* (bakers yeats) and the human pathogenic fungi, *Candida albicans* (Lay and Anderson, 2005; Tavares *et al*., 2008; Thevissen *et al*., 2012). Interestingly, the plant defensin RsAFP2 was shown to control candidiasis *in vivo* in mice and was at least as effective as the frequently used drug fluconazole (Travares *et al*., 2008). Furthermore, PTH1, a defensin peptide isolated from potato (*Solanum tuberosum*) killed *Leishmania donovani*, the protozoan parasite, responsible for causing human visceral Leishmaniasis (Berrocal-Lobo *et al*., 2009).

The best characterized biological activity of plant defensins are their ability to cause the growth inhibition of a broad range of fungi. The two radish seed (*R. sativus*) plant defensins, RsAFP1 and RsAFP2 were the first plant defensin peptides shown to possess antifungal activity (Terras *et al.*, 1993). Numerous studies have reported the ability of a variety of different plant defensins to inhibit a broad range of fungi, with varying potencies (Terras *et al*, 1992, Terras *et al*, 1993; Osborn *et al*, 1995; Broekaert *et al*., 1995; Aerts *et al*., 2008; Carvalho and Ghomes 2009; Stots *et al*., 2009; Wilmes *et al*, 2011).

According to the effects of the peptides on hyphal morphology, two groups of antifungal plant defensins can be distinguished, the morphogenic plant defensins and non-morphogenic plant defensins. The morphogenic plant defensins cause growth inhibition with distinct morphological changes on the treated hyphae, including multiple budding and swelling of germ tubes and hyper-branching. This group includes plant defensins from *Brassicaceae,* such as RsAFP1 and RsAFP2; and from *Saxifragaceae spp*, including HsAFP1 from *H. sanguine*. In contrast, the non-morphogenic plant defensins cause growth inhibition without the induction of visible morphogenic effects on treated hyphae. This group includes plant defensins from the *Asteraceae*, including DmAMP1 from *D. merckii*, AhAMP1 from *A. hippocastanum* and CtAmp1 from *C. ternatea*. Interestingly, these two different groups possess differential sensitivity towards fungal targets (Terras *et al.*, 1992; Osborn *et al.*, 1995).

2.5. Mode of action of antifungal plant defensins

Plant defensins possess potent antifungal activity against a wide variety of fungal species. However, for most of these plant defensins the molecular components involved in signalling and possible intracellular targets involved in this process, as well as the underlying mechanism of this potent antifungal activity remains unknown. Several common observations regarding their possible antifungal action has been made.

The antimicrobial activity of plant defensins is strongly antagonized by the addition of salts to the medium (Ameilda *et al*., 2000; Osborn *et al.*, 1995, Terras *et al*., 1992 and Terras *et al*., 1993). This sensitivity of defensins towards cations was illustrated in a study by Osborn *et al.* (1995) where five plant defensins were isolated from the seeds of *Aesculus hippocastanum* (Hippocastanaceae), *C. ternatea* (Fabaceae), *D. merckii* (Asteraceae) and *H. sanguine*

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(Saxifragaceae) and characterized (Table 2.2). In contrast to the antifungal activities of Hs-AFP 1 and Rs-AFP2 on *N. crassa* that were unaffected by 1 or 5 mM Ca^{2+} or Mg^{2+}, all of the other peptides showed reduced antifungal activity in the presence of Ca^{2+} or Mg^{2+} in the media (Osborn *et al.,* 1995) (Table 2.2).

Table 2.2 Effect of different divalent cations on the antifungal activity on *Fusarium culmorum* and *Neurospora crassa* of five plant defensins isolated from the seeds of *Aesculus hippocastanum* (Hippocastanaceae), *Clitoria ternatea* (Fabaceae), *Dahilia merckii* (Asteraceae) and *Heuchera sanguine* (Saxifragaceae) (taken form Osborn *et al.,* 1995).

Fungus	Antifungal protein/peptide	IC_{50} values (µg mL^{-1})				
		Reference medium supplemented with				
		Reference medium[a]	1 mM Ca^{2+}	5 mM Ca^{2+}	1 mM Mg^{2+}	5 mM Mg^{2+}
Fusarium culmorum	*Ah*AMP1	0.7	>50	>50	22	>50
	*Ct*AMP1	0.6	20	>40	7	>40
	*Dm*AMP1	1	23	>50	4	11
	*Hs*AMP1	0.9	1.5	20	1	2.5
	*Rs*AFP2	5	5.1	11	5	11
Neurospora crassa	*Ah*AMP1	0.5	3.3	18	0.8	4
	*Ct*AMP1	0.2	0.9	0.9	0.8	1.8
	*Dm*AMP1	1	4.5	22	3.4	9
	*Hs*AMP1	0.8	0.3	0.3	0.6	0.6
	*Rs*AFP2	5	5	5	5	5

[a]IC_{50} values for the peptides were determined on *F. cumarum* and *N. crassa* in a synthetic growth medium (SMF).

The characterization of *R. sativus* defensins (RsAFP1 and 2) revealed that the antifungal activity of RsAFPs is more strongly antagonized by divalent cations than monovalent cations (Terras *et al*., 1992). This antagonistic effect of cations on the antifungal activity appears to vary greatly between the fungal pathogens tested (Terras *et al*., 1992, Osborn *et al.*, 1995). Although the precise mechanism of this antagonistic effect has not been elucidated yet, the current thinking is, based on a proposal by Terres *et al*. (1992), that it is rooted in a electrostatic interaction between the organism and the cations. The possible effect of the cations on the conformation of the peptides as a cause to the antagonism is not favoured in this proposal (Terras *et al*., 1992). Furthermore, it was suggested that the interaction between the peptide and cations may interfere with morphogenic Ca^{2+} signalling, responsible for of hyphal elongation (Robson *et al*., 1991; Silverman-Gavrila and Lew, 2001).

Another observation made about the mode of antifungal action of plant defensins is that they were found to cause increased hyphal membrane permeabilization (Thevissen *et al*, 1996).

Furthermore, two types of permeabilization have been observed as a result of the interaction of plant defensins with fungal membranes, depending on the concentration of peptide and the

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cation composition of the medium. The first type of permeabilization occurs at high doses (10 to 40 µM) of plant defensins. This is a strong permeabilization and can be detected within 30 to 60 min of the addition of the defensin. Moreover, this type of permeabilization is sensitive to the presence of monovalent and divalent cations and is therefore referred to as cation-sensitive membrane permeabilization. This type of permeabilization occurs at plant defensin doses about 10-fold higher than those required for fungal growth inhibition and does not appear to be the major cause of growth inhibition in hyphae treated with plant defensins (Thevissen *et al.,* 1999). It was always thought that permeabilization was the major effect caused by plant defensins, responsible for the growth inhibition observed; however, permeabilization appears to be a secondary effect (Thevissen *et al.,* 1996). The second type of permeabilization occurs at lower plant defensin concentration (0.1 to 1 µM). This is a relatively weak permeabilization and can only be detected after 2 to 4 hours of incubation. Furthermore, this type of permeabilization is only slightly affected by the presence of 50 mM K^+ or 5 mM Mg^{2+} and is therefore referred to as cation-resistant membrane permeabilization. The concentration of defensin at which this cation-resistant membrane permeabilization occurs, significantly correlates with the concentration required to cause growth inhibition. This observation suggests that this effect is linked to the major cause of defensin-induced growth inhibition (Thevissen *et al.*, 1999).

The plant defensins, RsAFP2 and DmAMP1 induce a range of relatively rapid membrane responses in the fungus *N. crassa,* including Ca^{2+} uptake, K^+ efflux, alkalinization of the medium, as well as membrane potential changes (Thevissen *et al*., 1996). How these membrane responses are generated after an interaction with the plant defensin is, however, unknown. Insect and mammalian defensins form ion-permeable, voltage-dependent pores in artificial membranes (Kagan*et al*., 1990; Cociancich *et al*., 1993). Using model planar lipid bilayers, the pore-forming activity of plant defensins on artificial membranes were also assessed and found to neither form ion permeable pores in artificial membranes, nor change electrical potential (Thevissen *et al*., 1996). Thevissen *et al*. (1996) suggested that these membrane responses are either the consequence of the interaction with a membrane target that causes a transduction signal to endogenous membrane ion channels, or it might be the result of the insertion of the plant defensin peptide into the membrane that leads to ion channel formation (Thevissen *et al*., 1996).

Additional research by Thevissen *et al*. (1997) revealed the presence of specific high affinity binding sites for plant defensins. This was achieved by the radioactive labelling of the plant defensin Hs-AFP1 that resulted in a ^{35}S-labeled peptide, [^{35}S]HsAFP1, with unaltered antifungal activity. To assess the specificity of the binding of [^{35}S]HsAFP1 to *N. crassa* hyphae and microsomal membrane preparations, the labelled peptide was incubated with increasing concentrations of the native unlabeled HsAFP1. It was found that the labelled peptide could be competed out in the presence of excess unlabeled HsAFP1. The specificity of this binding was also assessed in the presence of defensins structurally related or unrelated to plant defensins. Some of

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the plant defensins structurally related to [^{35}S]HsAFP1 (such as RsAFP2, AhAMP1, DmAMP1 and CtAMP1), was able to compete for the binding to the hyphae and microsomal membranes. It is interesting to note that this competition was significantly weaker than that of the native unlabeled HsAFP1. However, the peptides that were structurally unrelated to plant defensins, were unsuccessful to displace [^{35}S]HsAFP1 from its binding site. Interestingly, in the presence of 20 mM $MgCl_2$ the binding of [^{35}S]HsAFP1 to *N. crassa* hyphae was reduced by over 95%. Thevissen *et al*., (1997) suggested that a receptor exists to which all the plant defensin peptides bind to, but with different affinities, or these defensin peptides bind to different sub-sites of the receptor (Thevissen *et al*., 1997).

The binding of [^{35}S]HsAFP1 to *N. crassa* hyphae and microsomal membranes was also found to be reversible. In addition, the saturability of the binding of [^{35}S]HsAFP1 to the targets was tested by the incubation of *N. crassa* hyphae and microsomal membranes with increasing concentrations of [^{35}S]HsAFP1. These results demonstrated that this binding was saturable. All these results point to the existence of a specific high affinity binding site for this plant defensin peptide. Interestingly, the dissociation constants obtained for binding of *N. crassa hyphae* and microsomal membranes were very similar, indicating that the binding sites for HsAFP1 was probably located in the plasma membrane (Thevissen *et al*., 1997).

2.5.1. Sphingolipids as targets of plant defensins

Sphingolipids are ubiquitous components of eukaryotic membranes. These lipids, along with sterols and phosphoglycerolipids, are one of the three major types of lipids found in eukaryotic membranes (Thevissen *et al*., 2000). Sphingolipids mediate cell adhesion/recognition, serve as lipid moieties for glycosyl phosphatidyl inositol (GPI)-anchored proteins and play an important role in intracellular vesicle transport, signalling, heat-stress response, Ca^{2+} homeostasis and transport of GPI-anchored proteins (Olsen and Jantzen, 2001; Warnecke and Heinz, 2002). Furthermore, sphingolipids are essential membrane components of yeast and like in higher eukaryotes, sphingolipids are highly enriched in the plasma membrane (Patton *et al*., 1991; Hechtberger *et al.*, 1994; Merrill *et al*., 1996). Sphingolipids contain a backbone of ceramide that is composed of a sphingoid backbone or a long chain base. This base is amide-linked to a fatty acid. Ceramide can contain different polar head groups at carbon atom C1. Depending on the type of head group, sphingolipids are classified in phosphosphingolipids and glycosphingolipids (Reviewed in Merrill *et al*., 1997). The length of the fatty acid-amide linked to the sphingoid determines the type of glycosphingolipid that will be formed, namely inositolphosphoryl-containing sphingolipids or glucosylceramide (GlcCer). Ceramide backbones, containing C_{16} or C_{18} fatty acids bound to sphingobase 9-methyl-4,8-sphingadienine, are only used as precursors for the biosynthesis of glucosylceramide (GlcCer), also known as the neutral lipid fraction (Figure 5), whereas ceramide backbones, containing long chain C_{24} or C_{26} fatty acids bound to the sphingobase 4-

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hydroxysphinganine, are solely precursors for the biosynthesis of inositol phosphoryl-containing sphingolipids, also known as the acidic lipid fraction (Conzelmann *et al*., 1992; Leipelt *et al*., 2001; Warnecke and Heinz, 2002; Thevissen *et al*., 2003). The three major classes of inositol phosphoryl-containing sphingolipids present in the yeast, *S. cerevisiae,* are inositol-p-ceramide (IPC), mannose-linositol-p-ceramide (MIPC) and mannose(inositol-P)$_2$-ceramide (M(IP)$_2$C) (Figure 2.7) (Patton *et al*., 1991; Hechtberger *et al*., 1994). M(IP)$_2$C is the most abundant sphingolipid in *S. cereviseae* (Daum *et al*., 1998).

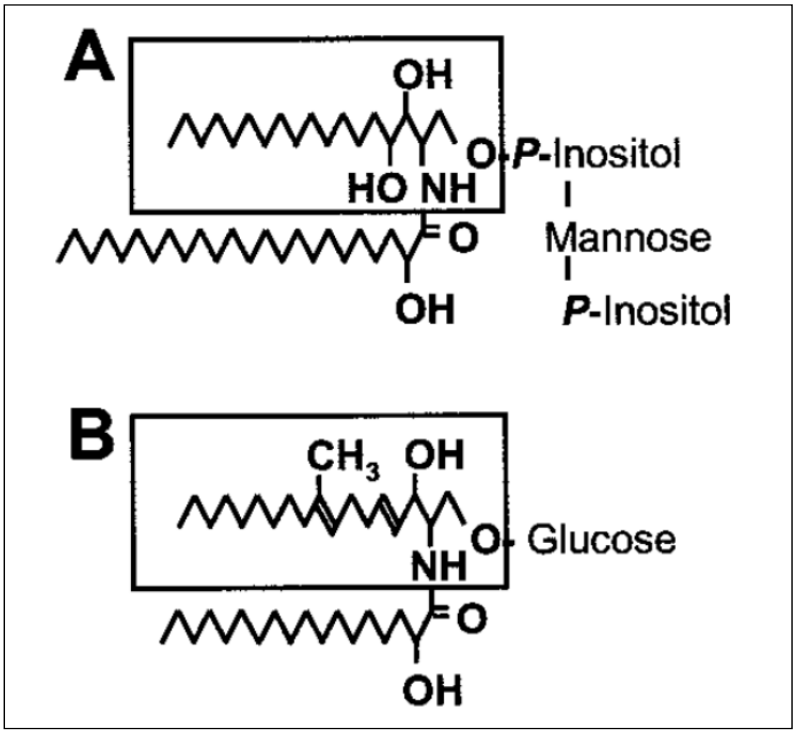

Figure 2.7 Structures of the sphingolipids (A) mannosyldiinositolphosphorylceramide M(IP)$_2$C and (B) glucosylceramide (GlcCer). The sphingobase moiety is boxed. (Thevissen *et al.*, 2003c).

DmAMP1, a plant defensin isolated from the seeds of *Dahlia merckii*, was shown to interact with M(IP)$_2$C (Thevissen *et al*., 2003a). This plant defensin induces an array of relatively rapid membrane responses in fungi (Thevissen *et al*., 1996). Via a genetic complementary approach, the gene determining the sensitivity of *S. cerevisiae* for DmAMP1 was identified as *IPT1*, encoding an enzyme called, inositol phosphotransferase 1 (Thevissen *et al*. 2000; Thevissen *et al*. 2003a). *S. cerevisiae IPT1*-deletion strains were deficient in M(IP)$_2$C, but did contain an increased amount of the precursor MICP (Dickson *et al.*, 1997). Strains containing a non-functional *IPT1* allele lacked M(IP)$_2$C in their plasma membranes. Furthermore, these strains were shown to bind significantly less DmAMP1, compared to the wild-type strains, and were extremely resistant to DmAMP1-mediated membrane permeabilization. However, DmAMP1 sensitivity is linked to the presence of M(IP)$_2$C and not to the presence of a functional *IPT1*-encoding protein (Ipt1p). This was shown when a DM1 yeast strain derivative, containing a deleted *SUR1* gene, was found to be sensitive to DmAMP1 at concentrations above 9 µM. The *SUR1* gene encodes the enzyme that catalyzes the

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conversion of IPC into MIPC. Membranes of a DM1 yeast strain mostly contain the sphingolipid MIPC, whereas the DM1*sur1*-deletion strain contain IPC, but lack both MICP and $M(IP)_2C$. Furthermore, both the DM1 and the deletion strain DM1Δ*sur1* do not contain a functional IPT1 protein and it was concluded that the DmAMP1 resistance has to do with the sphingolipid composition rather than with alterations in the IPT1 structure (Thevissen *et al*., 2000). In addition, DmAMP1-sensitivity of yeast strains is linked to the level of $M(IP)_2C$ present in their membranes. The sphingolipid $M(IP)_2C$ thus plays a crucial role in the DmAMP1 antifungal action (Im *et al*., 2003; Aerts *et al*., 2006).

The binding of DmAMP1 to $M(IP)_2C$ was confirmed by an enzyme-linked immunosorbent assay (ELISA)-based binding assay. DmAMP1 was shown to interact with $M(IP)_2C$ in a dose-dependent manner (Figure 2.8). This interaction between DmAMP1 and $M(IP)_2C$ was enhanced in the presence of equimolar concentrations of the fungal sterol, ergosterol (Thevissen *et al*., 2003a) (Figure 2.8c). This is in accordance with the fact that sphingolipids and sterols are enriched in specific domains in the outer plasma membrane and are called lipid rafts (Martin *et al.*, 2004; Thevissen *et al*., 2005).

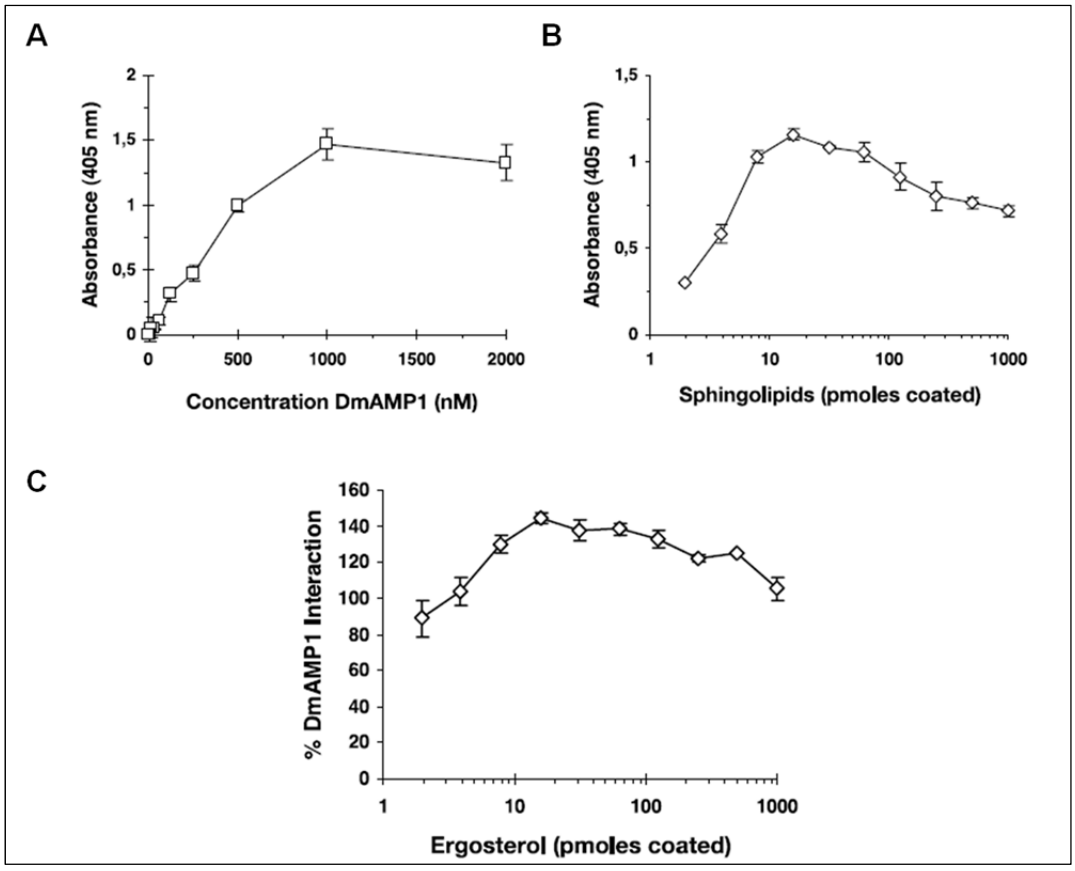

Figure 2.8 Interaction of DmAMP1 with sphingolipids isolated from *S. cerevisiae*. (A) A dose-response curve presenting the interaction of DmAMP1 with 15 pmol coated sphingolipids. (B) A dose-response curve presenting the interaction of 1 µM DmAMP1 with different concentrations of coated sphingolipids. (C) A dose-response curve presents the interaction of 1 µM DmAMP1 with 15 pmol of coated sphingolipids, in the presence of different concentrations of ergosterol (Adopted from Thevissen *et al.*, 2003a).

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Thevissen *et al*. (2003a) subsequently proposed a mode of action for DmAMP1, and defensins in general (Figure 2.9). As mentioned previously, sphingolipids are, together with sterols and phospholipids, one of the three major classes of eukaryotic membrane components. The plasma membrane of fungal cells has an asymmetric nature with phosphoglygolipids mainly present in the inner leaflet, whereas the sphingolipids and sterols are mainly present in the outer leaflet. As mentioned above, sphingolipids and sterols are enriched in specific domains in the outer leaflet of the plasma membrane to form what is known as lipid rafts. The interaction of DmAMP1 with these specific domains could result in high local concentrations of this defensin peptide to a small area on the fungal membrane. This interaction of the plant defensin with the fungal membrane leads to compromised membrane permeability that results in increased Ca^{2+} influx and K^+ efflux (Thevissen *et al*., 2003a and 2003b).

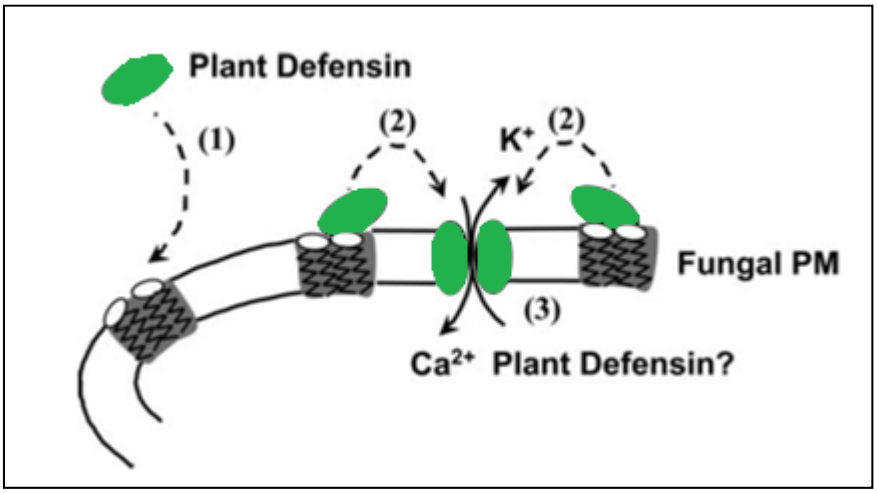

Figure 2.9 Model for the mode of action of plant defensins by Thevissen *et al.* (2003b): (1) Plant defensins, represented in the green ovals; (2) bind to the lipid rafts composed of sphingolipids (represented in grey shapes in the fungal plasma membrane (PM)) that result in the alteration of membrane integrity. (3) Modification of the membrane permeability results in an increased Ca^{2+} uptake and K^+ efflux (Adopted from Thevissen *et al*., 2003b).

Through the screening of a *S. cereviseae* deletion mutant library, Thevissen *et al.* (2005) identified a second putative DmAMP1-sensitivity gene, named *SKN1.* This finding was based on the observation of an 8-fold increase in DmAMP1-resistance for a Δ*skn1* mutant compared to the parental *S. cerevisea* strain. Furthermore, this Δ*skn1* mutant, like the *IPT1*-deletion strain, lacked the sphingolipid, $M(IP)_2I$, in its membranes. The *SKN1 gene* was thought to be involved only in β-1-6-glucan biosynthesis. However, this study identified a novel function of this gene, namely its involvement in the biosynthesis of sphingolipids (Thevissen *et al*., 2005).

This important relationship between sphingolipids and the activity of plant defensins was further confirmed when the membrane components of *N. crassa* mutants (MUT16 and MUT24), resistant to several plant defensin-induced membrane permeabilization, including RsAFP2 and DmAMP1, were analyzed by high performance thin layer chromatography (HPTLC) and compared

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to that of the wild type. The acidic fraction of the lipids of *N. crassa* mutants exhibited a distinct profile compared to that of the wild type. Furthermore, the natural lipid fraction analysis demonstrated that the *N. crassa* mutants contained structurally different GlcCer as well as different levels of steryl β-glycoside (GlcSte). This further emphasized the important role sphingolipids play in the activity of certain plant defensin peptides, as well as the vital role of the specific structure of sphingolipids (Ferket *et al*., 2003).

R. sativus antifungal peptide 2 (RsAFP2) was found to interact in a dose-dependent manner with the sphingolipid, glycosylceramide (GlcCer) of *Pichia pastoris* (Terras *et al.*, 1992; Thevissen *et al.*, 2003c). This interaction was found to cause membrane permeabilization and subsequent cell growth arrest (Thevissen *et al*., 2009). Moreover, this peptide was found to cause growth inhibition of *P. pastoris* and the drug resistant *C. albicans* at concentrations of 1-2 μM and higher, while the corresponding *gcs*-deletion strains were found to be at least 20-fold more resistant to RsAFP2. The gene *GCS* encodes the enzyme UDP-glucose:ceramide glucosyltransferases. This enzyme is responsible for the catalysis of the final step in the biosynthesis of the membrane lipid, glucosylceramide (GlcCer). RsAFP2 contains selective antifungal activity as it does not interact with GlcCer from humans. This interaction of RsAFP2 and GlcCer and the resulting fungal growth inhibition was further supported by the observation that two yeast species, namely *S. cerevisiae* and *Schizosaccharomyces pombe*, which do not contain GlcCer in their membranes are resistant to RsAFP2 activity (Thevissen *et al*., 2004), again emphasizing the important role of GlcCer in the antifungal activity of RsAFP2.

Further evidence was obtained to this effect when RsAFP2 was tested against different *Candida* species and isolates. All the species tested were found to be susceptible to RsAFP2 in a dose dependent manner, except the *C. glabrata* strains. The relationship between susceptibility to RsAFP2 and GlcCer content was investigated and the presence of GlcCer could not be detected in the *C. glabrata* strains (Tavares *et al*., 2008). Furthermore, the levels of GlcCer in three *C. albicans* strains, namely 2A, 78 and 12A, were extracted and analyzed with HPTLC and the amount of GlcCer were then determined. These *C. albicans* strains were found to be highly, moderately and weakly susceptible, respectively, to RsAFP2 (Figure 2.10), again confirming that there is a clear relationship between the GlcCer *content* and level of RsAFP2 susceptibility (Travers *et al*., 2008).

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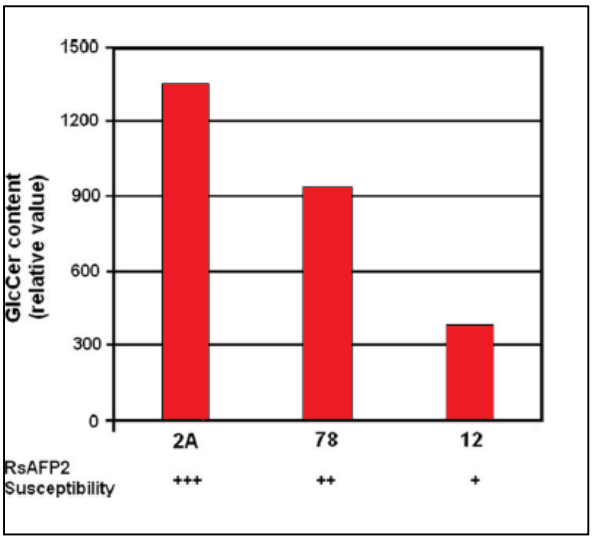

Figure 2.10 Densitometric analysis of extracted lipids analyzed with high-performance thin-layer chromatography of three *C. albicans* strains 2A, 78 and 12. These strains, 2A, 78 and 12 were highly, moderately and weakly susceptible to RsAFP2, respectively (+++ indicates more susceptible; ++, intermediate susceptibility and +, less susceptible). This analysis shows a clear link between the GlcCer and RsAFP2 susceptibility in *C. albicans.* The densometric analysis was performed using Scion image software (Travares *et al*., 2008).

A recent study provided further insight into the interaction of RsAFP2 with GlcCer, as presented by Thevissen *et al.*, (2012). *C. albicans* CAI4 was incubated with RsAFP2 (50 µg mL^{-1}) and an anti-RsAFP2 serum was added. The resistant *C. albicans* Δgcs, *C. glabrata* and *S. cerevisiae* were used as positive controls as they do not contain or produce any GlcCer. This interaction was visualized by immunofluorescence microscopy (Figure 2.11) and clearly showed that RsAFP2 interacted with the cell wall of *C. albicans* CAI4 to exert its potent antifungal activity. In contrast, RsAFP2 did not associate with the cell walls of the resistant controls that do not contain GlcCer. Furthermore, using a quantitative HPLC analysis, the internalization of RsAFP2 in *C. albicans* cells was assessed. Very low levels of intracellular RsAFP2 uptake were detected, indicating that RsAFP2 interacts with the cell membrane and that intracellular uptake of the peptide is not required for this peptide to exert its potent antifungal activity (Thevissen *et al*., 2012).

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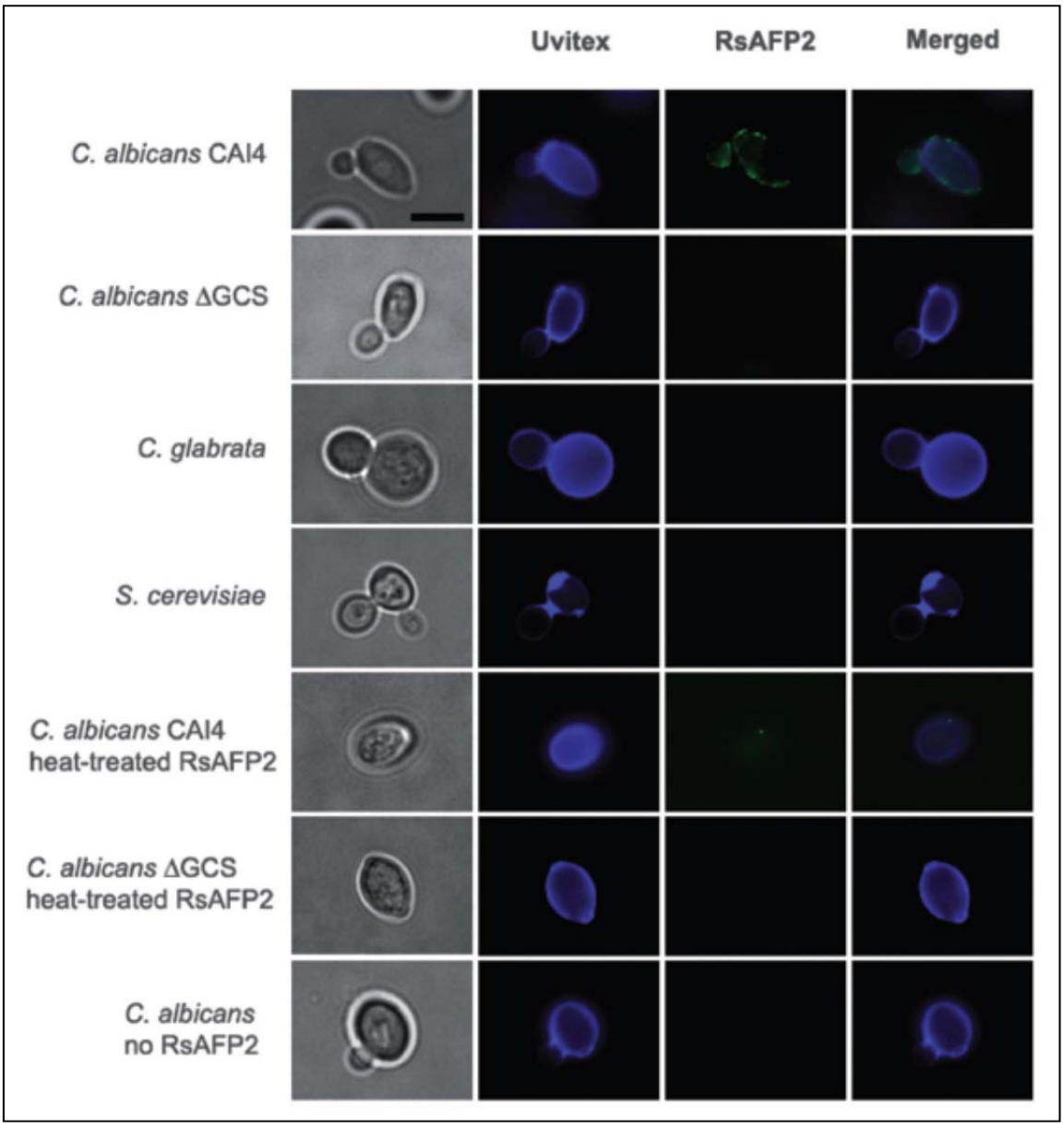

Figure 2.11 Localization of RsAFP2 at the fungal cell surface of *C. albicans* CAI4*, C. albicans* ∆gcs, *C.* glabrata and *S. cerevisiae.* Fungal species were treated with 50 µg mL^{-1} RsAFP2 for 3 hours and followed by Uvitex B2 (blue) and anti-RsAFP2 antibodies and FITC-labelled goat anti-rabbit IgG (green) (Adopted from Thevissen *et al*., 2012).

Interestingly, glucosylceramide was recently shown to be a virulence effector of *C. albicans.* This was done by creating a library of isogenic null mutants and screening the mutants for infectivity in a mouse model, as well as morphological switching and proliferation *in vitro* (Noble *et al*., 2010).

Sphingolipids have become promising, attractive new targets for the development of novel antimycotics, due to the distinctiveness of sphingolipids in mammalian and fungal cells. This will potentially enable the development of selective, non-toxic antifungal drugs. Furthermore, sphingolipids was once thought to be only structural components of eukaryotic membranes; but they are now known to play important roles in cell regulation, cell growth and cell stress responses and as stated above, have recently been found to be important pathogenicity determinants.

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2.5.2. Interaction of plant defensins with intracellular targets after the initial interaction with the cell wall components

Most plant defensins have been shown to act extracellularly of fungal cells, however, recently it has been demonstrated that certain plant defensins enter cells and affect intracellular targets. One of these plant defensins is NaD1 (*N. alata* defensin 1). This peptide is active against various fungal species including, *Leptospheria maculans, V. dahlia, Thielaviopsis basicola* and *Aspergillus nidulans*, but has no effect on the growth of *S. cerevisiae* and *C. albicans* and *P. pastoris*. It was demonstrated, by using immunofluorescence, that NaD1 binds to the cell surface of hyphae treated with this plant defensin (Figure 2.12). This interaction was further investigated to determine if the binding took place to the cell wall or the plasma membrane (Van Weerden *et al*., 2008).

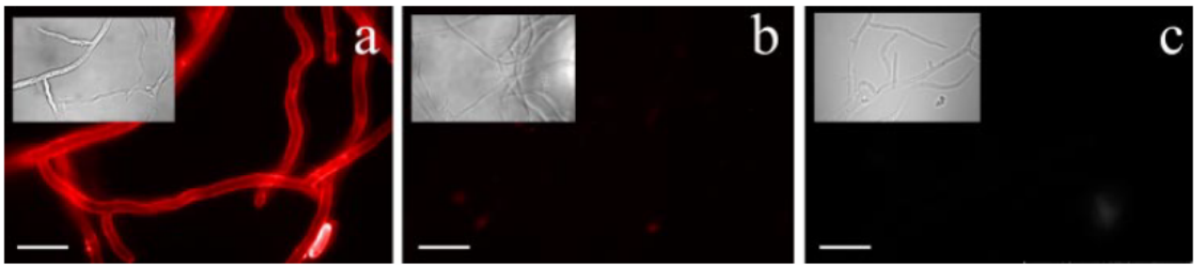

Figure 2.12 Investigation of the interaction between NaD1 and the cell surface using immunofluorecence using an anti-NaD1 antibody. (a) *Fusarium oxysporum f. sp. vasinfectum* (Fov) hyphae treated with 2 µM NaD1; (b) Fov hyhae incubated with water followed by anti-NaD1 antibody and (C) Fov hyphae incubated with the protease inhibitor C1 followed by anti-NaD1 antibody (Adopted from Van der Weerden *et al*., 2008).

The membrane permeabilization ability of NaD1 was assessed with a SYTOX green assay and it was found that the NaD1-induced permeabilization increased in a concentration dependent manner. More importantly, it was observed that this plant defensin lead to fluorescence across the cell that located in the nuclei. The cytoplasm appeared granular without distinguishable organelles (Figure 2.13) indicating that the internal membranes have lost their integrity and was no longer intact. NaD1 was shown to appear in the cytoplasm after 30 min of the incubation of the *Fusarium oxysporum f. sp. vasinfectum* (Fov) hyphae with the plant defensin. In addition, NaD1 was found to be present inside hyphae with granulated hyphae, but absent from hyphae that appeared healthy. Interestingly, NaD1 caused the formation of an aperture with an internal diameter between 14 and 22 Å, independent of the concentration of NaD1. NaD1 was also shown to induce the development of reactive oxygen species (ROS). These results supported the probability of an internal target for NaD1 (Van der Weerden *et al*., 2008).

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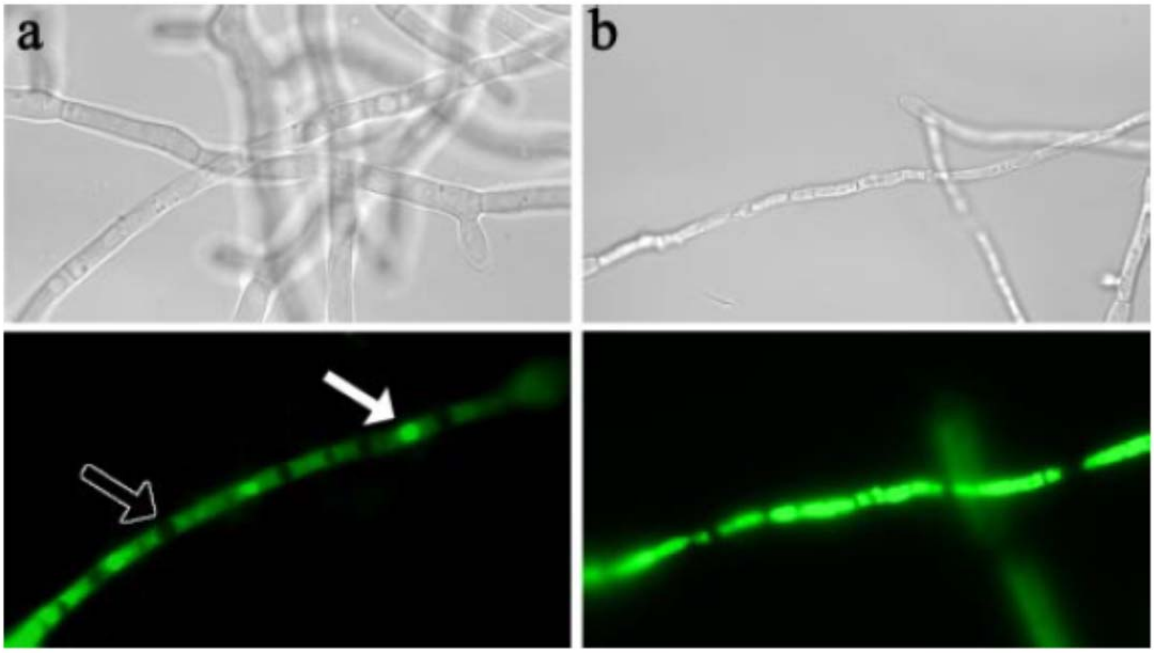

Figure 13. SYTOX green assay of NaD1-treated Fov hyphae. (a) Fov hyphae treated with 2 μM NaD1. SYTOX green can be seen located in the nuclei (block arrow) but not in vacuoles (unfilled arrow). (b) Fov hyphae treated with 0.1 μM NaD1. Fov hyphae were incubated with SYTOX green after the treatment with NaD1 (Adopted from Van der Weerden *et al*., 2008).

The permeablilization caused by NaD1 was further investigated by van Weerden *et al*., (2010). A SYTOX green assay indicated that the permeabilization of NaD1 was found to contain a lag time of 10 minutes before any permeabilization was visible and the permeabilization was found to be saturable. These results indicate the presence of a receptor on the cell wall and the lag time might represent the time needed by the NaD1 to interact with this receptor or to assemble in the membrane. The ability of NaD1 to disrupt artificial liposomes was assessed and, like other plant defensins, NaD1 was unable to distupt the artificial liposomes. Therefore these peptides are dependent on the cell wall to exert its potent antifungal activity. This dependence of NaD1 on the cell wall to cause membrane permeabilization was further assessed by modifying the cell wall. Treatment of the fungal cell walls with DTT and sodium hydroxide resulted in the removal of the (1-3)-β-glucanase layer and this prevented the permeabilization action of NaD1. Furthermore, the treatment of hyphae with β-glucanase inhibited NaD1 from killing Fov. These observations all indicated the existence of a receptor that is required for the antifungal activity of NaD1. Furthermore, it is likely that this receptor is located in the proteinacous layer of the cell wall (Van der Weerden *et al*., 2010). It was proposed that NaD1 exerts its antifungal activity by interacting with the cell wall, with subsequent permeabilization of the fungal membrane which allows NaD1 to enter the hyphal cells where it interacts with intracellular targets (Van der Weerden *et al*., 2008; 2010).

Another peptide reported to interact via intracellular targets is *Pisum sativum* defensin 1 (Psd1). It was shown that Psd1 internalize in the nucleus of *N. crassa* hyphae. Furthermore, it was

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demonstrated that this peptide has a direct physical interaction with an intracellular protein namely, *N. crassa* cyclin F, a cell cycle control protein (Lobo *et al*., 2007).

2.5.3. Downstream signalling pathways and processes taking place after initial interaction between plant defensins and their specific sphingolipid target

One of the consequences of the binding of a specific plant defensin to its specific sphingolipid target is the production of reactive oxygen species (ROS), resulting in apoptosis (Ames *et al*., 1993; De Zélicourt *et al*., 2000; Aerts *et al*., 2007; Aerts *et al*., 2008). ROS-like hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH), are produced as a consequence of normal aerobic respiration and causes damage to DNA, protein and lipids. This leads to maturation and the loss of cell viability (Ames *et al*., 1993; Aerts *et al*., 2007). Apoptosis in context of defence is an evolutionary conserved process whereby a cell commits suicide in order to protect the organism and eliminate dangerous, infected or damaged cells (De Brucker *et al*., 2011). The current available information on plant defensins and apoptosis is summarised in Table 2.3 and below some specific examples are discussed.

Table 2.3: Summary of the current knowledge of apoptosis-inducing plant defensin peptides

Defensin Peptide	Source	Antifungal spectrum	Interaction partners on fungal envelope	Mode of action	Interacting signalling cascades	References
RsAFP2	Radisch seed (*R. Sativus)*	*C. albicans; C. krusei; A.flavus; F.solani*	Sphingolipid; GlcCer	Hyperpololarising of the membrane potential; membrane permeabilization; ion fluxes; ROS; apoptosis	MAPK signalling pathways	De brucker et al., 2011
HsAFP1	Coral bells (*H. Sanguine a)*	*C. albicans; C. krusei; A.flavus; F.culmorum; B. cinerea*	n.d.	Membrane permeabilization; ROS; apoptosis	MAPK signalling pathways	De brucker et al., 2011
HaDEF1	Sunflowe r (*Helianth us annuus*)	*O. Cumana; O. Ramosa; S. cerevisiae; A. brassicicola*	n.d.	Membrane permeabilization; apoptosis	n.d.	De Zélicourt et al., 2007
MsDef1	*M. sativa*	*F. graminearum*	n.d.	Ion channal blocking; hyper-branching	MAPK signalling pathways	Spelbrink et al., 2004; Ramamoorthy et al., 2007; Sagaram et al., 2011

Abbreviations: n.d., not determined; ROS, reactive oxygen species

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The first evidence that the antifungal activity of the plant defensin RsAFP2 involves the generation of reactive oxygen species was reported by Aerts *et al.* (2007). It was demonstrated that RsAFP2 induced ROS species in a dose dependent manner in *C. albicans.* Furthermore, it was demonstrated that the induction of ROS species in *C. albicans* by RsAFP2 was dependent on the RsAFP2-binding site, GlcCer. It was demonstrated that there is a link between the RsAFP2-induced ROS generation and the antifungal activity of RsAFP2. The pre-incubation of *C. albicans* with the antioxidant, ascorbic acid (AA) prevented RsAFP2 induced ROS generation and caused a migration in the antifungal activity of RsAFP2.

Additional research by Aerts *et al.* (2008) revealed that RsAFP2 induced apoptosis in *C. albicans*. This was the first report of a defensin peptide to cause apoptotic cell death in its pathogen. It was also shown that this peptide induced apoptosis with the activation of caspases, but not *C. albicans* metacaspase 1 (CaMca1p), a putative caspase involved in the oxidative stress-induced cell death. This was demonstrated when a *C. albicans CaMCA*1 deletion mutant (*mca1*Δ/Δ mutant), the isogenic strains BWP17 and the *CaMCA1* complemented *C. albicans mca1*Δ/Δ mutant were treated with RsAFP2 and resulted in equal killing of all three strains and the activation of a significant amount of caspases (Figure 2.14) This finding indicated that RsAFP2 induces apoptosis by another caspases-like protease (Aerts *et al.*, 2008). Research by Ramamoorthy *et al.* (2007) revealed that Mgv1 and Gpmk1 MAP kinase (MAPK) signalling pathways are involved in the regulation of the growth inhibition caused by RsAFP2. Similar results were found for HsAFP1, shown to induce MAPK signalling pathways (Ramamoorthy *et al*., 2008). This is interesting since it has been reported that MAP kinases are essential for the early phases of infection of fungal pathogens like *B. cinerea,* specifically for the penetration of plant surfaces (Williamson *et al*., 2007).

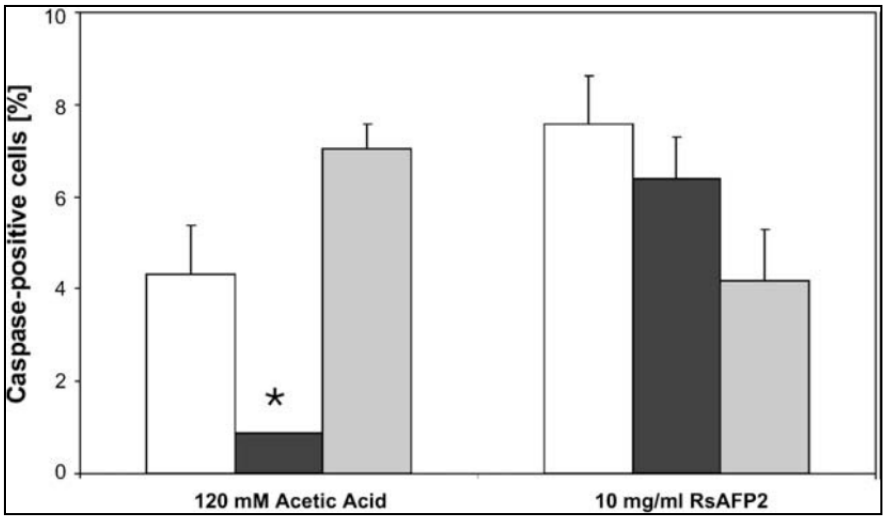

Figure 2.14. Percentage of caspase active cells after the treatment of 120 mM acetic acid for 2 h or with 10 µg/ml RsAFP2 for 2 h 30 min of the isogenic *C. albicans* BWP17 strains (white bar), the mutated *C. albicans mca1*Δ/Δ strains (black bar) and the *CaMA1* complimented *C. albicans mca1*Δ/Δ strains (grey bar) (Adopted from Aerts *et al*., 2009).

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MAPK is activated by cell wall stress induced by membrane disruption conditions in *C. albicans* (Blankenship *et al*., 2010; Thevissen *et al*., 2012). Interestingly, it was recently shown that RsAFP2 disrupts the cell wall integrity and causes abnormal septin localization (involved in septum formation) (Thevissen *et al*., 2012). These two phenomena are closely linked as an intact septum ring requires a normal cell wall structure and vice versa (Blankenship *et al*., 2010; Thevissen *et al*., 2012). RsAFP2 lead to a 1.5 fold increase of the average neck size of yeast cells, compared to the untreated control. Septins have several important functions including cell division, polarity determination, vesicle trafficking, and cytoskelethal dynamics (Thevissen *et al*., 2012). Furthermore, septins are essential for the polarized growth of filamentous fungi. Thevissen *et al*. (2012) suggested that the disruption of normal septin localization by RsAFP2 might be responsible for the RsAFP2-induced hyper-branching observed in *F. culmorum* (Terras *et al*., 1992; Thevissen *et al*., 2012). Thevissen *et al*. (2012) went further and tested the yeast-to-hyphae ratio as it has been reported that abnormal septin localization is linked with the inhibition of this ratio. In the presence of RsAFP2, the amount of hyphae was reduced in a dose dependent manner. Abnormal septin localization can be induced by the accumulation of ceramide in high concentrations. It was found that the *C. albicansi* treated with RsAFP2 lead to a significant increase in the concentration of C-24phytoceramide and (α-hydroxy)C-24phytoceramide (Thevissen *et al*., 2012). This is a vital discovery into the mechanism of the mode of antifungal action of RsAFP2. However, the precise enzymes and signalling pathways involved in these processes still need to be investigated.

HaDEF1, a plant defensin isolated from sunflower was found to be active against *Orobanche cumana*, a root parasitic plant. A transgenic sunflower over-expressing HaDEF1 was found to be resistant to *O. cumana*. This is the first plant defensin reported for having activity against plant cells. HaDEF1 induced the development of browning symptoms on *O. cumana* and *O. ramosa* after 24 hours of treatment and further investigation revealed that the cells located in the browning areas were dead. HaDEF1 was tested on the non-parasitic plant *Arabidopsis thaliana* and another parasitic plant, *Striga hermonthica*, but was found to be inactive. It is suggested that HcDEF1 might exert its mode of action via an interaction with the spingolipid, $M(IP)_2C$. This was proposed, because HaDEF1 is closely related to DmAMP1, CtAMP1 and AhAMP1 which are known to also interact with $M(IP)_2C$ (De Zélicourt *et al*., 2007).

2.6 Concluding remarks

Defensin peptides are endogenous in plants and are involved in biological activities that play a vital role in the protection of the plant. Although the precise mechanism of action for plant defensins are not yet known, more insight in the underlining molecular mechanisms of antifungal activity of the plant defensins RsAFP2 and DmAMP1 from radish and dahlia are becoming known and are providing a strong base to work from. The broad range and potency of the antifungal

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activity of defensin peptides makes them attractive potential candidates to be used in agribiotechnological and pharmaceutical applications.

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Chapter 3

Research results

The recombinant production and activity analysis of four *Heliophila coronopifolia* defensin peptides

Part of this chapter will be incorporated into a manuscript in preparation for **PLoS ONE .** The authors of the manuscript will be Helmien Barkhuizen, Abré de Beer, Marina Rautenbach and Melané A. Vivier.

The authors contributed as follows to the work presented: HB performed all experiments, data-interpretation and compiling of results under the guidance of AdeB, MR and MAV; HB drafted and finalized the chapter with inputs from all authors; AdeB, MR and MAV conceived of the study.

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RESEARCH RESULTS

The recombinant production and activity analysis of four *Heliophila coronopifolia* defensin peptides

3.1 INTRODUCTION

Plant defensins are small (45-54 amino acids), basic, cysteine-rich, cationic peptides. Most plant defensins have been reported to inhibit the growth of a broad range of phytopathogenic fungi at micromolar concentrations, but are nontoxic to plant cells (Broekaert *et al*., 1995; Osborn *et al*., 1995; Thevissen *et al*., 1997; Almeida *et al*., 2000). The defensin peptides are constitutively expressed, or can be induced in response to infection and play an essential role in the protection of plants against invading fungal pathogens (Broekaert *et al*., 1995; Osborn *et al*., 1995; Terras *et al*., 1995; Thevissen *et al*., 1997; Thomma *et al*., 2003; Lay and Anderson, 2005). These peptides are generally expressed in the peripheral cells of different plant organs which are the first barriers to pathogen invasion. These locations where plant defensins are generally expressed are consistent with their role as the first line of defense against invading pathogens (Broekaert *et al*., 1995; Terras *et al*., 1995).

The conserved three dimensional structures of plant defensins have been resolved with nuclear magnetic resonance. Plant defensins adopt a globular structure that consists of an α-helix, three helical turns and three anti-parallel β-sheets. This globular structure is stabilized by four disulphide bridges (Bruix *et al*., 1993 and Fant *et al*., 1998). Plant defensin peptides contain the distinctive, conserved structural feature, namely the cysteine-stabilized α-helix motif (CSαβ), that is found in all defensin peptides and is generally conserved in all peptides that posses antimicrobial activity (Bruix *et al*., 1993; Broekaert *et al*., 1995, Cornet *et al*., 1995, Fant *et al*., 1998 and Lay *et al*., 2003; Carvalho *et al*., 2009). Despite this conserved tertiary structure, plant defensins share limited sequence homology. This homology consists of eight cysteiene residues, two glycines at positions 13 and 34, an aromatic residue at position 11 and a glutamic acid at position 29 (numbering relative to Rs-AFP1) (Broekaert *et al*., 1995). The large variation in their primary amino acid sequences explains the numerous biological functions that have been attributed to these peptides (Lay at al., 2003a).

Recently four plant defensin peptides have been isolated from *Heliophila coronopifolia,* a native South African *Brassicaceae* species (De Beer and Vivier, 2011). Analysis of the deduced amino acid sequences showed that the peptides were 72% similar and grouped closest to defensins isolated from other Brassicaceae species. The Hc-AFP1 and 3 peptides shared high homology (94%) and formed a unique grouping in the *Brassicaceae* defensins, whereas Hc-AFP2 and 4 formed a second homology grouping with defensins from *Arabidopsis* and *Raphanus*.

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Homology modelling showed that the few amino acids that differed between the four peptides had an effect on the surface properties of the defensins, specifically in the α-helix and the loop connecting the second and third β-strands ($L\beta_2\beta_3$). These areas, and specifically $L\beta_2\beta_3$, are implicated in determining differential activities of defensins. Hc-AFP1 and 3 is predominantly expressed in the vegetative and reproductive tissues (leaves, stems and flowers) of *H. coronopifolia* and is only expressed in very low levels in the reproductive storage tissues (siliques and seed). Hc-AFP1 was the dominant transcript in the stem and flower tissues whereas Hc-AFP3 was the dominant transcript in the leaf tissue. Hc-AFP2 and 4 were predominantly expressed in the reproductive storage organs of *H. coronopifolia*; Hc-AFP2 mostly in green siliques and Hc-AFP4 in the seeds. These four defensin peptides were found to strongly inhibit *Botrytis cinerea* and *Fusarium solani* in *in vitro* tests (De Beer and Vivier, 2011).

The *H. coronopifolia* defensin peptides were previously produced and purified from a bacterial system; the system rendered active peptides, but in quite low yields. It is widely reported that bacterial expression systems often fail to produce sufficient yields of properly folded cysteine-rich proteins (Almeida *et al*., 2001; Marqués *et al*., 2009). Furthermore, previous work used *Escherichia coli* as a production system which resulted in the formation of inclusion bodies which required tedious methods to recover the active defensin peptides (De Beer and Vivier, 2011). These problems prompted the current study to optimize the production of the defensin peptides with higher yields and a simplified purification process. Our aim was to over-express these four *H. coronopifolia* plant defensins in the biologically active form, striving for a high yield of these peptides to perform subsequent characterization experiments. We report the sub-cloning, production and purification of the four plant defensin peptides form *H. coronopifolia* in *Pichia pastoris*. *Pichia* has been used to produce large quantities of plant defensin peptides previously (Almeide *et al*., 2001; Cabral *et al*., 2003; De-Paula *et al*., 2008; Marqués *et al*., 2008). This host organism is known for its ability to properly process and fold eukaryotic proteins (Cregg *et al*., 1985). Furthermore, *Pichia* only secretes low levels of its own proteins which should simplify the purification process. In the current study we report the successful production of three of the *H. coronopifolia* plant defensins in the biologically active form. Moreover, the *P. pastoris* production system rendered a significant increase in peptide yield. Hc-AFP2 was initially produced with a blocked N-terminal and we showed that the cyclization of the N-terminal glutamine affected the activity of this peptide. The production of Hc-AFP2 was subsequently successfully optimized by using a lower ionic buffer. The Hc-AFP4 peptide was not correctly produced with the *Pichia* system and requires further optimization.

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3.2 MATERIALS AND METHODS

3.2.1 Microbial strains and genetic material

Pichia pastoris strain X-33 (Invitrogen, USA) was obtained from the Department of Microbiology, University of Stellenbosch. The full length genes of Hc-AFP 1-4 were previously isolated (De Beer and Vivier, 2011) and served as a template to obtain the mature sequence. *Fusarium solani* and *Botrytis cinerea* cultures were obtained from the Department of Plant Pathology (DPP), Stellenbosch University. *F. solani* and *B. cinerea* cultures were maintained on potato dextrose agar at 25˚C until sporulation. Spores were harvested in sterile deionized H_2O and spore concentrations were determined with a haemocytometer.

3.2.2 Expression vector construction

A strategy was developed that would allow for the secretion of mature plant defensin peptide form the expression host without any additional amino acids being added during the cloning process. Primers were designed (Table 3.1) that allowed for the direct fusion of the gene of interest to the N-terminal *Saccharomyces cerevisiae* α-factor secretion signal (MFα) to yield the expression vector pGAPZαA (Invitrogen, USA) using the 5' - *XhoI* restriction site and the 3' - *XbaI* restriction site.

Table 3.1. Primers and their amplification products used in this study

Primer name	Sequence[a]	Tm (˚C)	Amplicon size	Amplification
pGAP-Hc1 5'	**CTCGAG**AAAAGAAGGTACTGTGAGAGATCGAG	60.4	208 bp	Amplification of the Hc-AFP1 coding region form *H. coronopifolia* with *Xho1* and *Xba1*
pGAP-Hc1 3'	**TCTAGA**TCAACATGGGTAGTAACAGA	54.8		
pGAP-Hc2 5'	**CTCGAG**AAAAGACAAAAGTTGTGTGAGAGACC	60.3	211 bp	Amplification of the Hc-AFP2 coding region form *H. coronopifolia* with *Xho1* and *Xba1*
pGAP-Hc2 3'	**TCTAGA**TTAACATGGGAAGTAGCAGA	55.0		
pGAP-Hc3 5'	**CTCGAG**AAAAGAAGGTACTGTGAGAGATCGAG	60.4	208 bp	Amplification of the Hc-AFP3 coding region form *H. coronopifolia* with *Xho1* and *Xba1*
pGAP-Hc3 3'	**TCTAGA**TTAACATGGGTAGTAACAGA	52.8		
pGAP-Hc4 5'	**CTCGAG**AAAAGACAGAAGTTGTGTGAGAGACCAAGT	63.0	215 bp	Amplification of the Hc-AFP4 coding region form *H. coronopifolia* with *Xho1* and *Xba1*
pGAP-Hc4 3'	**TCTAGA**TTAACATGGGAAGTAACAGA	53.3		
Mfa-HindIII	GCGCAAGCTTATGAGATTTCCTTCTATTTTT	63.3	404 bp	Amplification of the α-factor mating signal coding region

[a]The boldface bases represent the restriction sites

The mature coding regions of the various Hc-AFPs were PCR amplified using the corresponding pGAP-Hc 5' and pGAP-Hc 3' primer set (Table 3.2). The PCR reactions were performed in 25 µL reaction containing 1 x Expand buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 200

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nM pGAP-Hc-5' primer, 200 nM pGAP-Hc-3' primer, 10 ng template DNA and 1 U Expand high fidelity polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The PCR program was as follows: 94˚C for 5 min; followed by 35 cycles of 94˚C for 30 sec, 52˚C for 30 sec and 72˚C for 2 min. The PCR products obtained were cloned into pGEMt easy vector (Promega Corporation, Madison, USA) and this vector was transformed into *E. coli* (DH5α) cells by means of heat shock.

Table 3.2. Description of the plasmids used in this study

Name of plasmid	Description	Reference
pGEMt	Expression vector	Promega
pGEMt-HC-AFP1	Hc-AFP1 from *H. coronopifolia* and cloned into pGEMt-easy cloning vector	This study
pGEMt-HC-AFP2	Hc-AFP2 from *H. coronopifolia* and cloned into pGEMt-easy cloning vector	This study
pGEMt-HC-AFP3	Hc-AFP3 from *H. coronopifolia* and cloned into pGEMt-easy cloning vector	This study
pGEMt-HC-AFP4	Hc-AFP4 from *H. coronopifolia* and cloned into pGEMt-easy cloning vector	This study
pGAPZαA	Expression vector	Invitrogen
pGAPZαA-Hc-AFP1	Hc-AFP 1 cassette ligated into pGAPZαA-Hc-AFP1	This study
pGAPZαA-Hc-AFP2	Hc-AFP 2 cassette ligated into pGAPZαA-Hc-AFP2	This study
pGAPZαA-Hc-AFP3	Hc-AFP 3 cassette ligated into pGAPZαA-Hc-AFP3	This study
pGAPZαA-Hc-AFP4	Hc-AFP 4 cassette ligated into pGAPZαA-Hc-AFP4	This study

Positive transformants (pGEMt-Hc-AFP1-4) were selected on Luria Bertani (LB) agar plates containing 100 µg mL^{-1} ampicillin. Positive clones were confirmed by means of a restriction digest with *EcoR*I and the inserts verified by sequencing. The subsequent cloning was performed according to the pGAPZαA *Pichia* expression vector for constitutive expression and purification of recombinant proteins user manual (Invitrogen, USA, 2002). The mature Hc-AFPs coding regions were excised from the respective pGEMt easy vectors with *Xho*I and *Xba*I and ligated into the pGAPZαA vector prepared with *Xho*I and *Xba*I. This vector was transformed into *E. coli* and positive transformants (pGAPZαA-Hc-AFP1-4) were selected on low salt LB agar plates containing 25 µg mL^{-1} Zeocin (Invitrogen, USA). Plasmid DNA of (pGAPZαA-Hc-AFP1-4) was isolated and used to confirm positive clones by PCR using primers MFa-HindIII-F 5' and pGAP-Hc1-4 3'. These PCR reactions were performed in a 50 µL reaction volume with GoTaq Flexi DNA Polymerase (Promega, USA) and the above mentioned PCR program was followed. The fusion between MFα and the peptide was verified by sequencing with the MFα primer. pGAPZαA-Hc-AFP1-4 DNA (5-10 µg) was linearized by digestion with *Xma*JI *(AvrII)* (Fermentas, Thermo Scientific, USA) and transformed into *P. pastoris* strain X33 by means of electroporation (Pipes *et al.* 2005). Approximately 4 µL (50-100 ng) of linearized plasmid was mixed with 40 µL of competent *P. pastoris* X33 cells in a pre-cooled electroporation cuvette and incubated for 2 minutes on ice. The

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samples were electroporated using the following parameters: EasyjecT Untegrasser: cuvette gap, 2.0 mm; charging voltage, 1500 V; resistance, 200 Ω; capacitance, 25 µF. The samples were then resuspended in 0.5 mL 1.0 M sorbitol and 0.5 mL yeast extract petone dextrose broth (YPD) and incubated in a 30˚C shaker for 1 h. The samples were plated on yeast extract peptone dextrose medium (YPD) agar plates containing 100 µg mL^{-1} Zeocin (Invitrogen, USA). Genomic DNA from putatively positive transformants (*P. pastoris* Hc-AFP1-4) were isolated and the gene inserts were confirmed by PCR using primers MFa-HindIII-F 5' and pGAP-Hc1-4 3'. These PCR reactions were performed in a 50 µL reaction volume with GoTaq Flexi DNA Polymerase (Promega, USA) and the above mentioned PCR program was followed.

3.2.3 Recombinant production of defensin peptides

Three clones of each of the *P. pastoris* Hc-AFPs were inoculated into a 5 mL pre-culture of YPD containing 100 µg mL^{-1} Zeocin and grown for three days at 30˚C. A total of ten baffled 500 mL erlennmeyer flasks containing 100 mL buffered minimal glygerol medium (BMG: 100 mM potassium phosphate, pH 6.0; 20 mM MES, pH 6.0; 1.34 % (*w/v*) Yeast Nitrogen Base (YNB); 2 mL 500x biotin and 1% (*v/v*) glycerol) each per clone were inoculated with 100 µL of pre-culture and incubated at 30˚C with continuous shaking at 190 rpm for 4 days. The negative control consisted out of *P. pastoris* containing a pGAPZαA vector without a gene insert. To test the effect of a lower ionic buffer, Hc-AFP2 was also produced using reduced ion minimal glycerol (BMG) medium containing: 10x 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, 20 mM MES, pH 6.0; 1.34 % (*w/v*) Yeas nitrogen base (YNB); 2 mL 0.02% (*v/v*) biotin and 1% (*v/v*) glycerol.

3.2.4 Purification of recombinant defensin peptides

Recombinant Hc-AFPs were purified using cation exchange chromatography. Cells were removed from the media by centrifugation. The pH of the clear media was adjusted to 4.0 with 3 M NaOH. The media were passed over a 5 ml bed volume Sephadex SP fast flow column (Amersham Biosciences, Uppsala, Sweden), equilibrated with 20 mM MES buffer pH 6.0 (Buffer A) at room temperature. The column was loaded at a flow rate of 0.8 mL min^{-1} using a Biorad Econo Pump (Biorad, USA). The column was washed with 20 mM MES buffer, pH 6.0 (buffer A) at a flow rate of 1 mL min^{-1} by means of a Biorad Biologic LP instrument (Biorad, USA) system at room temperature until the baseline UV absorbance (280 nm) reached zero. Hc-AFPs were eluted using a linear NaCl gradient (Buffer B) ranging from 0% Buffer B – 100% Buffer B over 50 minutes at room temperature.

The presence and purity of the specific Hc-AFP produced was evaluated by separating 5.0 µL of the collected fractions on a 15% (*w/v*) Tris-Tricine gel (Schagger *et al.* 1978). The resulting peptide bands were then visualized my means of silver staining. Fractions 4-11 collected during the cation exchange purification of the specific Hc-AFP produced, were desalted on an Isolute C_8 (EC) column (Biotage AB, Switzerland). The desalted peptide was eluted with 50% (*v/v*)

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acetonitrile and freeze-dried. The concentration obtained from each production was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

Analytical HPLC was used to purify the Hc-AFPs to a purity of 95%. Samples were prepared in analytical grade water to the concentration of 1000 µg mL^{-1}. In order to remove impurities, samples were centrifuged for 10 minutes at 10000×*g*. Peptide purity was assessed on an analytical reverse phase C_2/C_{18} µRPC HPLC column (3 µm spherical particles, 120 Å pore size, 30 mm x 32 mm; Pharmacia Biotech, Uppsala, Sweden). A linear gradient at a flow rate of 300 µL min^{-1} was created over a time period of 10 min starting at 100% eluant A (0.1% (*v/v*) trifluoroacetic acid in analytical grade water) to 100% eluant B (90% (*v/v*) acetonitrile and 10% *(v/v*) A). An UV-MII UV-detector was used to monitor the chromatography at 280 nm.

3.2.5 Peptide preparation

The peptides were all dissolved in 50% (*v/v*) acetonitrile, in order to ensure sterility, and were then freeze-dried in pyrolyzed glassware. To ensure accuracy, all peptides were weighed according to an analytical protocol described by Rautenbach *et al*. (2007). The analytically weighed peptides were used to prepare stock solutions of 1.0 mg mL^{-1} in filtered analytical-grade water before each assay. The water used to prepare the peptide stock solution was filtered beforehand through a sterile 20 µm syringe filter to ensure sterility.

3.2.6 Characterization of recombinant *Heliophila coronopifolia* defensin peptides

Purified Hc-AFPs were subjected to MS analysis in order to confirm that the correct peptides were produced by the respective *P. pastoris* clones. Purified Hc-AFP (10 µL) was injected on a Waters API Q-TOF Ultima. Analays was carried out using the following settings: source, ESI+; capillary voltage, 3 kV; cone voltage, 20 V; RF1, 40 source temeperature, 100˚C; desolvation temperature, 350˚C; desolvation gas flowrate, 400 L h^{-1} . The experimentally determined mono-isotopic mass of Hc-AFPs with all cysteine residues in an oxidized state, were calculated as (M + H) by using the MassLynx MaxEnt3 function of the MassLynx version 4.1 software (Waters Corporation, 2008).

3.2.7 Characterisation by nano-liquid chromatography and mass spectrometry

This characterization was done by the MS unit, Proteomics laboratory of the Central Analytical Facilities (CAF) at Stellenbosch University using the following methodology as supplied by Dr. S Smit (CAF).

Gel pieces from the in gel trypsin digestion were cut into smaller cubes and washed twice with water followed by 50% (*v/v*) acetonitrile for 10 minutes. The acetonitrile was replaced with a 50 mM ammonium bicarbonate solution and incubated for 10 minutes. The process was repeated two more times. All gel pieces were then incubated in 100% acetonitrile until they turned white, after which the gel pieces were dried *in vacue*. Proteins were reduced with 10 mM DTT for 1 hour at 57

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˚C. This was followed by brief washing steps with ammonium bicarbonate followed by 50% acetonitrile before proteins/peptides were alkylated with 55 mM iodoacetamide for 1 hour in the dark. Following alkylation the gel pieces were washed with ammonium bicarbonate for 10 minutes followed by 50% acetonitrile for 20 minutes, before being dried *in vacuo.* The gel pieces were digested with 20 μL of a 10 ng μL^{-1} trypsin solution at 37˚C overnight. The resulting peptides were extracted twice with 70% acetonitrile in 0.1% formic acid for 30 minutes, and then dried and stored at -20˚C. All peptides were cleaned using stage tips (used for rapid desalting and step elution of peptide mixtures) before injection. Dried peptides were dissolved in a 5% acetonitrile in 0.1% formic acid solution and 10 μL injections were made for nano-LC chromatography.

All experiments were performed on a Thermo Scientific EASY-nLC II coupled to a LTQ Orbitrap Verlos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100μm, 5μm, C_{18}) pre-column followed by XBridge BEH130 NanoEase column (15 cm, ID 75μm, 3.5μm, C_{18}) column at a flow rate of 300 nL min^{-1}. The gradient used was from 5-40 % solvent B in 20 minutes, followed by 40-80% solvent B over 5 minutes and kept at 80% solvent B for 10 minutes (solvent A was 100% water in 0.1% formic acid, and solvent B was 100% acetonitrile in 0.1% formic acid). The mass spectrometer was operated in data-dependent mode automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. The precursor ion scan MS spectra (*m/z* 400-2000) were acquired in the Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 x 10^6. The 20 most intense ions were isolated and fragmented in a linear ion trap (number of accumulated ions 1.5×10^4) using collision induced dissociation. The lock mass option (polydimethylcyclosiloxane; *m/z* 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments, dynamic exclusion was used with 60s exclusion duration. Mass spectrometry conditions were 1.5 kV, capillary temperature of 200 ˚C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and and an activation Q-value of 0.25 anc activation time of 10 ms were applied for MS/MS.

Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) was used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK and Sequest) of all tandem mass spectra against the Swissprot database. Carbamidolmethyl cysteine was set as fixed modification, and oxidized methionine, N-acetylation and deamindation (NQ) was used as variable modifications. The precursor mass tolerance was set to 20 ppm, and fragment mass tolerance set to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 2 tryptic peptides per proteins. A Mascot of Sequest score of more than $p<0.05$ (determined by Percolator a decoy database) was searched with FDR (strict) of 0.2 FDR (relaxed) of 0.05 with validation based on the q-value.

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3.2.8 Structural characterization of peptides with circular dichroism spectroscopy

The circular dichroism (CD) spectroscopy analysis was performed on all the purified defensins with an Applied Photophysics Chirascan-plus CD spectropolarimeter (Applied Photophysics Limited, Leuterhead, United Kingdom). The Hc-AFPs were dissolved at a concentration of 100 µM in dH_2O. The CD spectrum was generated at 22 ˚C using a quartz cell with a path-length of 0.01 cm. Each experiment was performed with three scans per spectrum recorded from 185 to 260 nm with a 0.5 nm step and with a scan speed of 0.63 seconds per nm. Data was acquired at 0.5 nm per second. The water used in all experiments was analytical grade.

3.2.9 Antifungal activity of *Heliophila coronopifolia* defensin peptides

Antifungal assays were performed to assess the activity of the recombinant produced Hc-AFP defensin peptides. This was achieved by using a microspectrophotometric assay as described by Broekaert *et al.* (1990). The assays were performed in 96 well microtiter plates (Bibby Sterilin Ltd, Stone, Staffs, UK) where each well contained 1000 fungal spores in 100 µL half strength potato dextrose broth (PDB). The antifungal assay was conducted on *F. solani*, one of the pathogens previously shown to be sensitive to these peptides (De Beer and Vivier, 2011). The concentrations of purified Hc-AFP used in the biological assays were 25 µg mL^{-1} for Hc-AFP1 and Hc-AFP3, 15 µg mL^{-1} for Hc-AFP2 peptides, and 10 µg mL^{-1} for Hc-AFP4, with control reactions containing no peptide. These specific concentrations were chosen according to the results previously obtained with the peptides produced from *E. coli* and represent the previously determined IC_{50} values per peptide. Plates were incubated in the dark for two days at 23˚C and microspectrophotometric readings were taken after 36 hours at a wavelength of 595 nm to assess the antifungal activity of Hc-AFP defensin peptides and were expressed in terms of % growth inhibition as described by Broekaert *et al*. (1990). Statistical analysis was performed by a student's two-tailed paired T-test compared to the growth control. The effects of the defensins on hyphal growth were observed after 24 hours with a Leica inverted microscope. Images were captured with a DCMI30E microscope digital camera and analyzed with SchopeTec ScopePhoto software version x86, 3.1.475 (ScopeTec, Hnangzhou, China).

3.3 RESULTS

3.3.1 Sub-cloning of *Heliophila coronopifolia* defensin peptides

The mature coding regions of the Hc-AFPs were successfully PCR amplified and cloned into pGEM-T easy cloning vector (Figure 3.1) as confirmed by means of a restriction digest and verified by sequencing (results not shown). In order to confirm the presence of the Hc-AFP gene insert in *P. pastoris* the genomic DNA of positive transformants (*P. pastoris* Hc-AFP) were isolated and

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screened by a PCR reaction using specific primers for each Hc-AFP peptide, resulting in ~420-450 bp products (Figure 3.2).

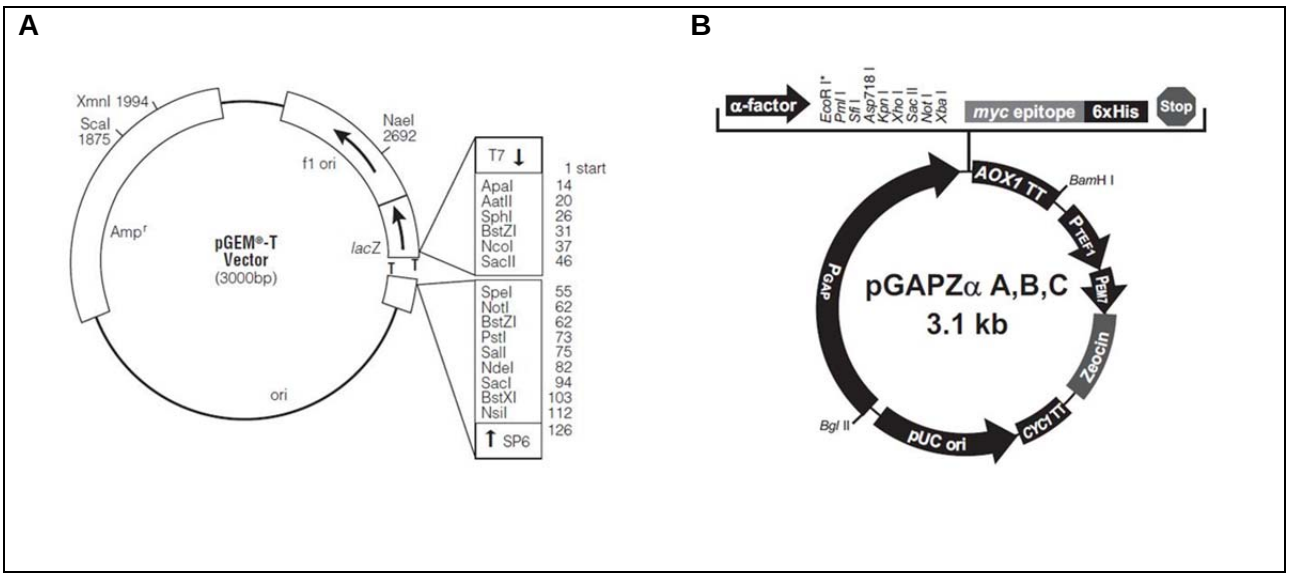

Figure 3.1. Vector maps of the bacterial and yeast expression vectors. pGEM-T from Promega (A) and pGAPZα from Invitrogen (B) are shown.

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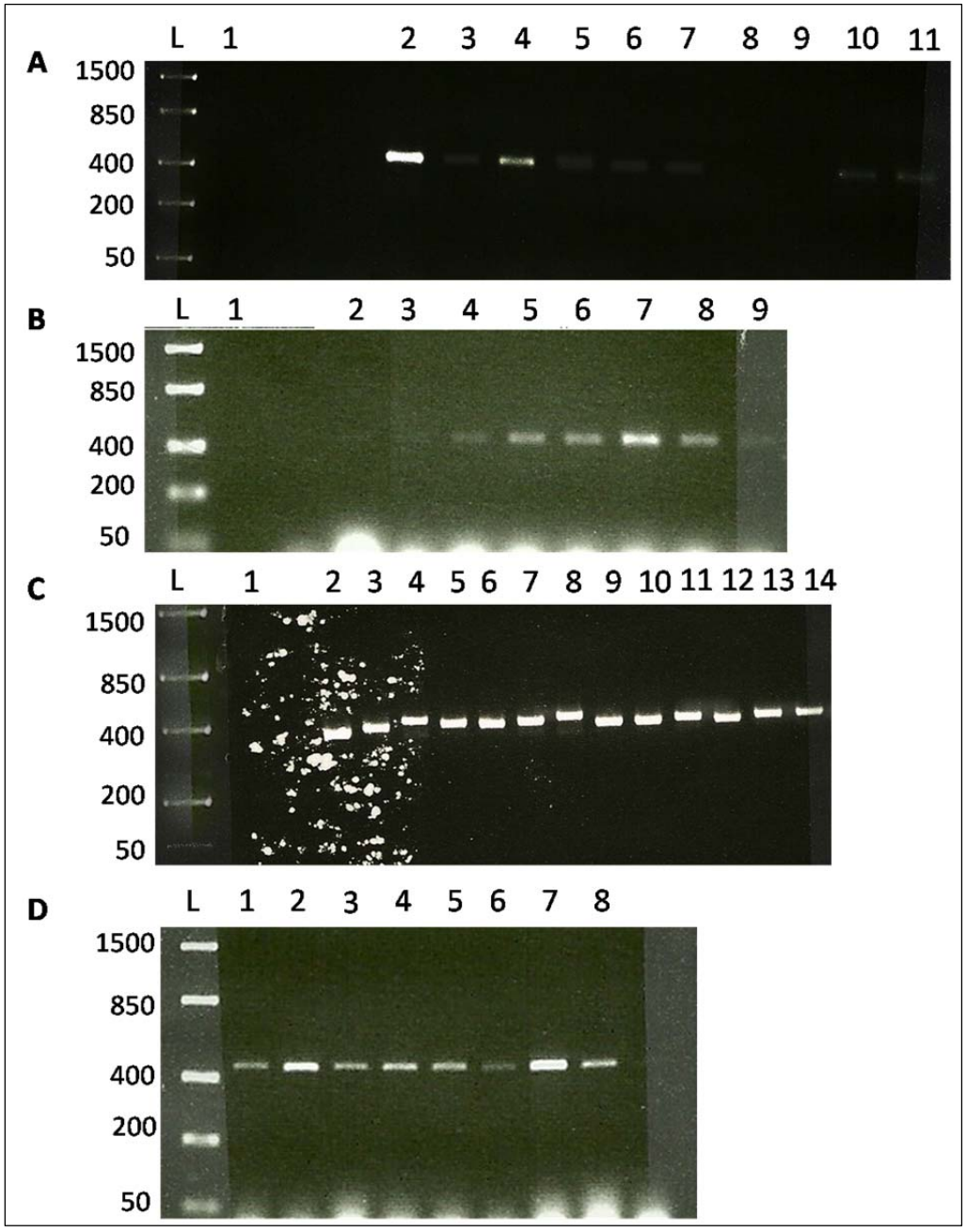

Figure 3.2. PCR analysis of the positive Hc-AFPs gene inserts into *Phicha pastoris*. Genomic DNA of positive transformants (*P. pastoris* Hc-AFP1-4) was isolated and the positive gene inserts were confirmed by a PCR reaction described under Materials and Methods. The PCR products corresponding to the Hc-AFPs (404 bp) were separated using a 1% agarose gels and visualized with gel red staining. (A) The results obtained from the reaction for each peptide is presented as follows: Hc-AFP1 (A), with lane L, FastRuler Low range DNA ladder (Fermentas, Thermo Scientific, Germany), lane 1, negative control; lane 2, positive control; lane 3-11 *Pichia* Hc-AFP1 positive clones. Hc-AFP2 (B), with lane L, FastRuler Low range DNA ladder (Fermentas, Thermo Scientific, Germany), lane 1, negative control; lane 2, positive control; lane 3-9 *Pichia* Hc-AFP2 positive clones. Hc-AFP3 (C) with lane L, FastRuler Low range DNA ladder (Fermentas, Thermo Scientific, Germany), lane 1, negative control; lane 2, positive control; lane 3-14 *Pichia* Hc-AFP3 positive clones. Hc-AFP4 (D) with lane L, FastRuler Low range DNA ladder (Fermentas, Thermo Scientific, Germany), lane 1, positive control; lane 2-9 *Pichia* Hc-AFP4 positive clones.

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3.3.2 Recombinant production of *Heliophila coronopifolia* defensin peptides, purification and characterization

The Hc-AFPs peptides were successfully produced in *P. pastoris* X33. The peptides were purified by cation exchange chromatography on a Sephadex SP fast flow column. The peptide fractions were eluted as single peaks at relatively high ionic strength (between 200 and 250 mM NaCl) (Figure 3.3). The fractions obtained through this purification that corresponded to the elution of the peptide peak were analyzed by Tris-Tricine SDS-PAGE (Figure 3.3). This analysis revealed that the different conformational forms characteristic to these peptides were present. Hc-AFP1, Hc-AFP2 and Hc-AFP 4 all displayed monomeric (5 kDa band), dimeric (10 kDa band) and trimeric (15 kDa band) forms of the defensin peptides (Fig 3.3B, D, H); however, Hc-AFP3 only displayed monomeric (5 kDa band) and dimeric (10 kDa band) forms (Figure 3.3F). This oligomerization is a known feature of the *Brassicaceae* plant defensins (Terras *et al*., 1992; Terras *et al*., 1993). In Figure 3.3 the cation exchange chromatography elution profile of each peptide and the analysis of the collected fractions by Tris-Tricine SDS-PAGE are indicated.

All the recombinant Hc-AFPs could be easily purified on a reverse-phase HPLC column, except for Hc-AFP4 that that exhibited a broad peak. All of the Hc-AFPs eluted at approximately 30% acetonitrile containing 0.1% trifluoroacetic acid (Figure 3.4).

Mass spectrometry analyses of the purified Hc-AFPs were used to confirm the success of the production system and to further confirm the molecular mass of the peptides (Table 3.3). The mass spectrometry analysis of Hc-AFP2 indicated the presence of two forms of the peptide, one peptide of the expected molecular mass and another with a molecular mass 17 Da less than the expected molecular mass. The molecular mass obtained for Hc-AFP4 was also 17 Da smaller than expected (Table 3.3). The Hc-AFP2 and Hc-AFP4 peptides were therefore mixtures of two peptides. The molecular masses of Hc-AFP1 and Hc-AFP3 corresponded to their theoretical molecular masses.

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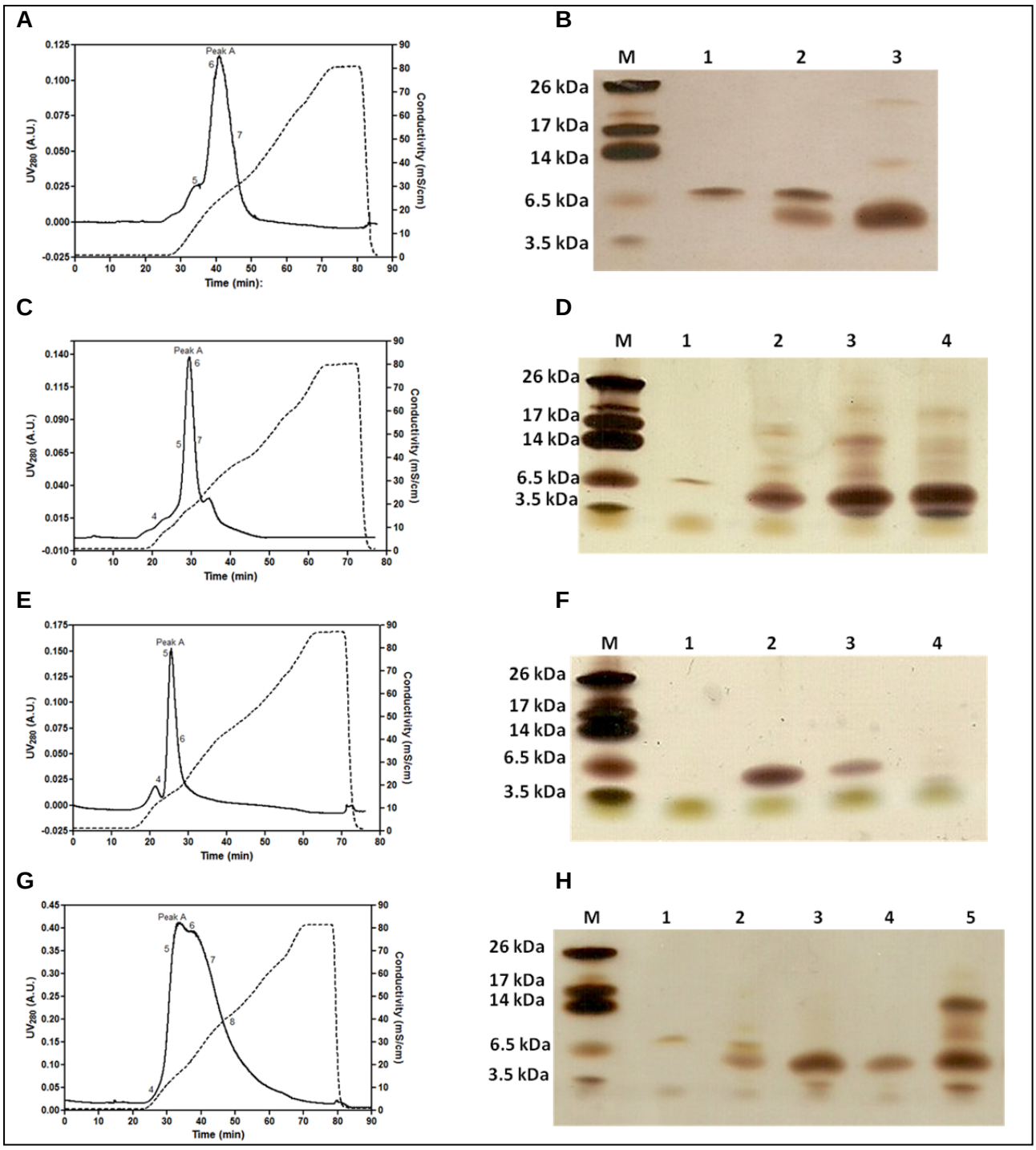

Figure 3.3. Cation exchange chromatograms and Tris-Tricine SDS-PAGE analyses of Hc-AFP 1-4 peptides. The results obtained from the cation exchange purification for each peptide is presented as follows: Hc-AFP1 (A), Hc-AFP2 (C), Hc-AFP3 (E) and Hc-AFP4 (G). The elution was performed by means of a gradient of 0.01-1M $NaCl_2$ (dashed line). The protein content was monitored by measurement of UV at 280 nm (solid line). Peak A indicated on the chromatogram contained the specific defensin peptide as was collected in the numbered fractions as indicated on the chromatograms. Tris-Tricine SDS-PAGE analysis of the peaks collected during the cation exchange purification of Hc-AFP1 (B), Hc-AFP2 (D), Hc-AFP3 (F) and Hc-AFP4 (G) with lane M, low molecular weight marker (Sigma, St. Louis, USA); lane 1, fraction 4; lane 2, fraction 5; lane 3, fraction 6; lane 4, fraction 7 and lane 5, fraction 8.

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Table 3.3. Predicted mono-isotopic molecular mass in Dalton (-8 Da because of oxidized cysteines) of each Hc-AFP peptide, the molecular mass expected and the molecular mass obtained by mass spectrometry analysis. The molecular mass was calculated by using the MaxEnt function of MassLynx 4.1.

Peptide	Molecular mass (Da)	[M + H] Expected (Da)	[M + H] Experimental (Da)
Hc-AFP1	5471.25	5472.25	5472.27
Hc-AFP2	5710.30	5711.30	5694.50/5711.54
Hc-AFP3	5416.0	5417.0	5417.23
Hc-AFP4	5724.4	5725.4	5707.15/6415.10

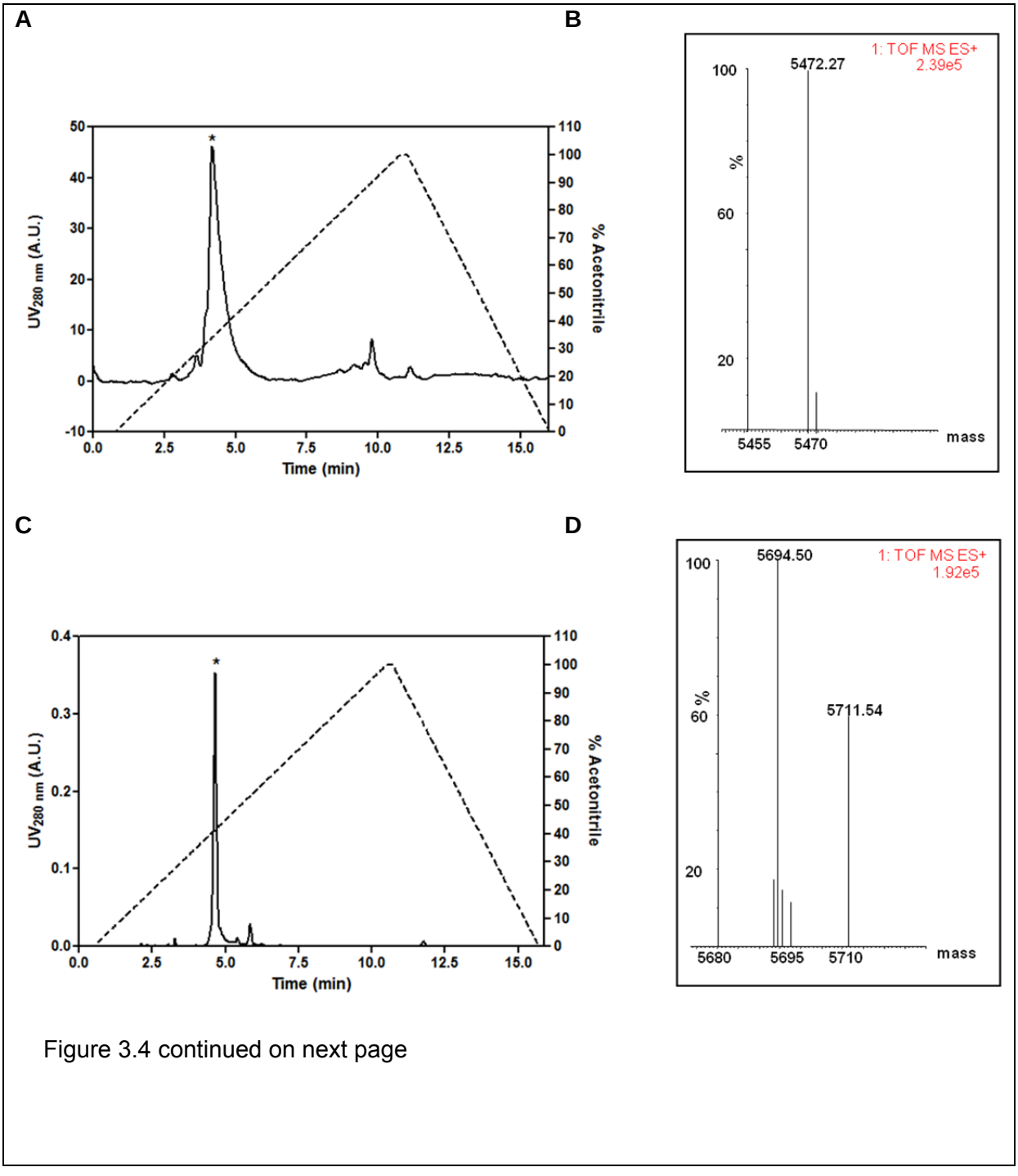

Figure 3.4 continued on next page

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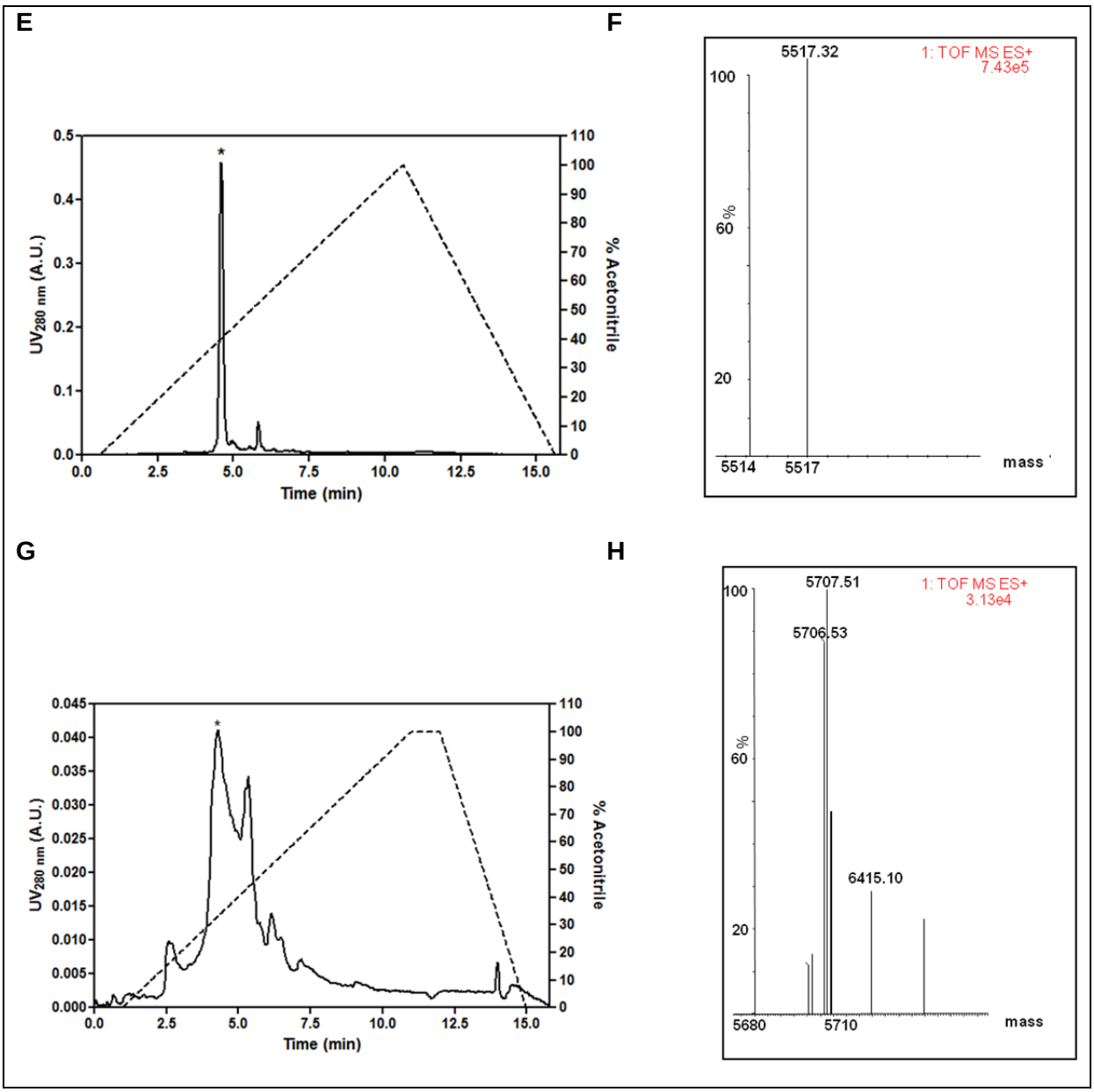

Figure 3.4. Purification of Hc-AFPs by reverse phase HPLC and the MALDI-TOF mass spectra of the corresponding purified Hc-AFPs. The bound peptides were eluted with a linear gradient of acetonitrile (dashed line) in 0.1% TFA and monitored by absorbance at 280 nm (solid line). The results obtained from the HPLC purification and the MALDI-TOF mass spectra respectively for each peptide is presented as follows: Hc-AFP1 (A) and (B); Hc-AFP2 (C) and (D); Hc-AFP3 (E) and (F) and Hc-AFP4 (G) and (H). The asterisk (*) indicates the peak corresponding to the specific Hc-AFP.

When comparing the previously used bacterial production system with the current yeast production system it is clear that there is a marked increase in yield of the produced peptides (Table 3.4). The yeast production system rendered a yield that was at 4-10 fold higher than the amount of peptide obtained with the bacterial production system.

3.3.3 The effect of ionic buffer strength on Hc-AFP2 production and characteristics from *Pichia*

It has been reported that antimicrobial peptides with an N-terminal glutamine undergo a common post-translational modification where the N-terminal glutamine cyclizes to form pyroglutamic acid

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(pGlu) (Kandke *et al*., 1989; Welker *et al*., 2007). The formation of pGlu would explain why Hc-AFP2 and Hc-AFP4 exhibited molecular masses that were 17 Da lower than their expected molecular mass. Furthermore it was reported that this cyclization was increased in phosphate buffer (Kandke *et al*., 1989; Welker *et al*., 2007). In an attempt to solve the problem of pGlu formation that has been detected with the production of Hc-AFP2 and Hc-AFP4, Hc-AFP2 was chosen as a test case where a reduced ion BMG medium was used.

The Hc-AFP2 was successfully produced in the *P. pastoris* X33 using the reduced ion BMG medium and was named Hc-AFP2 (Gln). Hc-AFP2 (Gln) was purified by cation exchange chromatography (Figure 3.5A). Tris-Tricine SDS-PAGE analysis of the Hc-AFP2 (Gln) peptide fractions displayed monomeric (5 kDa band), dimeric (10 kDa band) and trimeric (15 kDa band) forms of this recombinant defensin (Figure 3.5B).

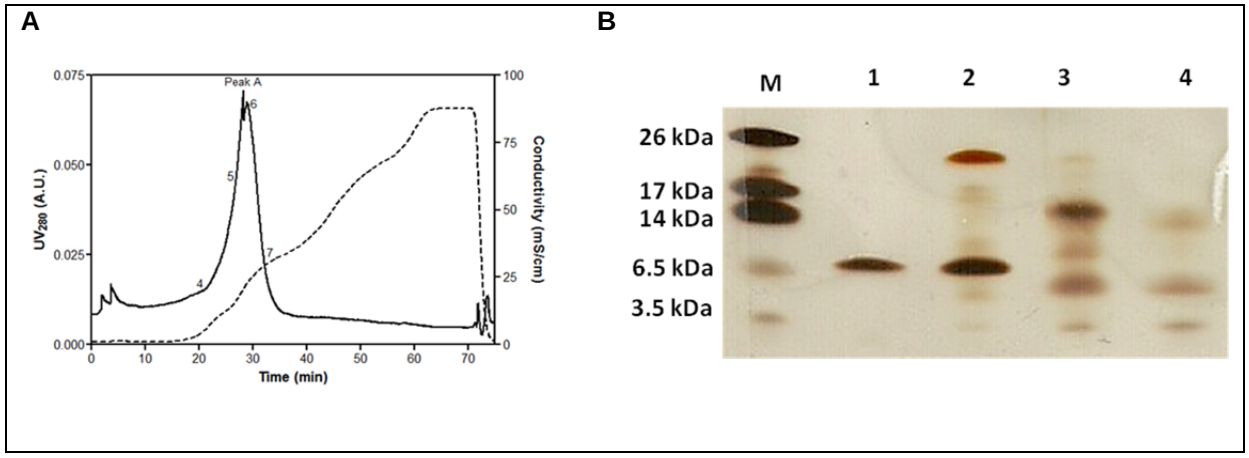

Figure 3.5. Cation exchange chromatograms and Tris-Tricine SDS-PAGE analyses of Hc-AFP2 produced with low ionic strength medium. The results obtained from the cation exchange purification for Hc-AFP2 is presented in (A). The elution was performed by means of a gradient of 0.01-1M $NaCl_2$ (dashed line). The protein content was monitored by measurement of UV at 280 nm (solid line). Peak A indicated on the chromatogram contained Hc-AFP2 defensin peptide as was collected in the numbered fractions as indicated on the chromatograms. Tris-Tricine SDS-PAGE analysis of the peaks collected during the cation exchange purification of Hc-AFP2 (B) with lane M, low molecular weight marker (Sigma, St. Louis, USA); lane 1, fraction 4; lane 2, fraction 5; lane 3, fraction 6 and lane 4, fraction 7.

Interestingly, compared to Hc-AFP2 produced in normal BMG (designated Hc-AFP2 (pGlu), Hc-AFP2 (Gln) produced in the reduced ion BMG medium eluted earlier under the same chromatographic conditions. This is a clear indication that Hc-AFP2 (pGlu), produced in normal BMG is more hydrophobic than Hc-AFP2 (Gln), possibly due to the N-terminal neutral pGlu.

Mass spectrometry analyses revealed a molecular mass of 5711.55 for Hc-AFP2 (Gln) (Figure 3.6B). This molecular mass obtained was the correct mass and indicates that this peptide was produced successfully and its folding was the correct conformation. Furthermore, this also indicated that four disulphide bridges were formed in this peptide. In addition, this peptide was derived from its respective gene in the *P. pastoris* expression vector and was therefore successfully produced.

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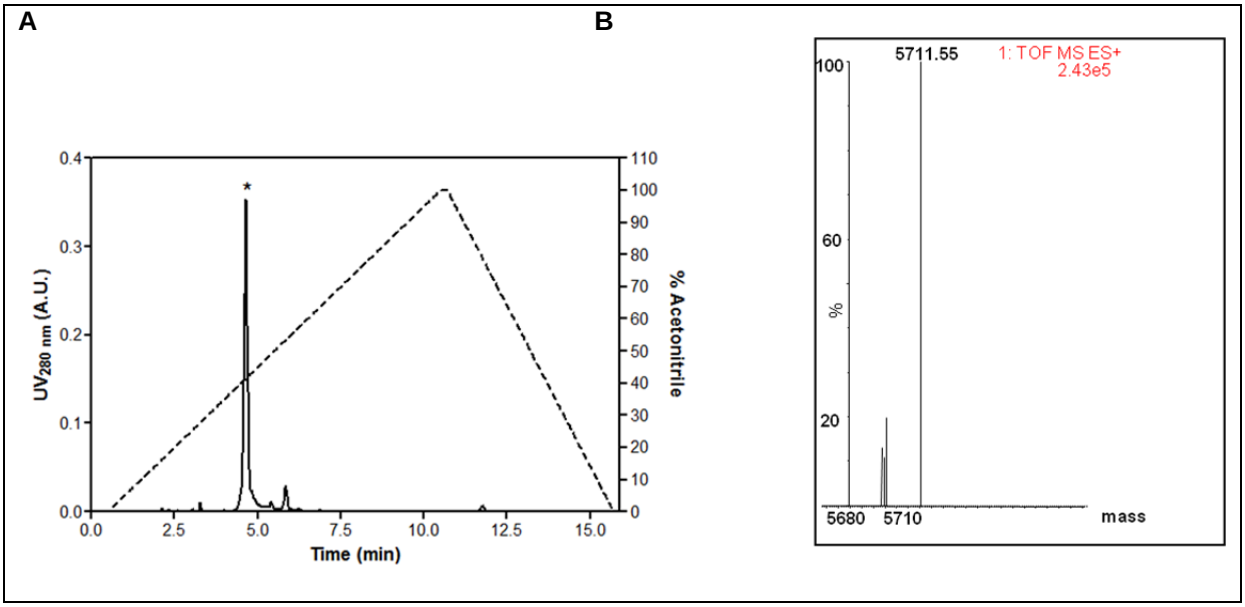

Figure 3.6. Purification of Hc-AFP2, produced in low-ionic BMG buffer, by reverse phase HPLC and the MALDI-TOF mass spectra of the corresponding purified Hc-AFP2. The bound peptide was eluted with a linear gradient of acetonitrile (dashed line) in 0.1% TFA and monitored by absorbance at 280 nm (solid line). The results obtained from the HPLC purification and the MALDI-TOF mass spectra respectively for Hc-AFP2 (Gln) (A) and (B). The asterisk (*) indicates the peak corresponding to the peptide.

Nano-Liquid chromatography and mass spectrometry were used to investigate structural differences between Hc-AFP2 (pGlu) and Hc-AFP2 (Gln). The chromatography of the trypsin digested Hc-AFP2 (pGlu) peptides revealed four peaks eluting at 14, 15 19.5 and 23 minutes respectively. The trypsin digested Hc-AFP2 (Gln) presented a similar chromatographic profile, however, with two extra peaks eluting at 11 and 20.5 minutes. Although the MS/MS fragmentation of the correlating peaks were identical, it is clear that these two peptides fragment differently with trypsin digestion. An additional trypsin digestion takes place in Hc-AFP2 (Gln), indicating that there is a difference in the tertiary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln), with the former being more trypsin resistant, possibly due to the N-terminal modification. Furthermore, the intensities in the peaks of these two trypsin digested peptides differed. The two peaks eluting at 14 and 15 minutes of Hc-AFP2 (Gln) were 3.4 fold higher than that of the corresponding eluting peaks of Hc-AFP2 (pGlu), a further indication of a difference in the conformation between these two peptides.

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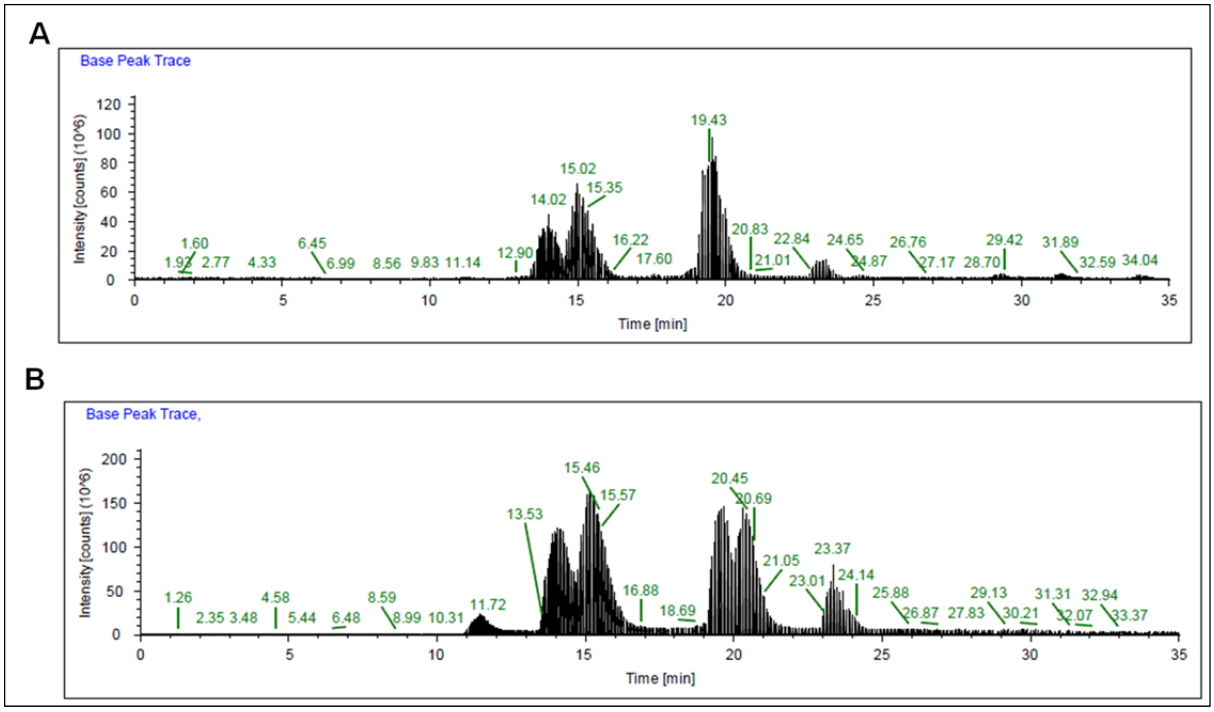

Figure 3.7. The nano-Liquid chromatography and mass spectrometry spectra of Hc-AFP2 (pGlu) (A) and Hc-AFP2 (Gln) (B).

3.3.4 Structural characterization of *H. coronopifolia* defensin peptides with circular dichroism spectroscopy

Circular dichroism (CD) analysis was performed to investigate the secondary structure of the Hc-AFP defensin peptides. All the Hc-AFPs presented CD spectra that are characteristic to structured peptides (Figure 3.8). The Hc-AFPs presented spectra with maxima and minima respectively at 192 nm and 211 nm for Hc-AFP1, 192 nm and 210/215 nm for Hc-AFP3, 191 nm and 207 nm for Hc-AFP2 (pGlu), 189 nm and 206 nm for Hc-AFP2 (Gln) and 186 nm and 199 nm for Hc-AFP4. These detected CD spectral maxima and broad minima for Hc-AFP1-3 are indicative of predominantly β-sheet combined with α-helical structures. This is consistent with the conserved structure of a short three helical turn α-helix with three anti-parallel β-sheets found for plant defensins (Bruix *et al*., 1993; Fant *et al*., 1998). Furthermore, CDspectra of several other plant defensin peptides presented similar spectral characteristics (Almeida at al., 2001; Carbral *et al*., 2003; Marqués *et al*., 2009). Hc-AFP1 and Hc-AFP3 share structural similarities, with Hc-AFP3 possibly having more α-helical structure. Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) also have similar structures, with Hc-AFP2 (pGlu) having less ordered structures in the aqueous solution. In this analysis, Hc-AFP4 differed from the rest of the Hc-AFP defensin peptides, displaying a typical unordered structure, confirming the failure to produce this peptide correctly in the *Pichia* system. A more detailed CD study will be presented in Chapter 4.

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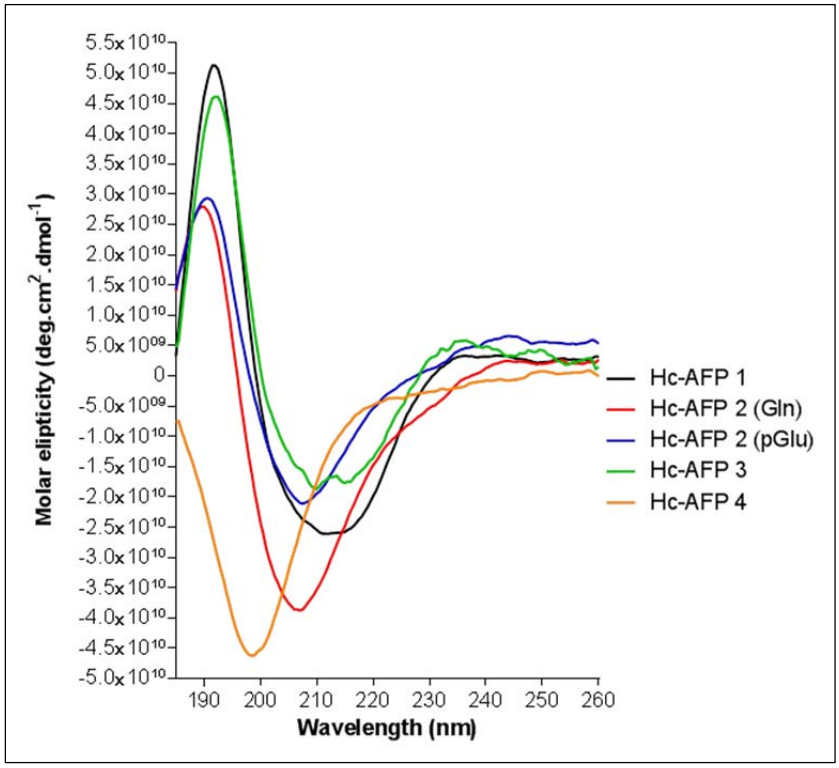

Figure 1. Circular dichroism spectra of Hc-AFP peptides. Circular dichroism spectra were recorded form 185 nm to 206 nm for Hc-AFP1 (black), Hc-AFP2 (pGlu) (blue), Hc-AFP2 (Gln) (red); Hc-AFP3 (green) and Hc-AFP4 (orange). The protein concentration was 100 μM.

3.3.5 Functional characterization of the antifungal activity of *Heliophila coronopifolia* defensin peptides

The recombinant Hc-AFP defensins were tested against *F. solani* to determine if these peptides are biologically active. Liquid plate assays revealed that the different Hc-AFPs had variable activity against *F. solani* and also exhibited some well-known defensin-induced morphological changes on the spores and hyphae. In Figure 3.6, panel A shows the typical growth of *F. solani* in the absence of peptides. Examples of hyper-branching, tip swelling and disruption/lysis, when this pathogen was exposed to the peptides, are shown in panels B-E. The Hc-AFPs were tested at the concentrations known to inhibit 50% of the growth of *F. solani* (according to results obtained by bacterially produced Hc-AFPs and reported in De Beer and Vivier, 2011).

Hc-AFP1 was active against *F. solani* and caused 70% growth inhibition at a concentration of 25 μg mL^{-1}. This was 1.4 fold higher that the growth inhibition caused by the bacterially produced Hc-AFP1 at a concentration of 25 μg mL^{-1} (Figure 3.7 and Table 3.4). Microscopic analysis conducted on *F. solani* revealed that, like the bacterially produced Hc-AFP defensins, these Hc-AFP defensins also induce morphological changes on the hyphae of *F. solani* compared to the untreated control (Figure 3.7A vs Figure 3.6A). Compared to the untreated control, Hc-AFP1 induced severe hyper-branching, swollen tips and lysis of the treated hyphae, whereas only mild hyper-branching on Hc-AFP1 treated *F. solani* hyphae was reported (De Beer and Vivier, 2011) from the bacterially produced Hc-AFP1. This difference may be due to differences in experimental

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procedures and conditions ie spore count, growth rate and analytical weighing procedures. However, the difference in activity could also be due to the amount of correctly folded peptide in the preparation, with the *Pichia* produced preparation containing more correctly folded peptide molecules exerting activity.

Hc-AFP2 (pGlu) reduced the percentage growth of *F. solani* by only 19% at a concentration of 15 μg mL^{-1} and caused a very slight hyperbranching phenotype, confirming a low activity for this modified Hc-AFP2 peptide (Figure 3.7 and Table 3.4). However, the Hc-AFP2 (Gln) form demonstrated stronger antifungal activity and inhibited the growth of *F. solani* by 43% at 15 μg mL^{-1}. Furthermore, this form of the peptide induced severe hyper-branching and lysis of the hyphae compared to the untreated control. These observed morphological effects of Hc-AFP2 (Gln) are consistent with those observed from bacterially produced Hc-AFP2 (Table 3.4).

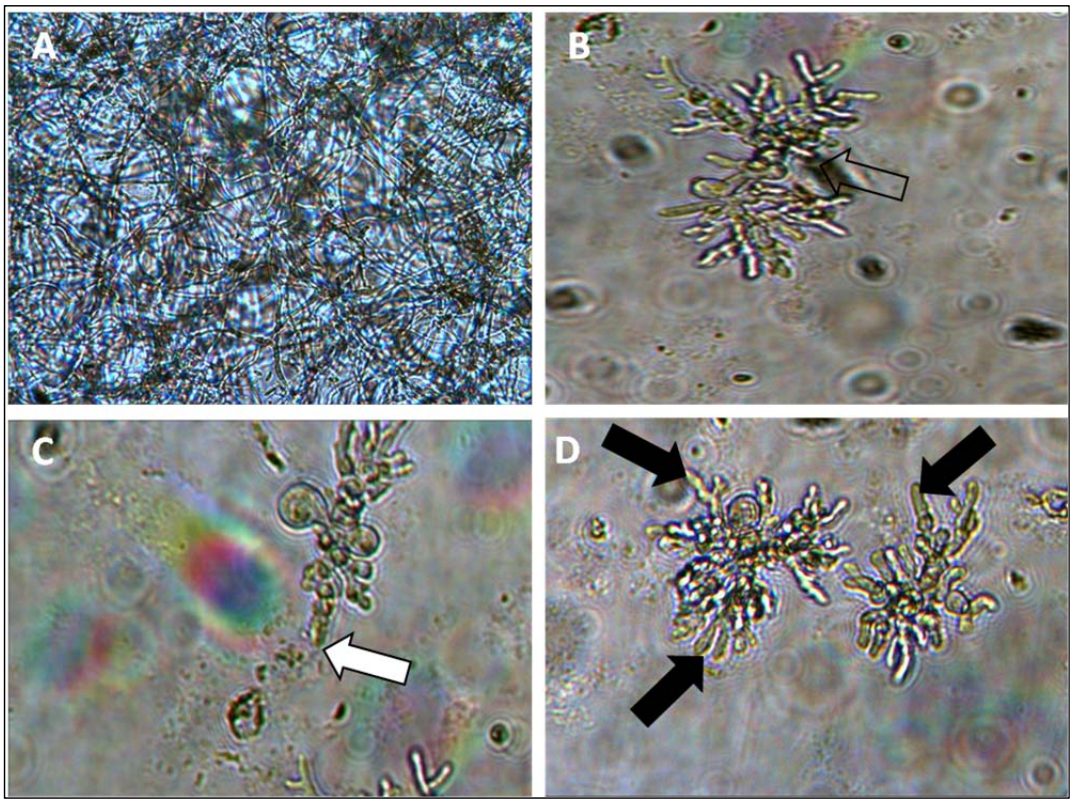

Figure 3.6. Typical growth of F. solani in the absence of peptides at 40x magnification (A) and some examples of hyper-branching (B), disruption/lysis (C) and tip swelling (D). The unfilled arrows indicate hyper-branching, the black arrows indicate swollen tips and the white arrows indicate lysis.

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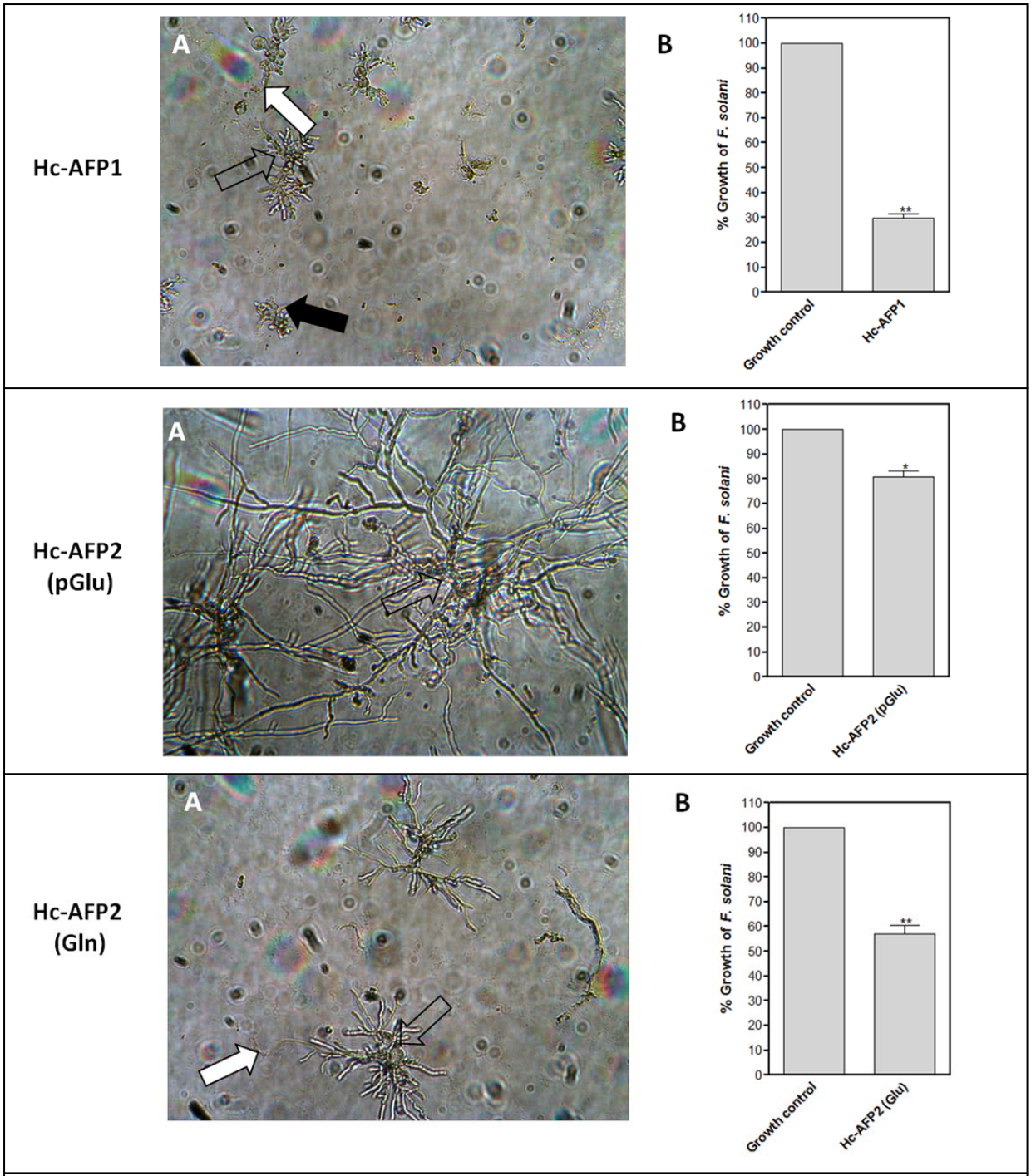

Figure 3.7 continued on next page

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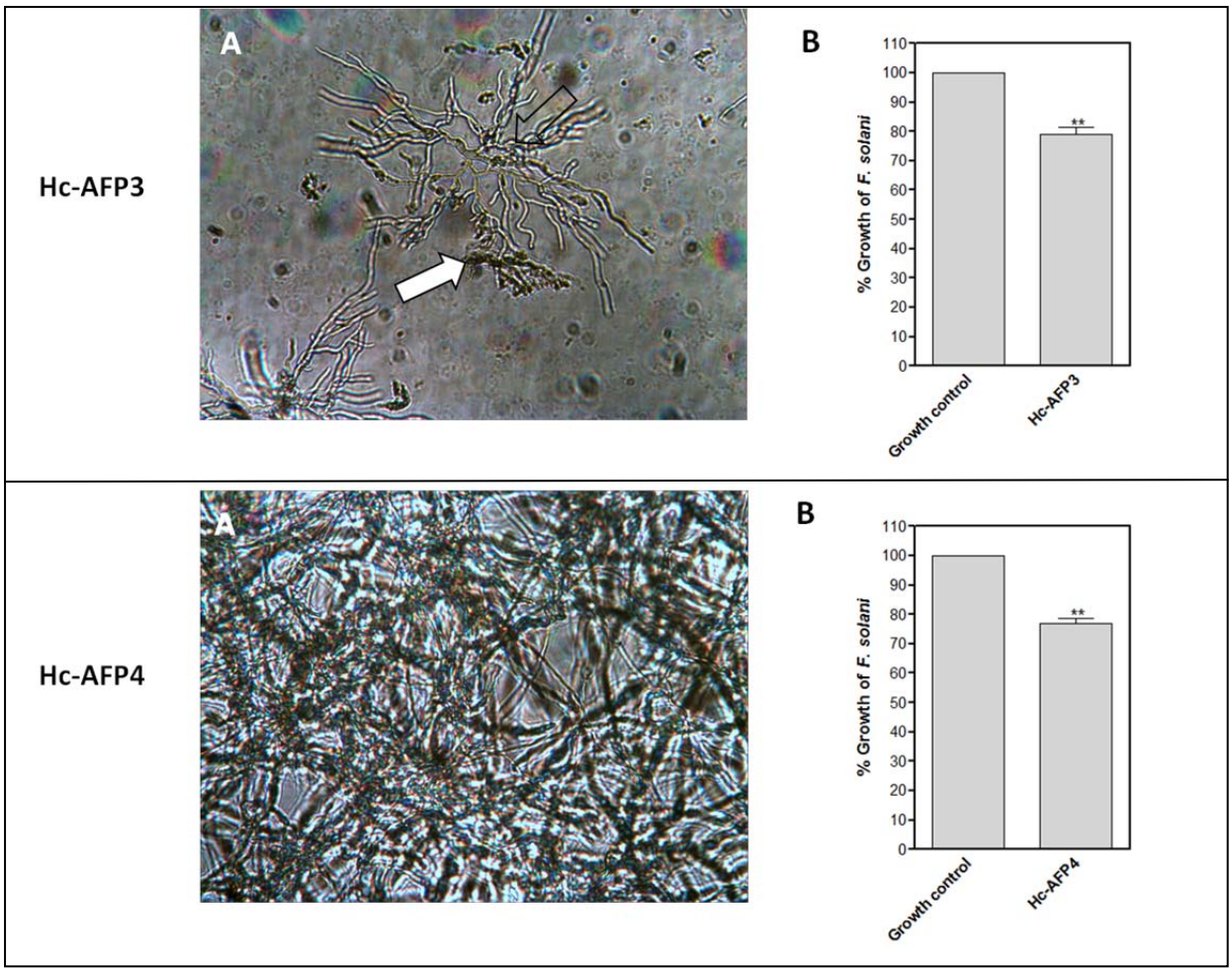

Figure 3.7. Antifungal activity of the recombinant *H. coronopifolia* peptides against *F. solani*. *F. solani* was grown in the presence of Hc-AFPs for 36 h at 23˚C. *F. solani* treated with the Hc-AFPs at 40x magnification (A) and the respective measured percentage growth (B). The concentrations of Hc-AFPs used were 25 µg ml^{-1} for Hc-AFP1, 15 µg ml^{-1} for Hc-AFP2 (pGlu) and Hc-AFP2 (Gln), 25 µg ml^{-1} for Hc-AFP3 and 10 µg ml^{-1} for Hc-AFP 4. The unfilled arrows indicate hyper-branching, the black arrows indicate swollen tips and the white arrows indicate lysis. Statistical analysis was performed by a T-test compared to the control with $p< 0.01$ (*) and $p< 0.001$ (**).

The *Pichia*-produced Hc-AFP3 only mildly inhibited the growth of *F. solani*, but induced severe hyper-branching and lysis of the *F. solani* hyphae (Figure 3.7 and Table 3.4). Hc-AFP4, however, did not show strong inhibitory activity against *F. solani* at a concentration of 10 µg mL^{-1} and no morphological changes on the treated *F. solani* hyphae could be observed at this concentration (Figure 3.7 and Table 3.4). A comparative summary of the activities and morphological effects observed of the peptides produced in *Pichia* (this study) versus the data generated from bacterially produced peptides (according to De Beer and Vivier, 2011) is presented in Table 3.4.

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Table 3.4. Antifungal activity of the Hc-AFP defensins

	***E. coli* production system**				***P. pastoris* production system**			
Defensin	**Yield (mg mL^{-1}) from 1.6L**	**Conc. tested µg/mL**	**% Growth inh.**	**Hyphal morpho-logy**	**Yield (mg mL^{-1}) from 1 L**	**Conc. tested µg/ mL**	**% Growth inh.**	**Hyphal morpho-logy**
Hc-AFP1	0.2	25	<50	Mild hyper-branching	1.95	25	70	Severe hyper-branching Tip swelling Lysis
Hc-AFP2 (pGlu)	nd	-	-	-	1.40	15	19	Mild hyper-branching
Hc-AFP2 (Gln)	0.2	10-15	50	Severe hyper-branching	1.53	15	43	Severe hyper-branching Lysis
Hc-AFP3	0.2	25	<50	Mild hyper-branching	0.91	25	21	Severe hyper-branching Lysis
Hc-AFP4	0.2	5-10	50	Severe hyper-branching	1.02	10	23	none

nd not determined

3.4 DISCUSSION

Here we describe the production, purification and activity analysis of the *Heliophila coronopifolia* plant defensins, Hc-AFP1-4, using *Pichia* as a production system.

3.4.1 *P. pastoris* as a production system for *H. coronopifolia* plant defensin peptides

Due to the tedious purification process required during the purification of the *H. coronopifolia* plant defensin peptides produced in a bacterial system and the low yields obtained (De Beer and Vivier, 2011), an alternative strategy was selected for the production and purification of these peptides that involved the use of *Pichia* as heterolgous host. This production system uses a pGAPZαA vector that constitutively express recombinant proteins from the GAP promoter in *P. pastoris.* Furthermore this vector produces proteins fused to an N-terminal peptide encoding the *S. cerevisiae* α-factor secretion signal. One of the benefits of using this yeast for expression includes the tightly regulated and efficient promoters as well as a strong tendency for respiratory growth instead of fermentative growth (Cregg *et al*., 2009). Furthermore this production system has the ability to produce foreign proteins at a high level and supports many eukaryotic post-translational modifications. In addition, one of the major advantages of this system is that *P. pastoris* secretes low levels of native proteins, greatly facilitating the purification procedures. The limitations of this

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production system include the fact that the *P. pastoris* system lack promoters for moderate expression, sometimes leading to toxic levels of expression. Moreover, the protein-handling machinery of the cell could be overwhelmed by the overexpression, leading to misfolding and unprocessing of some of the cellular protein. However, despite these limitations, *P. pastoris* has become a very popular host system for the production of heterologous proteins (Cregg *et al*., 1993; Waterham *et al*., 1997; Balamurugan *et al*., 2007) and has been used, amongst others, for the development of human and animal vaccines. These include the expression of the human papilloma virus (HPV) type 6 L1 protein, turnicated bovine herpes virus-1 (BHV-1) gd gene and Mb86 antigen from the cattle tick, *Boophilus microplus* in *P. pastoris* (Balamurugan *et al*., 2007). Similarly, several plant defensin peptides have been successfully expressed in *P. pastoris.* A few examples of these defensins include the pea *Pisum sativum* defensin 1 (Psd1); the mungbean *Vigna radiata* defensin 1 (VrD1); the tomato pistil-predominant 3 (TPP3) defensin and thePlant defensin corn 1 (PDC1) (Almeida *et al*., 2001; Chen *et al*., 2004; Kant *et al*., 2009; Lay *et al*., 2012).

The Hc-AFP defensin peptides previously isolated were shown to contain typical plant defensin tertiary structure as they harbour eight cysteine residues engaged in four disulphide bridges that are essential for the biological activities. This complex tertiary structure complicated the production of these peptides in a bacterial system, but was successfully produced in *Pichia* in this study in a higher yield. After four days of growth in baffled flasks, the *P. pastoris* Hc-AFP 1-4 strains produced approximately ~1 mg Hc-AFP defensin peptides from a starting volume of 1000 mL. This increased yield is one of the advantages of the *P. pastoris* production system and in line with what was reported for the mungbean defensin, VrD1 (Chen *et al*., 2004; Balamurugan *et al*., 2007) and the expression of the *Chondrus crispus* Hexose oxidase (HOX) (Wolff *et al*., 2001).

P. pastoris was also selected as a heterologous host system for its known ability to correctly process and fold eukaryotic proteins (Larroque *et al*, 2011) and importantly, also for the processing and folding of cystein-rich peptides and proteins (Larroque *et al*, 2011; Yan *et al*, 2003; and Cregg *et al*, 1985). The Hc-AFP defensin peptides were easily purified by a few chromatographic steps.

The mass spectrometry analysis of the purified Hc-AFP1 and Hc-AFP 3 corresponded to the predicted mono-isotopic mass, confirming the successful production of these two Hc-AFP defensins. Furthermore, this also revealed that the four disulphide bridges of these peptides were formed. Intact disulphide bridges are necessary for the stabilization of the oligomeric structure of these peptides (Lay and Anderson, 2005).

The mass spectrometry analyses of Hc-AFP2 and Hc-AFP4 showed that the peptide preparations contained peptides with masses that were 17 Da less than the expected and predicted mono-isotopic mass. A common post translational modification that may explain this occurrence is the cyclization of the amino terminal glutamine residue to form pyroglutamic acid (Blomback *et al*., 1967). This is probable since the Hc-AFP2 and Hc-AFP4 peptide sequences both contain an N-terminal glutamine and the purified peptides differed from their predicted counterparts in mass corresponding to the loss of 17 atomic mass units or an OH group. Interestingly, the

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purified preparation of Hc-AFP2 contained two species, one with the expected mono-isotopic mass and another that was 17 Da less, so it is possible that the preparation contained a mixture of peptides, some with and some without the post translational modification. This is a reported disadvantage of the *P. pastoris* production system, where the high level of expression of the GAP promoter may overwhelm the protein-handling machinery of the cell and leading to the misfolding or unprocessing of a significant portion of the cellular proteins (Balamurugan *et al*., 2007). Furthermore, this post translational modification event has been reported to be dependent on reaction conditions such as the buffer, temperature and time of incubation (Khandke *et al*., 1989). Although this is a familiar post translational modification in defensin peptides, it has not yet been demonstrated to have an effect on the activity of plant defensins (Mendez *et al*., 1990; Terras *et al*., 1992). However, for certain other proteins this cyclization has been demonstrated to negatively affect the enzymatic stability, conformational properties and biological activity (Khandke *et al*., 1989; Welker *et al*., 2007; Burov *et al*., 2009), whereas an increase in the activity and stability of the onconase protein, an anti-cancer chemotherapeutic agent (Welker *et al*., 2007) was reported and the N-terminally modified Alzheimer's disease-related amyloid-β peptide has been shown to have an increased aggregation propensity and showed increased toxicity (Sun *et al*., 2012).

3.5.2 A low ionic buffer resolved the post-translational modification that caused the presence of a terminal pyroglutamic acid in Hc-AFP2

Hc-AFP2 was used to evaluate if a buffer change could solve the cyclization post-translational modification of the N-terminal glutamine. It has been reported that ions influence this cyclization and, more importantly, that phosphate buffer accelerates the conversion of glutamine to pyroglutamic acid (Khandke *et al*., 1989). The lower ionic strength buffer produced an Hc-AFP2 with the expected mass (Hc-AFP2 (Gln)) and was useful to compare with the pyroglutamic acid-containing peptide (Hc-AFP2 (pGlu)). Mass spectrometry analysis demonstrated the expected Hc-AFP2 molecular mass for Hc-AFP2 (Gln), indicating that this peptide was produced successfully and that the purified plant defensins were derived from their respective genes present in the yeast expression vectors. Furthermore this indicated that the fundamentally important four disulphide bonds were formed. SDS-PAGE analysis also revealed that the different expected conformational forms were present in Hc-AFP2 (pGlu). This oligomerization is a known feature of the *Brassicaceae* plant defensins (Terras *et al*., 1992; Terras *et al*., 1993). Interestingly, compared to Hc-AFP2 (pGlu), Hc-AFP2 (Gln) eluted earlier in the HPLC purification step, indicating that Hc-AFP2 (pGlu) is more hydrophobic than Hc-AFP2 (Gln). It has been reported that peptides containing an N-terminal glutamine has a significantly higher retention time compared to their pGlu cyclized counterparts due to the increased hydrophobicity afforded by cyclization (Khandke *et al*., 1988).

Nano-liquid chromatography and mass spectrometry were used to investigate structural differences between Hc-AFP2 (pGlu) and Hc-AFP2 (Gln). The fragmentation spectra of Hc-AFP2

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(pGlu) and (Gln) differed in that Hc-AFP2 (Gln) contained an additional trypsin digestion. This demonstrated that the tertiary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) differ and can be explained by the possible N-terminal modification of Hc-AFP2 (pGlu), resulting in a more trypsin resistant peptide. Furthermore, the intensities of the peaks in the fragmentation spectra differed, a. clear indication that these two peptides are structurally different.

3.4.3 The characterization of *H. coronopifolia* defensin peptides in terms of structural stability

To further evaluate the *Pichia* production system the secondary structures of Hc-AFP defensin peptides were investigated by CD spectroscopy. The spectra obtained for the Hc-AFPs were characteristic to structured peptides. Furthermore, the structures of Hc-AFPs induced a CD spectrum that is indicative of predominantly β-sheet combined with α-helical structures. This is consistent with the conserved structure previously determined for plant defensins that consists out of a α-helix with three helical turns and three anti-parallel β-sheets (Bruix *et al*., 1993; Fant *et al*., 1998). In addition, several other plant defensin peptides that have been characterized by CD presented similar circular dichroism spectra (Almeida at al., 2001; Carbral *et al*., 2003; Marqués *et al*., 2009). Interestingly, the CD spectra of Hc-AFP1 and Hc-AFP3 shared similarities, consistent with *in silico* analysis conducted on these peptides that revealed that Hc-AFP1 and Hc-AFP3 share 94% amino acid sequence homology. Combined homology models and alignment analysis of Hc-AFPs showed that most of the amino acid differences occurred in the putative α-helical regions of the peptides. Hc-AFP3 has a less polar α-helical region as compared to Hc-AFP1.

Analysis of the amino acid sequences of Hc-AFP2 and Hc-AFP4 showed that these two peptides also share 94% similarity and only differed in the α-helical region of the peptides. The predicted tertiary structure of Hc-AFP1 and Hc-AFP3 (designated homology group 1) differs from that of Hc-AFP2 and Hc-AFP4 (designated homology group 2) due to the unique amino acids present in the putative α-helical region (De Beer and Vivier, 2011). Although it was expected that the CD spectra of the secondary structures of Hc-AFP2 and 4 should demonstrate similarity, the Hc-AFP4 spectrum indicated an unordered structure. This confirmed that Hc-AFP4 was not produced correctly in the *Pichia* production system. In analysing the results of Hc-AFP2, it was concluded that the N-terminal glutamine was converted into pyroglutamic acid producing the Hc-AFP2 (pGlu) form of the peptide rather than the “normal” and more active Hc-AFP2 (Gln) form. Although the primary and secondary structures of these two forms of Hc-AFP2 were similar, the differences in the spectra indicated conformational differences. This was corroborated by the differences in trypsin sensitivity of the two peptides with the N-terminal modified peptide being less sensitive to trypsin as discussed above.

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3.4.4 Three of the four *Pichia*-produced *H. coronopifolia* plant defensins showed activity against *F. solani*

In order to further evaluate the recombinant production system and the peptides produced, the biological activity of the Hc-AFP defensin peptides were determined against *F. solani.* Both Hc-AFP 1 and 3 showed strong activity against *F. solani,* inducing changes in the morphology of the *F. solani* hyphae, including severe hyper-branching and lysis of the treated hyphae at a concentration of 25 µg mL^{-1}. In addition, Hc-AFP1 also induced tip swelling. The activity of Hc-AFP 1 and 3 observed in this study is consistent with the activity reported for Hc-AFP 1 and 3 produced in a bacterial system (De Beer and Vivier, 2011). This further confirms that the peptide preparations contained the correct foldamers and that the production of these two peptides in *Pichia* was successful. Nevertheless, Hc-AFP1 and Hc-AFP3, produced in *P. pastoris,* showed a higher level of activity as that reported for the Hc-AFP1 and Hc-AFP3, produced in the bacterial system, particularly with regards to the severity of the hyper-branching effect on treated hyphae at a concentration of 25 µg mL^{-1}. This effect was not reported for bacterially-produced Hc-AFP 1 and 3 treated hyphae at the same concentration. Similarly Hc-AFP1 and Hc-AFP3 produced by *P. pastoris* had a severe effect on the integrity of the *F. solani* spores, causing disintegration of the membranes of spores, leading to the leakage of the cytoplasmic content into the surrounding environment. This lytic activity was not reported for the bacterially-produced corresponding peptides and might be due to the enhanced folding ability of the eukaryotic system (Balamurugan *et al*., 2007). Although the growth inhibitory effect of Hc-AFP3 was not reflected in the liquid plate assays (this aspect requires further investigation), the strong activity of this peptide was clear in the microscopical analysis, specifically with regards to the lytic activity.

Our data clearly showed that the post-translational modification that produced a terminal pyroglutamic acid versus a glutamine, negatively affected the biological activity of Hc-AFP2. The inhibitory effect of Hc-AFP2 (Gln) was double that of Hc-AFP2 (pGlu) (refer to Figure 3.7, Table 3.4). Furthermore, Hc-AFP2 (Gln) induced similar morphological effects on the hyphae of *F. solani* that has previously been reported for this peptide (De Beer and Vivier, 2011). These include the severe hyper-branching and lysis of *F. solani* hyphae. Despite showing low inhibition of fungal growth, subtle morphological effects were induced by the Hc-AFP2 (pGlu) peptide. It is unclear whether these effects were due to the two species of peptides identified in the initial purification when phosphate buffer was used, as observed in the mass spectrometry analysis.

Hc-AFP4 did not demonstrate the strong morphogenic inhibitory activity against *F. solani* that has been previously reported for the bacterially-produced peptide and had no morphological effect on the hyphae. This corroborates the low abundance of a correctly folded Hc-AFP4 peptide in the preparation, as determined by the mass spectrometry analysis. It remains to be seen whether this peptide can be produced correctly by using the low ionic strength buffer that was used to produce Hc-AFP2 (Gln).

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Although the *P. pastoris* produced peptides Hc-AFP1, Hc-AFP2 (Gln) and Hc-AFP3 demonstrated a higher activity than that of the bacterially produced peptides, caution is necessary when interpreting these results since the concentrations of the Hc-AFP defensin peptides used were based on the IC_{50} concentrations determined with bacterially-produced Hc-AFP defensin peptides. Future studies need to include a determination of the IC_{50} values of the *P. pastoris* produced Hc-AFP defensin peptides.. Furthermore, future studies should include an activity assay over time since this will provide a better understanding of the specific activity of each peptide. As in most cases, the *P. pastoris* produced plant defensins have demonstrated to be more active than that of the *E. coli* production system (Chen *et al*., 2004; Balamurugan *et al*., 2007). The difference could be that more correct foldamers are produced in the eukaryotic than in the prokaryotic expression system, leading o a higher overall activity in the peptide preparation.

In conclusion, although Hc-AFP4 was not successfully produced in *P. pastoris*, this system was successful in the production of active forms of Hc-AFP1, 2 and 3, with higher yields and using a simplified purification process than the bacterial system. Moreover, the structural analysis of the peptides with CD confirmed their deduced structural features based on sequence analysis and homology modelling.

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Chapter 4

Research results

The characterization of *Heliophila coronopifolia* defensin peptides in terms of structural stability and activity in the presence of cations

Part of this chapter will be incorporated into a manuscript in preparation for **PLoS ONE .** The authors of the manuscript will be Helmien Barkhuizen, Abré de Beer, Marina Rautenbach and Melané A. Vivier.

The authors contributed as follows to the work presented: HB performed all experiments, data-interpretation and compiling of results under the guidance of AdeB, MR and MAV; HB drafted and finalized the chapter with inputs from all authors; AdeB, MR and MAV conceived of the study.

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RESEARCH RESULTS

The characterization of *Heliophila coronopifolia* defensin peptides in terms of structural stability and activity in the presence of cations

4.1 INTRODUCTION

Plant defensins are ubiquitous in the plant kingdom and have been isolated from a broad range of plant species and organs, including seeds, leaves, pods, flowers and tubers (Broekaert *et al*., 1997). These peptides form an important part of the innate immune system of plants and play a vital role in the protection of plants from invading pathogens. These peptides can be easily produced and are delivered rapidly to the location of infection without excessive energy as they are produced as single gene products (Bowman and Hultman, 1981; Broekaert *et al*., 1995; Hancock, 2001; Aerts *et al*., 2008).

The structure of plant defensins, as determined by nuclear magnetic resonance (NMR) is known to contain a distinctive structural feature, namely the cysteine-stabilized α-helix motif (CSαβ), conserved in all peptides that possess antimicrobial activity (Bruix *et al*., 1993; Broekaert *et al*., 1995, Cornet *et al*., 1995, Fant *et al*., 1998 and Lay *et al*., 2003; Carvalho *et al*., 2009). Plant defensins are strikingly structurally conserved, although they share limited sequence homology (Broekaert *et al*., 1995).

Defensins can be categorized as morphogenic and non-morphogenic peptides, based on their influence on fungal structures. Morphogenic plant defensins cause reduced hyphal elongation with an increase in hyphal hyperbranching. The *Brassicaceae* plant defensins, including *Raphanus sativus* antifungal peptide 1 and 2, belong to this group (Terras *et al*., 1992; Terras *et al*., 1993; Osborn *et al*., 1995). In comparison, non-morphogenic plant defensins slows down hyphal extension without inducing marked morphological distortions. These include plant defensins from *Asteraceae* including *Dahlia merckii* antimicrobial peptide 1. Moreover, these morphogenic and non-morphogenic plant defensins also differ in their spectrum of antifungal properties (Osborn *et al*., 1995).

Apart from their defence roles, plant defensins have been linked to other functions in plants as well. These functions include translation inhibitors, ion channel blockers and enzyme inhibitors (Carvalho et al., 2009).

The antifungal activity of plant defensins have been shown to be reduced in the presence of monovalent and divalent cations in the medium. Cations play a vital role in the growth and development of plants. It is clear that there is a constant fluctuation in the concentrations of cations in plant cells as the plant grow and respond to intrinsic and extrinsic signals. These changes occur in compartments where AMPs will typically be found. Generally the antifungal activity of defensins is more strongly antagonized by divalent cations than monovalent cations (Ameilda *et al*., 2000;

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Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). This antagonistic effect of cations on the antifungal activity appears to depend strongly on the fungal pathogens tested (Terras *et al*., 1992, Osborn *et al.*, 1995), although the precise mechanism of this antagonistic effect is still unknown. It is proposed that the effect is the result of an electrostatic interaction between the fungus and the cations rather than a conformational change in the peptide imparted by the cations (Terras *et al*., 1992).

Most plant defensins induce a range of relatively rapid membrane responses in the fungal species, as well as membrane potential changes (Thevissen *et al*., 1996). The exact mechanism of how these membrane responses are generated after an interaction with the plant defensin is, however, unknown. Plant defensins have never been found to form ion permeable pores in artificial membranes, nor change the electrical potential (Thevissen *et al*., 1996). Recently some of the molecular components involved in the antifungal inhibitory activity have been revealed for two of the best studied plant defensins, namely RsAFP2 isolated form *R. sativus,* and DmAMP1, isolated form *D. merckii*. RsAFP2 was demonstrated to interact with glucosylceramide (GlcCer), a sphingolipid present in the membranes of susceptible fungi. Interestingly RsAFP2 does not interact with the structurally related GlcCer from mammals, pointing out their selective antifungal activity (Thevissen *et al*., 2004). Moreover, glucosylceramide was recently shown to be a virulence effector of *C. albicans* (Noble *et al*., 2010). The interaction of RsAFP2 with GlcCer leads to membrane permeabilization and subsequent cell growth arrest (Thevissen *et al*., 2007). After interacting with GlcCer, RsAFP2 induces a MAPK signalling pathway involving the induction of reactive oxygen species (ROS) in a dose dependent manner and apoptosis with concomitant activation of caspases (Aerts *et al*., 2007; Aerts *et al*., 2008; Blankenship *et al*., 2010; Thevissen *et al*., 2012).

DmAMP1, was shown to interact with $M(IP)_2C$, the most abundant sphingolipid in *S. cereviseae* (Daum *et al*., 1998; Thievissen *et al*., 2003a). This plant defensin induces an array of relatively rapid membrane responses in fungi including Ca^{2+} uptake, K^+ efflux, alkalinization of the medium and membrane potential changes (Thevissen *et al*., 1996). The gene determining the sensitivity of *S. cerevisiae* for DmAMP1 was identified as *inositol phosphotransferase 1* (*IPT1*), encoding the enzyme that is involved in the terminal step in the synthesis of the major sphingolipid $M(IP)_2C$ (Dickson *et al*., 1997; Thevissen *et al*. 2000; Thevissen *et al*. 2003a). DmAMP1 sensitivity is linked to the presence of $M(IP)_2C$ and not to the presence of a functional *IPT1*-encoding protein (Ipt1p) (Thevissen *et al*., 2000). Moreover, DmAMP1-sensitivity of yeast strains is linked to the level of $M(IP)_2C$ present in their membranes which therefore plays a crucial role in the DmAMP1 antifungal action (Im *et al*., 2003; Aerts *et al*., 2006).

Recently four native plant defensin peptides have been isolated from *Heliophila coronopifolia,* a native South African *Brassicaceae* species (De Beer and Vivier, 2011). In this study we focused on two of these native plant defensins, namely Hc-AFP1 and Hc-AFP2 (both the Hc-AFP2 (Gln) and Hc-AFP2 (pGlu) forms; refer to Chapter 3 for details). These peptides were selected as representatives of the two homology groups that have been identified by De Beer and Vivier,

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2011). The Hc-AFP1, Hc-AFP2 (Gln) and Hc-AFP2 (pGlu) peptides were characterized in terms of structural stability in the presence of monovalent and divalent cations, by using circular dichroism spectroscopy. It was clear that monovalent and divalent cations induced conformational changes in the secondary structures of all the peptides, with subtle differences between the peptides. Furthermore, the activity of Hc-AFP1 against *B. cinerea* (as a known organism sensitive to the defensin), in the absence and presence of monovalent and divalent cations was evaluated. The biological activity of Hc-AFP1 was severely reduced in the presence of these cations, especially in the presence of the divalent cations. However, the permeabilization activity of Hc-AFP1 on the membranes of *B. cinerea* hyphae was not reduced in the presence of the cations. The observed conformational changes in the secondary structure of Hc-AFP1, as induced by the presence of cations could be correlated with a reduction in the biological activity of Hc-AFP1 against *Botrytis*.

4.2 MATERIALS AND METHODS

4.2.1 Microbial strains used

Botrytis cinerea was used as a sensitive organism to test the activity of the Hc-AFP1 peptide. Refer to Materials and Methods in Chapter 3 section 3.3.1 for details on the growth and preparation of the organism.

4.2.2 Hc-AFP peptides

Purified Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln), peptides were used in structural analysis (with CD spectroscopy) and activity analyses (the latter was only performed on Hc-AFP1). Refer to Materials and Methods in Chapter 3 for a description of the production, purification and preparation of the peptides.

4.2.3 Structural characterization of peptides with circular dichroism spectroscopy in the presence and absence of TFE and cations

The circular dichroism (CD) spectroscopy analysis was performed with an Applied Photophysics Chirascan-plus CD spectropolarimeter (Applied Photophysics Limited, Leuterhead, United Kingdom). Refer to Material and Methods in Chapter 3 section 3.2.8 for the description of the circular dichroism method used. Trifluoroethanol (TFE) (Sigma Aldricht Biotechnologies, USA), NaCl (Merk Chemicals, Gauteng, RSA), KCl (Merk Chemicals, Gauteng, RSA), $CaCl_2$ (Merk Chemicals, Gauteng, RSA) and $MgCl_2$ (Merk Chemicals, Gauteng, RSA) used for sample preparation were of the highest grade from commercial sources. The water used in all experiments was analytical grade water, prepared by filtering water from a reverse osmosis plant through a Millipore Milli-Q® water purification system (Milford, USA).

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The effects of monovalent and divalent cations were tested on Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) peptides by adding 1 µL of 250 mM chloride salt to 60 µL of the peptide (100 µM) dissolved in water to obtain a final concentration of 5 mM of NaCl, KCl, $CaCl_2$ and $MgCl_2$. The Hc-AFP1 and Hc-AFP2 peptides were chosen as representative examples of the two homology groupings identified in the four *Heliophilia* peptides, whereas Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) was compared for the potential impact of the terminal pyroglutamic acid on the structure of this peptide. The effects of 50% TFE:water (*v/v*) were tested on Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln). A reference spectrum, for each salt solution and 50% TFE:water (*v/v*) in analytical quality water was recorded. Results were expressed in terms of molar ellipticity ([θ]) in units of cm^2dmol^{-1} that is determined by the following equation:

$$[\theta] = 100 \times \theta/(c \times l)$$

, where θ corresponds to the measured ellipticity angle at wavelength λ (mdeg), l is the optical path length (m) and c is the protein/peptide concentration (M). A maximum (187-197 nm) and a minimum (207-220 nm) range were selected that represent the wavelengths where changes in the spectra of the selected peptides occurred. Change in secondary structure of the Hc-AFP defensin peptides in all the analyses was analysed by plotting the average of the molar ellipticity of the maxima and minima ranges and by calculating the $\theta_{max}/\theta_{min}$ ratio of these ranges. All spectra were obtained by subtracting buffer base-line spectra and smoothing was used to reduce noise levels.

4.2.4 Antimicrobial activity and membrane permeabilization of Hc-AFP1 in the absence and presence of cations

Antifungal assays were performed in order to assess the activity of the recombinant produced Hc-AFP defensin peptides; this experiment used only the Hc-AFP1 peptide as representative example. The assays were conducted by using a microspectrophotometric assay (Broekaert *et al,* 1990), performed in a 96 well microtiter plate (Bibby Sterilin Ltd, Stone, Staffs, UK) where each well contained 1000 fungal spores in 100 µL half strength potato dextrose broth (PDB). The antifungal activity of Hc-AFP1 against *B. cinerea* was assessed in the presence of monovalent and divalent cations. The purified Hc-AFP1 concentration used for this analysis was 25 µg mL^{-1}, whereas control samples contained no peptide. *B. cinerea* spores were incubated in 100 µL half strength potato dextrose broth (PDB) supplemented with 5 mM NaCl, KCl, $CaCl_2$ or $MgCl_2$ and 25 µg mL^{-1} Hc-AFP1 defensin peptide. There were also control reactions where *B. cinerea* was incubated with each of the salt solutions to assess the impact of the cations on the growth and morphology. Plates were incubated in the dark at 23˚C and readings were taken after 48 hours at a wavelength of 595 nm, whereas the morphology of the spores and hyphae was visualized microscopically after 24 hours with a Leica inverted microscope. Images were captured with a DCMI30E digital camera and analyzed with SchopeTec ScopePhoto software version x86, 3.1.475

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(ScopeTec, Hnangzhou, China). The antifungal activity of Hc-AFP1 defensin peptides were scored after 48 h and expressed in terms of % growth as described by Broekaert *et al*. (1990). The experiment consisted out of five biological repeats and two technical repeats. Statistical analysis were performed with the Student t-test with $p < 0.01$ (*) and $p < 0.001$ (**).

The ability of Hc-AFP1 to cause membrane permeabilization in the presence of monovalent and divalent cations was also assessed by using a Propidium Iodide (PI) uptake assay (Gangwar *et al*., 2006) conducted on *B. cinerea*. The Hc-AFP1 control consisted of 2× 100 µL half-strength PDB containing fungal spores (1000 spores/100 µL) and Hc-AFP1 peptide at a concentration of 25 µg mL^{-1}. Fungal strains were incubated at 23 ˚C in the presence of Hc-AFP1 for 24 hours. Control samples contained no Hc-AFP1. The salt control reactions consisted out of *B. cinerea* spores incubated in 100 µL half strength PDB supplemented with 5 mM NaCl, KCl, $CaCl_2$ and $MgCl_2$, whereas the same composition was used, but with the addition of 25 µg mL^{-1} of Hc-AFP1 to test the possible inhibitory effect of the cations on peptide action. Two incubation regimes were used: In the first, *B. cinerea* was exposed to the peptide over a 24 hours incubation period (designated “long exposure”) and in the second, peptide was added to a *Botrytis* culture that were grown for 24 hours without the peptide and incubated for 10 minutes (designated “short exposure”), before the PI assay was conducted. These incubation regimes were conducted on samples in the presence and absence of the cations. After incubation, the PDB was removed and replaced with a PI staining solution (25 µg mL^{-1} PI in half-strength PDB). The samples were viewed under an Olympus IX 81 inverted fluorescent microscope. Images were captured with a CelliR® digital camera and software system (Olympus Soft Imaging Solutions GmbH). The value of fluorescence background subtraction was determined according to the control image. The same value of background subtraction was applied to all the photos. The amount of fluorescence was determined by measuring the average grey value of the maximum of the total area on each image. The measured average grey value was normalized by the measured average grey value of the control reactions containing no salts or peptide. This experiment contained 2 biological repeats.

4.3 RESULTS

4.3.1 Structural characterization of *H. coronopifolia* defensin peptides with circular dichroism spectroscopy

Since Hc-AFP1 and Hc-AFP2 are each a representative of the two homology groups recognized among the Hc-AFP defensin peptides (as outlined in De Beer and Vivier, 2011), a more detailed structural characterization using circular dichroism was performed with these two peptides to evaluate the effects of a secondary structure inducing solvent, as well as monovalent and divalent chloride salts (cations) on peptide structure.

Comparing the spectra of Hc-AFP1 and Hc-AFP2 (Gln), the representatives of the two homology groups, it is clear that there are differences in their structures. Hc-AFP1 presented a secondary structure with a maximum molar elipticity of 192 nm and a minimum at 211 nm, whereas Hc-AFP2 (Gln) presented a secondary structure with both the maximum and minimum molar elipticities blue shifted to 189 nm and 206 nm respectively. The difference in $\theta_{max}/\theta_{min}$ ratios of these two peptides in water also indicated that Hc-AFP1 has different H-bonded structure(s) to that of Hc-AFP2 (Gln).

Furthermore, Hc-AFP1 and Hc-AFP2 (Gln) both demonstrated and increase in the maximum and decrease in the minimum molar ellipticity values with the exposure to TFE. This leads to a significant increase in the $\theta_{max}/\theta_{min}$ ratio, indicating a change in the secondary structures of these two peptides (refer to discussion below). TFE, as expected, induced more defined secondary structures of Hc-AFP1 and Hc-AFP2 (Gln) (Figure 4.1). However, TFE had a more prominent structure inducing effect on Hc-AFP2 (Gln) compared to Hc-AFP1. From these results it is clear that the secondary structures of Hc-AFP1 and Hc-AFP2 (Gln) are different in water and differently affected in the presence of TFE, indicating that the secondary structures of these two peptides are dissimilar (Figure 4.1).

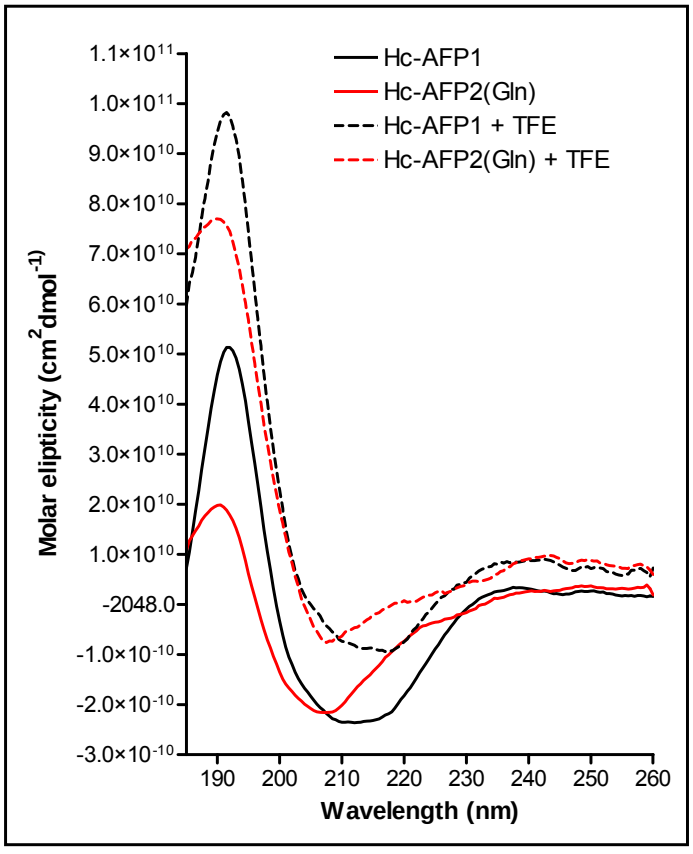

Figure 4.1. Comparative circular dichroism spectra of the Hc-AFP peptides in water and 50% TFE. Circular dichroism spectra were recorded form 185 nm to 260 nm for Hc-AFP1 (black) and Hc-AFP2 (Gln) (red). The protein concentration was 100 μM.

The influence of the structure inducing solvent, TFE, on the secondary structure of Hc-AFP1 is reported in more detail in Figure 4.2. Compared to the spectra recorded in water, which indicated a

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mixture of β-sheet and α-helical structures, the presence of 50% TFE significantly increased the ellipticity and in effect the amount of H-bonded structures (Figure 4.2A). The average of the molar ellipticity values of the maxima and minima ranges clearly indicated a significant increased maximum molar ellipticity and a significant decrease of the minimum molar ellipticity values in the presence of 50% TFE, compared to water (Figure 4.2B). The presence of TFE lead to a significant increase in the $\theta_{max}/\theta_{min}$ ratio of Hc-AFP1, which is indicative of the increase in hydrogen bonded secondary structure of Hc-AFP1 in the presence of the secondary structure inducing solvent (Figure 4.2C). TFE also led to a red shift in the minimum from 211 nm to 218 nm, indicating a change in type of secondary structure, to include more β-sheet-like structures, or higher order oligomeric structures.

The secondary structures of Hc-AFP2 (pGlu) as well as Hc-AFP2 (Gln) were similarly investigated and are reported in more detail in Figures 4.3 and 4.4. The secondary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) presented circular dichroism spectra with a maximum around 190 nm and minimum around 207 nm, indicative of β-sheet and some α-helical structures (Figure 4.3). There was not a major difference in the maximum molar ellipticity values observed between the two forms of Hc-AFP2. However, there was a significant difference in the minimum molar ellipticity values between Hc-AFP2 (pGlu) and Hc-AFP2 (Gln), where the minimum molar ellipticity of Hc-AFP2 (Gln) was significantly higher than that of Hc-AFP2 (pGlu). This lead to a significant decrease in the $\theta_{max}/\theta_{min}$ ratio of Hc-AFP2 (Gln) compared to Hc-AFP2 (pGlu) indicating significant structural difference between the two preparations. Hc-AFP2 (Gln) has more ordered structures than the modified Hc-AFP2 (pGlu), corroborating our previous results that the N-terminal modification has an influence on the secondary structure of the peptide.

There was a marked increase in the molar elipticity of both Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) in the presence of 50% TFE, indicating an increase in the overall amount of H-bonded structure (Figure 4.4B). Although the specific changes observed were different for the two forms of the peptides in the presence of 50% TFE, both the maximum and minimum molar ellipticity of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) increased, with the maxima increasing more drastically (Figure 4.4B). This lead to an overall increase in the $\theta_{max}/\theta_{min}$ ratios, due to a change in the H-bonds and the secondary structure of the two peptides (Figure 4.4C). However, the increase in the amount of structure induced by TFE in Hc-AFP2 (Gln) was significantly more than that of Hc-AFP2 (pGlu).

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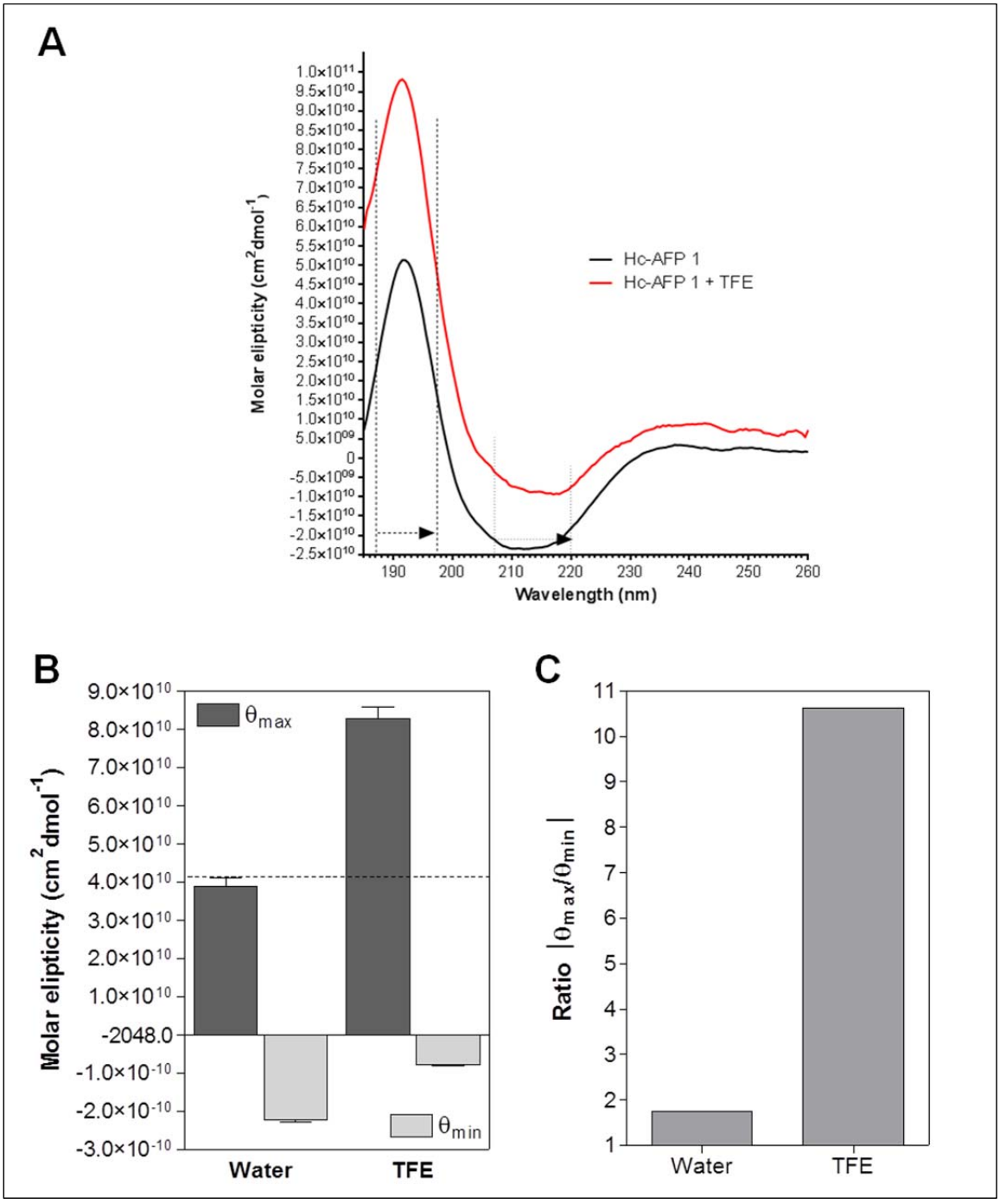

Figure 4.2. Circular dichroism spectroscopy analyses of the effects of TFE on the secondary structure of Hc-AFP1. The CD spectra were obtained at 22˚C in analytical grade milliQ water and 50% TFE. The peptide concentration used in this analysis was 100 µM. (A) Circular dichroism spectra of Hc-AFP1 in water (black line) and 50% TFE (red line). The wavelength range defining the maximum peak is 187-197 nm (dashed lines and arrow) and the wavelength range defining the minimum peak is 207-220 nm (dotted lines and arrow) (B) The maximum and minimum molar elipticity of Hc-AFP1 in the presence of water and 50% TFE. Error bars indicates the standard deviation. (C) The absolute values of the $\theta_{max}/\theta_{min}$ ratio of the Hc-AFP1 circular dichroism spectra in water and 50% TFE.

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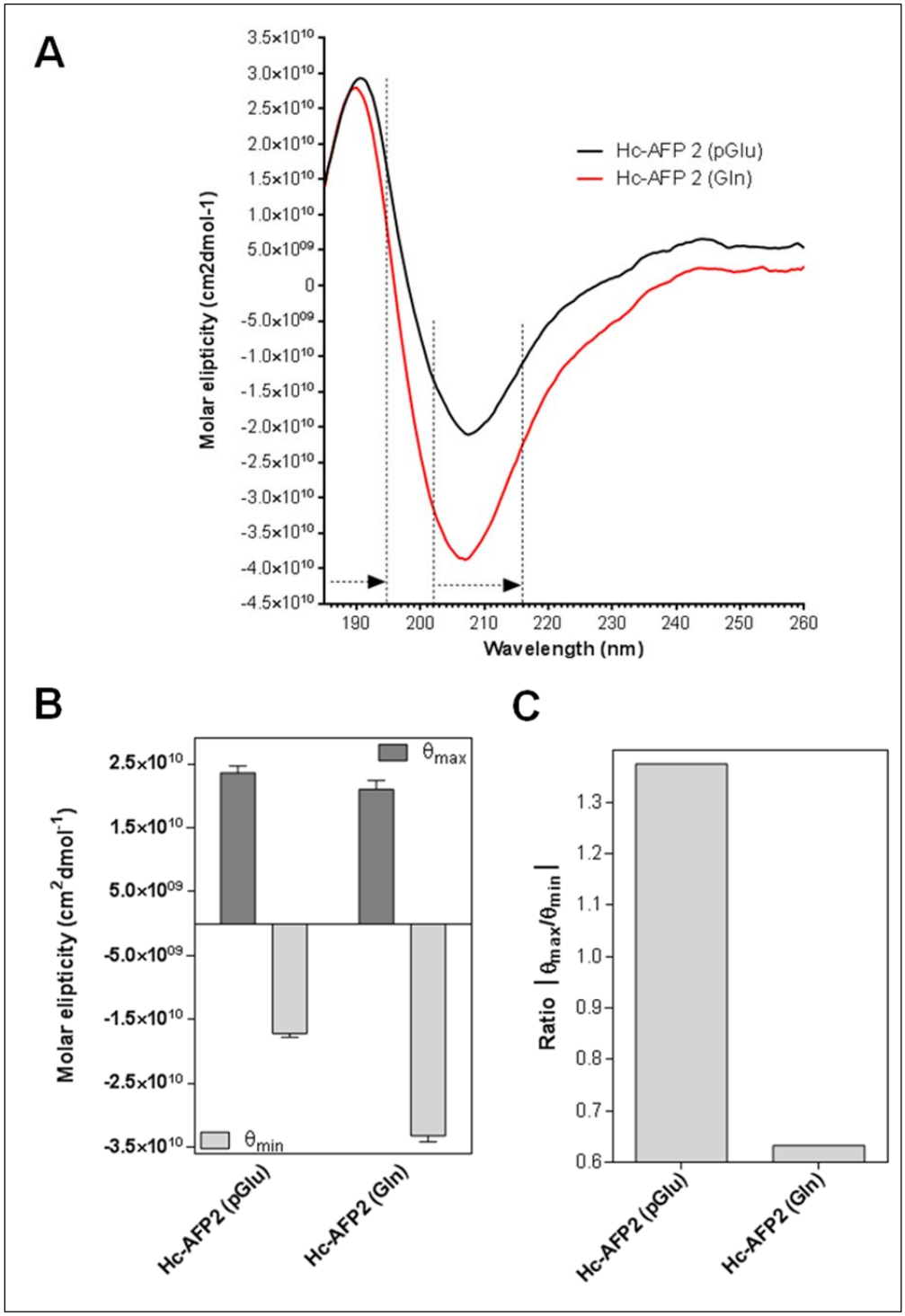

Figure 4.3. Circular dichroism spectroscopy analysis of the secondary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln). The CD spectra were obtained at 22 ˚C in analytical grade milliQ water. The peptide concentration used in this analysis was 100 µM. (A) Circular dichroism spectra of Hc-AFP2 (pGlu) (black line) and (Gln) (red line) in water. The wavelength range defining the maximum peak is 185-195 nm (dashed lines and arrow) and the wavelength range defining the minimum peak is 202-216 nm (dotted lines and arrow) (B) The maximum and minimum molar ellipticity of Hc-AFP2 (pGlu) and (Gln) in the presence of water. Error bars indicates the standard deviation. (C) The absolute values of the $\theta_{max}/\theta_{min}$ of Hc-AFP2 (pGlu) and (Gln) circular dichroism spectra in water.

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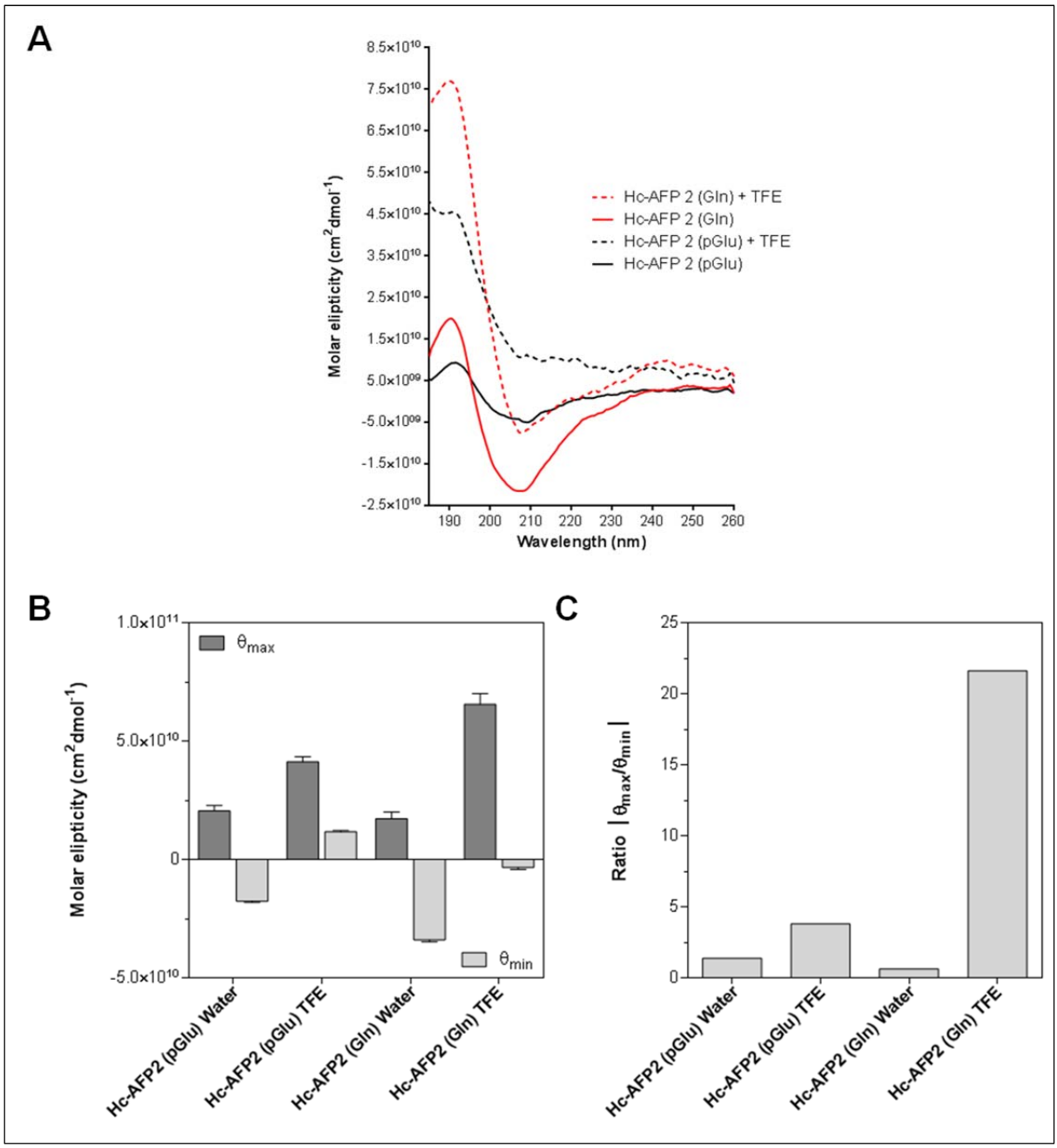

Figure 4.4. Circular dichroism spectroscopy analysis of the effects of TFE on the secondary structure of Hc-AFP2 (pGlu) and (Gln). The CD spectra were obtained at 22 ˚C in analytical grade milliQ water and 50% TFE. The peptide concentration used in this analysis was 100 µM. (A) Circular dichroism spectra of Hc-AFP2 (pGlu) (black lines) in water (black solid line) and 50% TFE (black dashed line) and Hc-AFP2 (Gln) (red lines) in water (red solid line) and 50% TFE (red dashed line). The wavelength defining the maximum peak is 185-195 nm and the wavelength defining the minimum peak is 202-216 nm. (B) The maximum and minimum peaks in molar ellipticity of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) in the presence of water and 50 %TFE. Error bars indicates the standard deviation. (C) The absolute values of the $\theta_{max}/\theta_{min}$ of the Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) circular dichroism spectra in water and 50% TFE.

To determine the effect of monovalent and divalent cations on the secondary structures of Hc-AFP1, Hc-AFP2 (Gln) and Hc-AFP2 (pGlu) circular dichroism spectroscopy analyses were also conducted on the peptides in the presence of biologically important chloride salts, NaCl, KCl, $MgCl_2$, and $CaCl_2$ (Figure 4.5A and B). These salts were tested at a biological relevant

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concentration of 5 mM and the same concentration was used for all the salts in order to compare their effect on the CD spectra of a specific peptide. A maximum (187-197 nm) and minimum (207-220 nm) range were selected that represent the wavelengths where the major changes in the spectra of the peptides occurred. It was clear that, compared to water, the presence of 5 mM of both NaCl and $MgCl_2$ lead to a significant increase in the maximum molar ellipticity and a decrease in the minimum molar ellipticity (Figure 4.5A). These changes increased the $\theta_{max}/\theta_{min}$ ratio of the Hc-AFP1 secondary structure, suggesting that Hc-AFP1 hydrogen-bonded structures are moderately affected in the presence of these cations (Figure 4.5B). In contrast, 5 mM $CaCl_2$ and KCl induced a significant decrease in the maximum molar ellipticity and an increase of the minimum molar ellipticity, which lead to an overall decrease in the $\theta_{max}/\theta_{min}$ ratio of the secondary structure of Hc-AFP1. These changes indicated a more radical change in secondary structure of Hc-AFP1 in the presence of 5 mM $CaCl_2$ and KCl.

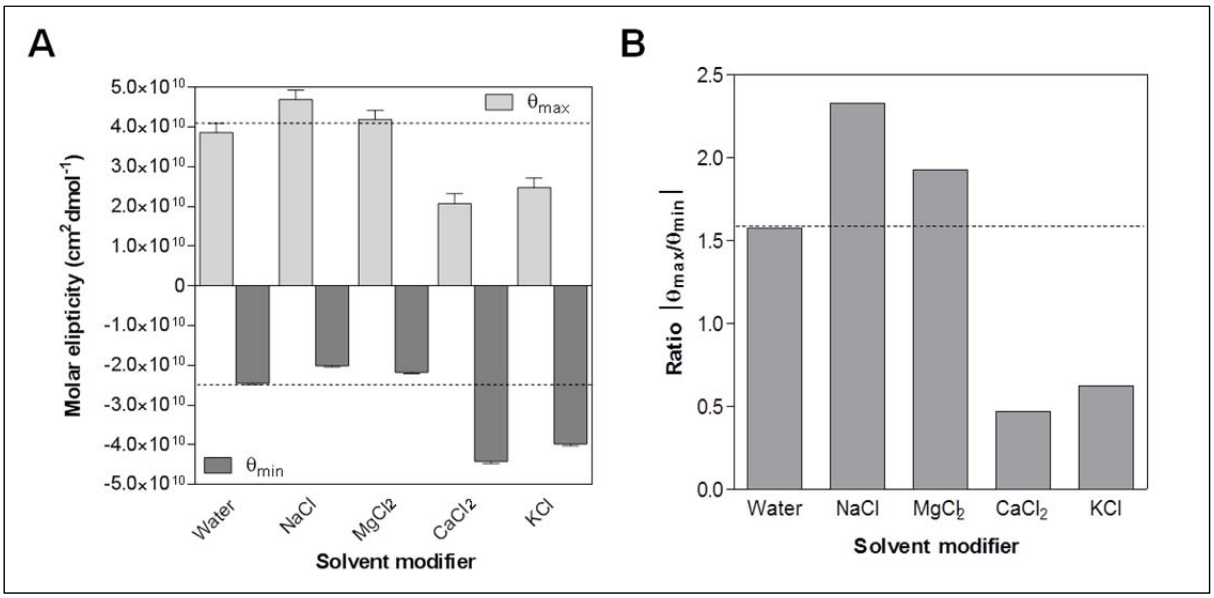

Figure 4.5. Effect of divalent and monovalent cations in the form of biological salts on the secondary structure of Hc-AFP1. (A) The effect of divalent and monovalent ions on the change in the maximum (187-197) and minimum (207-220) molar ellipticity**.** Error bars indicate the standard deviation. (B) The absolute values of the $\theta_{max}/\theta_{min}$ of the Hc-AFP1 circular dichroism spectra in water and in the presence of 5 mM NaCl, $MgCl_2$, $CaCl_2$ and KCl.

The same CD analysis was also done for Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) (Figures 4.6 and 4.7). Maxima (185-195 nm) and minima (202-216 nm) ranges were selected to represent the wavelengths where major changes in spectra of the two analogous peptides occurred. In the presence of NaCl, the maximum molar ellipticity of the secondary structure of Hc-AFP2 (pGlu) increased slightly and there was little or no effect in the minimum molar elipticity (Figure 4.6A and 4.7A). This lead to a slight increase in the $\theta_{max}/\theta_{min}$ ratio of the secondary structure of Hc-AFP2 (pGlu), indicating a slight change in structure (Figure 4.6B). The presence of the divalent cation $MgCl_2$ caused a slight decrease in the maximum molar ellipticity and an increase in the minimum molar ellipticity. This indicates a decrease of the secondary structure and in the $\theta_{max}/\theta_{min}$ ratio of

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Hc-AFP2 (pGlu). KCl and $CaCl_2$ had a more pronounced effect on the secondary structure of Hc-AFP2 (pGlu), where there was a decrease in the maximum molar ellipticity and an increase in the minimum molar ellipticity indicating a loss of the secondary structure. This caused a significant decrease in the $\theta_{max}/\theta_{min}$ ratio, indicative of a conformational change of the secondary structure of Hc-AFP2 (pGlu), or a loss of peptide signal due to aggregation.

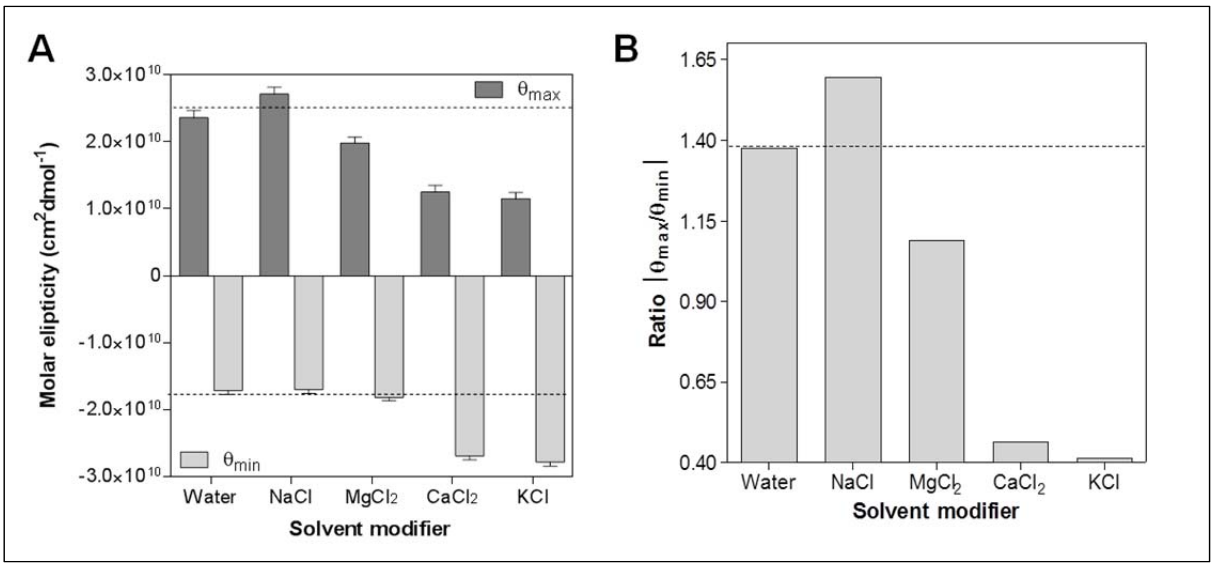

Figure 4.6. Effect of divalent and monovalent cations in the form of biological salts on the secondary structure of Hc-AFP2 (pGlu). (A) The effect of divalent and monovalent ions on the change in the maximum (185-195) and minimum (202-216) molar ellipticity. Error bars indicate the standard deviation. (B) The absolute values of the $\theta_{max}/\theta_{min}$ of the Hc-AFP2 (Gln) circular dichroism spectra in water and in the presence of 5 mM NaCl, $MgCl_2$, $CaCl_2$ and KCl.

The effects of the salts on Hc-AFP2 (pGlu) were similar to that of the salts on Hc-AFP1, but quite dissimilar to that of its analogue, Hc-AFP2 (Gln). In the presence of NaCl and $MgCl_2$, the maximum molar ellipticity of Hc-AFP2 (Gln) increased and minimum molar elipticity decreased (Figure 347). This lead to a major increase in the $\theta_{max}/\theta_{min}$ ratios indicating structural change. KCl and $CaCl_2$ had similar effects as with Hc-AFP2 (pGlu) and Hc-AFP1 on the secondary structure of Hc-AFP2 (Gln), where there was a decrease in the maximum molar ellipticity and increase in the minimum molar ellipticity of the secondary structure. This indicates a conformational change of the secondary structure of Hc-AFP2 (Gln) to a less defined secondary structure or loss of peptide from solution. This change also resulted in a significant decrease in the $\theta_{max}/\theta_{min}$ ratio, again indicating a structural change. The overall effect of the monovalent and divalent cations on the secondary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) therefore differed between these two forms of the peptide.

When comparing the effect of monovalent and divalent cations on the secondary structure of Hc-AFP1, and the two forms of Hc-AFP2, it is obvious that the secondary structures of these peptides were affected differently. Although the presence of NaCl possibly lead to an overall increase in secondary structure of these peptides, the increase in structure was much greater for Hc-AFP2 (Gln). The presence of $MgCl_2$ lead to an decrease in the $\theta_{max}/\theta_{min}$ ratio of Hc-AFP2 (pGlu)

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and an increase in the $\theta_{max}/\theta_{min}$ ratio of Hc-AFP1 and Hc-AFP2 (Gln). However, $MgCl_2$ had a greater conformational effect on Hc-AFP2 (Gln) as the increase in the $\theta_{max}/\theta_{min}$ ratio of the secondary structure was much higher than that of the secondary structure of Hc-AFP1. These results again indicate that the secondary structures of Hc-AFP1 and the two analogous Hc-AFP2 preparations are different.

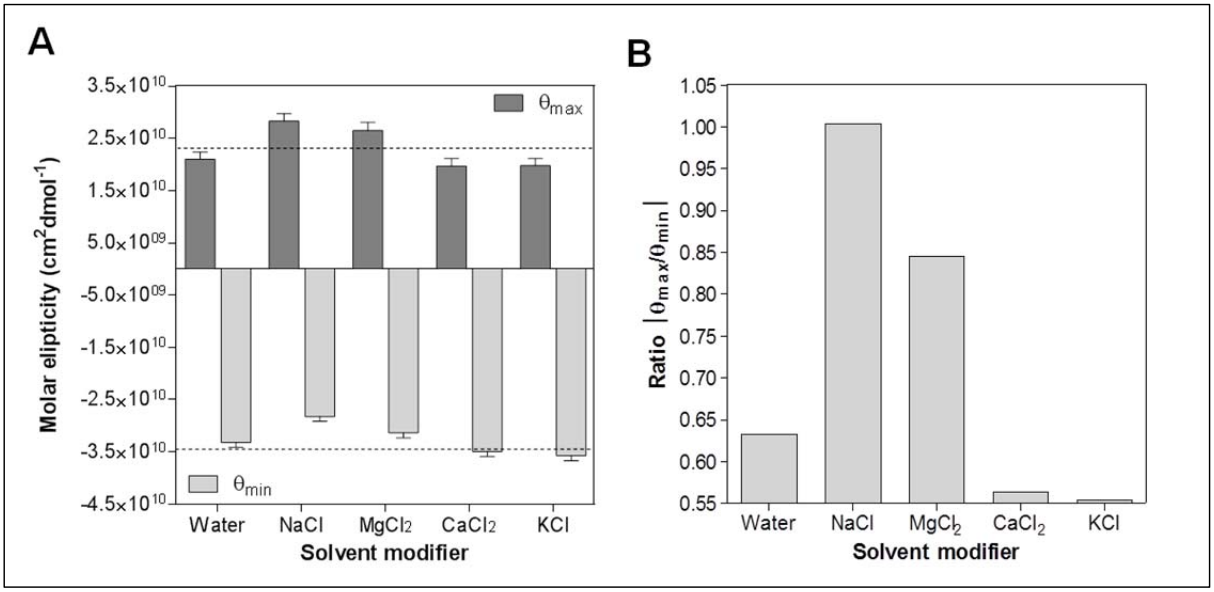

Figure 4.7. Effect of divalent and monovalent cations in the form of biological salts on the secondary structure of Hc-AFP2 (Gln). (A) The effect of divalent and monovalent ions on the change in the maxima (185-195) and minima (202-216) molar ellipticity. Error bars indicate the standard deviation. (B) The absolute values of the $\theta_{max}/\theta_{min}$ of the Hc-AFP2 (Gln) circular dichroism spectra in water and in the presence of 5 mM NaCl, $MgCl_2$, $CaCl_2$ and KCl.

4.3.2 Effect of monovalent and divalent ions on the biological activity of Hc-AFP1

The effect of monovalent and divalent ions on the antifungal activity of one of the peptides, Hc-AFP1, was assessed as a representative example. These assays were performed by incubating *B. cinerea* spores in the absence of peptide and cations, as a control to observe normal *Botrytis* growth; in the presence of 25 μg mL^{-1} Hc-AFP1, as the Hc-AFP1 peptide control; in the presence of 5 mM NaCl, $MgCl_2$, $CaCl_2$, and KCl each as the salt controls (without peptide); and in the presence of 5 mM NaCl, $MgCl_2$, $CaCl_2$, KCl and 25 μg mL^{-1} Hc-AFP1 to test the effect of the salts on peptide action. Interestingly, there was no significant inhibitory effect on the biomass production of *B. cinerea* grown in the presence of 5 mM NaCl, $MgCl_2$, $CaCl_2$ or KCl (Figure 4.8), although microscopic analysis of the fungal hyphae revealed that the presence of these cations induced very slight morphological changes in the hyphae and the formation of reproductive structures that was not observed in the control reaction without supplemented cations. Figure 4.9 shows typical examples of morphological effects induced by the presence of the salts and/or peptide, including tip swelling, hyphal and spore lysis/disruption and hyperbranching.

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In the presence of Hc-AFP1, the growth of *B. cinerea* was reduced by at least 90% and microscopic analysis demonstrated that this peptide induced severe hyper-branching of the *B. cinerea* hyphae (Figure 4.10 and Table 4.1). In the presence of NaCl, Hc-AFP1 inhibited the growth of *B. cinerea* by 61% with a severe hyper-branched morphology, comparable to the Hc-AFP1 control (Figure 4.10B and C). Hc-AFP1 in the presence of the other monovalent cation tested, KCl, reduced *B. cinerea* growth only by 32% (2.8 fold less than that of the Hc-AFP1 control without salt), but was unaltered in its ability to cause severe hyperbranching. Therefore, although the monovalent cations antagonised some of Hc-AFP1 activity on the growth of *B. cinerea*, strong morphological effects were still observed on the hyphae of *B. cinerea* (Table 4.1).

The divalent cations had a more pronounced antagonism on the activity of Hc-AFP1 towards *B. cinerea*. Hc-AFP1, in the presence of $CaCl_2$, or $MgCl_2$ no longer inhibited the growth of *B. cinerea* hyphae. However, the morphology of the fungal hyphae was still affected, showing hyper-branching and hyphal tip swelling (Figure 3.10G-I and J-L and Table 4.1).

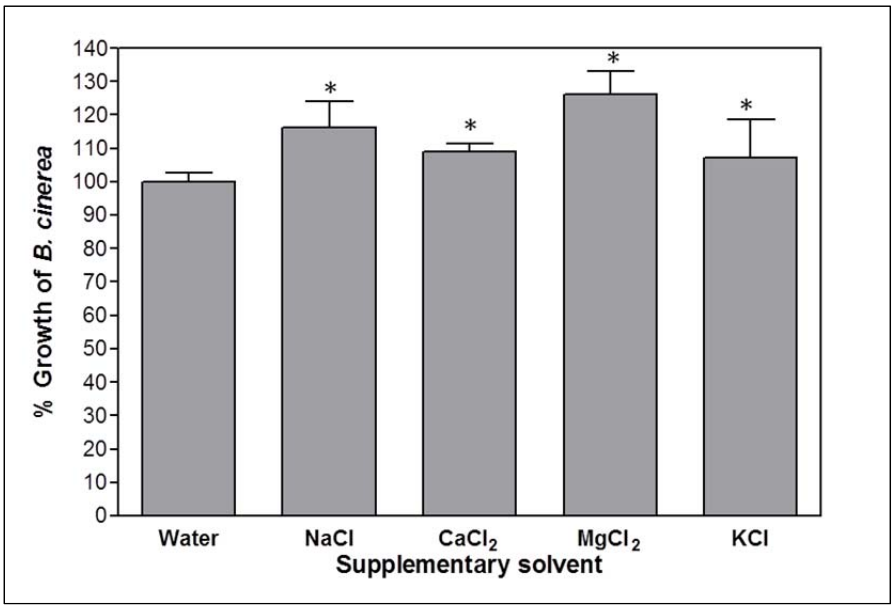

Figure 4.8. Comparison of the effect of monovalent and divalent cations on the growth of *B. cinerea*. Fungal spores were incubated in 5 mM NaCl, $CaCl_2$, KCl and $MgCl_2$ for 48 hours. The data is a representation of the percentage growth as compared to the water control. Growth inhibition was determined after 48 hours. Bars represent the average of four biological repeats and the standard error of mean. Statistical analysis was performed with a t-test showing similar growth in the presence of the salts than the control ($p < 0.05$).

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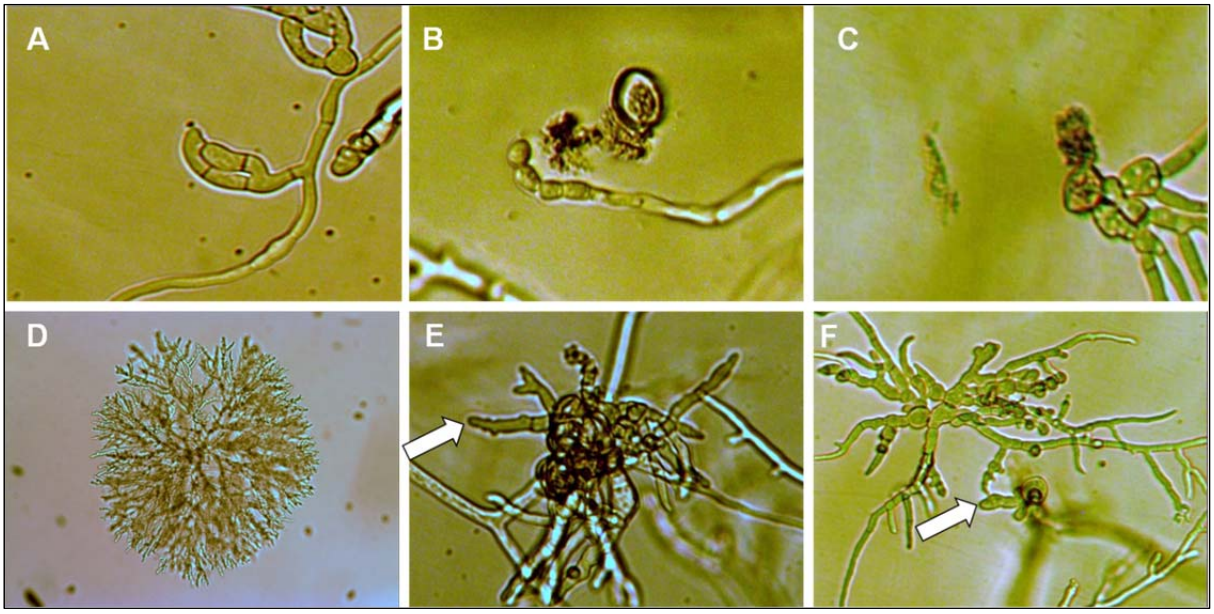

Figure 4.9. Examples of morphological structures/effects observed in the hyphae and spores of *B. cinerea* in the presence of biological salts and/or plant defensins. (A) Micro conidia reproductive structures, (B) and (C) spore lysis, (D) hyper-branching, (E) and (F) tip swelling (indicated in white arrows).

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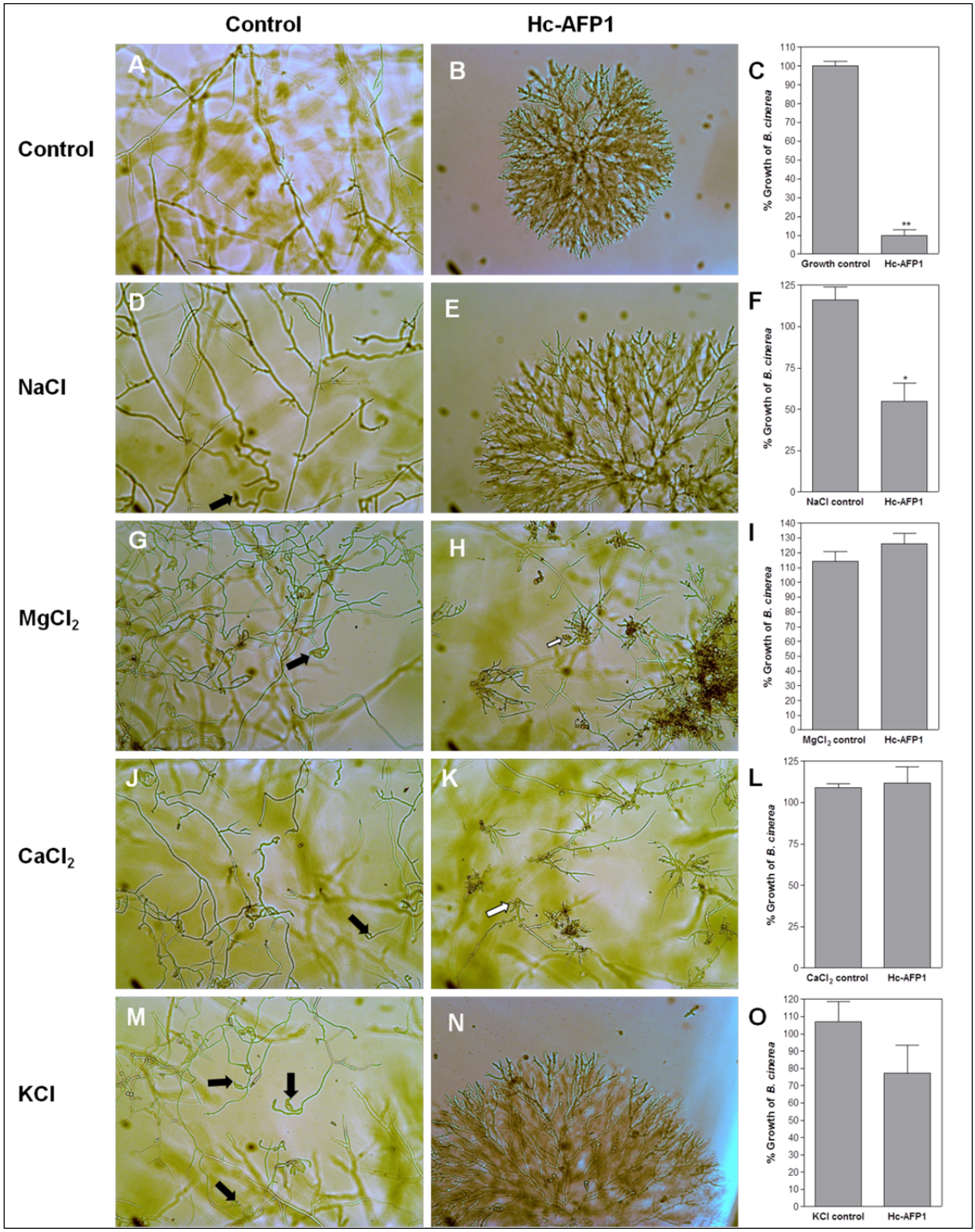

Figure 4.10. Microscopic analysis of the effect of monovalent and divalent cations on the antifungal activity of Hc-AFP1 against *B. cinerea*. Left hand photo panels: Control reactions of *B. cinerea* without Hc-AFP1 peptide at 10× magnification in the presence of water (A), 5 mM NaCl (D), 5 mM $MgCl_2$ (G), 5mM $CaCl_2$ (J) and 5 mM KCl (M). Right hand photo panels: *B. cinerea* treated with 25 µg mL^{-1} Hc-AFP1 in the presence of water (B); 5 mM NaCl (E); 5 mM $MgCl_2$ (H); 5 mM $CaCl_2$ (K) and 5 mM KCl (N) at 10x magnification. The black arrows indicate reproductive structures and the white arrows indicate hyphal tip swelling. The respective growth assays results are shown in the bar graphs in (D, F, I, L, O). The bars represents the average of four biological repeats with the standard error of mean. Statistical analysis was performed with a t-test with $p < 0.01$ (*) and $p < 0.001$ (**).

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Table 4.1. Summary of the observed antifungal activity and effect on hyphal morphology of 25 μg mL^{-1} Hc-AFP1 on *B. cinerea* in the presence and absence of cations.

Treatment	% *Botrytis* growth	Hyphal morphology
Control	100	Normal hyphae
Peptide	9.9	Severe hyper-branching
NaCl	116	Hyphae and reproductive structures
NaCl + peptide	39	Severe hyper-branching
KCl	100	Hyphae and reproductive structures
KCl + peptide	68	Severe hyper-branching
$CaCl_2$	100	Hyphae and reproductive structures
$CaCl_2$ + peptide	100	Tip swelling
$MgCl_2$	100	Hyphae and reproductive structures
$MgCl_2$ + peptide	100	Hyper-branching, tip swelling

To determine the effect of monovalent and divalent cations on the ability of Hc-AFP1 to induce membrane permeabilization, fluorescent microscopy was conducted on PI stained *B. cinerea* hyphae and spores exposed to 25 μg mL^{-1} Hc-AFP1 in two regimes. A long exposure of 24 hours and a short exposure of 10 minutes were assessed (Figures 4.11, 3.12, 3.13 and 3.14 and Table 4.2). During the long exposure period we monitored the effect of Hc-AFP1 on *B. cinerea* spore germination and subsequent hyphal growth, since the culture was started and incubated in the presence of the peptide and/or salt, depending on the treatment. During the short exposure period the effect of the peptide on actively growing *B. cinerea* hyphae in the presence or absence of the cations was assessed, since these spores were allowed to germinate and grew in the absence of Hc-AFP1 for the first 24 hours. From Figure 4.11 it is clear that the long exposure of *B. cinerea* to the peptide, without any added salts, caused a significant reduction in growth as expected. Furthermore, the measured fluorescence (average grey value) for the short exposure period was higher than that of the long exposure period (Figure 4.14). In other words, Hc-AFP1 has a greater permeabilization effect on the growing hyphae than on the spores of *B. cinerea.* It is clear from Figure 4.12 that the presence of the monovalent ions did not dramatically impede the effects of Hc-AFP1 on *B. cinerea,* corroborating previous results. Furthermore, the *B. cinerea* cultures, grown in the presence of the salts alone (NaCl and KCl), did not show increased fluorescence, when compared to the control reaction that contained no added salt or peptide (results not shown). In the presence of NaCl, Hc-AFP1 increased permeabilization of *B. cinerea* spores by 2.6 fold during the long exposure period. Furthermore, short exposure of *B. cinerea* hyphae to Hc-AFP1 and NaCl lead to a 10 fold increase in the detected fluorescence. This indicated that NaCl supported the lytic

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mode of action of the peptide possibly by stabilising secondary structure, as indicated by the CD studies. In the presence of KCl and Hc-AFP1 membrane permeabilization could be observed for both the short and long exposure periods, although long exposure lead to a 50% decrease in fluorescence, whereas the short exposure lead to a 2.1 fold increase in fluorescence.

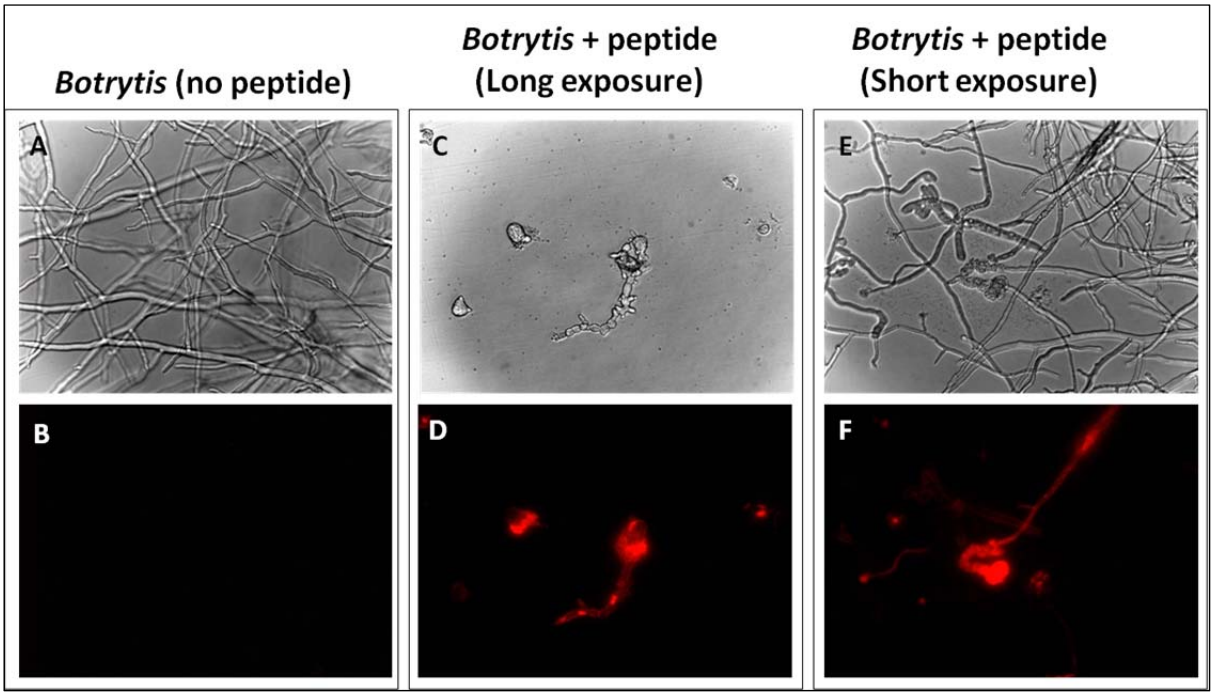

Figure 4.11. Fluorescent microscopic analysis of propidium uptake during membrane permeabilization assay in the presence of 25 ug mL^{-1} Hc-AFP1. Light microscope images (A, C, E) and fluorescent images (B, D, F) of *B. cinerea*, containing no peptide (A, B), exposed to 25 μg mL^{-1} Hc-AFP1 for a long period (C, D) and for a short period (E, F). Fungal hyphae were stained with propidium iodide for 10 min prior to fluorescent microscopic analysis.

The $CaCl_2$ and $MgCl_2$ salt control reactions showed no increase in the fluorescence, compared to the control reaction that contained no added salt or peptide (Figure 4.13). It was clear that the divalent cations had a more pronounced antagonism on the growth inhibitory activity of Hc-AFP1 towards *B. cinerea.* There was 1.5 fold decrease in the amount of fluorescence during the long exposure period of *B. cinerea* to Hc-AFP1 in the presence of $CaCl_2$. During the short exposure period of Hc-AFP1 to *B. cinerea,* in the presence of $CaCl_2$, there was an increase of 1.7 fold in the detected fluorescence (Figure 4.14 and Table 4.2). As expected, in the presence of $MgCl_2$ Hc-AFP1 did not induce a growth inhibitory affect on *B. cinerea* as was seen in the presence of Hc-AFP1 without any added salt. Furthermore this effect was comparable to that observed in the presence of $CaCl_2$ and Hc-AFP1 (Figure 4.13). In the presence of $MgCl_2$ 2.5 fold increase in fluorescence was seen during the long exposure of *B. cinerea* to Hc-AFP1 and in the presence of $MgCl_2$ there was a 2.4 fold increase in the amount of fluorescence during the short exposure period. Overall it is clear that, in the presence of monovalent and divalent cations, Hc-AFP1 has a greater permeabilization effect during the short exposure period accentuating the effect on the hyphae of *B. cinerea* (Figure 4.14 and Table 4.2).

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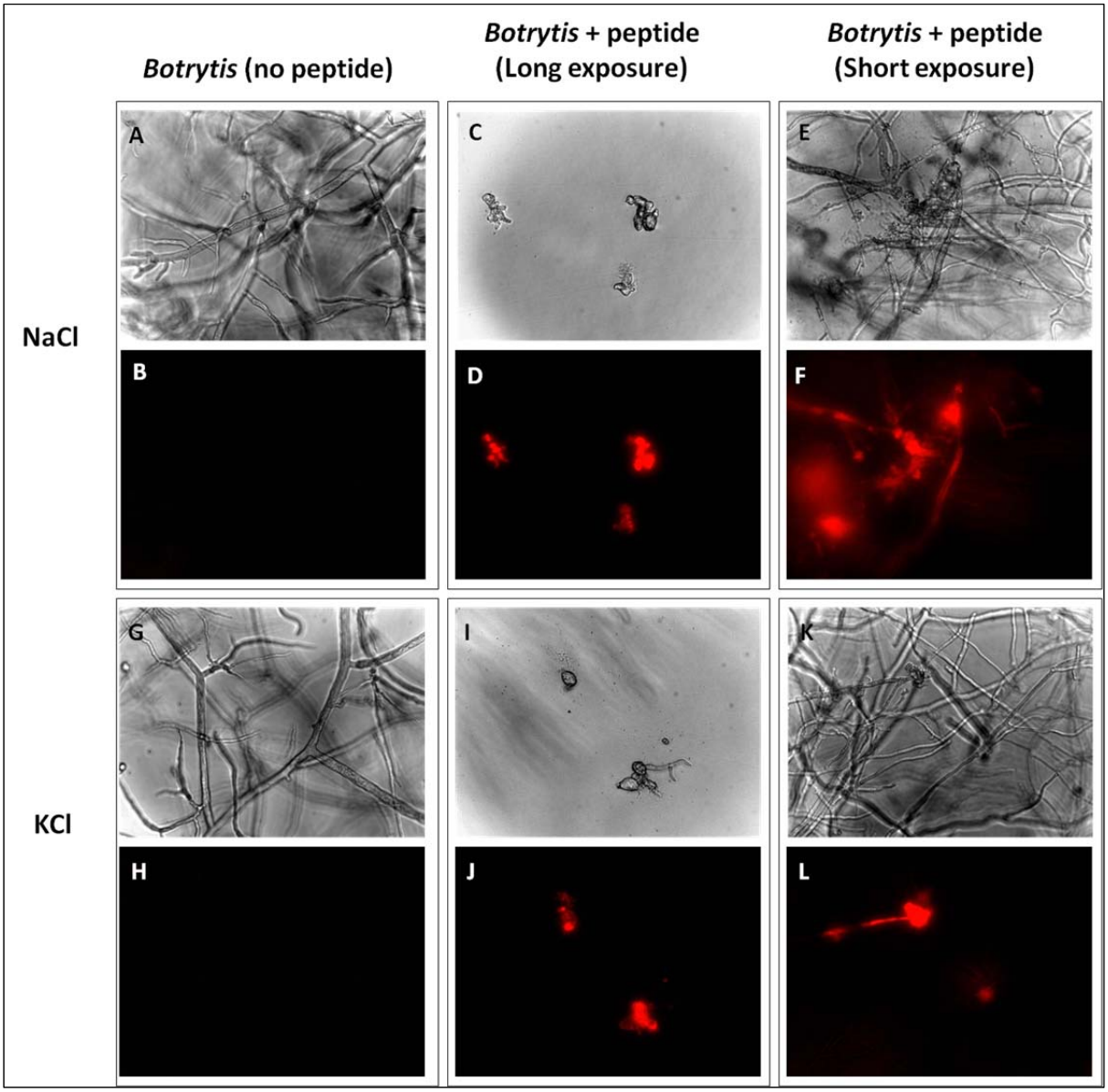

Figure 4.12. Fluorescent microscopic analysis of propidium uptake during membrane permeabilization assay in the presence of movovalent cations and of 25 μg mL^{-1} Hc-AFP1. Light microscope images (A, C, E) and fluorescent images (B, D, F) of *B. cinerea* grown in the presence of 5 mM NaCl, containing no peptide (A, B), exposed to 25 μg mL^{-1} Hc-AFP1 for a long period (C, D) and for a short period (E, F). Light microscope images (G, I, K) and fluorescent images (H, J, L) of *B. cinerea* grown in the presence of 5 mM KCl, containing no peptide (G, H), exposed to 25 μg mL^{-1} Hc-AFP1 for a long period (I, J) and for a short period (K, L). Fungal hyphae were stained with propidium iodide for 10 min prior to fluorescent microscopic analysis.

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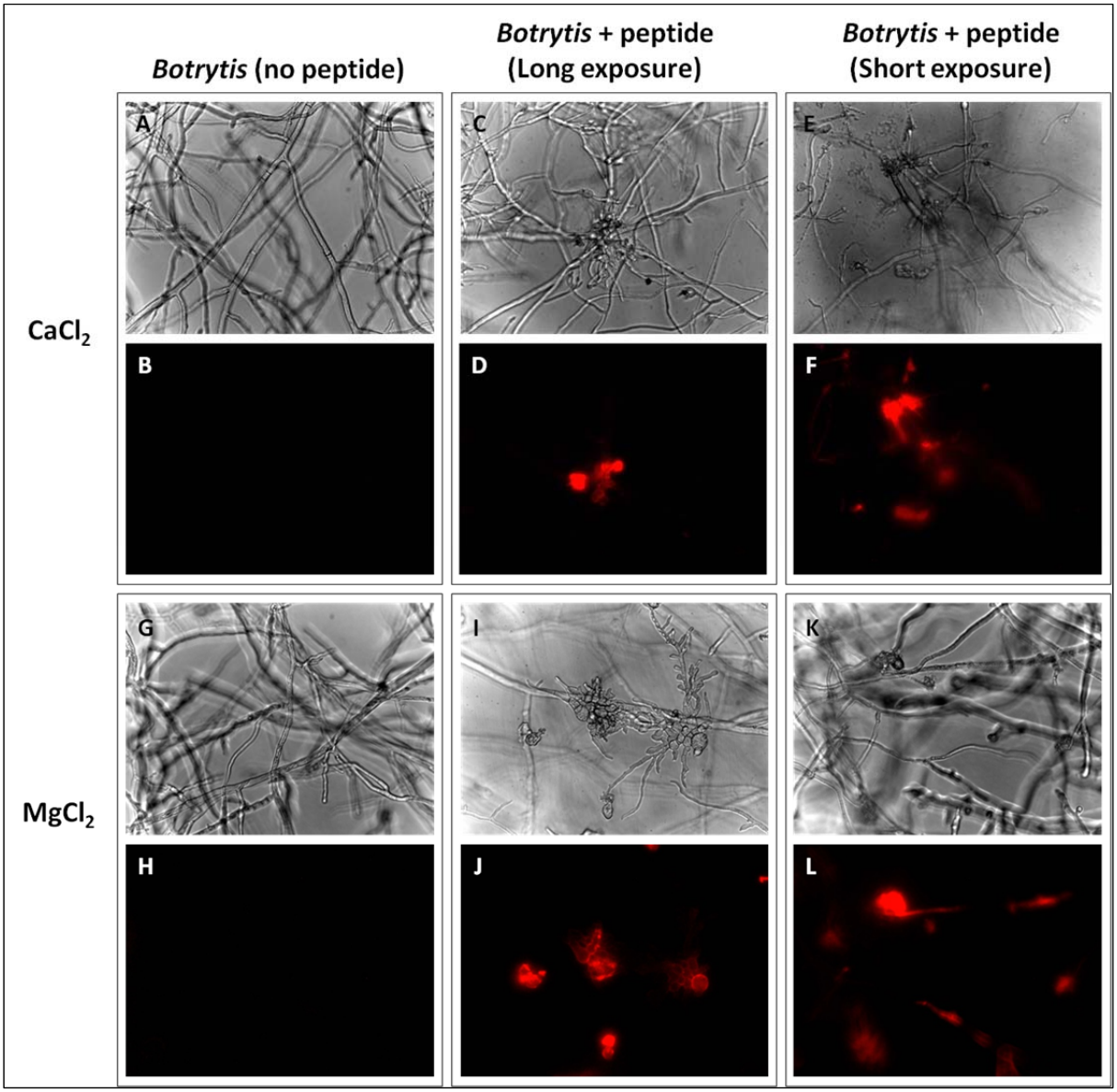

Figure 4.13 Fluorescent microscopic analysis of propidium uptake during membrane permeabilization assay in the presence of divalent cations and of 25 μg mL^{-1} Hc-AFP1. Light microscope images (A-C) and fluorescent images (D-F) of *B. cinerea* grown in the presence of 5 mM $CaCl_2$, containing no peptide (A, D), exposed to 25 μg mL^{-1} Hc-AFP1 for a long period (B, E) and for a short period (C, F). Light microscope images (G-I) and fluorescent images (J-L) of *B. cinerea* grown in the presence of 5 mM $MgCl_2$, containing no peptide (G, J), exposed to 25 μg mL^{-1} Hc-AFP1 for a long period (H, K) and for a short period (I, L). Fungal hyphae were stained with propidium iodide for 10 min prior to fluorescent microscopic analysis.

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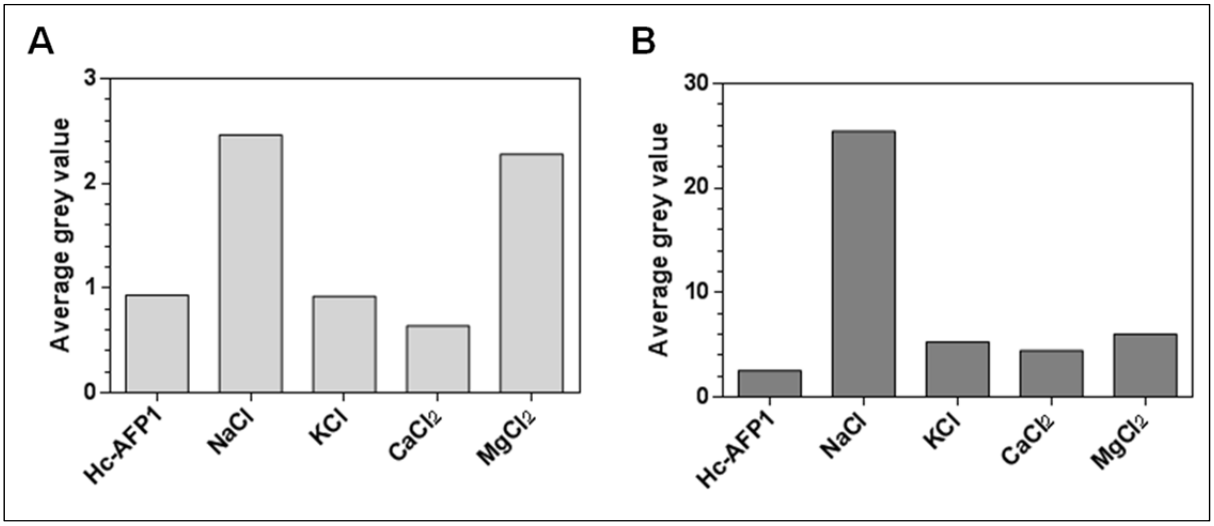

Figure 4.14. Permeabilization of *B. cinerea* hyphae induced by 25 μg mL^{-1} Hc-AFP1 in the presence of 5 mM NaCl, KCl, $CaCl_2$ and $MgCl_2$, represented as the average grey value. Fluorescent microscope analysis of propidium uptake during membrane permeabilization assay of *B. cinerea* grown in the presence of 5 mM NaCl, $CaCl_2$, $MgCl_2$ and KCl containing no peptide and exposed to 25 μg mL^{-1} Hc-AFP1 for a long period (A) and for a short period (B). The measured average grey value was normalized by the measured average grey value of the control reactions containing no peptide.

Table 4.2. Effect of biological salts on the permeabilization activity of Hc-AFP1 expressed in fold chances of the measured fluorescence in terms of the average grey value. The measured fluorescence was normalized to the control reactions containing no peptide.

	Long exposure		Short exposure	
Biological salt	**Increase or decrease in fluorescence (+/-)**	**Fold change**	**Increase or decrease in fluorescence (+/-)**	**Fold change**
NaCl	+	2.6	+	10.0
KCl	-	1.0	+	2.1
$CaCl_2$	-	1.5	+	1.7
$MgCl_2$	+	2.5	+	2.4

4.4 DISCUSSION

Plant defensins from *Brassicaceae* species have been shown to have potential to be used in the agricultural and pharmaceutical field (Thomma *et al*., 2003; Lay and Anderson, 2005). These peptides are small, basic cysteine rich peptides that exhibit a broad range of antimicrobial activities. Furthermore, they form an important part of the innate immune system of plants and play a major role in the protection of plants from invading pathogens (Broekaert *et al*., 1995, Broekaert *et al*., 1997; Lay and Anderson, 2005, Thevissen *et al*., 2007 and De Oliceria Carvalho and Gomes, 2000). Several *Brassicaceae* plant defensins have been transformed into plants and the recipient plants demonstrated to have enhanced resistance towards specific pathogens (Thomma *et al*., 2003; Lay and Anderson, 2005). Although these peptides are promising to be used in the agricultural and pharmaceutical field, their precise mechanisms of action (MOA) are still actively

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studied and particularly the intracellular targets and molecular components involved in the signalling events linked to peptide action still remain to be discovered for many of the defensins.

Four defensins were previously isolated from *Heliophilla coronopifolia* (Hc-AFP1-4) and partially characterized (De Beer and Vivier, 2011). Here we report a more in-depth characterization of the four peptides with regards to their structures, as well as the effects of monovalent and divalent cations on their secondary structures and inhibitory activities. This study follows the initial *in silico* analysis that was conducted on the peptides that revealed that most of the amino acid differences between the peptides resided in a putative α-helical region; this lead to the formation of two structurally defined groups with Hc-AFP1 and Hc-AFP3 in the first group and Hc-AFP2 and 4 in the second group (De Beer and Vivier, 2011).

4.4.1 The characterization of *H. coronopifolia* defensin peptides in terms of structural stability

It was decided to perform the structural characterization of the *H. coronopifolia* defensin peptides with Hc-AFP1 and Hc-AFP2 (Gln) as representatives of the two homology groups of *Heliophilia* peptides. The Hc-AFP2 (pGlu) form was also included to evaluate the impact of the post-translational modification, since it was shown earlier that this form was less active (refer to Chapter 3).

Since there is still a lack of knowledge on the precise MOA of plant defensins, it is of interest to determine the molecular structure and conformation of these plant defensins in a membrane like environment. Here we used circular dichroism to monitor the changes in the secondary structures of Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) in the presence of the membrane mimicking and structure-inducing solvent, TFE, to investigate changes in secondary structure of these peptides. In the presence of TFE the secondary structure of Hc-AFP1 adopted a more ordered structure. For both Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) there was an increase in the molar elipticity in the presence of 50% TFE. This indicates an increase in the concentration of ordered H-bonded structure in the presence of TFE for both Hc-AFP2 (pGlu) and Hc-AFP2 (Gln). When comparing Hc-AFP1 and Hc-AFP2 (Gln), the representative peptides from the two homology groups, TFE had a greater structure inducing effect on Hc-AFP2 (Gln) compared to Hc-AFP1. The secondary structures of Hc-AFP1 and Hc-AFP2 (Gln) are differently affected in the presence of TFE, further supporting the initial *in silico* analysis indicating that the secondary structures of these two peptides are dissimilar. Generally the majority of cationic antimicrobial peptides are in an unordered state in aqueous solution and are able to adopt folded conformations in hydrophobic and hydrophilic environments. These environments include membranes that are by definition bilayers that are amphipathic (both hydrophobic and hydrophilic) (Yeaman and Yount, 2003; Bowman and Free, 2006). Plant defensins have been demonstrated to interact with the fungal membrane components named sphingolipids (Almeida *et al*., 2000; Thevissen *et al*., 2003a; Thevissen *et al*., 2003b; Carvalho and Gomes, 2009; Wilmes *et al*., 2011). Furthermore, plant defensins are expressed in

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environments like the epidermis, vasvular lamellae and apoplast, generally tissues that are the first barriers to pathogen invasion. These areas are both hydrophobic and hydrophilic environments. TFE is a well known for stabilizing secondary structures. Furthermore, it is a weak proton donor, compared to water and this solvent allows the formation of intramolecular hydrogen-bonding (Dyson *et al*., 1988). Several antimicrobial peptides have been shown to adopt a more defined, secondary structure in the presence of a membrane like environment (Barrow *et al*., 1992; Syvitski *et al*., 2005; Gopal *et al*., 2012). These conformation dynamics of antimicrobial peptides subsequent to initial binding contribute to the antimicrobial peptide activity in selected membrane environments (Yount and Yeaman, 2003). Our results show that the *Heliophilia* plant defensins conform to what is known for other AMPs and adopt a more active conformation that consists out of a more defined secondary structure in the presence of a membrane like environment. Divalent cations, such as Ca^{2+}, and monovalent cations, such as K^{+}, have been shown to have an antagonistic effect on the activity of defensin peptides (Ameilda *et al*., 2000; Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). To determine if this is the result of a conformational change in the peptide, due to the direct interaction between the peptide and the cations, we characterized the effect of monovalent and divalent cations on the structure of Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) with CD spectroscopy. The CD spectroscopy data revealed that, in the presence of NaCl, Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) adapted a more defined secondary structure conformation. $MgCl_2$ had a variable effect on these three defensin peptides where the secondary structures of Hc-AFP1 and Hc-AFP2 (Gln) adapted a more defined secondary structure conformation and Hc-AFP2 (pGlu) adapted a less defined secondary structure conformation. In the presence of $CaCl_2$ and KCl the secondary structures of all three peptides adapted a less defined hydrogen bonded conformation.

The precise mechanism of the antagonistic effect on the antifungal activity is still unknown. Terras *et al.* (1992) proposed that this antagonistic effect is the result of an electrostatic interaction between the fungus and the cations rather than a conformational change in the peptide due to the direct interaction between the peptide and the cations (Terras *et al*., 1992). Our results, however, show that cations directly induced conformational changes in the secondary structures of the plant defensins tested. Moreover, there was a difference in effect between the monovalent and divalent cations, with the latter having the stronger effect.

The two representatives of the two homology groupings were differently affected in the presence of monovalent and divalent cations. These results indicate that the secondary structures of Hc-AFP1 and the two forms of Hc-AFP2 clearly differ as previously determined by the *in silico* analysis. Peptides from homology group 1 are predominantly expressed in vegetative and reproductive tissues whereas peptides form the homology group 2 are predominantly expressed in the storage tissues (De Beer and Vivier, 2011). The fact that these two groups of peptides are differently affected by these different environments could be related to their different *in vivo* environments. Our results indicated that the secondary structures of Hc-AFP2 (pGlu) and Hc-AFP2

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(Gln) were differently affected by the presence biological salts, again confirming that these two peptides differ in their conformations. It was previously established that the Hc-AFP2 (Gln) form was the more active form against a fungal pathogen, although the other form also induced some morphological abnormalities in the fungal hyphae (see Chapter 3 for details). Future studies will entail activity assays of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) in the presence of biological salts to determine if these induced structural changes due to the presence of the cations will have an effect on the biological activity of these peptides.

4.4.3 Effect of biological salts on the activity of *H. coronopifolia* defensin peptides

The antifungal activity of plant defensins have been shown to be strongly antagonized in the presence of monovalent and divalent cations in the medium (Ameilda *et al*., 2000; Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). It is important to understand the underlying mechanism of this antagonistic effect on the antifungal potency of defensin peptides in order to evaluate the possible contribution to defence reactions against microorganisms *in planta*. K^+ is the most abundant cellular and apoplastic monovalent cation found in plant cells. This monovalent cation can reach levels of approximately 100 mM in the cytosol, vacuoles and apoplast. Ca^{2+} and Mg^{2+} are the most abundant divalent cations found in plant tissues where their concentrations can reach up to 1 µM and 1 mM, respectively in the cytosol (Clarkson and Hanson, 1980; Flowers and Läuchli, 1983; Grignon and Sentenac, 1991; Terras *et al*., 1992; White and Broadly, 2003). Although these are the natural occurring concentrations of these cations, fungal infection leads to the disruption of intact plant cells and affects the concentrations of these cations. Furthermore, pathogen defence in plants consists of a complex network that involves the opening of specific ion channels. Following pathogen recognition, the first reactions involves the opening of specific ion channels and the formation of reactive oxygen intermediates. These include the transient influxes of H^+ and Ca^{2+} and the effluxes of K^+ and Cl^- over the plasma membrane. These reactions are required for the further signal transduction events that consist of a complex signalling network that triggers the overall defence response (Somssich and Hahlbrock, 1998). In defence against *B. cinerea* infection, plants also produce nitric oxide (NO). This NO production triggered by oligogalacturonide that is dependent on the influx of Ca^{2+} that leads to cystolic-free Ca^{2+} and results in NO production (Rasul *et al*., 2012). It is thus very difficult to predict the exact ionic conditions under which these plant defensin peptides interact with invading fungal hyphae *in planta*, but it is a fair assumption that there will be shifts in ionic strengths in the compartments where defensins are present. These ionic changes in the environments might influence the activity and stability of plant defensin peptides and might partly explain some reports of *in vitro* antifungal activities of defensins without observable enhancement of defence phenotypes in *in planta* analysis.

This is an important feature to fully understand in order to sustain the potential application of these peptides and understand how these peptides will behave and interact *in planta* for the

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engineering of disease resistant crops. Liquid plate antifungal assays demonstrated that the biomass production of *B. cinerea* is not negatively influenced by the addition of biological salts to the media. However, in the presence of the salts, *B. cinerea* hyphae formed reproductive structures more readily. Our results show that the growth inhibition activity of Hc-AFP1 against *B. cinerea* was reduced in the presence of monovalent and divalent cations, correlating with previous results on the sensitivity of defensins to cations (Ameilda *et al*., 2000; Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). However, the effect of divalent cations was far more pronounced; the growth inhibition activity of Hc-AFP1 against *B. cinerea* was completely abolished whereas the monovalent cations had a limited effect. This correlates well with the significant loss in secondary structure of Hc-AFP1, observed with CD in the presence of the divalent cations. Several studies have reported that divalent cations had a greater antagonistic effect when compared to the monovalent cations (Ameilda *et al*., 2000; Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). Furthermore, Hc-AFP1 in the presence of these divalent cations induced morphological effects on the hyphae. These included hyper-branching and hyphal tip swelling. This is interesting since the growth inhibition caused by morphological plant defensins are usually achieved by causing morphological effects on hyphae (Terras *et al*, 1992; Osborn *et al*, 1995). This interesting feature will require further investigation. Although there was a slight reduction in the growth inhibition activity of Hc-AFP1 in the presence of NaCl and KCl, the peptide was still able to cause the characteristic morphological effects of a morphological defensin peptide that included severe hyper-branching of the *B. cinerea* hyphae. This is in agreement with reported results that the effect of monovalent cations is less antagonistic than divalent cations (Terras *et al*., 1992; Terras *et al*., 1993; Osborn *et al*., 1995).

The permeabilization activity of Hc-AFP1 on *B. cinerea* hyphae and spores were investigated by fluorescent microscopy conducted on propidium iodide stained *B. cinerea*. In the presence of all the biological salts the ability of Hc-AFP1 to permeabilize *B. cinerea* hyphae was in fact increased. In particular, NaCl induced a 10-fold increase of permeabilization of hyphae by HcAFP1. This correlated with the CD results that showed that NaCl stabilized or supported the conformation of Hc-AFP. This can also be explained by mycelial fungal cell walls that are relatively thin, typically 0.2 µm, that may be more sensitive to peptide action. However, the ability of Hc-AFP1 to affect and/or permeabilize *B. cinerea* spores was decreased in the presence of KCl and $CaCl_2$, again correlating with the loss of conformation in the presence of these salts. This can also be explained since fungal spores consist out of a thick wall that protects the organism against the environment and these structures can survive extreme physical conditions. These fungal cell wall is typically composed out of a matrix containing β(1→3)-, α(1→3)-, and β(1→6)-glucans, glycoproteins, and lipids, reinforced by chitin (polyacetyl glucoseamine) or cellulose fibres. The outer cell wall of spores is hydrophobic and contains lipids and proteins, whereas the inner cell wall is similar to the mycelial wall and consists of a β(1→3)-glucan matrix reinforced with chitin fibrils. A slightly lower active peptide, induced by certain salts, could therefore abolish the observed peptide activity. It is

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thus easier for Hc-AFP1 to permeabilize the thinner hyphal cell wall than that of the spore and therefore less peptide in its active conformation may be necessary to exert its activity.

Two types of permeabilization have been observed as a result of the interaction of plant defensins with fungal membranes, depending on the concentration of peptide and the cation composition of the medium. The first type of permeabilization occurs at high doses (10 to 40 µM) of plant defensins. Moreover, this is a strong permeabilization and can be detected within 30 to 60 min of the addition of the defensin. Furthermore, this type of permeabilization is sensitive to the presence of monovalent and divalent cations and is therefore referred to as cation-sensitive membrane permeabilization. This permeabilization occurs at plant defensin concentrations about 10-fold higher than those required for fungal growth inhibition. The second type of permeabilization occurs at lower plant defensin concentration (0.1 to 1 µM). Furthermore, this permeabilization is a relatively weak and can only be detected after 2 to 4 hours of incubation. This type of permeabilization is only slightly affected by the presence of cations and is therefore referred to as cation-resistant membrane permeabilization. The concentration of defensin at which this cation-resistant membrane permeabilization occurs, significantly correlates with the concentration required to cause growth inhibition (Thevissen *et al.,* 1999).

Furthermore, the fact that the permeabilization activity of Hc-AFP1 on the hyphae of *B. cinerea* was not reduced and that this permeabilization occurs at concentrations consistent with concentrations that cause growth inhibition, might indicate that this permeabilization correspond to the cation-resistant type. However, this type of permeabilization demonstrated to be a strong permeabilization and occurred immediately and this does not correlate with the cation-resistant permeabilization described by Thevissen *et al.* (1999). The fact that growth inhibition activity of Hc-AFP1 was reduced in the presence of the biological salts indicates that the permeabilization is not the sole activity responsible for growth inhibition caused by Hc-AFP1 and that the peptide probably has an alternative/primary target and more complex MOA. It has been previously reported that permeabilization of fungal membranes is not the primary event responsible for growth inhibition of fungi, but is in fact only a secondary effect in the presence of high plant defensin concentrations (Thevissen *et al*., 2003a; Thevissen *et al*., 2003b). Furthermore, it has been reported that the ability of plant defensins to permeabilize plasma membranes is not a direct indication of the antifungal activity and potency of these peptides (Sagaram *et al.*, 2011). These aspects should be explored further by performing permeabilization experiments using a wide range of peptide concentrations to determine if permeabilization is part of the mode of action of this peptide of if it is a secondary effect occurring at high concentration of peptide.

It has been proposed that the antagonistic effect of cations on the biological activity of Hc-AFP1 is the result of an electrostatic interaction between the fungus and the cations rather than a conformational change in the peptide due to the direct interaction between the peptide and the cations (Terras *et al*., 1992). However, our results clearly indicate that there is a definite conformational change in the secondary structure of Hc-AFP1, Hc-AFP2 (pGlu) and Hc-AFP2 (Gln)

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in the presence of divalent and monovalent cations. These conformational chances in the secondary structure may be the cause of the resulting reduction in the biological activity of Hc-AFP1 in the presence of monovalent and divalent cations, or at least a contributing factor. This study has provided significant progress towards the structure-function analysis of the *Heliophilia* peptides as such and as examples of plant defensins in general.

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Chapter 5

General discussion and conclusions

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GENRAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION AND CONCLUSIONS

Plant diseases, caused by fungi, are currently one of the major factors limiting crop production worldwide (Salmeron and Vernooij, 1998; Montesinos, 2007). The effective and durable control of pathogens of crops by a safe and natural alternative has therefore become one of the major concerns in modern agriculture (Gao *et al.*, 2000; Kaur *et al.*, 2011). Antimicrobial peptides like plant defensins are considered attractive and promising candidates to be used in agricultural biotechnology due to their broad antifungal activity and their possible ability to move away from the negative impacts of current fungicides (Van der Biezen, 2001; Lay and Anderson, 2005; Stotz *et al.*, 2009). These peptides have a broad spectrum antifungal activity and form part of the innate immune system and play a vital role in the protection of plants against invading fungal pathogens (Broekaert *et al.*, 1995; Osborn *et al.*, 1995; Terras *et al.*, 1995; Thevissen *et al.*, 1997; Thomma *et al.*, 2003; Lay and Anderson, 2005). Plant defensins have known potent activities *in vitro* against important pathogens and several defensin genes have been successfully transformed into various plant hosts with resulting significantly enhanced disease resistance of the hosts under greenhouse-conditions and even field-conditions (Terras *et al.*, 1995; Gao *et al.*, 2000; Koike *et al.*, 2002; Lay and Anderson 2005; Portieles *et al.*, 2010; Kaur *et al.*, 2011). Despite their application potential, the exact mechanism of antifungal action of these peptides to date is unknown. Previous findings have shown that the antimicrobial activity of plant defensins is strongly antagonized by the addition of monovalent and divalent cations to the medium (Ameilda *et al.*, 2000; Osborn *et al.*, 1995, Terras *et al.*, 1992 and Terras *et al.*, 1993). However, the precise mechanism of this antagonistic effect on the antifungal activity is also still unknown. It is proposed that the antagonistic effect is the result of an electrostatic interaction between the fungus and the cations, rather than a conformational change in the peptide due to the direct interaction between the peptide and the cations (Terras *et al.*, 1992). Furthermore, ion fluxes occur naturally in plant cells and during pathogen infection, a series of ion fluxes is initiated in the plasma membrane (de Bruxelles and Roberts, 2001; Yeaman and Yount, 2003). These locations in the plant cells typically house defensins and they would be affected by changes in cation levels. It is therefore important to understand how these peptides play their reported vital role in the defence of their hosts, as well as the the mode of antagonism of cations.

In the Institute for Wine Biotechnology, four plant defensin peptides have recently been isolated from *Heliophila coronopifolia,* a native South African *Brassicaceae* species. Analysis of the deduced amino acid sequences of Hc-AFP1-4 showed that the peptides were 72%

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similar and grouped closest to defensins isolated from other *Brassicaceae* species. The Hc-AFP1 and Hc-AFP3 peptides formed a unique grouping amongst the Brassicaceae defensins, sharing high sequence homology (94%) whereas Hc-AFP2 and 4 formed a second homology grouping with defensins from *Arabidopsis* and *Raphanus*. Furthermore, homology modelling showed that the amino acid differences between the four peptides had an effect on the surface properties of the defensins, specifically in the alpha helix and the loop connecting the second and third beta strands ($L\beta_2\beta_3$). These four defensin peptides demonstrated to exhibit high inhibitory activity against *Botrytis cinerea* and *Fusarium solani*. The antifungal activities and morphogenic effects induced by the four peptides against the two pathogens were extremely diverse, despite their high sequence similarity. All the defensin peptides were found to be morphogenic plant defensins and induced changes in hyphal morphology of *B. cinerea*, including hyper-branching, fungal tip swelling, increased granulation of hyphae and spores, as well as hyphal and spore disruption. Furthermore, all four of these plant defenins was associated with membrane permeabilization activity on both, or at least one of the pathogens (De Beer and Vivier, 2011). These peptides have great potential to be used in agriculture, especially in viticulture, since they are potent against *Vitis Verifera* pathogens. However, the precise mechanism of action and how these peptides exert their action *in planta* during defence is still unknown. In this study we set out to characterize the effects of monovalent and divalent cations on the secondary structure and biological activity of plant defensins in order to gain more insight on how these peptides exert their potent antifungal action and react in an *in planta* environment. Furthermore, in order to perform these characterization experiments, a production system was required that produced active peptides in sufficient yield and involved simple and rapid purification processes. *Pichia pastoris* was selected as host for the production of the *H. coronopifolia* peptides.

Pichia pastoris **demonstrated to be a successful host for the recombinant production of** ***Heliophila coronopifolia*** **peptides**

P. pastoris was selected as a host system to produce the *H. coronopifolia* peptides. Several other plant defensins have been successfully produced in *P. pastoris* with large yields (Almeida *et al*., 2001; Chen *et al*., 2004; Kant *et al*., 2009; Lay *et al*., 2012). Hc-AFP1 and Hc-AFP3 was successfully produced in *P. pastoris* with a significant increase in yield compared to the previously used bacterial production system (De Beer and Vivier, 2011). This production system, however, had its limitations in the production of Hc-AFP2 and Hc-AFP4, where these peptides demonstrated molecular masses 17 Da less than expected. Our analysis lead us to the conclusion that these peptides underwent a post-translational

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modification common to peptides containing a N-terminal glutamine where the cyclization of the amino terminal glutamine residue forms pyroglutamic acid (Blomback *et al*., 1967). This is one of the limitations of this production system due to the fact that the *P. pastoris* system lacks promoters for moderate expression which can lead in toxic levels of expression. These toxic levels of expression can influence the protein-handling machinery of the organism, leading to misfolding and unprocessing of some of the cellular protein (Balamurugan *et al*., 2007). We also showed that this limitation of the production system could be overcome by the modification of the production buffer to a reduced ion buffer since it has been reported that ions influence this cyclization and more importantly that phosphate buffer accelerates the conversion of glutamine to pyroglutamic acid (Khandke *et al*., 1989). This was proven with Hc-AFP2 which was produced with both the normal and reduced ion buffer, yielding the Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) forms of the peptide.

The *P. pastoris* production system was able to successfully produce three of the four *H. coronopifolia* peptides. Circular dichroism spectroscopy showed that Hc-AFP1, Hc-AFP3 and Hc-AFP2 (Gln) presented CD spectra that are characteristic to structured peptides. Furthermore, these CD spectra were indicative of predominantly β-sheet combined with α-helical structures. This is consistent with the conserved structure of a short three helical turn α-helix with three anti-parallel β-sheets found for plant defensins (Bruix *et al*., 1993; Fant *et al*., 1998). Furthermore, this confirmed that this system successfully produced Hc-AFP defensin peptides in the correct structural conformation. In addition, the evaluation of the biological activity of these peptides revealed that these peptides were produced in a biologically active form. The peptides reduced the growth inhibition of *F. solani* and induced morphological effect on the hyphae of this pathogen characteristic to morphogenic plant defensin peptides. These morphological effects included hyper-branching, swollen tips and lysis, further confirming the success of the *P. pastoris* production system in the production of *H. coronopifolia* plant defensins.

In this study the successful production of three *H. coronopifolia* defensin peptides were achieved with an increased yield. Furthermore this was achieved with simple and rapid purification methods. Moreover, this study provided a simple, rapid and effective production system and aids in the overcoming of post-translational modification limitations experienced with this system. Future studies could also involve the use of a methanol inducible promoter to further increase the yield of the Pichia production system.

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Pyroglutamic acid alters the secondary structure of Hc-AFP2 and affects the biological activity

A post translational modification common to peptides involves the cyclization of the amino terminal glutamine residue to form pyroglutamic acid (Blomback *et al*., 1967). Furthermore, this post translational modification event has been reported to be influenced by reaction conditions such as the buffer, temperature and time of incubation (Khandke *et al*., 1989). This is a very familiar post translational modification in defensin peptides, however, it has not yet been demonstrated to have an effect on the activity of plant defensins (Mendez *et al*., 1990; Terras *et al*., 1992). This study, however, clearly demonstrated that this post-translational modification influenced the biological activity of Hc-AFP2, where Hc-AFP2 (Gln) inhibited the growth of *Fusarium solani* by 43% at a concentration of 15 µg mL^{-1} while Hc-AFP2 (pGlu) only inhibited the growth of this pathogen by 19% at the same concentration. Trypsin digestion of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) yielded different fragments where Hc-AFP2 (Gln) contained an additional trypsin digestion. This finding confirms that the tertiary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) differ, a finding which can be explained by the N-terminal modification of Hc-AFP2 (pGlu), resulting in a more trypsin resistant peptide.

To investigate how this post-translational modification alters the secondary structure of Hc-AFP2 and how the secondary structures of Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) differs and reacts in different environments, circular dichroism spectroscopy was used. Although the primary and secondary structures of these two forms of Hc-AFP2 were similar, Hc-AFP2 (pGlu) was demonstrated to have less ordered structures in the aqueous solution that clearly indicates conformational changes. Furthermore, in the presence of the structure inducing solvent, TFE, both Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) exhibited an increase in the concentration of ordered H-bonded structure, however, the increase in the amount of ordered structure, induced by TFE in Hc-AFP2 (Gln), was significantly more than that of Hc-AFP2 (pGlu). In the presence of NaCl the secondary structure Hc-AFP2 (pGlu) and Hc-AFP2 (Gln) adapted a more defined secondary structure conformation. $MgCl_2$, however, had a variable effect on these two defensin peptides where the secondary structures of Hc-AFP2 (Gln) adopted a more defined secondary structure conformation and Hc-AFP2 (pGlu) adopted a less defined secondary structure conformation. In the presence of $CaCl_2$ and KCl the secondary structures of these two peptides adopted a less defined hydrogen bonded conformation. It is clear that these two peptides adopt different conformations in different environments. Therefore, it can be concluded that the secondary structures of Hc-AFP2 (pGlu) and (Gln) differ significantly. Furthermore, Hc-AFP2 (Gln) contains a more defined secondary structure in aqueous solution and in the presence of the structure inducing

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solvent TFE. This can explain why Hc-AFP2 (Gln) was the more active form against a fungal pathogen, *F. solani*. It is important to note that several antimicrobial peptides have shown to adopt a more defined, secondary structure in the presence of a membrane like environment (Barrow *et al*., 1992; Syvitski *et al*., 2005; Gopal *et al*., 2012).

Cations influence the structural conformation and activity of plant defensins

To investigate if the antagonistic effect of cations on the activity of Hc-AFP1 is linked to structural conformation change the effect of cations on the secondary structure of Hc-AFP1 was investigated by circular dichroism spectroscopy. Circular dichroism spectroscopy is one of the most widely used techniques used for the determination of secondary structure (Raussens *et al*., 2003). Furthermore, this is an excellent technique to rapidly evaluate the secondary structure conformation changes of antimicrobial peptides (Greenfield, 2006; Gopal 2012). This technique can be used to differentiate between unordered (random coil) and ordered (α-helix or β-sheet) secondary structures (Kelly *et al.*, 2005; Gopal *et al.*, 2012). The biological activity of Hc-AFP1 was evaluated against *Botrytis cinerea* by a liquid plate assays and the morphological effects were microscopically visualized.

The CD spectroscopy data revealed that the different biological salts had variable effects on the secondary structure of Hc-AFP1. In the presence of NaCl and $MgCl_2$ Hc-AFP1 adopted a more defined secondary structure conformation. Whereas in the presence of $CaCl_2$ and KCl the secondary structures of all three peptides adopted a less defined hydrogen bonded conformation.

The growth inhibitory activity of Hc-AFP1 against *B. cinerea* was shown to be reduced in the presence of monovalent and divalent cations. This is consistent with previous results reporting the antagonistic effect of cations on the activity of defensin peptides (Ameilda *et al*., 2000; Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). The effect of divalent cations was far more pronounced; the growth inhibition activity of Hc-AFP1 against *B. cinerea* was completely abolished, whereas the monovalent cations had a limited effect. This correlates with several studies that have reported that divalent cations have a far more severe antagonistic effect than of monovalent cations (Ameilda *et al*., 2000; Osborn *et al*., 1995, Terras *et al*., 1992 and Terras *et al*., 1993). Interestingly, although Hc-AFP1 was unable to induce growth inhibition of *B. cinerea* in the presence of divalent cations, the peptide still had the ability to induced morphological effects on the hyphae. These included hyperbranching and hyphal tip swelling. In the presence of the monovalent cations, there was a slight reduction in the growth inhibition activity of Hc-AFP1 in the presence of NaCl and KCl, however, the peptide was still able to cause the characteristic morphological effects of a morphological defensin peptide that included severe hyper-branching of the *B. cinerea*

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hyphae. The precise mechanism of the antagonistic effect observed on the antifungal activity is still unknown. It has been proposed that this antagonistic effect is the result of an electrostatic interaction between the fungus and the cations rather than a conformational change in the peptide due to the direct interaction between the peptide and the cations (Terras *et al*., 1992). However, the results of this study clearly showed that cations directly induced conformational changes in the secondary structures of the plant defensins tested. Cations naturally occur in plant cells and fluxuate continuously throughout the growth and developments of plants. Furthermore, abiotic stressed like fungal infection leads to the disruption of intact plant cells and affects the concentrations of these cations. Moreover, pathogen defence processes in plants consists out of a complex network that involves the opening of specific ion channels (Somssich and Hahlbrock, 1998). It is of vital importance to understand the underlying mechanism of this antagonistic effect on the antifungal potency of defensin peptides in order to evaluate the possible contribution to defence reactions against microorganisms *in planta* as well as to sustain the potential application of these promising peptides.

The permeabilization activity of Hc-AFP1 on *B. cinerea* hyphae and spores were investigated by fluorescent microscopy conducted on propidium iodide stained *B. cinerea*. The presence of the biological salts tested did not reduce the ability of Hc-AFP1 to permeabilize *B. cinerea* hyphae; this ability was rather increased by the salts. NaCl had the greatest increase on the permeabilization activity of Hc-AFP1, where permeabilization of *B. cinerea* hyphae was increased 10-fold. This correlated with the CD results that showed that NaCl stabilised or supported the secondary structure conformation of Hc-AFP1. This permeabilization activity of Hc-AFP1 was therefore insensitive to cations.

Thevissen *et al.* (1999) reported the existence of two types of permeabilization for plant defensins depending on the concentration of peptide and the cation composition of the medium. The first type of permeabilization is sensitive to the presence of monovalent and divalent cations and is referred to as cation-sensitive membrane permeabilization. This type of permeabilization occurs at plant defensin concentrations about 10-fold higher than those required for fungal growth inhibition. Moreover, this is a strong permeabilization and can be detected within 30 to 60 min of the addition of the defensin. The second type of permeabilization occurs at lower plant defensin concentrations that is required to cause membrane permeabilization. This type of permeabilization is called cation-resistant membrane permeabilization since it is only slightly affected by the presence of cations. Moreover, this permeabilization is a relatively weak and can only be detected after 2 to 4 hours of incubation (Thevissen *et al.,* 1999).

The permeabilization caused by Hc-AFP1 on the hyphae of *B. cinerea* was not reduced in the presence of cations and permeabilization occurs at concentrations consistent with

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concentrations that cause growth inhibition. This might indicate that this type of permeabilization can correspond the cation-resistant permeabilization described by Thevissen *et al.* (1999), however, this type of permeabilization was demonstrated to be a strong permeabilization. Furthermore, this membrane permeabilization occurred immediately and this does not correlate with the cation-resistant permeabilization described by Thevissen *et al.* (1999). Therefore, permeabilization is not the sole mode of action of Hc-AFP1, since the growth inhibition activity of Hc-AFP1 was reduced in the presence of the biological salts. It is suspected that that this peptide contains an alternative target and MOA. This correlates with literature that have reported that permeabilization of fungal membranes is not the primary event responsible for growth inhibition of fungi, but is in fact only a secondary effect in the presence of high plant defensin concentrations (Thevissen *et al*., 2003a; Thevissen *et al*., 2003b). This work should be followed by a more in-depth analysis to test the effect of a range of concentrations (from low to high) on the effect of permeabilization in order to fully understand the role of permeabilization in the activity of this peptide.

In conclusion, it is clear that cations have a significant effect on the secondary structure conformation of Hc-AFP1. Although it is common knowledge that cations antagonise the activity of plant defensins, this is the first known report of the investigation of the influence of cations on the structure of plant defensin peptides. This change in structural conformation may be linked to the antagonism of the biological activity observed of this peptide or may contribute to the antagonistic effect observed. This study aids in a better understanding of the antagonism of cations on the biological activity of plant defensins and has provided more insight into the structure-function analysis of the *Heliophilia* peptides as such and as examples of plant defensins in general.

Future prospects

Future studies will focus on the elucidation of the mode of action of the four *H. coronopifolia* peptides. This will involve structural studies with nuclear magnetic resonance (NMR) spectroscopy to characterize their interactions with the fungal membrane. These studies will also involve the investigation of a potential membrane target using confocal microscopy with labelled *H. coronopifolia* peptides in order to determine their initial location when exposed to fungal hyphae as well as their final location in order to determine if these peptides have an intracellular target. Furthermore, the dependence of these peptides to inhibit fungal growth on the fungal cell wall will be investigated by cell wall modifications in order to determine if there is a possible membrane target and the location of this target. This all will contribute to a better understanding of the mode of action of these peptides.

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