

**AN INVESTIGATION INTO THE PROTEOLYTIC
DEGRADATION OF ANTIMICROBIAL PEPTIDES BY
PLANT EXTRACTS AND LOCALISATION OF
PLEUROCIDIN IN TRANSGENIC *SACCHARUM*
HYBRID SPECIES**

BY

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PREFACE

The experimental work described in this thesis for M.Sc was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, under the supervision of Professor John Hastings.

This document represents original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is dually acknowledged in the text.

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TABLE OF CONTENTS

ABSTRACT	i
LIST OF FIGURES	ii
LIST OF TABLES	vii
ABBREVIATIONS	viii
ACKNOWLEDGEMENTS	ix
CHAPTER ONE: LITERATURE REVIEW	
1.1 General Introduction	2
1.2 Cationic Antimicrobial Peptides	3
1.2.1 Mammalian Antimicrobial Peptides	4
1.2.1.1 Classic or α defensins	4
1.2.1.2 β -defensins	5
1.2.2 Amphibian Antimicrobial Peptides	6
1.2.2.1 Magainins	6
1.2.2.2 Dermaseptins	6
1.2.2.3 Brevinins	7
1.2.2.4 Bombinins	7
1.2.3 Insect Antimicrobial Peptides	8
1.2.3.1 Cecropins	9
1.2.3.2 Sarcotoxins	9
1.2.3.3 Sapecins	10

1.2.3.4	Attacins	10
1.2.3.5	Apidaecins	10
1.2.3.6	Abaecins	11
1.2.3.7	Mellitins	11
1.2.3.8	Drosomycin	11
1.2.4	Fish Antimicrobial Peptides	11
1.2.4.1	Pleurocidin	11
1.2.4.2	Other antimicrobial peptides of fish origin	13
1.2.5	Plant Antimicrobial Peptides	14
1.2.5.1	<i>Mirabilis jalapa</i> antimicrobial peptides (Mj-AMP)	16
1.2.5.2	<i>Amaranthus caudatus</i> antimicrobial peptides (Ac-AMPs)	17
1.2.5.3	<i>Raphanus sativus</i> antifungal proteins (RS-AFPs)	17
1.2.5.4	<i>Raphanus sativus</i> non-specific lipid transfer protein (Rs-nsLTP)	17
1.2.5.5	<i>Raphanus sativus</i> 2S albumins (Rs-2S)	17
1.2.5.6	Thionins	18
1.2.6	Synthetic Antimicrobial Peptides	18
1.2.6.1	ESF1-GR7	19
1.3	Mode of Action for Cationic Antimicrobial Peptides	20
1.4	Potential Applications for Antimicrobial Peptides	23
1.4.1	Therapeutic Applications	23
1.4.1.1	Tropical antibiotic agents	23

1.4.1.2	Systemically administered antibiotics	24
1.4.1.3	Wound healing stimulants	24
1.4.1.4	Anticancer agents	24
1.4.2	Antimicrobial Peptides as Agents of Crop Protection	25
1.4.3	Stability of Antimicrobial Peptides in Transgenic Plants	25
1.4.3.1	Proteolytic instability	27
1.4.3.2	Post-translational modification	27
1.4.3.3	Gene silencing	28
1.5	Strategies for Introducing Antimicrobial Peptides into Plants	28
1.5.1	Direct Methods of Gene Transfer	29
1.5.1.1	Fusion strategy	31
1.5.2	Indirect Methods of Gene Transfer	31
1.5.2.1	<i>Agrobacterium</i> gene transfer systems	31
1.5.2.2	Gene cloning using plant viruses	32
1.6	Objectives of Current Research	34

CHAPTER TWO: PROTEOLYTIC DEGRADATION OF ANTIMICROBIAL PEPTIDES BY PLANT EXTRACTS 36

2.1	INTRODUCTION	38
2.2	MATERIALS AND METHODS	40
2.2.1	Culture Conditions	40
2.2.2	Leaf Intercellular Fluid Extraction	40

2.2.3	Treatment and Bioassay of Peptides	41
2.2.3.1	ESF1-GR7	41
2.2.4	Analysis for Peptide Degradation	41
2.2.4.1	Retention of antimicrobial activity bioassay	41
2.2.4.2	Gel electrophoresis analysis	42
2.2.4.3	Reverse-phase high pressure liquid chromatography	42
2.3	RESULTS AND DISCUSSION	43
2.3.1	Proteolytic Degradation of Antimicrobial Peptides	43
2.3.1.1	Retention of antimicrobial activity assay	43
2.3.1.2	Gel electrophoresis of breakdown products	45
2.3.1.3	Reverse-phase HPLC analysis	46
2.3.1.4	Protease identification	50
CHAPTER THREE: LOCALIZATION OF PLEUROCIDIN IN TRANSGENIC SUGARCANE		53
3.1	INTRODUCTION	55
3.2	MATERIALS AND METHODS	58
3.2.1	Protein Extraction	58
3.2.2	Tricine SDS-PAGE Analysis	58
3.2.3	Electroblotting onto Nitrocellulose Membrane	59
3.2.4	Western Blotting	59

3.2.5	Immunocytochemistry	60
3.2.5.1	Sample preparation	60
3.2.5.2	Immunogold labelling	60
3.2.6	Electroblotting of Peptide onto PVDF membrane	61
3.2.7	Amino Acid Sequencing	62
3.3	RESULTS AND DISCUSSION	62
3.3.1	Extraction of Pleurocidin from Transgenic Leaf Material	62
3.3.2	Localization of Pleurocidin in Leaf Tissue	65
3.3.3	Amino Acid Analysis	66
3.3.4	Fate of Pleurocidin in Transgenic Sugarcane	66
	CHAPTER FOUR: CONCLUSION	70
	CHAPTER FIVE : LIST OF REFERENCES	75

ABSTRACT

Two cationic antimicrobial peptides, ESF1-GR7, and pleurocidin, were assessed for their stability in plant intercellular fluid, the targeted locale for their expression in transgenic plants. Incubation of ESF1-GR7 and pleurocidin with intercellular fluid (ICF) extracted from sugarcane, tomato and tobacco leaves reduced their biotoxicity towards various pathogens, namely *Carnobacterium mobile* DMSO and *Xanthomonas campestris*. It was concluded that it may be necessary to modify the amino acid structures of the peptides in order to ensure that endogenous proteases would not degrade the peptides once expressed in a transgenic environment. The presence of pleurocidin was detected in transgenic sugarcane transformed (in a previous study) with pleurocidin gene cloned into the pUBI 510 plasmid. ICF was extracted from four month old transgenic *Saccharum* hybrid species (sugarcane). Western blotting verified the presence of the transgenic protein in crude protein extracts. Immunogold labelling and transmission electron microscopy were performed to investigate the localisation of transgenic pleurocidin. The peptide was localized predominantly in the intercellular spaces and cell wall sugarcane leaves.

LIST OF FIGURES

- Figure 1.1:** Helical wheel diagrams of ESF1 GR7 and Pleurocidin showing amphipathic helical form. ESF1 GR7 is a synthetic peptide that mimics the charge distribution and amphipathic nature of the modified magainin pGLa. Pleurocidin is a 2.7kDa, 25 residue antimicrobial peptide that has been isolated from the winter flounder (*Pleuronectes americanus*) **Page 3**
- Figure 1.2:** β -stranded human defensin is a typical example of β -sheet type antimicrobial peptides. **Page 20**
- Figure 1.3:** Proposed mechanism of microbial killing by cationic peptides: the positively charged peptides (1) bind to the external surface of the negatively charged phospholipid bilayer (2) leading to a localized thinning of the cytoplasmic membrane. Under the influence of membrane potential, the peptide inserts into the membrane and forms channels (3) which result in leakage of the cytoplasmic molecules and subsequent death of the cell. **Page 21**
- Figure 1.4:** Self-promoted uptake of cationic peptides pathway showing positively charged peptides interact with the negatively charged divalent-cation-binding sites on the surface of the Lipopolysaccharide. This action disrupts these sites and leads to enhanced uptake of the cationic peptides across the membrane. **Page 22**
- Figure 1.5:** Overview of mechanisms for genetic modification of plants. **Page 30**

Figure 2.1: ESF1-GR7 (40µg) was incubated with an equal volume of intercellular fluid (ICF) vacuum infiltrated from tomato leaves for times ranging from 0 to 30 minutes. 10µl from each mixture were spotted onto 0.7% agar overlays inoculated with *Carnobacterium mobile*. The plates were incubated at 30°C overnight, and zones of inhibition were observed. Control ICF extract and untreated peptide were included on the plate.

Page 44

Figure 2.2: Aliquots (20µl) of ICF extract from tomato plants were placed on 1% Milk Agar Plates and incubated overnight at 37°C. Proteolytic degradation of milk was observed as zones of clearing. A 20µl aliquot of Proteinase K (20mg/ml) were also placed on the milk agar as a control.

Page 44

Figure 2.3: ESF1-GR7 (40µg) was incubated with an equal volume of tomato ICF for times ranging from 0 to 60 minutes with each reaction being separated by tricine-SDS PAGE gel electrophoresis and stained with Coomassie Blue R250 (Sigma) showing the peptide band disappearing over time as follows: Lane 1, Low Range molecular weight marker (Promega); Lane 2, Control Peptide ESF1-GR7; Lane 3, Proteinase K digested peptide after 30 minutes; Lanes 4-9, ESF1-GR7 incubated with tomato ICF at time 0, 5, 10, 15, 30 and 60 minutes; and Lane 10, tomato ICF extract only.

Page 46

Figure 2.4: Samples of ESF1-GR7 exposed to intercellular fluid (ICF) extract from tomato leaves were subjected to reverse-phase HPLC analysis using an acetonitrile/TFA gradient.

Chromatograms of the eluents show the gradual disappearance of the peptide peak over a period of 30 minutes, while two new peaks were observed following five minutes incubation with ICF. One of the new peaks was further degraded after 30 minutes incubation with ICF.

Page 48

Figure 2.5: Pleurocidin was incubated together with sugarcane ICF at 37°C for one hour; and 50µl aliquots were removed after 0, 10, 30 and 60 minutes, and analysed using reverse-phase HPLC. The peptide was no longer detected within 10 minutes. Prolonged incubation of the mixture, resulted in the appearance of new chromatogram peaks which corresponded to breakdown products.

Page 49

Figure 2.6: Intercellular fluid (ICF) from sugarcane leaves was treated with protease inhibitors on ice for 60 minutes prior to incubation with pleurocidin at 37°C for 60 minutes. The samples were separated by electrophoresis using the Tricine SDS-PAGE system to determine the presence of the pleurocidin, as follows: Lane 1, Promega Low Range Protein Molecular Weight Marker; Lanes 2-9, 10µl aliquots of the different treatments were taken at times 0 and 60 minutes and separated; Lane 2, 5µg untreated pleurocidin; Lane 3, peptide with untreated ICF at time 0; Lane 4, Peptide incubated with ICF treated with a cocktail of protease inhibitors at time 0; Lane 5, Peptide incubated with ICF treated with PMSF at time 0; Lane 7, Peptide and cocktail protease inhibitor treated ICF after 60 minutes; Lane 8, Peptide and PMSF treated

ICF after 60 minutes; and Lane 9, Peptide and untreated ICF after 60 minutes.

Page 50

Figure 3.1: Genetic map of pUBI 510 showing restriction sites BAM HI and EcoRI into which the 317 nucleotide gene for pleurocidin was cloned. The 35s-Ubi hybrid promoter consisting of the CaMV and maize ubiquitin systems was used because of its high efficiency. The signal sequence ensured extracellular localisation of the peptide

Page 57

Figure 3.2: Crude protein extract from transgenic sugarcane leaves were separated by electrophoresis on Tricine SDS-PAGE gels as follows: **A:** Lane 1, low range molecular weight marker (Promega); Lane 3, 5µg control pleurocidin; Lanes 4-6, total cell protein from transgenic sugarcane leaves; and **B:** the nitrocellulose onto which a gel run concurrently with gel A was blotted as follows: Lane 3, 5µg of pleurocidin; Lanes 4 and 5, transgenic pleurocidin. This blot was developed using the alkaline-phosphatase conjugated secondary antibody.

Page 63

Figure 3.3: Crude protein extract from transgenic sugarcane was separated by electrophoresis on a tricine SDS-PAGE gel as follows: **A:** Lane 1, low range molecular weight marker (Promega); Lane 2, 50µg pleurocidin (positive control); Lane 3, intercellular fluid extract from transgenic leaf material; Lanes 4 and 5, crude protein extract from transgenic leaf material. Transgenic protein observed as band corresponding in size

to the control pleurocidin. ECL analysis of electroblotted membrane was carried out using Horse-radish peroxidase-conjugated secondary antibody to confirm identity of band as pleurocidin; and **B**: Lane 2, control pleurocidin; Lane 4, transgenic pleurocidin.

Page 64

Figure 3.4: Electron micrograph of transgenic (1) and normal (2) sugarcane leaves after immunogold detection of pleurocidin using primary antibody at a dilution of 1:300 followed by secondary labeling with a 10nm gold probe (Sigma) diluted to 1:300. **IS:** Intercellular Space, **CW:** Cell Wall, **CH:** Chloroplast

Page 68

Figure 3.5: Electron micrograph showing immunogold labeling controls treated as follows: 1. Primary antibody only (1:300 dilution 0.5% Bovine Serum Albumin in Tris buffered Saline) with no gold probe added. 2. Gold Probe only (1:300 dilution in 0.5% Bovine Serum Albumin in Tris buffered Saline) with no primary antibody added. 3. Pre-immune serum used at a dilution of 1:300 in 0.5% Bovine Serum Albumin in Tris buffered Saline. The pre-immune control was also probed with the Protein A gold probe at a dilution of 1:300. **IS:** Intercellular Space, **CW:** Cell Wall, **CH:** Chloroplast, **V:** Vacuole

Page 69

Figure 3.6: Electron micrograph of organelles in transgenic sugarcane indicating the presence of pleurocidin. The gold probes were concentrated within the cell wall and intercellular spaces (1), small amounts of peptide were also detected in the chloroplast (2) and the nucleus (3). **IS:** Intercellular Space, **CW:** Cell Wall, **CH:** Chloroplast, **V:** Vacuole, **N:** Nucleus

Page 70

LIST OF TABLES

Table 1.1:	Antimicrobial peptides from the skin of different amphibian species*	Page 8
Table 1.2:	Bacteria against which pleurocidin is active	Page 12
Table 1.3:	Description of key groups of pathogenesis-related proteins in plants	Page 15
Table 1.4:	Comparison of ESF1 and its analogues	Page 19
Table 1.5:	Examples of cationic peptides that have been used in making transgenic plants	Page 26
Table 1.6:	Summary of constitutive promoters that have been used for transgenic cereals	Page 33
Table 2.1:	List of indicator organisms and culture conditions used	Page 40
Table 2.2:	Determination of reduction in bioactivity of ESF1-GR7 following incubation with tobacco intercellular fluid (ICF)	Page 47
Table 3.1:	Comparison of pleurocidin content in ICF and total leaf extracts	Page 65

ABBREVIATIONS

A ₂₂₀	Absorbance at 220nm
BCIP	5-Bromo-4 Chloro- 3 Indoly Phosphate
BSA	Bovine Serum Albumin
BSA-TBS	Bovine Serum Albumin in Tris Buffered Saline
Da	Daltons
DNA	Deoxyribonucleic Acid
HPLC	High Pressure Liquid Chromatography
HRPO	Horse Radish Peroxidase
IAA	Iodo-acetamide
ICF	Intercellular Fluid
kDA	kilo Dalton
MIC	Minimum Inhibitory Concentration
mRNA	messenger Ribonucleic Acid
NBT	Nitroblue Tetrazolium Chloride
OD	Optical Density
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PMSF	Phenylmethylsulfonyl Fluoride
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	Tris Buffered Saline
TEM	Transmission Electron Microscopy

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 General Introduction

Plant protection is a major challenge to agriculture worldwide. Bacterial diseases of crop and fruit plants are a serious problem which needs to be solved in agronomy (Ohshima *et al.*, 1999). The option of a system of integrated pest management based on improved plant cultivars exhibiting resistance to microbial disease would reduce losses due to disease. Plants naturally respond to microbial attack by a complex network of defense mechanisms. These include the synthesis of: polymers forming physical barriers (cutins, lignin, callose); antimicrobial proteins (phytoalexins); and, pathogenesis related proteins (PR proteins), (Jach *et al.*, 1995). Potent antimicrobial peptides have been isolated and characterized from many plant and animal species. Antimicrobial peptides with broad spectrum activity provide a rich source of genes for transformation of plants for disease resistance. Natural defense proteins provide numerous candidates for antifungal genes to be used in transgenic crops. The challenge to biotechnology is to utilize these “naturally” occurring defense mechanisms in development of disease-resistant or -tolerant crops of commercial importance.

Defense proteins have been the focus of numerous studies aiming to develop genetically engineered fungal resistance. Introduced genes should not in any way influence plant growth once introduced into host plants. In the future there will be a need to evaluate the potential and limitations of “multi-transgene” tolerance strategy produced by combining different genes. Combinatorial expression of different antifungal proteins can lead to improved protection against a broad range of phytopathogenesis (Jach *et al.*, 1995).

The generation of plants resistant to various phytopathogens will go a long way towards reducing losses due to disease. Okamoto *et al.*, (1998) reported that as much as 90 percent of infectious diseases in plants are due to bacterial and fungal infections and that a third of all agro-chemicals in current use are germicides. Research efforts are, therefore, being aimed at enhancing a plant's defense potential against microbial infection through

expression of foreign antimicrobial peptides. The overall objective would be to produce transgenic plants that possess broad spectrum resistance against phytopathogens.

1.2 Cationic Antimicrobial Peptides

Cationic antimicrobial peptides are defined as endogenous antimicrobial peptides of plant and animal innate defense systems (Hancock and Lehrer, 1998). They are typically cationic (that is they contain excess lysine and arginine residues) amphipathic molecules composed of 12 to 45 amino acid residues (Hancock, 1997). Cationic antimicrobial peptides may be produced by the host organism constitutively or in response to infection or injury.

There are two basic structural classes distinguished - α (alpha)helical and β (beta) sheets. α -helical peptides usually adopt this configuration in the presence of structure enhancing solvents such as trifluoroethanol or when mixed with anionic phospholipid membranes (Hancock and Lehrer, 1998). β -sheets on the other hand usually contain structure stabilizing cysteine disulfide bonds.

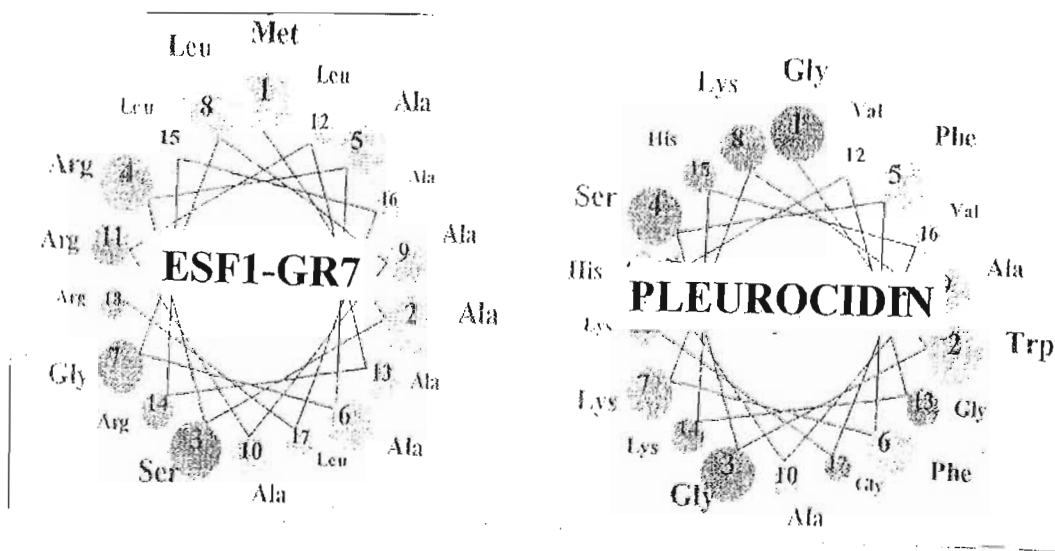


Figure 1.1 Helical wheel diagrams of ESF1 GR7 and Pleurocidin showing amphipathic helical form. ESF1 GR7 is a synthetic peptide that mimics the charge distribution and amphipathic nature of the modified magainin pGLa. Pleurocidin is a 2.7 kDa, 25 residue antimicrobial peptide that has been isolated from the winter flounder (*Pleuronectes americanus*).

Cationic antimicrobial peptides have been recognized as important constituents of nonspecific host defense mechanisms in both invertebrates and vertebrates alike. As a result, numerous antimicrobial peptides from an extensive variety of species have been isolated, characterized and studied.

1.2.1 Mammalian Antimicrobial Peptides

Defensins is the term used to refer to antimicrobial proteins produced by mammals in response to invasion by foreign organisms. Defensins have been isolated from the granules of neutrophils belonging to a broad range of mammals including humans, rabbits, rats, guinea pigs and hamsters. Defensins are produced as a first line of defense against invading pathogens. In general, mammalian defensins consist of approximately 29 to 35 amino acids, including six cysteine residues which provide three intermolecular disulfide bonds. Defensins of mammalian origin have been divided into two distinct classes according to the positions and bonding pairs of their cysteine. The two classes are the α or classic defensins and the more unusual β -defensins.

Defensins are most commonly found within the cytoplasmic granules of neutrophils where they may constitute between 5 and 15 per cent of total cellular protein (Hancock *et al.*, 1995). There are, however, several exceptions which include: two defensins, MCP-1 and MCP-2 that are expressed in elicited rabbit alveolar macrophages; cryptidins, found in the Paneth cells of mouse small intestine; and, the human defensins HNP-5 and HNP-6 also found in cells of the small intestine (Hancock *et al.*, 1995).

1.2.1.1 Classic or α defensins

Lactoferrin is a granule-associated glycoprotein present in mammalian fluids such as milk or tears that has long been linked with infant defense systems against both gram negative and gram positive bacteria. Lactoferrin is cationic with a high proportion of arginine and lysine residues at the N-terminal region (Salmon *et al.*, 1998). Its potent activity against

pathogenic bacteria has resulted in this peptide being exploited for potential use in the production of transgenic tobacco plants (Salmon *et al.*, 1998).

Human neutrophils are rapidly deployed phagocytic blood cells that constitute a first line of defense against invading pathogenic microorganisms. Four α -defensins (HNP1 to 4) have been isolated from the cytoplasmic granules of neutrophils (Hancock and Lehrer, 1998). Production of α -defensins by neutrophils appears to be constitutive and is regulated by the cell's intrinsic maturation programme.

1.2.1.2 β -defensins

Protegrins and tachyplesins are cationic peptides that consist of 16 to 18 amino acid residues that form an antiparallel β -sheet held together by two intramolecular disulfide bridges (Hancock and Lehrer, 1998). This group of antimicrobial peptides has been isolated from porcine leukocytes and from horse shoe crabs respectively. Both protegrins and tachyplesins display activity against fungi, bacteria and certain viruses, including extracellular HIV-1 (Hancock and Lehrer, 1998). Tachyplesin was first isolated from acid extracts of the Japanese horseshoe crab, *Tachyplesus tridentatum* haemocytes. The peptide has two disulfide bridges which result in its rigid structure and stability at low pH and high temperatures (Iwanaga *et al.*, 1994). Tachyplesin is typically comprised of 17 to 18 amino acid residues with an arginine amide at the C-terminal. Iwanaga *et al.*, (1994) reported that the peptide show potent antibacterial activity against both gram positive and gram negative bacteria owing to its ability to interact with the lipopolysaccharide layer of bacterial membranes.

The more unusual β -defensins have only been recently characterized from bovine neutrophils. Examples of these peptides include the bovine tracheal antimicrobial peptide (TAP), isolated from the epithelial layer of the trachea (Diamond *et al.*, 1991). TAP exhibits a broad spectrum of activity against several different species of both bacterial and fungal origin. Indolicidin, a tryptophan-rich peptide comprised of 13 amino acid residues that is stored in bovine neutrophils in its mature form (Selsted *et al.*, 1993). The primary structure of indolicidin is unique in that it contains five tryptophan residues among proline

residues (Subbalakshmi *et al.*, 1996).

1.2.2 Amphibian Antimicrobial Peptides

Several biologically active peptides have been isolated from the skin of a range of amphibians, those from frogs being the most common. Many of these peptides have been found to be homologous to hormones and neurotransmitters of mammals (Kreil, 1994). A number of amphibian peptides are positively charged and have a tendency to form amphipathic helices. Three categories of amphibian antimicrobial peptides have been distinguished on the basis of sequence and structure characteristics. Briefly, these are the linear amphipathic helix forming peptides such as the magainins, linear peptides containing two cysteine residues linked by a disulfide bridge at the carboxyl terminus as exemplified by the brevinins; and finally, very small peptides ranging in size from 10 to 13 amino acid residues which include temporins isolated from the frog *Rana temporari*.

1.2.2.1 Magainins

More information is known about the linear amphipathic helical magainins than any other of the amphibian antimicrobial peptides. Zaslof (1987), isolated the first two magainins, namely magainins-1 and -2, from the ventral skin of the frog *Xenopus laevis*. Magainins have been described as precursor molecules containing a highly conserved 20 amino acid signal sequence that includes an amino terminal processing motif, NH₂-ARGXXXVALARG (Bevins and Zaslof 1990). It was therefore proposed that this motif indicated a similar enzymatic cleavage activity in the processing of mature peptide.

1.2.2.2 Dermaseptins

Also included in the linear amphipathic helix group of amphibian antimicrobial peptides are the dermaseptin family originally isolated from the skin secretions of the South American frog *Phyllomedusa sauvagei* by Mor and Nicolas (1994). These peptides are very active against a variety of fungi although they have been found to be cytotoxic to mammalian cells (Kreil, 1994).

1.2.2.3 Brevinins

The brevinins comprise another well characterized group of amphipathic helices of amphibian origin. These peptides possess a net positive charge and contain 24 amino acids including two prolines. Brevinins typically have two cysteine residues present at positions 18 and 24 which form a disulfide bridge. This motif of two cysteines has also been found in a number of peptides present in the skin of the Ranidae family of frogs (Kreil 1994).

1.2.2.4 Bombinins

Studies on the antimicrobial and hemolytic peptides on the skin secretions of *Bombina variegata* led to the characterization of bombinin. Members of this group have been identified from analysis of skin secretions from two related species, namely, *B. variegata* and *B. orientalis*. These peptides have been found to all contain 27 amino acid residues with a constant C-terminal region and a variable N-terminal segment.

Table 1.1 lists examples of various amphibian antimicrobial peptides. The peptides listed are positively charged with a propensity to form amphipathic helices that encompass all or part of their sequences (Kreil, 1994). Therefore, although none of the peptides show any significant sequence homology, they do possess a common structure linked to their antimicrobial activity (Kreil, 1994).

Table 1.1: Antimicrobial peptides from the skin of different amphibian species

Species	Peptide	Sequence
Amphipathic peptides		
<i>Xenopus laevis</i>	Magainin/PG	NH ₂ -GIGKFLHSAGKFGKAFVGEIMKS
	S	
	PGLa	NH ₂ -GMASKAGAIAGKIAKVALKAL
<i>Rana brevipoda</i>	Brevenin-2	NH ₂ -GLLDSLKGFAATAGKGVLSLLSTASCKLAKTC
<i>Phyllomedusa sauvagei</i>	Dermaseptin I	NH ₂ -ALWKTMLKKLGTMALHAGKAALGAAADTISQGTO
<i>Bombina variegata</i>	Bombinin	NH ₂ -GIGGALLSAGKSALKGLAKGLAEHFAN
Other Peptides		
<i>Rana esculenta</i>	Brevenin-IE	NH ₂ -FLPLLAGLAANFLPKIFCKITRKC
<i>Bombina variegata</i>	Bombinin-H5	NH ₂ -IIGPVLGLVGSALGGLLKKI

From Antimicrobial Peptides 1994, Wiley, Chichester (Ciba Foundation Symposium 186)

1.2.3 Insect Antimicrobial Peptides

Antimicrobial peptides of insect origin are among the most studied and best understood of the cationic antimicrobial peptides. Hoffmann and Hoffmann (1990), report that antimicrobial activity of the haemolymph of insects appears to be induced after the insect is challenged by microbial penetration or by injuries within the humoral immune system. Insect peptides are moderately cationic, non-glycosylated peptides comprising between 38 and 43 amino acid residues (Lepage *et al.*, 1991). In general, the insect peptide structure, may be described as an anti-parallel β - sheet linked to the amino terminal loop by a disulfide bond. The α -helix is stabilized to the second strand of the β - sheet by the other two disulfide bonds (Hoffmann and Hetru, 1992). Defense peptides from insects have been divided into four groups according to various factors. The different categories of insect defense peptides are described below.

1.2.3.1 Cecropins

Cecropins are a key component of the immune response in insects (Cavallarin *et al.*, 1998). Cecropins are a family of homologous antibacterial peptides of 35-37 amino acid residues derived from the Cecropia moth *Hyalophora cecropia*. Like most cationic peptides, cecropins possess potent activity against both gram positive and gram negative bacteria. The antibacterial activity of cecropins has been linked to their structural features which include a strongly basic N- terminus, an intermediate hinge region containing glycine, proline or both and a hydrophobic C terminus (Rao, 1995). Their potential use as agents of crop protection stems from the fact that these peptides have no effect on animal and plant cells. It has also been found that the cecropins have no effect on eukaryotic cells but rather affect prokaryotic cells through the formation of time-variant and voltage-dependent ion channels (Christensen *et al.*, 1981). These peptides have, therefore, been earmarked for transgenic crop development. Jaynes *et al.*, (1993), reported attempts to produce transgenic tobacco expressing Shiva-1, a synthetic analogue of cecropin B, that exhibited superior biological activity and approximately 46% homology with the natural peptide.

1.2.3.2 Sarcotoxins

The Sarcotoxin family is a group of glycine-rich cecropin-like antibacterial proteins, isolated from the flesh fly *Sarcophaga peregrina*. Sarcotoxin IA is comprised of 24 amino acid residues and affects the morphology of gram positive cells by inhibiting cell wall synthesis. Sarcotoxin also has activity against gram-negative bacteria. The N-terminal half of this molecule is rich in positively charged amino acids and is hydrophilic whereas the C-terminal is predominantly hydrophobic (Okada and Natori, 1985). This enables the molecule to be amphipathic and thereby interact directly with bacterial membranes.

1.2.3.3 Sapecin

Sapecin is another antibacterial peptide commonly found in the *Sarcophaga* family. This peptide consists of 40 amino acid residues including six cysteine residues that form three intramolecular disulfide bridges (Natori, 1994). Like the sarcotoxins, the sapecin primary target is the bacterial membrane of gram positive bacteria. Two homologues of sapecin termed sapecin A and B, have also been isolated. Sapecin B is only active against gram positive bacteria owing to structural modifications that render it unable to interact with gram negative bacteria and fungi (Yamada and Natori, 1993).

1.2.3.4 Attacins

The Attacin family of antimicrobial peptides is mostly found in insects and is comprised of at least six members, all considerably large molecules of about 180 amino acid residues in size. Forms A to D are basic with similar sequences, while forms E and F are acidic with identical sequences that are slightly different from forms A to D (Hultmark *et al.*, 1982). Like cecropins, mature attacins are made from pre-pro-forms in the fat bodies of insects after post-translational modification during transport to the haemolymph (Boman and Hultmark 1987). The structural features of attacins indicated by cDNAs are more like Sarcotoxin II in form, but lack extra domains (Okada and Natori, 1985).

1.2.3.5 Apidaecins

Apidaecins are a family of proline-rich peptides that have been isolated from the haemolymph of honeybees. These peptides have been demonstrated to be primarily active against gram negative and plant-associated bacteria (Hancock *et al.*, 1995). Apidaecins are thought to be specifically induced and owing to their high proline content they are stable at high temperatures and low pH.

1.2.3.6 Abaecins

Abaecins are similar to apidaecins in that they also have a relatively high proline content. The major contrast lies in the fact that abaecins appear to have delayed action against target organisms whereas apidaecins exhibit more immediate activity (Hancock *et al.*, 1995).

1.2.3.7 Mellitins

Another well-characterized member of the antimicrobial peptides of bee origin is the venom component mellitin. Mellitin comprises approximately 50 per cent of bee venom (Hancock *et al.*, 1995). It is a 26 residue peptide which has a broad spectrum of biological effects including bactericidal properties and membrane permeabilization leading to cell lysis (Hancock *et al.*, 1995).

1.2.3.8 Drosomycin

Drosomycin is an inducible antifungal peptide produced by the fruit fly *Drosophilla*. The peptide is comprised of 44 amino acid residues, including eight cysteine that form four disulfide bridges paired identically to those found in plant defensins. Drosomycin has been demonstrated to have 38 percent sequence homology with the antimicrobial peptide Rs-AFP1, a plant defensin found in raddish seeds (Fehlbaum *et al.*, 1994).

1.2.4 Fish Antimicrobial Peptides

1.2.4.1 Pleurocidin

Pleurocidin has been characterized as a novel 25-residue antimicrobial peptide that is produced in the epidermal mucous cells of the winter flounder (*Pleuronectes americanus*). The primary amino acid sequence analysis of pleurocidin indicates homology with other

characterized antimicrobial peptides, namely the dermaseptins and ceratotoxin classes (Cole *et al.*, 1997). The dermaseptins and ceratotoxins have been proposed to form amphipathic α -helices. Pleurocidin is predicted to form an amphipathic α -helical structure similar to many other antimicrobial peptides which exert their function by forming holes in the bacterial membranes. The structure and function of pleurocidin differs greatly from other classes of antimicrobial molecules from marine species.

Pleurocidin is 2.7 kDa in size and is glycosylated. The amino acid sequence reads:



Pleurocidin has been tested for efficacy against 11 gram positive and gram negative bacteria for bactericidal and bacteriostatic activity. Examples of bacteria tested are detailed in figure 1.2. Results showed that the peptide is active against both gram negative and gram positive bacteria. Pleurocidin has also been noted to be salt-insensitive at physiological concentrations (Cole *et al.*, 1997).

Table 1.2: Bacteria against which pleurocidin is active

Gram Positive Bacteria	Gram Negative Bacteria
<i>Leucothrix mucor</i>	<i>Escherichia coli</i>
<i>Bacillus subtilis</i>	<i>Aeromonas salmonicidae</i>
<i>Staphylococcus aureus</i>	<i>Pasteurella haemolytica</i>
	<i>Xanthomonas species</i>
	<i>Pseudomonas aeruginosa</i>
	<i>Salmonella typhimurium I</i>
	<i>Salmonella typhimurium II</i>

At present, pleurocidin is being investigated for potential use as a means of crop protection in sugarcane. Research efforts are at an advanced state, with the peptide being successfully cloned into sugarcane by the use of a biolistic gun. Pleurocidin is also being investigated for its potential use as a therapeutic agent in transgenic Coho salmon owing to its potent activity against other fish pathogens (Jia *et al.*, 2000). Pleurocidin-amide, a C-terminally amidated derivative of pleurocidin, is more active than the native peptide and hence has

potential use as a therapeutic agent (Jia *et al.*, 2000). Since it has been established that genes derived from as close a species as possible to the host are most efficiently expressed in transgenic fish pleurocidin-amide becomes a potential agent for exploitation in transgenic research (Devlin, 1997).

1.2.4.2 Other Antimicrobial Peptides of Fish Origin

Cationic antimicrobial peptides with potent activity against both gram negative and gram positive have also been isolated from the Atlantic Hag fish, *Myxine glutinosa*. Three related peptides from hematopoietic cells in intestinal mucosa. have been isolated and characterized (Shinner *et al.*, 1996). The linear α -helical peptides which are composed of 30 to 37 amino acid residues each have been named HAP-MXGL are approximately 3.4 kDa in size.

Pardaxin is a 33 amino acid pore-forming antimicrobial peptide isolated from the Red Sea Moses sole *Pardachins marmoratus*, and has been found to have a helix-hinge-structure (Oren and Shai, 1996). Although this peptide has been shown to exhibit significant similarities to the bee venom melittin as well as cecropin it has less hemolytic activity against human red blood cells (Oren and Shai, 1996). This feature renders pardaxin a more suitable candidate for therapeutic applications.

Penaeidins are antimicrobial peptides isolated from the plasma and haemocytes of the tropical shrimp *Penaeus vannamei*. These peptides are estimated to range in size from 5.5 to 6.6 kDa (Destoumieux *et al.*, 1999). These molecules are unique in that they are composed of a proline rich N-terminus and of a C-terminus containing six cysteine-rich engaged in three disulfide bridges (Destoumieux *et al.*, 1999). Penaeidins have also been shown to have impressive activity against gram negative bacteria as well as filamentous fungi.

1.2.5 Plant Antimicrobial Peptides

Unlike other higher vertebrates, plants do not possess an immune system to protect themselves against attack by pathogens. Their resistance is, therefore, mainly based on a dynamic defense system composed of antimicrobial agents of varying molecular weight sizes. The first group is comprised of secondary products of metabolism such as phytoalexins. Phytoalexins are low molecular weight compounds that rapidly accumulate at the sites of infection to act as antibiotics against invading pathogens (Vigers *et al.*, 1991). Higher molecular weight defense-related proteins, include structural proteins such as hydroxyproline-rich glycoprotein that incorporate into the cell wall to participate in confinement of pathogens. The best known group of stress-induced defense proteins are the pathogenesis-related proteins which include the chitinases and β -1,3-glucanases. These proteins are thought to combat fungal pathogens by hydrolyzing their cell walls (Cammue *et al.*, 1994). The current classification system groups pathogenesis related proteins into fourteen families, as detailed in Table 1.3, based on sequence homology (Jakobs *et al.*, 1999).

Table 1.3: Description of key groups of pathogenesis-related proteins in plants

PR Class	Properties	Examples	Reference
PR-1	no mode of action is known for this class and function is still unclear	tobacco PR-1a	van Loon and van Strien, 1999
PR-2	β -1,3-glucanases	tobacco PR-2	van Loon and van Strien, 1999
PR-3	chitinases	tobacco P, Q	van Loon and van Strien, 1999
PR-4	chitin-binding proteins	Tobacco 'R'	van Loon et al., 1994
PR-5	thaumatin-like proteins that tend to have acidic isoelectric points and found in the extracellular space	VvTI 1 in ripening grapes	Jakobs <i>et al.</i> , 1999
PR-6	proteinase inhibitors implicated in defense against insects, herbivores, microorganisms, nematodes	Tomato inhibitor 1	Koiwa <i>et al.</i> , 1997
PR-7	only characterised in tomato and acts as an endoproteinase	Tomato P ₆₉	Goldman and Goldman, 1998
PR-8	endochitinases whose basic isoforms possess substantial lysozyme activity	Cucumber chitinase	Brunner <i>et al.</i> , 1998
PR-9	peroxidases that strengthen plant cell walls by catalyzing lignin deposition in response to microbial attack	tobacco lignin-forming peroxidase	Vigers <i>et al.</i> , 1991

PR-10	structurally related to ribonucleases intracellular	Parsley PR-1	Moiseyev <i>et al.</i> , 1997
PR-11	endochitinases	tobacco class V chitinases	
PR-12	pathogenesis induced plant defensins similar to those of insect origin	Rs-AFP3	Loon and van Stien, 1999
PR-13	thionins that act by permeabilising the membranes of target microorganisms	Arabidopsis THI2.1	Loon and van Stien, 1999
PR-14	lipid transfer proteins that exert their effect on the plasma membrane of target microorganism	Barley LTP4	Loon and van Stien, 1999

This discussion will focus on those plant peptides that have been found to be structurally related to cationic antimicrobial peptides in insects and mammals. These peptides tend to be cysteine rich and range in size from 3 to 14kDa. Plant defensins exhibit potent antimicrobial activity in vitro and have now been isolated from over twenty different plant species from various monocot and dicot species (Carvalho *et al.*, 2001).

1.2.5.1 *Mirabilis jalapa* antimicrobial peptides (Mj-AMP)

The first type of plant antimicrobial peptide characterized was found in the seeds of the *Mirabilis jalapa* (four o'clock plant). Two isoforms of the peptide differing from each other by only four amino acids have been identified. The peptides are comprised of 36 and 37 residues, and have been designated Mj-AMP1 and Mj-AMP2 respectively (Cammue *et al.*, 1994). Both peptides are highly basic and their structure is stabilized by three disulfide bridges. The structural and biological properties of the Mj-AMPs resemble those

of defensins from both insect and mammalian sources (Cammue *et al.*, 1994).

1.2.5.2 *Amaranthus caudatus* antimicrobial peptides (Ac-AMPs)

A second type of plant antimicrobial peptides was isolated from the seeds of *Amaranthus caudatus* by Broekaert *et al.*, 1992). Two basic isoforms of approximately 3025 and 3181 Da respectively were distinguished. Three disulfide bridges formed by six cysteine residues stabilize the Ac-AMP molecules. These peptides, therefore, have structural similarities to the Mj-AMPs, but as Table 1.3 demonstrates, there is no sequence homology between the two classes.

1.2.5.3 *Raphanus sativus* antifungal proteins (RS-AFPs)

Two highly homologous antifungal proteins (RS-AFP1 and RS-AFP2) have been discovered in the imbibition medium of germinating radish. These peptides are approximately 5kDa in size and each contain four disulfide bridges (Terras *et al.*, 1992). These peptides have also been shown to striking amino acid sequence homology to with other plant proteins such as γ - thionins (defensins), thought to be involved in plant defense mechanisms (Cammue *et al.*, 1994).

1.2.5.4 *Raphanus sativus* non-specific lipid transfer protein (Rs-nsLTP)

This is a 9kDa protein that was partially purified during the isolation of the Rs-AFPs (Terras *et al.*, 1992). This peptide was the first lipid transfer protein from plant seeds reported to have antifungal activity (Cammue *et al.*, 1994).

1.2.5.5 *Raphanus sativus* 2S albumins (Rs-2S)

Another type of antifungal protein isolated from radish seeds was identified as one of the well-characterized seed storage proteins called 2S albumins (Terras *et al.*, 1992). This protein has a molecular weight of about 14 kDa and exists in a number of isoforms. The protein can be found in small (4kDa) and large (10kDa) subunits (Terras *et al.*, 1992).

1.2.5.6 Thionins

Thionins are a group of cysteine-rich plant peptides generally located intracellularly in the endosperm of monocotyledonous plants such as wheat (purothionins), barley (hordothionins), rye, and maize (Cammue *et al.*, 1994). Thionins are compact (5kDa), L-shaped molecules, whose long arm is formed by two disulfide-linked α -helices and with the short arm containing two antiparallel β -sheets. More recently 5kDa molecules with 45 to 54 amino acid residue named γ -thionins, have been isolated from the seeds of many plants of both mono- and dicotyledonous form, and characterized.

1.2.6 Synthetic Antimicrobial Peptides

The emergence of resistant strains has resulted in there being a constant search for more potent and efficient antibiotic agents. The purpose of designing synthetic peptides would therefore be to optimize their desired activity as gene products prior to applying them as either therapeutic agents or cloning them into plants. The synthetic peptide designed would therefore need to mimic the activity of the natural peptide while incorporating other desirable qualities such as increased potency and reduced hemolytic activity. In order to design highly potent antimicrobial peptides with lowered or no side effects, novel peptides should be based on the sequence and α -helical wheel diagrams, in the case of amphipathic peptides, of natural antimicrobial peptides. Design of synthetic peptides therefore requires full knowledge of the structure-function relationships of the natural peptide.

Synthetic peptides also play a crucial role in studying structure-function relationships. This is because, often it is easier to study and better understand the nature of antimicrobial peptides by making synthetic analogues. Synthetic peptides allow considerable studies to be carried out on chain length, hydrophobicity and charge distribution which are not always possible with native peptides (Oh *et al.*, 1998). Using synthetic peptides it is possible to not only enhance specific aspects such as efficacy and reduced cytotoxicity but to also make peptides more amenable to genetic manipulation.

The solid phase method on an automated peptide synthesizer is the most common means employed to synthesize peptides. The peptides are then purified using reverse-phase high pressure liquid chromatography (RP-HPLC), (Matsudura, 1987).

1.2.6.1 ESF1-GR7

ESF1- GR7 is a synthetic peptide which contains a single amino acid substitution of arginine for glycine at position 7 for improved efficacy (Dykes *et al.*, 1998). The mother peptide, ESF1 is a synthetic peptide that mimics the charge distribution and amphipathic on the α -helical structure of the modified magainin pGLa. ESF1 differs from pGLa in two major way. Firstly, ESF1 has a completely different amino acid sequence to pGLa, and secondly, ESF1 is shorter than pGLa by one amino acid. This 20 amino acid, 2.052kDa peptide is a member of a series of pGLa based variants, chemically synthesized for the specific purpose of optimizing its desired activity as a gene product prior to cloning it into plant (Powell *et al.*, 1995). Modifications to the ESF1 peptide sequence producing in the synthesis of the analogue ESF1-GR7, encouraged the formation of a tighter α -helical structure that decreased the minimum inhibitory activity of the peptide. Table 1.4 below illustrates the increased antimicrobial activity in the ESF1 analogues. Dykes *et al.*, 1998, found that the elimination of positively charged residues destroyed bioactivity. It was also observed by Powell *et al.*, (1995) that shortening of the peptide from 20 to 18 residues also contributed to the reduction in bioactivity against the test organism.

Table 1.4: Comparison of ESF1 and its analogues (from Dykes *et al.*, 1998)

Peptide	Sequence	MIC μ M against <i>Carnobacterium mobile</i>
ESF1	MASRAAAGLAARLARLALRAL	50nM
ESF1-GR7	MASRAAA <u>R</u> LAARLARLALRAL	2.5nM
ESF1-SA7	MA <u>A</u> RAAA <u>R</u> LAARLARLALRAL	2.5nM

1.3 Mode of Action for Cationic Antimicrobial Peptides

The antimicrobial function of cationic antimicrobial peptides appears to be related to the amphipathic structure that arises from the distribution of bulky hydrophobic groups at the “head” of the anti-parallel β -pleated sheet structure linked by a turn to a hydrophilic “tail” comprising six cationic residues derived from the N and C termini of proteins (Hancock and Lehrer, 1998). Biological activity appears to be associated with membranolytic function that arises from an amphipathic structure stabilized by four disulfide bonds (Oh *et al.*, 1999). Antimicrobial peptides are thought to work in two main ways by either targeting the physiology of the pathogen or by disrupting the cellular structure of the microorganism.

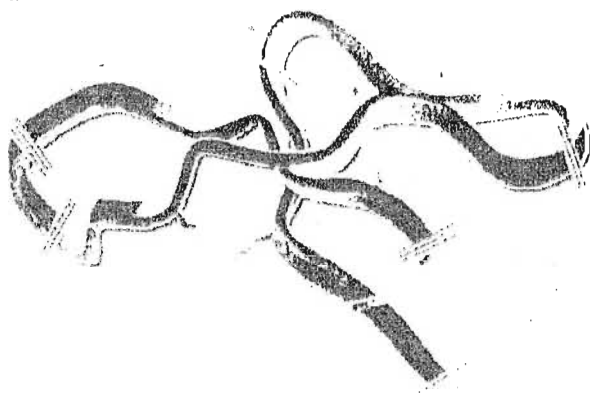


Figure 1.2 β -stranded human defensin is a typical example of β -sheet type antimicrobial peptides.

Studies conducted on various native and synthetic antimicrobial peptides emphasize the importance of an amphipathic α -helical structure and a net positive charge for antimicrobial activity (Oh *et al.*, 1999). The net positive charge is important for facilitating interaction with the negatively charged membranes while the α -helical structure is essential for lytic activity (Andreu *et al.*, 1985). It has also been found that increasing α -helicity increases the bioactivity of the peptide (Christensen *et al.*, 1988). This structure is also responsible for their association with lipid bilayers and thus antimicrobial activity (Boman,

1995).

A model of the predicted mode of action for cationic antimicrobial peptides was proposed by Christensen *et al.*, (1988). This model proposed that there was an initial electrostatic interaction between the positively charged cationic peptide and the negatively charged phospholipid bilayer of the cytoplasmic membrane of the microorganism. The electric potential of the bacterial membrane is thought to facilitate the transition of the peptide from an unstructured form to a structured or “active” form. An aggregation of peptides in this manner would result in the hydrophobic faces of the peptides being directed toward the interior of the membrane, while their hydrophilic sides would form the channel pointing inwards. This process would result in weakening of the membrane and subsequent cell death as a result of cytoplasmic leakage. Figure 1.3 below illustrates the three steps entailed in mechanism of bacteria killing by cationic peptides.

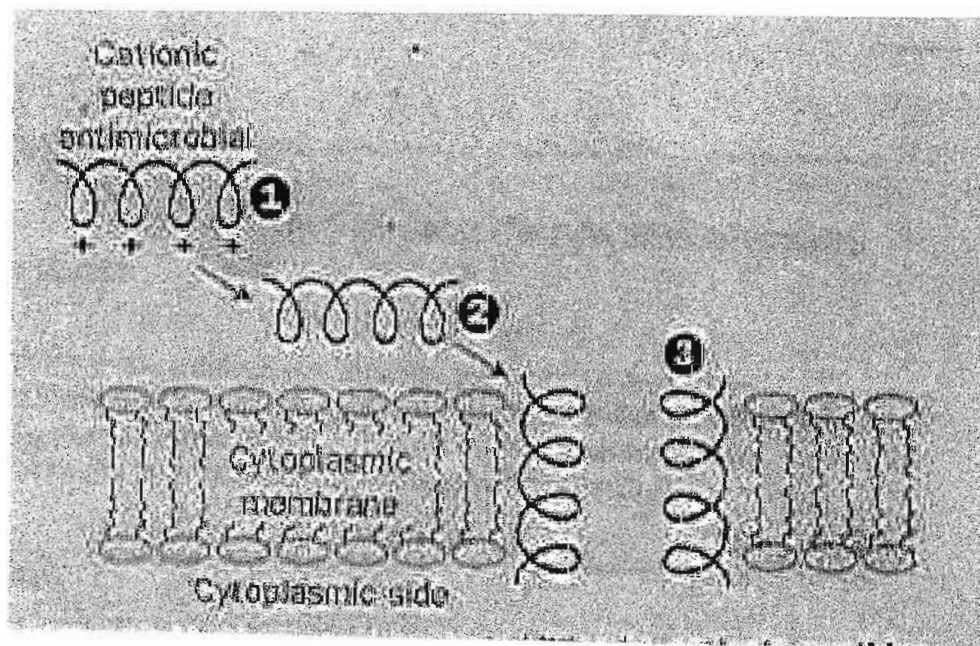


Figure 1.3: Proposed mechanism of microbial killing by cationic peptides: the positively charged peptides (1) bind to the external surface of the negatively charged phospholipid bilayer (2) leading to a localized thinning of the cytoplasmic membrane. Under the influence of membrane potential, the peptide inserts into the membrane and forms channels (3) which result in leakage of the cytoplasmic

molecules and subsequent death of the cell. (From Hancock ,1997)

It has been suggested that in the case of gram negative bacteria, cationic antimicrobial peptides interact with the lipopolysaccharide (LPS) layer of the outer membrane, and are taken up by a “self-promoted uptake” pathway (Hancock and Lehrer, 1998). The initiation of the “self-promoted uptake” pathway is thought to occur when the cationic peptide interacts with the anionic divalent cation-binding sites on the LPS as illustrated in Figure 1.4. This interaction results in the outer membrane being disrupted, forming pores that allow the antimicrobial peptide access to the cell (Hancock and Lehrer, 1998).

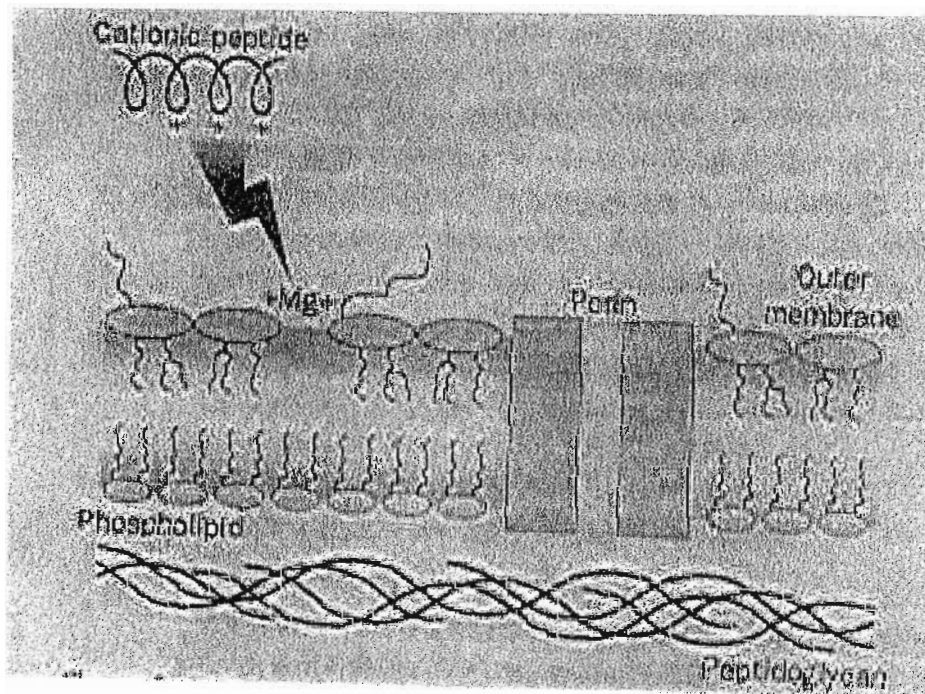


Figure 1.4: Self-promoted uptake of cationic peptides showing positively charged peptides interact with the negatively charged divalent-cation-binding sites on the surface of the lipopolysaccharide. This action disrupts these sites and leads to enhanced uptake of the cationic peptides across the membrane. (From Hancock , 1997)

It should be noted that the modes of action described above do not refer to all cationic antimicrobial peptides. Other modes of action for antimicrobial peptides have also been

proposed suggesting that mechanisms of action are a function of structure (Oh *et al.*, 1999). Studies on lantibiotics have shown that antimicrobial activity appears to be associated with inhibition of enzymatic reactions rather than impairment of the cell wall (Brötz *et al.*, 1998). Experiments on mersacidin, a globular lantibiotic, indicated that bactericidal action occurred as a result of inhibition of the transglycosilation level of peptidoglycan biosynthesis through the interaction of the lipid II component of peptidoglycan (Brötz *et al.*, 1998).

Further research on modes of action for the various classes of antimicrobial peptides is therefore required in order for their to be a more fuller understanding of antimicrobial peptides, and an increase in their potential uses.

1.4 Potential Applications for Antimicrobial Peptides

1.4.1 Therapeutic Applications

Cationic antimicrobial peptides have potential widespread application as therapeutic agents. The novel nature and mode of action of antimicrobial peptides suggests that they may be utilized as antibiotics against serious bacterial and fungal infections that are resistant to conventional antibiotics.

1.4.1.1 Topical antibiotic agents

Owing to the fact that antimicrobial peptides tend to be involved in a localized response to infection, it has been assumed that their application may be limited to the treatment of topical infections (Hancock and Lehrer, 1998). Various pharmaceutical companies have reported successful preclinical and clinical studies to assess the efficacy of several antimicrobial peptides as therapeutic agents for common infections. Magainin Pharmaceuticals have taken their α -helical magainin variant MSI-78 into phase III clinical trials with 926 patients in an attempt to reduce dense bacterial flora indigenous to the

perineal area (<http://www.pslgroup.com/dg/2168e.htm>). The company has reported that these trials demonstrated equivalent efficacy with orally administered ofloxacin.

1.4.1.2 Systemically administered antibiotics

Magainin has been shown to act synergistically with certain “standard” antibiotics in the chemotherapy of gram negative bacterial infections (Jacobs and Zaslof 1994, cited in Ciba Foundation Symposium 1994). The authors suggest that the administration of an antibiotic peptide along with a traditional antibiotic in the treatment of infections in clinical settings where immune function has been depressed may be successfully achieved. As human therapeutics, peptide antibiotics will be developed which exhibit both antibiotic and endotoxin-neutralizing activity, a combination of properties that might be beneficial in the chemotherapy of bacterial infections (Ciba Foundation, 1994). Cole *et al.*, (2000), reported that pleurocidin, a peptide of fish origin showed significant activity against clinical fungal isolates, again suggesting that this peptide could also be administered for systemic infections.

1.4.1.3 Wound healing stimulants

MS1-420 is a 14 residue magainin analogue that has been found to be effective in the re-epithelialization process in the cornea of rabbits (Jacobs and Zaslof, 1994 in Ciba Symposium). Future clinical studies will therefore be focused on evaluating the potential of this peptide as a wound healing stimulant in humans. Destoumieux *et al.*, (1999) suggested that penaeidins can potentially represent a new generation of therapeutic agents for human applications in order to overcome the problems of antimicrobial resistance strains as well as for veterinary and agronomic use.

1.4.1.3 Anticancer agents

A pharmaceutical company, IntraBiotics (Pvt. Ltd) are utilizing a protegrin-derived peptide IB-367 against oral polymicrobial (oral mucositis) in cancer patients (Hancock and Lehrer, 1998). A cecropin-mellitin hybrid has also been shown to have topical activity

against *Pseudomonas aeruginosa* eye infections of rabbits. Several animal studies have also been conducted to evaluate the use of magainin peptides in cancer-bearing animals by Jacobs and Zasloff (1994). Promising results were obtained with MSI-511, a 14 residue magainin analogue composed of all-D-amino acids. This peptide was able to induce tissue damage around tumour while at the same time arrest development of the initial melanoma tumour (Jacobs and Zasloff, 1994).

1.4.2 Antimicrobial Peptides as Agents of Crop Protection

The idea of antimicrobial peptides as agents of crop protection has been widely accepted as a possible cost-effective means of improving the resistance of various crops of commercial importance to microbial infection. Transgenic technology may be used to facilitate the expression of such peptides in plants. Table 1.5 below illustrates some of the attempts that have been made to successfully integrate the genes from a broad range of sources into the genomes of various plants.

Current research has demonstrated that cationic antimicrobial peptides such as cecropins and magainins are promising peptides with potential for plant genetic engineering initiatives (Jaynes *et al.*, 1993). This is because not only do these peptides possess wide antibacterial spectra against both gram positive and negative bacteria, but also their small size requires small DNA coding sequences suitable for synthesis and further manipulation.

1.4.3 Stability of Antimicrobial Peptides in Transgenic Plants

The fate of antimicrobial peptides expressed in transgenic plants requires careful understanding. Knowledge of the expression of transgenes in plants would be useful in predicting the expression of agronomically useful genes, Curtis *et al.*, (2000). There are several factors that play an important role in transgenic protein expression. These factors include low protein levels that can be attributed to poor translation, incorrect folding of protein into its tertiary structure, inefficient post-translational modification, protein instability in the targeted locale or a combination of these factors.

Table 1.5: Examples of cationic peptides that have been used in making transgenic plants

Transformed Species	Peptide Utilized	Peptide Expression Level	Target Organism	Reference
Tobacco	Barley α -hordothionin	2-60 ng mg ⁻¹ leaf protein	<i>Clavibacter michiganensis</i>	Florack, D (PhD thesis) cited by Hancock and Lehrer, 1998
Tobacco	Barley β -hordothionin	2-60 ng mg ⁻¹ leaf protein	<i>Clavibacter michiganensis</i>	Florack, D (PhD thesis) cited by Hancock and Lehrer, 1998
Tobacco	cecropin B	not examined	<i>Pseudomonas syringae</i> pv. <i>tabaci</i>	Huang <i>et al.</i> , 1997
Potato	cecropin B	none	<i>Erwinia</i> spp	Allefs <i>et al.</i> , 1995
Potato	tachyplestin 1	low expression recorded	<i>Erwinia</i> resistance	Allefs <i>et al.</i> , 1996
Tobacco	Rs-AFP2	0.2-2.4 μ g mg ⁻¹ leaf protein	Fungal <i>Alternaria longipes</i>	Eppel <i>et al.</i> , 1997
Tobacco	Ac-AMP2	0.6-1.1 μ g mg ⁻¹ leaf protein	Antifungal resistance	De Bolle <i>et al.</i> , 1996
Tobacco	Mj-AMP2	0.9-1.4 μ g mg ⁻¹ leaf protein	Antifungal resistance	Jaynes <i>et al.</i> , 1993
Tobacco	*SB-37 Shiva-1	Up to 1 μ g mg ⁻¹ leaf protein	<i>Burkholderia solanacearum</i>	Jaynes <i>et al.</i> , 1993

*cecropin B analogues

1.4.3.1 Proteolytic instability

Low levels of enhanced resistance in transgenic plants has been attributed to proteolytic instability in transgenic plant environments (Cavallarin *et al.*, 1998). In plants engineered to secrete antimicrobial peptides to the intercellular compartment of leaves, the degradative activity of proteases present in the intercellular fluid could be critical to achieving the expected transgene function (Owens and Huette, 1997). Hightower *et al.*, (1994), reported that while it had been possible to generate tobacco plants which had high levels of cecropin mRNA, the corresponding cecropin protein was barely detectable in leaf tissue. Further analysis by Hightower *et al.*, (1994) revealed that cecropin was rapidly degraded by leaf protein extracts indicating that the low protein expression levels *in planta* was due, in this case, to protein instability rather than translational inefficiency. Owens and Huette (1997) reported that when MB39 (a structural analogue of cecropin B), was degraded less rapidly than cecropin B by intercellular fluid from 10 different crops.

The small size of the cationic antimicrobial peptides expressed in transgenic plants may play an important role in their instability in proteolytic environments. Fusion of the sarcotoxin 1A gene with the GUS gene protected the introduced gene product from degradation by plant proteases (Okamoto *et al.*, 1998). This conclusion was reached after it was observed that tobacco plants transformed in the absence of the β -glucuronidase (GUS) fusion constructs had lower sarcotoxin accumulation levels.

The accumulation of transgenic proteins in plants may also be influenced by the rate at which synthesized proteins are degraded. Consequently, engineering a protein to resist degradation by proteases could, conceivably, enhance the accumulation of that protein in the transgenic plant (Owens and Huette, 1997).

1.4.3.2 Post-translational modification

Post-translational processing may result in the truncation of the introduced peptide. When Mitra and Zhang (1994), attempted to overexpress human lactoferrin in transgenic tobacco callus, the gene product appeared to undergo post translational processing, resulting in a

truncated protein of approximately 48 kDa in place of the full length 80 kDa protein. This truncated protein exhibited strong bactericidal activity. Further research carried out by Zhang *et al.*, (1998), produced intact full length lactoferrin in transgenic tobacco plants. This fact was attributed to the fact that cells in the callus stage undergo different physiological changes that may result in modification of proteins (Zhang *et al.*, 1998). Both the truncated and full length proteins were found to be active against the target organism, *Ralstonia solanacearum*. The smaller molecule was named lactoferricin and it was postulated that this molecule was released following proteolytic degradation of the lactoferrin molecule by plant proteases (Mitra and Zhang, 1994).

1.4.3.3 Gene silencing

The stability of transgenic protein expression is just as important as the expression level (De Neve *et al.*, 1999). Silencing of the introduced genes has frequently been observed in plants (Curtis *et al.*, 2000), where young transformants may show high accumulation of the introduced protein, while older plants can have accumulation levels that are 100-fold lower (De Neve *et al.*, 1999). The exact mechanism by which gene silencing occurs is not clearly understood. It has been suggested that this reduction in heterologous protein expression may be caused by a reversible inactivation of highly expressed homozygous transgene, or by a progressive transcriptional inactivation of the transgene (De Neve *et al.*, 1999). In order to ensure success of transgenic expression systems, it may be necessary to obtain further insight into the mechanism of gene silencing in order to prepare gene constructs and vectors with low susceptibility to gene silencing.

1.5 Strategies for Introducing Antimicrobial Peptides into Plants

Rapid development in recombinant DNA technologies and plant genetic transformation have made it possible to introduce selected genes into plants to confer novel phenotypes (Huang *et al.*, 1997). Genetic engineering may be used to modify the expression of genes already present in plants; to introduce new genes from species the target plant does not breed with conventionally; or totally novel or synthetic genes may be added. Genetic engineering overcomes the limitations of traditional breeding and allows scientists to use

new traits from many kinds of plants and other living things such as fish, insects, bacteria and even humans. In spite of the universal nature of the genetic code, it is not always possible to efficiently express bacterial genes in plants. The *Bacillus thuringiensis cry* genes, for example, is not easily expressed in plants owing to the problem of expressing bacterial prokaryotic genes in higher plants or any other eukaryotic organism (de la Riva and Adang, 1996). The expression problem has been associated with preferential codon usage. Expression levels of bacterial genes in plants are therefore enhanced by the introduction of preferential codons used by plants. This is because the codon usage patterns in plants are different to those used in bacteria. Bacteria tend to be AT-rich while plants are more likely to prefer GC-rich codons (de la Riva and Adang, 1996). This results in low expression levels of bacterial genes in plants. Figure 1.5 illustrates the process of genetic engineering in plants.

This technology has revolutionized disease resistance in agricultural crops (Huang *et al.*, 1997). Using transgenic methods, biotechnologists can be certain that if they have selected the correct gene, the new characteristic will be permanently established in the new crop variety. Transgenic technology has therefore made it possible to create a whole new range of genetic diversity.

Some methods for transforming plant cells are now sufficiently well established to be exploitable. There are, however, many emerging techniques of DNA transfer that are promising.

1.5.1 Direct Methods of Gene Transfer

Direct DNA transfer can be achieved by a range of physical methods. Included among these techniques are micro particle bombardment, electroporation, polyethylene glycol (PEG) treatment, abrasion with fibres, micro-injection and laser mediated approaches. A wide range of these methods, especially electroporation and PEG-mediated transformation, have been successfully used to introduce DNA into plant protoplasts. It should be noted however that protoplast regeneration to whole plants provides a major bottle neck in genetic engineering because it is very difficult for some species and is often very genotype-dependent (Henry, 1997).

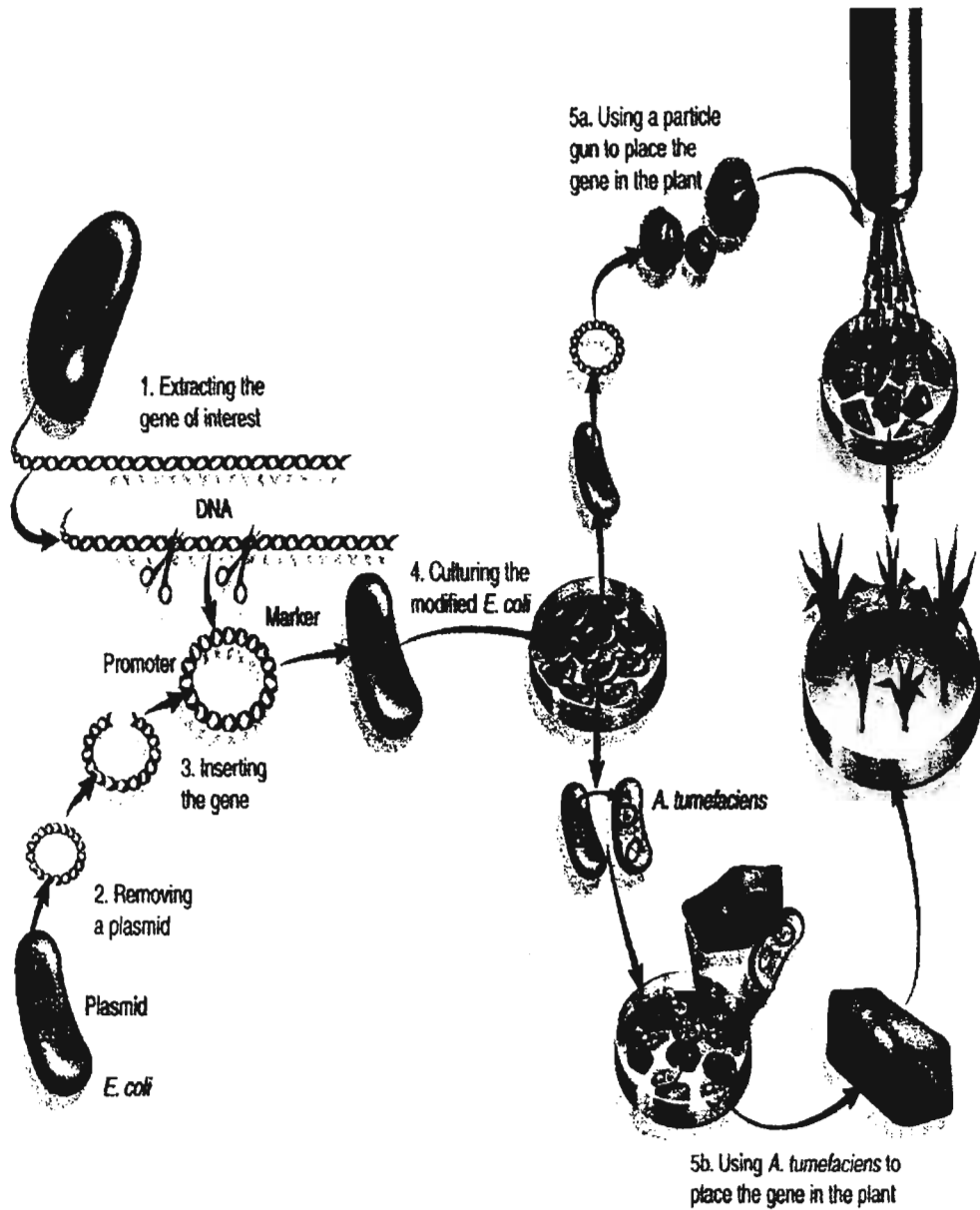


Figure 1.3: Overview of mechanisms for genetic modification of plants (From Levidow, 1999)

Micro particle bombardment is the most amenable technique for monocots and most widely used on cell and organ cultures. For this technique the DNA is coated on tungsten or gold micro particles approximately 1 µm in size and projected into the cells to be transformed using a gun powered with gunpowder or an inert gas such as helium. This technique allows the cell wall to be penetrated and provides a generally more successful approach. Coating fibres with DNA represents a variation to micro particle bombardment technique (Henry, 1997).

Vortex mixing of the suspended plant cells and fibres results in transformation associated with penetration of cell membranes by the fibres. Laser beams provide yet another mechanism for facilitating DNA uptake through plant cell membranes. Introduction of DNA into pollen provides a route for DNA transmission into seeds. Electroporation may be used to transfer DNA into the pollen and pollination with such pollen may generate transgenic plants. Micro targeting allows the specific transformation of cells like meristem cells that are totipotent by targeting specific tissues or cell types (Henry, 1997).

1.5.1.1 Fusion Strategy

The fusion strategy has been found to increase production of antimicrobial peptides in plants (Okamoto et al 1998). β-glucuronidase (GUS) may be used as the fusion partner because it is stably expressed in plants and the assay of its activity is simple and has high sensitivity. In addition, the use of GUS enables rapid screening of high level expression transformants.

1.5.2 Indirect Methods of Gene Transfer

1.5.2.1 *Agrobacterium* gene transfer systems

Agrobacterium-mediated transformation is the most effective approach to transforming dicot plants.. For the purposes of this technique two different species, *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, may be considered as natural genetic engineers. As a soil pathogen, this bacterium causes crown gall tumours in wounded plants. Gall-

inducing strains contain a single copy of a plasmid known as the transfer or Ti plasmid, that includes a segment (T DNA) that is stably incorporated into the plant genome. Vectors for plant transformation may be produced by replacing the T-DNA with the DNA to be introduced into the plant. It should be noted that the range of species that can be transformed using *Agrobacterium* is restricted but species previously considered outside the range of this technique have progressively been successfully transformed (Henry, 1997).

1.5.2.2 Gene cloning using plant viruses

There are two kinds of DNA viruses known to infect higher plants namely the caulimoviruses and geminiviruses. Cauliflower Mosaic Virus (CaMV) is a typical example which has attracted considerable interest as a cloning vector as it has been completely characterized by genetic sequencing (Brown, 1990). The CaMV has been found to have a single intergenic region in which additional DNA may be inserted without destroying the effective capability of the virus. Owing to the nature of caulimoviruses which allows them to be spread through a plant from a single infection point, these viruses are used to transform plants without the need to regenerate them from cell cultures (Brown, 1990).

Geminiviruses are important agents for cloning in higher plants as their host range includes plants of commercial importance such as maize and wheat. Geminiviruses have small genomes of up to 6 kb in size typically comprising two DNA molecules (Brown, 1990). These viruses have one major drawback as potential cloning vectors in higher plants. It has been noted that during the natural infection cycle, geminiviral genomes undergo rearrangements and deletions which would scramble up any additional DNA that had been inserted. Future research is therefore aimed at finding ways to stabilize these viruses for their manipulation in transgenic technology.

Successful expression of foreign genes in plants requires the preparation of a suitable gene construct for introduction into the plant. In addition to the sequences encoding the gene product, appropriate promoter and termination sequences must be added to the construct. Care must be taken when designing vectors to ensure that the coding region is in frame

and that translation initiation and termination codons are included. If necessary, introns may be added to enhance gene expression (Henry, 1997). Transformation of crop plants with a specific gene controlled by a highly active constitutive promoter has been the general approach for obtaining transgenic plants with disease resistance against particular pathogens (Huang *et al.*,1997). The availability of suitable promoters may also restrict some applications. The table below lists some of the more common promoters used in transgenic cereal technology.

Many of the direct DNA transfer methods are highly genotype-specific because of the need for tissue culture (Henry,1997). Gene expression may not be stable in some systems because of gene silencing, especially with homologous genes (Matzke and Matzke, 1995). The high yields of production of foreign proteins in transgenic plants by efficient gene expression remains one of the most important aspects in plant molecular biology. The 35S Cauliflower Mosaic Virus (CaMV) is widely used in plant genetic engineering and is considered to be one of the strongest promoters now available (de la Riva and Adang, 1996).

Table 1.6: Summary of constitutive promoters that have been used for transgenic cereals

Promoter	Source	Relative Activity in Cereals	Use in Transgenic Cereals
35S	Cauliflower Mosaic Virus 35S RNA transcript	low	Rice Maize
35S- <i>Adh1</i> intron 1	Cauliflower Mosaic Virus 35S promoter and first intron of maize alcohol dehydrogenate 1 gene	Low	Maize Oats Wheat
Emu	Modified maize alcohol dehydrogenate 1 promoter and first intron	Moderate	Sugarcane Rice
<i>Act1-Act1</i> intron 1	Rice actin 1 gene	Moderate	Rice
<i>Ubi1-Ubi1</i> intron 1	Maize ubiquitin 1 gene	High	Wheat Barley Rice

from McElroy and Brettell, 1995

1.6 Objectives of This Research Study

In light of the ongoing interest in expression of antimicrobial peptides in plants, this project was undertaken to ascertain the suitability of two antimicrobial peptides ESF1-GR7 and pleurocidin, for use in transgenic plants. For these studies to be successful, it is essential that the toxicity of the peptide be negligible to the plant and that the peptide retain its bactericidal activity in the targeted locale.

Two aspects of importance to transgenic studies were examined. Firstly, the peptides were examined for their *in vitro* stability in plant systems. This was important as one of the major prerequisites of transgenic expression systems is that the introduced peptide be stable in the targeted locale (Mills *et al.*, 1994). ESF1-GR7 was selected for its potent antifungal activity against most plant fungal pathogens. Pleurocidin was chosen for its broad spectrum antibacterial activity against both gram positive and gram negative bacteria. In particular, pleurocidin was selected for its activity against *Xanthomonas* bacteria which are responsible for a number of commercially important diseases in sugarcane including gumming and leaf scald (Agrios, 1988).

The second aspect that was investigated was linked to the efficiency of the expression and secretion of pleurocidin in transgenic sugarcane. Expression systems play a pivotal role in transgenic studies. There are several different expression systems that have been widely used in transgenic plant studies. Table 1.6 lists some of the promoters that have previously been successfully utilized. This study assessed the presence and localization of pleurocidin in the cells of a transgenic sugarcane hybrid. The mature pleurocidin has a signal sequence attached to the coding sequence, for extracellular localisation. This study assessed the efficiency of this system.

CHAPTER TWO

PROTEOLYTIC DEGRADATION OF ANTIMICROBIAL PEPTIDES BY PLANT INTERCELLULAR FLUID

ABSTRACT

Two antimicrobial peptides that have potential use in transgenic plants for protection against bacterial and fungal diseases were studied to determine their stability in the presence of plant intercellular fluid extracts. ESF1-GR7, a synthetic peptide of magainin origin, and pleurocidin, a peptide isolated from the skin mucosa layer of the *Pleurocentes americanus*, were treated with intercellular fluid (ICF) extracts from sugarcane, tomato and tobacco leaves. Biototoxicity against *Carnobacterium mobile* DMSO, *Escherichia coli* W.T and *Xanthomonas campestris* was reduced after five minutes incubation with intercellular fluid from the plant extracts. Reverse-phase High Pressure Liquid Chromatography analysis was used to assess the effect of the ICF on peptide elution profiles. HPLC elution profiles generated indicated the emergence of new peaks which may be attributed to breakdown products. Proteinase inhibitors were used to determine the identity as possible serine proteases. It was concluded that it the current form of the proteases makes them vulnerable to proteolytic degradation.

2.1 INTRODUCTION

The incorporation of antibacterial and antifungal peptides into plants as a means of crop protection is becoming increasingly interesting to plant biotechnologists. In order for this technology to be successful it is important to first establish two crucial factors. Firstly, it is essential that the toxicity of the peptide to the host plant be negligible and secondly, the peptide should retain its activity in the targeted locale. It has been suggested that the limited success achieved in controlling plant pathogens by introducing genes encoding various peptides may in fact reflect a failure to meet one or both of these requirements (Mills *et al.*, 1994).

Proteolysis in plants involves not only the important recycling system of amino acids but also the final step of a complex cascade of regulatory events. Proteolysis therefore plays a role in physiological processes such as germination, senescence and environmental stress. Of major interest to this research work, is the potential role of proteolysis in the breakdown of foreign protein products expressed in transgenic plants.

Antimicrobial peptides have at least three advantages over other antimicrobial agents as potential factors for use in production of transgenic crops. Firstly, because of the nature of the mode of action (more detergent like than receptor mediated interaction) of most antimicrobial peptides, it is difficult for pathogenic bacteria to develop resistance to these peptides. Furthermore, the small sizes of these peptides are very convenient for new design, synthesis, testing and manipulation at both peptide and DNA coding levels. Most antimicrobial peptides are distinguished by their small size, which makes it relatively simple to synthesize synthetic analogues with improved or predetermined activity. Finally, they appear to be relatively non-toxic to genetically engineered plants expressing these peptides because a higher lethal concentration is generally required to kill plant cells than that required for bacterial or fungal cells (Mills *et al.*, 1994). Antimicrobial peptides also have the added advantage of there being no apparent deleterious environmental effect once they are produced

transgenically.

A number of peptides have been assessed for their potential for use in transgenic expression in plants (Jaynes *et al.*, 1993, Mitra and Zhang, 1994). Cecropins have been widely tested owing to their status as one of the most well characterized of the cationic antimicrobial peptides (Jaynes *et al.*, 1993). This is because not only do they possess wide antibacterial spectra against both gram negative and gram positive bacteria (Natori, 1994), but also their convenient small size requires small DNA coding sequences suitable for synthesis and further manipulation. However, the involvement of the both the C- and N-terminal portions in antimicrobial activity suggests that they may lose their potency, once truncated or covalently modified during post-translational modification in plants (Mitra and Zhang, 1994). This was demonstrated by Hightower *et al.*, (1994) who attempted to generate transgenic tobacco plant containing the cecropin gene. A synthetic cecropin gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter was introduced into tobacco using *Agrobacterium* mediated gene transfer. Tobacco plants with high levels of cecropin mRNA were generated, but the corresponding cecropin protein was barely detectable.

The overall aim of the research would be to produce transgenic plants which would constitutively express antimicrobial peptides. Ideally, the peptide would be secreted into the intercellular space, the point of contact between the invading pathogen and the plant cell. This study analyses the stability of ESF1-GR7 and pleurocidin in the presence of intercellular fluid (ICF) extracted from tomato, tobacco and sugarcane leaves.

2.2 MATERIALS AND METHODS

2.2.1 Culture Conditions

The bacterial strains used as indicator organisms in this study are listed in Table 2.1 together with their culture condition.

Table 2.1: List of indicator organisms and culture conditions used

Peptide	Indicator Organism	Culture Conditions	Reference
Pleurocidin	<i>E. coli</i> B Wild type	Grown in Luria-Bertani (LB) medium with vigorous agitation at 37°C	Sambrook <i>et al.</i> , 1989
	<i>Xanthomonas campestris</i>	Grown in Nutrient Broth at 37°C over three days	
ESF1-GR7	<i>Carnobacterium mobile</i>	Grown in Brain heart infusion medium at 30°C	Dykes <i>et al.</i> , 1998

2.2.2 Leaf Intercellular Fluid Extraction

Leaf apoplastic fluid (ICF) was extracted from detached tobacco, tomato and sugercane leaves following the procedure of De Wit and Spikman(1982). An average of 0.3g of leaf material were first soaked in distilled water for 15 minutes to remove surface debris before being surface sterilized in 70% ethanol. Leaves were then vacuum infiltrated under sterile deionised millipore water for one hour in the case of tobacco leaves and overnight for sugarcane leaves. Whole leaves were then centrifuged (3000 x g) for 30 minutes. The expressed intercellular fluid was collected and filter-sterilized using a 0.22µm pore low-protein binding Cameo filter and used immediately or stored at -20°C until ready for use. The protein content of the ICF was determined by the method of Bradford (1978) using bovine

serum albumin as a standard. Low protein concentrations were anticipated so the standard was used at very low concentrations.

2.2.3 Treatment and Bioassay of Peptides

2.2.3.1 ESF1-GR7

ESF1-GR7 (1 μ g/ μ l) was mixed with untreated tobacco and tomato ICF or alternatively ICF that had been incubated on ice for one hour with proteinase inhibitors. Two proteinase inhibitors were used: i) 0.1M Phenylmethylsulfonyl fluoride (PMSF) obtained from Sigma was used to inhibit any serine proteases present in ICF; and ii) Iodoacetamide (IAA) was used to detect the presence of cysteine proteases. The ESF1-GR7-ICF mixtures were incubated for various times (0, 5 to 60 minutes) at 37°C. Protease activity was arrested by freezing the samples at -70°C. The samples were then stored at -20°C until ready for analysis.

2.2.4 Analysis for Peptide Degradation

2.2.4.1 Retention of antimicrobial activity bioassay

Overnight cultures of the indicator organisms cultured as described in Table 2.1, were grown to an optical density of 1 measurement at 600nm. The 0.7% agar overlay (10ml) was seeded with 100 μ l of the bacterial culture and overlaid onto bottom agar. Samples (10 μ l) in duplicate, were spotted onto the overlays and incubated for 24 hours at the appropriate temperature for each indicator organism. Inhibition zones appearing on the lawn of bacteria were observed.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibits the growth of the indicator organism. Liquid MIC were determined by growing the bacteria up to an optical density (O.D) of between 0.5 and 1 at an absorbance of 600nm. The

following formula was used to calculate the inoculum to be added to each well in the microtitre plate. $X = OD/10$.

X μ l of inoculum were made up to a volume of 1ml in broth and a 100 μ l aliquot was added to each well of the microtitre plate. 100 μ l aliquots of each sample at the various test times was added to the first well and a doubling dilution series was performed. The microtitre plates were incubated at the appropriate temperatures for each test organism overnight. Growth of the test organisms was observed and the MIC calculated.

2.2.4.2 Gel electrophoresis analysis

Tricine -SDS Page using the method of Schagger and von Jagow (1987), was used to analyse degradation of the peptides after treatment with plant ICF. The gel was constituted by separating (10% T, 3% C), spacer (10%T, 3% C) and stacking layers (4%T, 3%C). A low range protein molecular weight marker (Promega) was used to determine the size of the peptide and resultant fragments. Electrophoresis was performed at 30V for 2.5 hours after which the gel was fixed in 10% formaldehyde for one hour. The gel was washed for a further one hour in five changes of distilled water before being stained overnight in Coomassie Brilliant Blue R-250 (Sigma). The gel was then left in destain solution (7% acetic acid, 5% methanol) overnight.

2.2.4.3 Reverse-phase high pressure liquid chromatography (RP-HPLC)

Reverse-phase HPLC was performed using acetonitrile with 0.1% trifluoroacetic acid (TFA) as the solvent and water containing 0.1% TFA using a Biorad C₁₈ column (4.6-250 mm). Eluents corresponding to chromatographs generated from the breakdown products were manually collected and the solvent was removed by freeze drying under vacuum overnight. The dehydrated peaks were stored in a desiccator or resuspended in a small volume of water

containing 0.1% TFA for further analysis.

2.3 RESULTS AND DISCUSSION

Prediction of potential *in vivo* performance of an antimicrobial peptide to be expressed in any plant, although difficult, is beneficial for the selection of an appropriate peptide candidate. This information should be used in conjunction with a sound understanding of the properties and mode of action of the peptide to its suitability to transgenic expression. The study undertaken, sought to investigate the nature of the interaction between ESF1-GR7 and pleurocidin when exposed to intercellular fluid (ICF) extracted from various plants.

2.3.1 Proteolytic Degradation of Antimicrobial Peptides

2.3.1.1 Retention of antimicrobial activity assay

The effect of incubating the peptide of interest with the extracted ICF from the plants of interest was investigated using several methods. The initial approach was to assess the effect of the plant extract on activity of the peptide. For this, the spot-on-lawn assay was employed. It was found that both peptides showed reduced activity against their target bacteria (Figure 2.1). This was observed as a reduction in the size of the inhibition zones over time until no activity was observed at all. At this point, it was not possible to ascertain whether the reduction in activity was caused as a result of the action of proteases or inhibitors present in the plant ICF extract. Further analysis using the milk agar assay showed that the plant ICF extract contained proteases that may have been responsible for the loss of activity observed. The ICF extract was able to degrade 0.2% milk agar overnight (Figure 2.2).

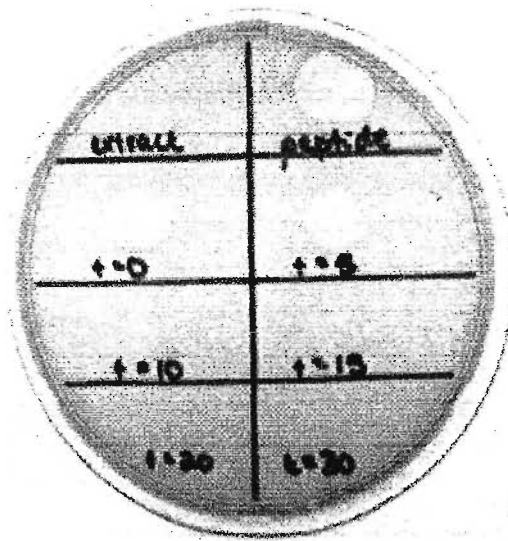


Figure 2.1: ESF1-GR7 (40 μ g) was incubated with an equal volume of intercellular fluid (ICF) vacuum infiltrated from tomato leaves for times ranging from 0 to 30 minutes. 10 μ l from each mixture were spotted onto 0.7% agar overlays inoculated with *Carnobacterium mobile*. The plates were incubated at 30°C overnight, and zones of inhibition were observed. Control ICF extract and untreated peptide were included on the plate.

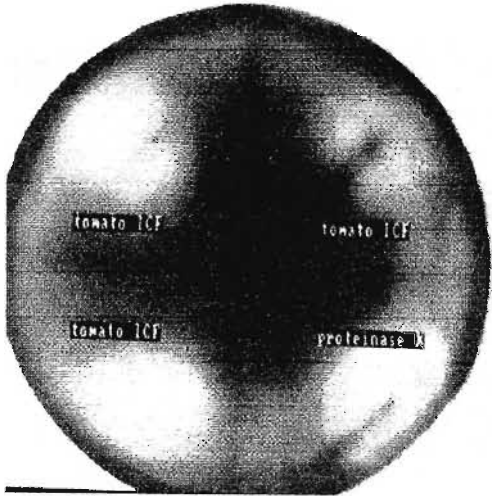


Figure 2.2: Aliquots (20 μ l) of ICF extract from tomato plants were placed on 1% Milk Agar Plates and incubated overnight at 37°C. Proteolytic degradation of milk was observed as zones of clearing. A 20 μ l aliquot of Proteinase K (20mg/ml) were also placed on the milk agar as a control.

2.3.1.2 Gel electrophoresis of breakdown products

Following incubation of the peptides with ICF extracts from various plants, it was observed that the polypeptide band corresponding to either peptide (Pleurocidin or ESF1-GR7), gradually disappeared over time resulting in the band becoming fainter until it disappeared completely (Figure 2.3). The breakdown products that resulted from the ICF activity were not detected by gel electrophoresis. This is understandable given that the peptides are small in size and difficult to resolve on tricine gels with pleurocidin being 2.7 kDa and ESF1-GR7 which is only 2.3 kDa in size.

The results observed by the study are similar to those reported using other antimicrobial peptides. Mills *et al.*, (1994) attempted to simulate *in vitro* the intercellular environment of a transgenic peach leaf to determine its effect on cecropin bioactivity. Further work done on the expression of cecropin B and its analogs in plants also revealed differences in the phenotypes of transgenic plants (Cavallarin *et al.*, 1998). It was observed that whereas transgenic tobacco plants producing a cecropin B analog (Shiva-1), were resistant to *Pseudomonas solanacearum*, no enhanced resistance was observed in plants producing another cecropin B analog, SB37 (Jaynes *et al.*, 1993). Tobacco plants engineered to secrete cecropin B into the intercellular spaces of leaves showed no enhanced resistance to target bacterial diseases (Florack *et al.*, 1995). The failure was attributed to proteolytic degradation of the peptide by proteases present within the intercellular spaces of the host plant. Owens and Huette (1997) were able to attribute this degradation to endopeptidase cleavage. Altogether these results suggested that cecropin sequences containing appropriate structural determinants might be applicable for plant transformation, provided that the plant does not degrade the peptide (Cavallarin *et al.*, 1998). In cases where the cecropin peptide is unstable due to the presence of peptide-degrading protease activities, the peptides might still be applicable by using substitution analogs in which protease-sensitive sites have been removed.

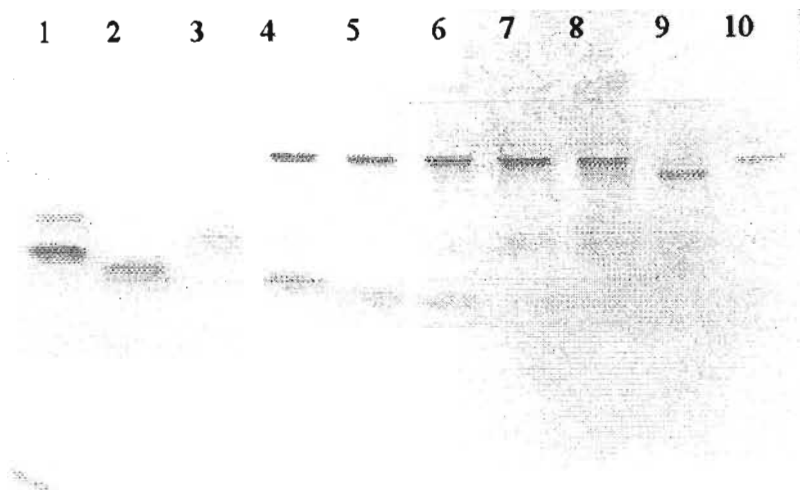


Figure 2.3: ESF1-GR7 (40 μ g) was incubated with an equal volume of tomato intercellular fluid for times ranging from 0 to 60 minutes with each reaction being separated on a tricine-SDS PAGE gel stained with Coomassie Blue R250 (Sigma) showing the peptide band disappearing over time, as follows: Lane 1, Low Range molecular weight marker (Promega); Lane 2, Control Peptide ESF1-GR7; Lane 3, Proteinase K digested peptide after 30 minutes; Lanes 4-9, ESF1-GR7 incubated with tomato ICF at time 0, 5, 10, 15, 30 and 60 minutes respectively; and Lane 10, tomato ICF extract only

2.3.1.3 Reverse-phase HPLC analysis

From the reverse phase HPLC analysis it was observed that the initial degradation of the peptide by ICF produced one major peak, which was followed by the appearance of a second peak approximately 30 minutes later. This result suggests that degradation of the peptide is specific. Endopeptidase activity appears to result in an initial break down product that is eventually broken down over time. The proteolytic degradation process produces two major products as observed on the resultant HPLC profiles. The profiles also depict the gradual reduction in the initial peptide peak as the two breakdown product peaks increase in size. From this result, it may be assumed that peptide degradation is rate limited by the initial breakdown process, and that

should this point be identified, alteration of the specific point would therefore render the peptide stable against proteolytic cleavage. Further knowledge on the amino acid sequence of this site may enable the design of a less labile molecule. Of interest, was the fact that this result was consistent in spite of the plant extract used. This further suggests that the assumption made that the peptide was being degraded in a specific manner by a particular enzyme may have been accurate.

The time taken for the peptides to be fully degraded can be used to make a few assumptions about the behaviour of the peptide once introduced into a host plant. Although degradation was observed instantaneously, it is important to note that once a peptide is constitutively expressed within host plant conditions it would be continuously produced. This means that although the peptide was eventually fully degraded under *in vitro* conditions, it is likely to be more stable *in vitro* owing to the constant supply of the overexpressed peptide. Table 2.2 below demonstrates the reduction in bioactivity of ESF1-GR7 over time following incubation with tobacco ICF.

Table 2.2: Determination of reduction in bioactivity of ESF1-GR7 following incubation with tobacco intercellular fluid (ICF)

Time of Incubation with Tobacco ICF	Minimum Inhibitory Concentration
Control Peptide(60 minutes)	512 000
t=0	25 600
t=5	3 200
t=10	3 200
t=15	400
t=20	-
t=30	-

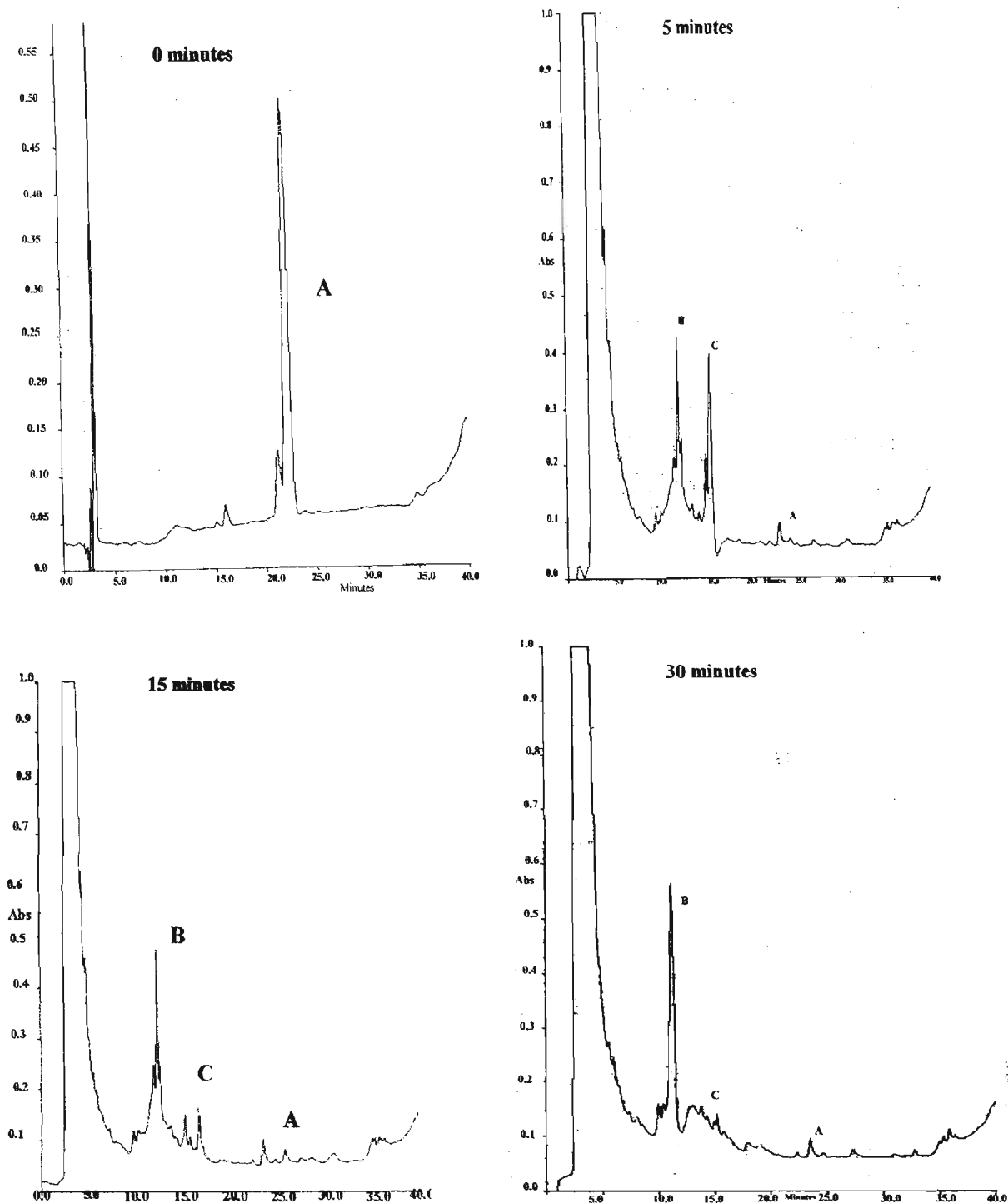


Figure 2.4: Samples of ESF1-GR7 exposed to intercellular fluid (ICF) extract from tomato leaves were subjected to reverse-phase HPLC analysis using an acetonitrile/TFA gradient. Chromatograms of the eluents show the gradual disappearance of the peptide peak (A) over a period of 30 minutes, while two new peaks were observed. Peaks B and C were observed following five minutes incubation with ICF. Peak C was further degraded after 30 minutes incubation with ICF. Abs= absorbance at 220nm

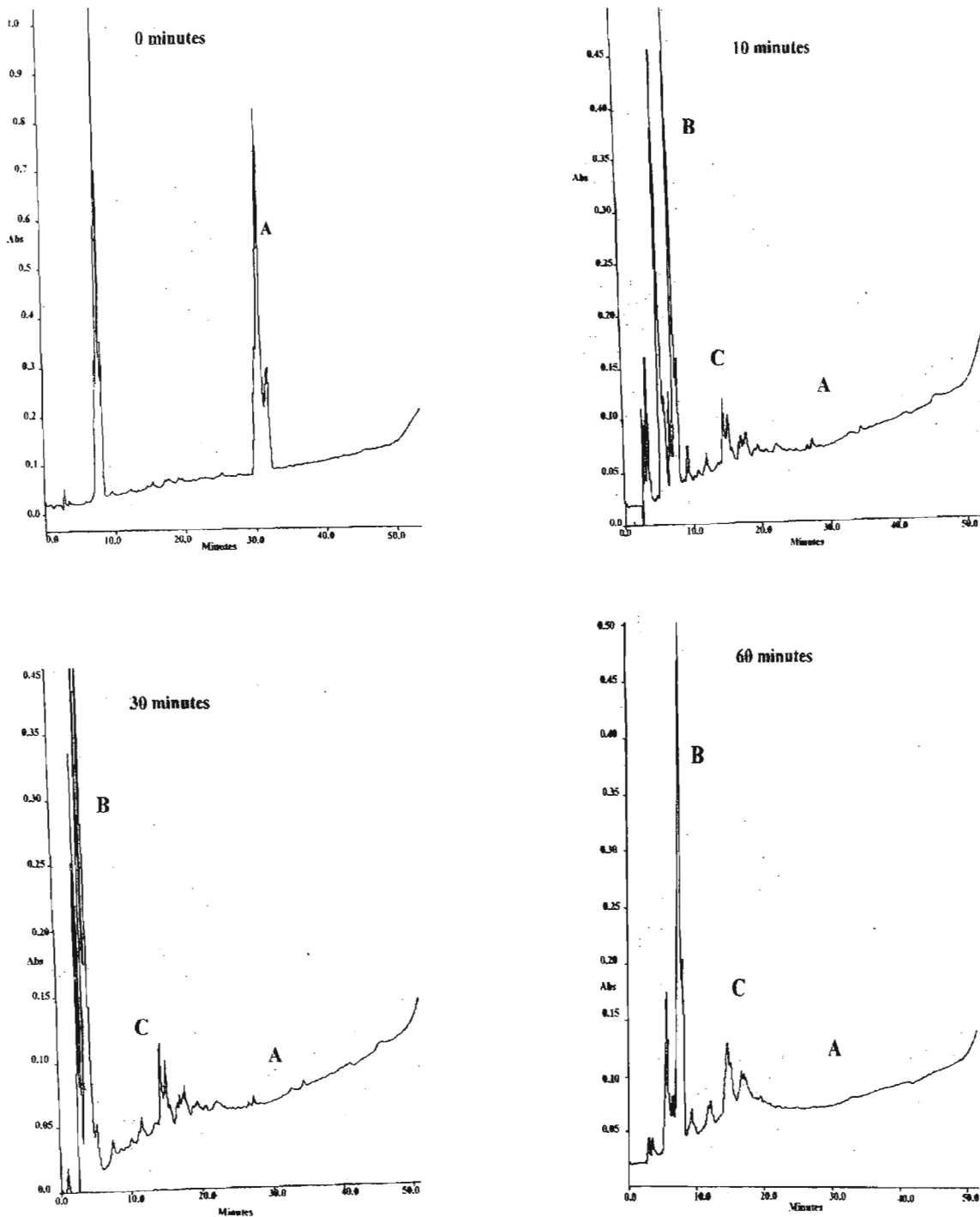


Figure 2.5: Pleurocidin was incubated together with sugarcane intercellular fluid at 37°C for one hour. 50µl aliquots were removed after 0, 10, 30 and 60 minutes, and analysed by reverse-phase HPLC. The initial peptide peak was reduced in size within the first 10 minutes. Prolonged incubation of the mixture resulted in the appearance of new chromatogram peaks which may correspond to breakdown products. Abs= Absorbance at 220 nm

2.3.1.4 Protease identification

The study also investigated the nature of the proteases responsible for the proteolytic cleavage of the peptides. This was done in an attempt to predict potential endopeptidase cleavage sites. Serine proteases, as exemplified by papain and ficin, are the common proteases found in plants (Benyon, 1989). Using this information, PMSF was selected as it is known to inhibit the activity of serine proteases by blocking their active sites. It was observed that ICF previously incubated with PMSF was unable to degrade either peptide. Incubation with Iodoacetic acid, a cysteine protease inhibitor, had no effect on the activity of the ICF extract thus providing further evidence suggesting nature of the endopeptidases involved.

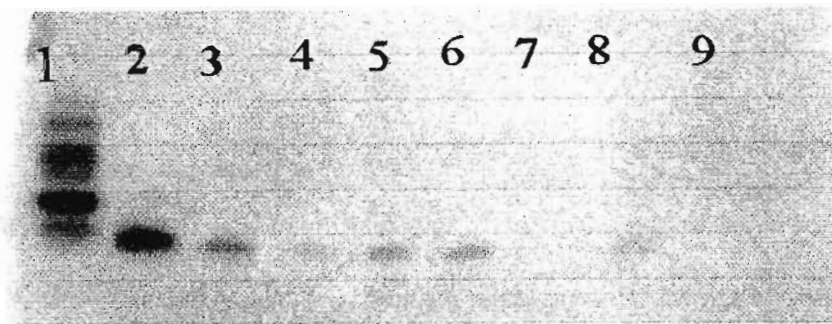


Figure 2.6: Intercellular fluid (ICF) from sugarcane leaves was treated with protease inhibitors on ice for 60 minutes prior to incubation with pleurocidin at 37°C for 60 minutes. The samples were separated by electrophoresis using the Tricine SDS-PAGE system to determine the presence of the pleurocidin, as follows: Lane 1, Promega Low Range Protein Molecular Weight Marker; Lanes 2-9, 10µl reaction volume aliquots of different treatments were taken at times 0 and 60 minutes and separated, as follows: Lane 2, 5µg untreated pleurocidin ; Lane 3, peptide with untreated ICF at time 0; Lane 4, Peptide incubated with ICF treated with a cocktail of protease inhibitors at time 0; Lane 5, Peptide incubated with ICF treated with PMSF at time 0; Lane 7, Peptide and cocktail protease inhibitor treated ICF after 60 minutes; Lane 8, Peptide and PMSF treated ICF after 60 minutes; Lane 9, Peptide and untreated ICF after 60 minutes.

CHAPTER THREE

Localization of Pleurocidin in Transgenic Sugarcane

ABSTRACT

One of the challenges of transgenic technology lies in ensuring that the transgene is properly expressed in the targeted locale and that it possesses the desired *in situ* activity. Immunogold labelling and transmission electron microscopy were used to investigate the transgenic expression of Pleurocidin, an antimicrobial peptide, from the fish, *Pleurocentes americanus*, in a sugarcane hybrid plant. Pleurocidin was extracted from crude homogenate of plant material as well as from the intercellular spaces of sugarcane leaves. Various methods were used to assess the expression of pleurocidin in transgenic sugarcane plants. Western blots, analysed using the alkaline-phosphatase and enhanced chemiluminescence detection systems, were used to confirm the presence of pleurocidin in transgenic plants. Transmission Electron Microscopy (TEM) was utilized to view the peptide following immunogold labelling. This preliminary investigation on four month old plants found that the pleurocidin was located predominantly in the cell wall and intercellular space. Trace amounts of pleurocidin were detected in other organelles such as the nucleus, chloroplasts and cytoplasm.

3.1 INTRODUCTION

Genes that are introduced into plants must be expressed in a stable and active form. The presence of the transgene in the as well as the final product in the targeted locale should be confirmed.

Several successful attempts have been made to produce transgenic plants expressing various antimicrobial peptides as a means of enhancing plant protection. Florack *et al.*, (1995), made several cecropin B gene constructs either for cytosol expression or for secretion and found that a gene construct lacking the N-terminal signal peptide was poorly expressed at the mRNA level, whereas the construct containing the insect signal peptide showed increased cecropin B mRNA levels in transgenic tobacco plants. However, cecropin B was not detected in any of the transgenic plants. Transgenic tobacco plants expressing the cecropia derivative shiva-1 have also been developed by Jaynes *et al.*, (1993). These plants exhibited an average delay of two weeks for symptom development when challenged by *Pseudomonas solanacearum*. Hightower *et al.*, (1994), demonstrated that transgenic tobacco containing a cecropia peptide was not resistant to *P. syringae tabaci in vivo*, irrespective of its potent *in vitro* antibacterial effect. Abnormal phenotypes have also been reported in some instances. Okamoto *et al.*, (1998), reported the appearance of some deformed tobacco leaves when sarcotoxin IA was introduced without an external secretion sequence attached. The problem was averted by the fusion of the peptide gene to the pathogenesis related (PR) protein gene to enable extracellular expression of the peptide. This system is useful in that it facilitates inducible expression of the transgene by ensuring that the introduced peptide becomes a part of the plant's innate defence system. The introduced peptide therefore acts in a synergistic fashion together with the pathogenesis related proteins released by the plant in response to infection.

Bacterial diseases of sugarcane are responsible for major crop losses in the sugarcane industry. These diseases are particularly important in tropical and subtropical environments

For the successful expression of antimicrobial peptides in transgenic plants, the peptides must be stable in the intercellular spaces where they are targeted for expression. This experiment assessed the suitability of Pleurocidin and ESF1-GR7 for transgenic expression. It was interesting to note that proteolysis of the peptides appeared to proceed via an initial endopeptidase cleavage that generated two major fragments. In the case of pleurocidin, the breakdown products themselves did not appear to be very stable. This would indicate that perhaps, a mixture of proteases is present in the sugarcane ICF. The initial degradation of the peptide results in the formation of breakdown products that can be further degraded by other proteases present in the ICF. Susceptibility to proteolysis is greatly influenced by the amino acid sequence of the protein (Owens and Huette, 1997). It is therefore likely that the initial endopeptidase cleavage results in new fragments that contain recognition sequences for other proteases.

Proteases are naturally produced by plants in response to a number of physiological processes. One such process is environmental stress that may result from damage to leaf tissue. In the case of the current study, while every effort was made to use whole leaves, the process of removing the leaves from the plant results in induced stress which may lead to the production of proteases not normally present in the leaf. These proteases may also have been responsible for the observed proteolytic degradation.

From the various data presented it was concluded that while stability of the peptides in transgenic plants was questionable. Further analysis needs to be done on identifying cleavage points for endopeptidase activity in order to protect the peptides from endopeptidase cleavage. It has been observed that relatively small changes in amino acid sequence can greatly affect the rate of degradation of antimicrobial peptides by ICF (Owens and Huette, 1997). Similar studies carried out on cecropin B suggested that a single amino acid substitution in the cecropin B peptide resulted in enhanced stability in the presence of leaf ICF (Owens and Heutte, 1997). This suggests that analogs of ESF1-GR7 and pleurocidin, both small peptides, may be rapidly synthesised and tested for efficacy, before being incorporated into gene constructs.

such as those experienced in South Africa, where warm, humid conditions are ideal for bacterial growth. *Xanthomonas campestris* pathovars cause leaf spots which are localized lesions, each developing from a separate infection event. *X.campestris* enters hydathodes to gain access to the vascular system. This results in the blockage of the vascular system and subsequent wilting of the sugarcane.

In a previous study conducted at the South Africa Sugar Experiment Station, a *Saccharum* species hybrid plant was made transgenic by introducing the gene taken from the fish *Pleurocentes americanus* which encodes the production of a 2.7 kDa peptide known as pleurocidin. The 317 nucleotide gene for pleurocidin was cloned into the BamHI and EcoRI sites of a specially constructed plasmid pUbi 510 (Figure 3.1). Both the 22 amino acid signal sequence and the propeptide sequence comprising 20 amino acid residues were included. Once expressed in the plant, the signal and propeptide sequences should be cleaved to release the mature peptide. Three basic procedures were used to generate transgenic sugarcane plants expressing the pleurocidin gene. Following the preparation of the gene construct, the DNA was precipitated onto tungsten or gold particles, by the method described Klein *et al.*, (1988). Microparticle bombardment, using a particle inflow gun, was used to transform embryogenic (type III) sugarcane callus according to the method described by Snyman *et al.*, (1996). Pleurocidin is a cationic peptide that has been demonstrated to have potent broad spectrum antibacterial activity against a range of both gram positive and negative bacteria that affect sugarcane. The relatively small size, 25 amino acids, that allows ease of manipulation and the available knowledge of its gene structure made this peptide an ideal candidate for transgenic expression in sugarcane.

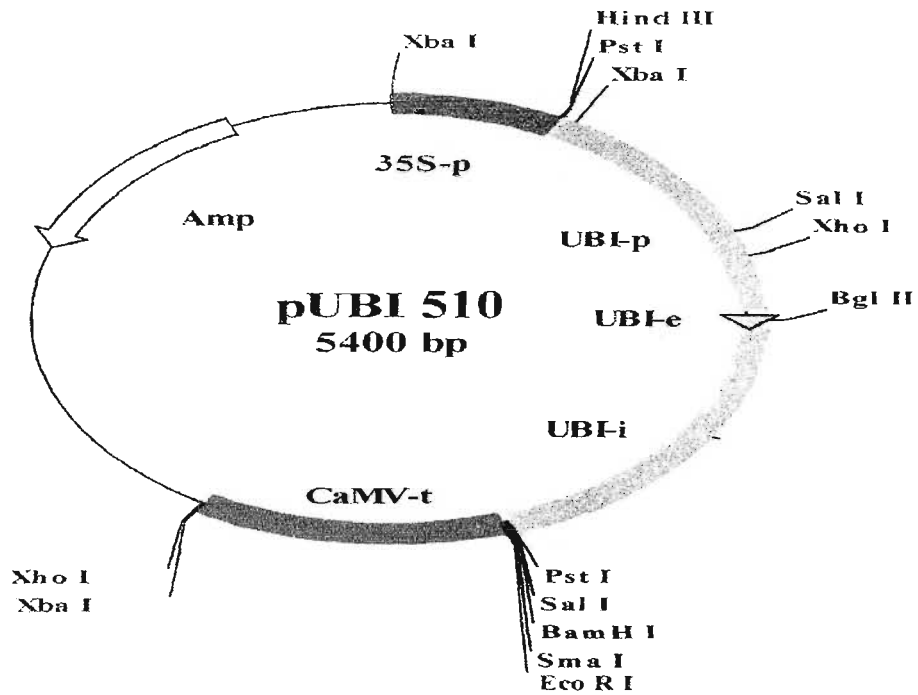


Figure 3.1: Genetic map of pUBI 510 showing restriction sites Bam HI and EcoRI into which the 317 nucleotide gene for pleurocidin was cloned. The 35s-Ubi hybrid promoter consisting of the CaMV and maize ubiquitin systems was used because of its high efficiency. A signal sequence attached ensured extracellular localisation of the peptide.

3.2 MATERIALS AND METHODS

3.2.1 Protein Extraction

Crude protein extract from the transgenic and control plants was obtained by homogenising leaves of 4 month old plants using Protein Extraction Buffer (100mM Tris-HCl pH 7.3, 5mM EDTA, 0.05% β - mercaptoethanol) according to the method of Siebler *et al.*, (2000). The extract was clarified by centrifugation. The protein extract was precipitated by incubating with 20% trichloroacetic acid on ice for 30 minutes followed by centrifugation at maximum force at 4°C and subsequent removal of the supernatant. The protein extract was stored at -20°C until further analysis.

Protein expressed in the intercellular spaces was extracted using the vacuum infiltration method of De Wit and Spikman (1982). Leaves were placed in protein extraction buffer and a vacuum was applied for two hours. Release of the vacuum facilitated the infiltration of the leaf material by the protein extraction buffer. Intercellular fluid was recovered by centrifugation of the leaves at 3000 x g for 15 minutes. Total protein content was quantified using the Bradford assay.

3.2.2 Tricine SDS-PAGE Analysis

Tricine SDS-PAGE gels were run according to the method of Schagger and von Jagow, 1987 to separate the proteins extracted. Three layers were prepared according to the following T:C ratios: Separating layer was 4:3 and contained glycerol; the spacer layer was 10:3; and the stacking layer was prepared to have a final T:C ratio of 4:3. Electrophoresis was carried out at a constant current of 30 AMP with unlimited voltage for two hours. Following electrophoresis, the gel was fixed in 15 % formaldehyde (Mills *et al.*, 1994), for one hour and then rinsed in several changes of distilled water before being stained for one hour in Coomassie Brilliant Blue R-250 (Sigma). Destaining was carried out overnight in a 7% acetic acid, 5 %

methanol destain solution. The Promega low range molecular marker was used as a size standard.

3.2.3 Electroblotting onto Nitrocellulose Membrane

Electroblotting was carried out at 200 amps constant current using a BIO-RAD Mini-TransBlot system for 20 minutes. The Towbin Transfer Buffer (25mM Tris, Glycine, Methanol, SDS) was utilized to allow effective transfer of the proteins onto Hybond-C nylon membrane (Amersham Life Sciences, Cleveland, Ohio). The membranes were allowed to dry overnight before detection.

3.2.4 Western Blotting

Both transgenic and control plant samples were run on the gel and the presence of the pleurocidin in transgenic sugarcane was confirmed using the Western Blotting technique. Transfer of the protein was verified by staining the membrane with Ponceau S stain. The membrane was washed in Tris Buffered Saline (TBS) containing 10mM sodium hydroxide to destain the membrane. Available sites on the membrane were blocked for one hour using 5% low fat milk in TBS followed by three five minute washes in TBS. The membrane was then incubated with the anti-pleurocidin immunoglobulin diluted to 1 in 5000 in 0.5% BSA (Sigma) in TBS for two hours followed by a brief wash to remove excess antibody. A pre-immune control using the same concentration of primary antibody was included in the experiment.

Presence of the peptide of interest was determined using the alkaline phosphatase detection system. For the alkaline phosphatase detection system, the secondary antibody, Goat anti-rabbit alkaline phosphatase conjugate (Sigma), was diluted 1 in 5000 in 0.5% BSA/TBS and incubated with the membrane for one hour rinsed in three by five washes of TBS before being detected. The colour substrate used was BCIP/NBT(Sigma) dissolved in Buffer 3 (0.1M Tris-Cl, pH 9.5; 0.1M NaCl; 50mM MgCl₂). The membrane was exposed to the substrate in the

dark for one hour before being washed in distilled water, dried and stored. The Amersham Enhanced Chemiluminescence (ECL) kit was also used as a detection system for pleurocidin on the western blot. For this system, the Horse-radish hydrogen Peroxidase (HRPO)-linked secondary antibody at a dilution of 1 in 5000 was used. Following incubation with the secondary antibody for one hour the membrane was washed and the mixture of detection solutions (mixed in equal volumes) was added to the protein face of the membrane, and incubated for precisely one minute. The excess detection buffer was then washed off and the blots were placed protein side up in the film cassettes in the dark. Hyperfilm-ECL was exposed to the blot overnight before results were interpreted.

3.2.5 Immunocytochemistry

3.2.5.1 Sample preparation

Plant leaves were cut into small pieces and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in sodium cacodylate buffer (0.1 M sodium phosphate pH 7.4) overnight at 4°C. Tissues were rinsed in the sodium cacodylate buffer for 30 minutes and then treated with 0.1M NH₄Cl for 30 minutes. After rinsing the tissues in sodium cacodylate buffer, the tissues were dehydrated in a graded series of ethanol ranging from 10 percent to 100 percent over a period of six hours, and then infiltrated with LR White (London Resin Company) at 55°C for 24 hours. Thin sections were sliced with a glass knife and placed on formval-coated 200-mesh nickel grids.

3.2.5.2 Immunogold labeling

The sections were blocked in 1% BSA in 10mM phosphate buffered saline (PBS) for 15 minutes, and then incubated with anti-pleurocidin immunoglobulin at a dilution of 1 in 300 for one hour at room temperature. Residual primary antibody was removed by several washes with PBS. The sections were then probed with the protein A-gold complex (10nm probe, Sigma)

at a dilution of 1:300, prepared by the method of Slot and Geuze (1981) for 1 hour. The tissues were then washed with PBS before being fixed on 1 percent glutaraldehyde in PBS, and finally rinsed in several changes of distilled water. The sections were stained with 2% uranyl acetate and with lead citrate for 2 minutes and examined under a Phillips JOEL Transmission Electron Microscope.

To exclude non specific binding of gold particles, incubation with the primary antibody was omitted, and the rest of the labeling procedure was performed. The second control involved the omission of the gold probe, and the final labeling control involved replacement of the primary antibody with the pre-immune serum. The experimental control was the non-transgenic control plant treated in an identical manner with the transgenic plant.

3.2.6 Electroblothing of Peptide onto Polyvinylidene Fluoride (PVDF) Membrane

Electroblothing of the peptide on to PVDF membrane was performed in order to facilitate amino acid sequencing of the peptide. The Mini Transblot Cell (Biorad) electroblotting apparatus was used. The protocol described by LeGendre and Matsudaira, 1989 was followed. Briefly, the 0.2 micron Transblot Transfer Medium (Biorad) PVDF membrane was immersed in 100% methanol for 2 to 3 seconds before being equilibrated in transfer buffer (10mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 10% methanol, pH adjusted to 11) for 30 minutes. After electrophoresis was completed, the gel was rinsed in transfer buffer before being assembled together with the membrane into the electroblotting apparatus. The transfer was carried out at 90V for 90 minutes. Once transfer was complete the membrane was washed in several changes of distilled water before being visualizing the proteins using Coomassie blue R-250 (0.1% in 50% methanol) for five minutes and destained over night in several changes of detain solution (50% methanol). Bands corresponding in size to pleurocidin were excised using a scalpel blade.

3.2.7 Amino Acid Sequencing

A Perkin Elmer Applied Biosystems Procise 491 automated protein sequencer was used to determine N-terminal amino acid sequence analysis of the band that corresponded in size to pleurocidin obtained from the crude protein extract from transgenic sugarcane leaves. These bands were blotted onto PVDF membrane prior to amino acid sequencing. The pulse field liquid PVDF peptide method (Hewick *et al.*, 1981) was used by the University of Natal Molecular Biology Unit.

3.3 RESULTS AND DISCUSSION

3.3.1 Extraction of Pleurocidin from Transgenic Sugarcane

Attempts to re-isolate pleurocidin from the crude protein in the transgenic plant produced low protein yields. Using the Bradford assay, the total cellular protein isolated from the leaf material was estimated to be 187 μ g per gram of fresh leaf material. From the Western Blot Analysis it was concluded that pleurocidin was indeed being produced by the transgenic sugarcane plant (see Figure 3.2) which depicts the Tricine SDS-PAGE gel which shows the total protein isolated from both the transgenic plant and the control plant. From the gels it is apparent that pleurocidin was being expressed in the transgenic plant only and not in the control plant. Confirmation of the peptide corresponding in size to pleurocidin was obtained using the western blotting procedure (see Figure 3.2b).

Reverse-phase HPLC analysis also detected small amounts of pleurocidin being expressed in the transgenic plants. Very little peptide was recovered from this analysis. There is therefore, need to optimize the protein isolation technique.

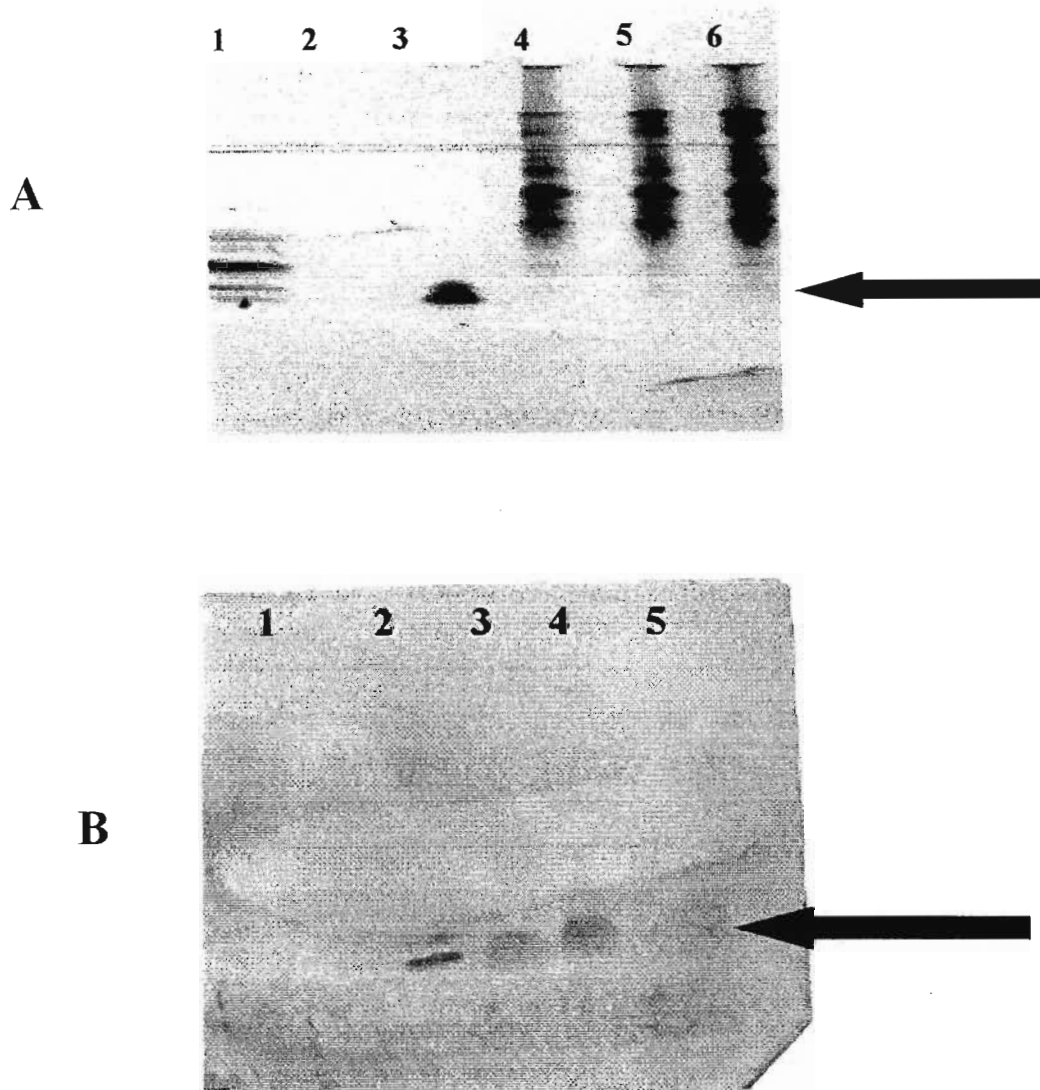


Figure 3.2: Crude protein extracts from the transgenic sugarcane leaves were separated by electrophoresis on tricine-SDS-PAGE gels. **A** : Lane 1: low range molecular weight marker (Promega); lane 3: 5 μ g control pleurocidin; lanes 4-6: total cell protein from transgenic sugarcane leaves. **B**: the nitrocellulose onto which a gel run concurrently with gel A was blotted. Lane 3: 5 μ g of pleurocidin, lanes 4 and 5, transgenic pleurocidin. This blot was developed using the alkaline-phosphatase conjugated secondary antibody.

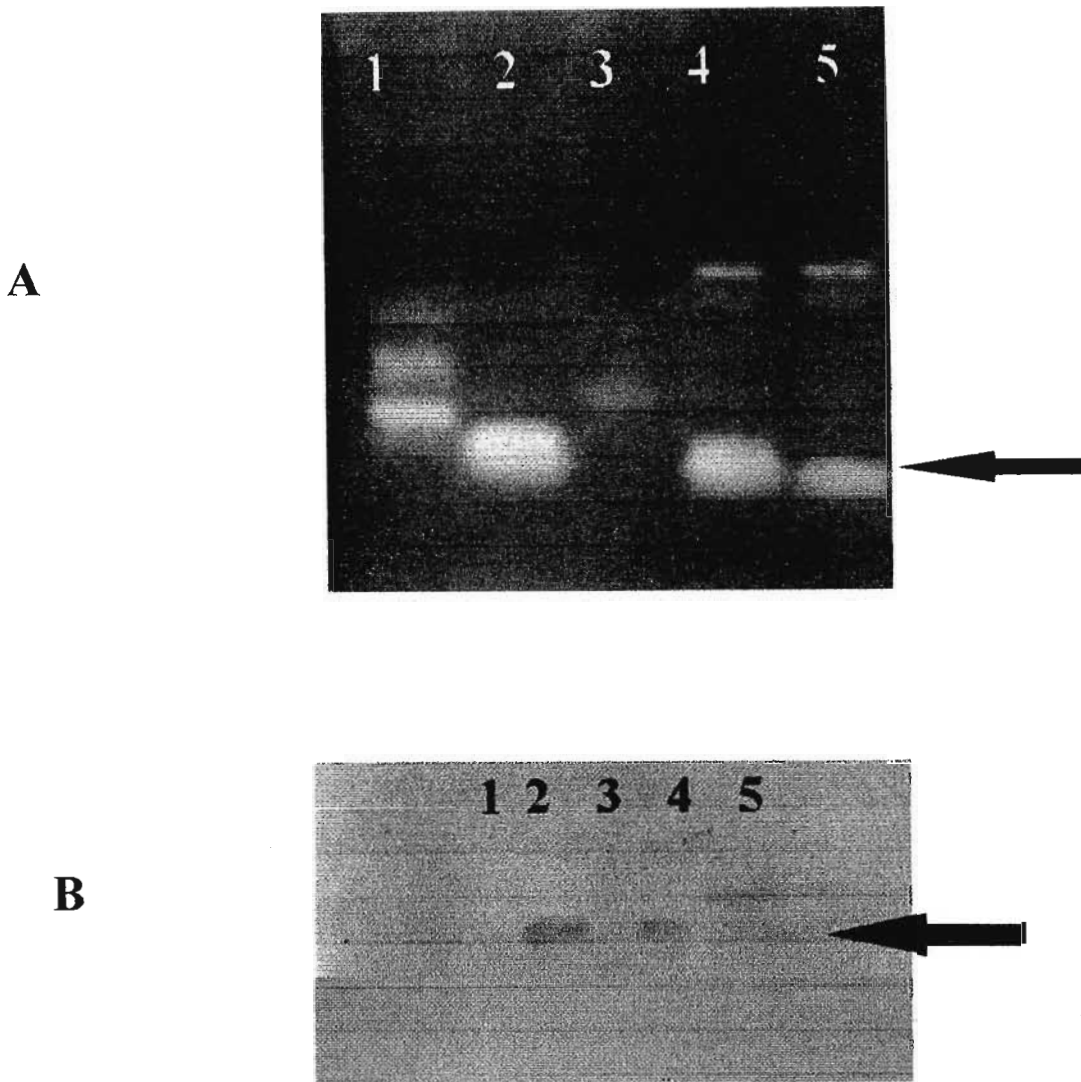


Figure 3.3: Crude protein extract from transgenic sugarcane was separated by electrophoresis on a SDS-PAGE gel. (A) Lane 1: Low range molecular weight marker (Promega); Lane 2: 50 μ g Pleurocidin (positive control); Lane 3: intercellular fluid extract from transgenic leaves; Lanes 4 and 5: crude protein extract from transgenic leaf material. Transgenic protein observed as band corresponding in size to the control pleurocidin. ECL analysis of electroblotted membrane was carried out using Horse radish peroxidase-conjugated secondary antibody to confirm identity of band as pleurocidin (B). Lane 2: Control pleurocidin; Lane 4:

Pleurocidin expression in crude and ICF extracts was compared. The ICF extract produced less peptide than the crude extract. This was demonstrated by the crude homogenate producing 1.6mg/g from a total protein content of 32.9mg/g, while the ICF extract yielded 0.27mg/g from a total protein content of 18.7mg. Reverse-phase HPLC analysis however showed that the ICF extract produced a clearer peak than the crude extract. This fact can be attributed to the peptide being expressed primarily in the intercellular spaces and there being fewer contaminating peptides present. The figures given in the table above were based on RP-HPLC analysis using pure pleurocidin of known concentration as the calibration control. The crude homogenate and ICF extract were both obtained from 1g of leaf material. In the case of the crude homogenate, it was suspended in 1 ml Protein Extraction Buffer, while the ICF extract was used undiluted. On average, approximately 0.16ml of ICF were obtained for each gram of fresh leaf material.

3.3.2 Localization of Pleurocidin in Leaf Tissue

Immunocytochemistry was employed to determine the point of expression of pleurocidin in the transgenic sugarcane hybrid species. Based on the construction of the plasmid vector into which the gene for pleurocidin was cloned, it was anticipated that the peptide would be expressed within the intercellular space. This is because the expression system used the Ubiquitin transport system. Ubiquitin is a protein found abundantly in the intercellular spaces of cells in a diverse range of organisms including mammals, yeast, and plants (Rechsteiner, 1988).

The gold probes indicating the presence of pleurocidin in the leaves were detected in the transgenic plant and not in the normal plant (see Figure 3.4). From the labeling controls (Figure 3.5) it was apparent that the labeling was very specific and as a result, the gold labels obtained were a true reflection of the distribution of pleurocidin within transgenic sugarcane. It was observed that gold probes depicting the presence of pleurocidin were concentrated in the areas around the cell wall and the intercellular space. It was noted however, that

pleurocidin was also detected within the plant cell (see Figure 3.6). Although the peptide was concentrated mostly in the cell wall, significant amounts were also detected in other parts of the plant cell including the cytoplasm, nucleus and chloroplasts. This implies that the transport system may not be efficient and as a result not all the peptide produced was being excreted out of the cell. It is also possible that the peptide detected within the plant cells was a result of diffusion of the peptide back into the cell following excretion into the intercellular space.

3.3.3 Amino Acid Analysis

Amino acid sequencing of the first five residues confirmed that the protein extracted from the transgenic sugarcane was indeed pleurocidin. The amino acid sequence obtained was NH₂-G W G S F, which corresponds with the first five amino acids in the pleurocidin sequence. This result also helped to confirm the fact the mature peptide was being correctly expressed as the signal and propeptide sequences cloned with it were not.

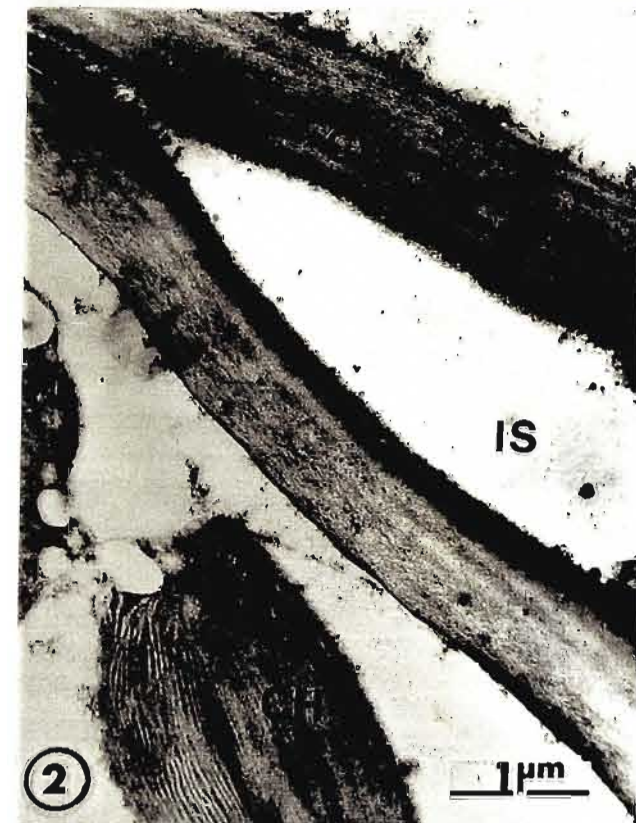
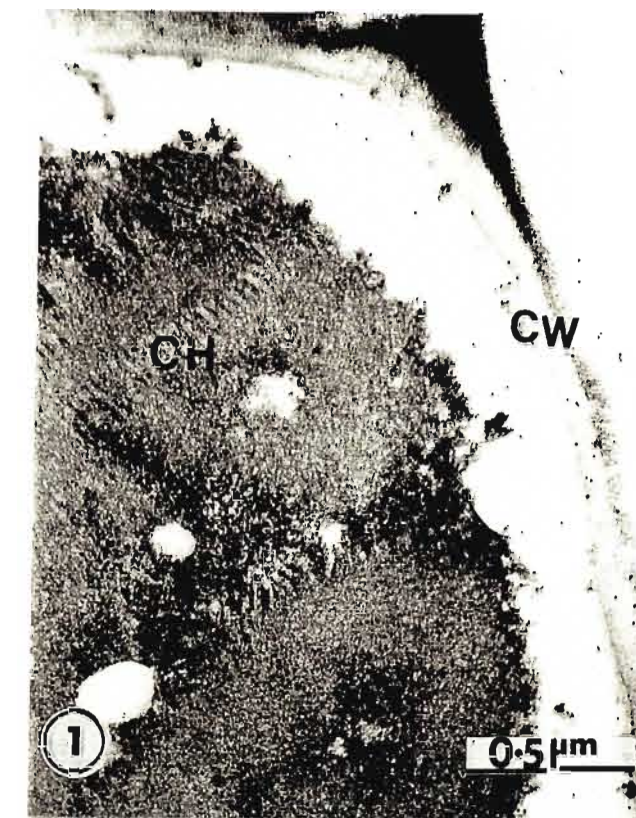
3.3.4 Fate of pleurocidin in transgenic sugarcane

The localization of pleurocidin in the intercellular spaces was observed by an immuno-electron microscopy technique. An antibody reacting to pleurocidin was used to identify the presence of the peptide in the plant leaves. As the antibody reacted only to pleurocidin and not the other proteins from the leaf tissue, the number of gold labels observed here reflect the summation of the total peptide expressed. No gold label was detected in the control sections from the normal sugarcane plants. However, a number of gold particles were also found in the cytoplasm and organelles within the plant cells. Extensive studies carried out on the secretory pathway for α -amylase have shown the secretion of the protein through the endoplasmic reticulum, Golgi apparatus and secretory vesicles before being secreted outside the cells (Hosokawa and Ohashi, 1988). The small number of gold particles found in the cytoplasm and organelles could therefore be indicative of pleurocidin being transported through the secretory

system.

The pleurocidin gene was cloned in sugarcane together with the pre-peptide and pro-peptide sequences flanking the mature peptide. The Ubiquitin expression system used in the pUBI 510 plasmid facilitated extracellular excretion of the mature peptide. During the course of this study, the reaction of the prepeptide and propeptide with the antibody were not established. These immature peptide sequences could possibly be immunoreactive to the antibody used and were as a result detected together with the mature peptide in the targeted locale. It is therefore possible that the peptide detected within the sugarcane cells can be attributed to the presence of immature pleurocidin that has not yet had its pre- and pro-peptide sequences cleaved.

Figure 3.4: Electron micrograph of transgenic (1) and normal (2) sugarcane leaves after immunogold detection of pleurocidin using primary antibody at a dilution of 1:300 followed by secondary labeling with a 10nm gold probe (Sigma) diluted to 1:300.
IS: Intercellular Space, CW: Cell Wall, CH: Chloroplast



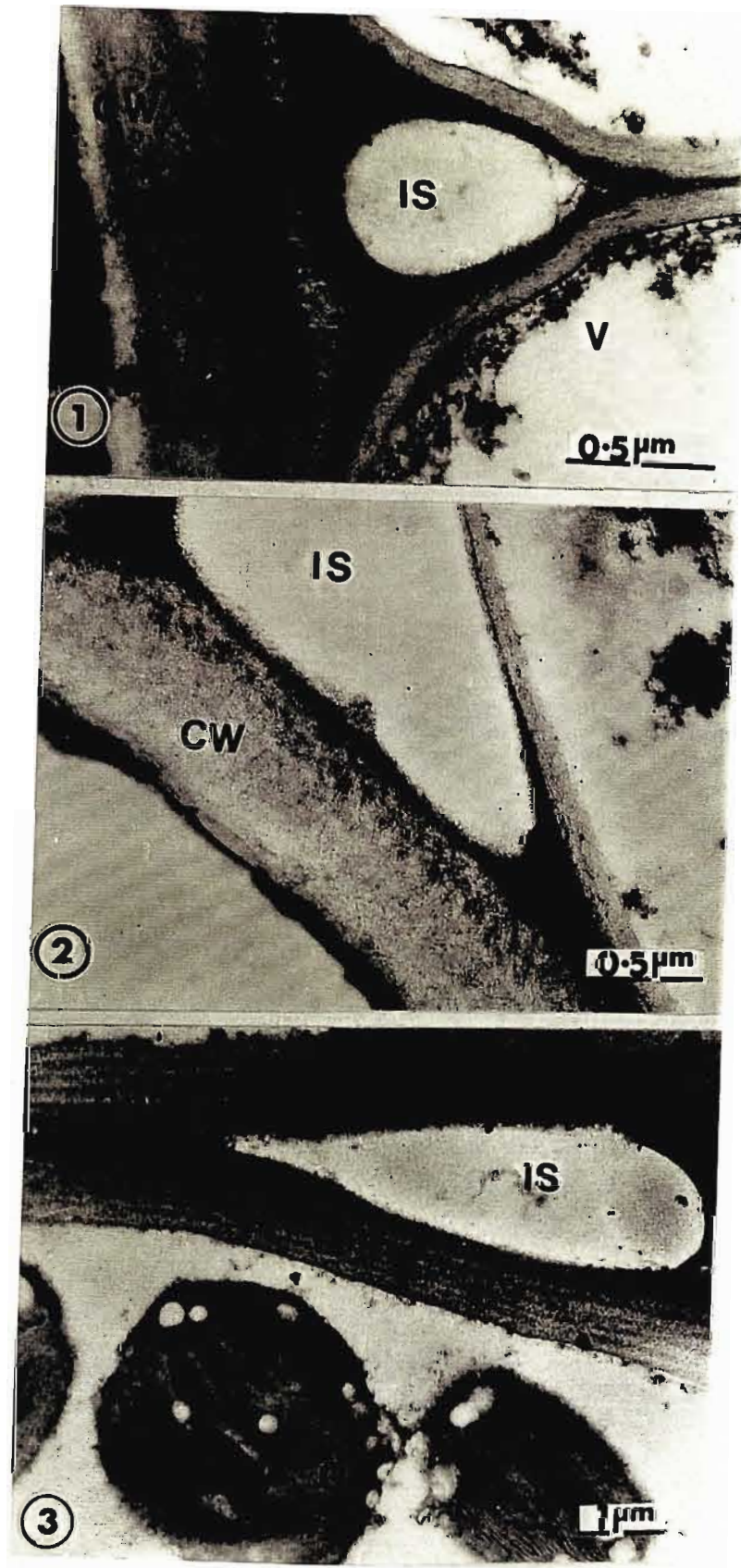


Figure 3.5: Electron micrograph showing immunogold labeling controls treated as follows: **1.** Primary antibody only (1:300 dilution 0.5% BSA in TBS) with no gold probe added; **2.** Gold Probe only (1:300 dilution in 0.5% BSA in TBS) with no primary antibody added; and, **3.** Pre-immune serum used at a dilution of 1:300 in 0.5% BSA in TBS. The pre-immune control was also probed with the Protein A gold probe at a dilution of 1:300. **IS:** Intercellular Space, **CW:** Cell Wall, **CH:** Chloroplast; **V:** Vacuole

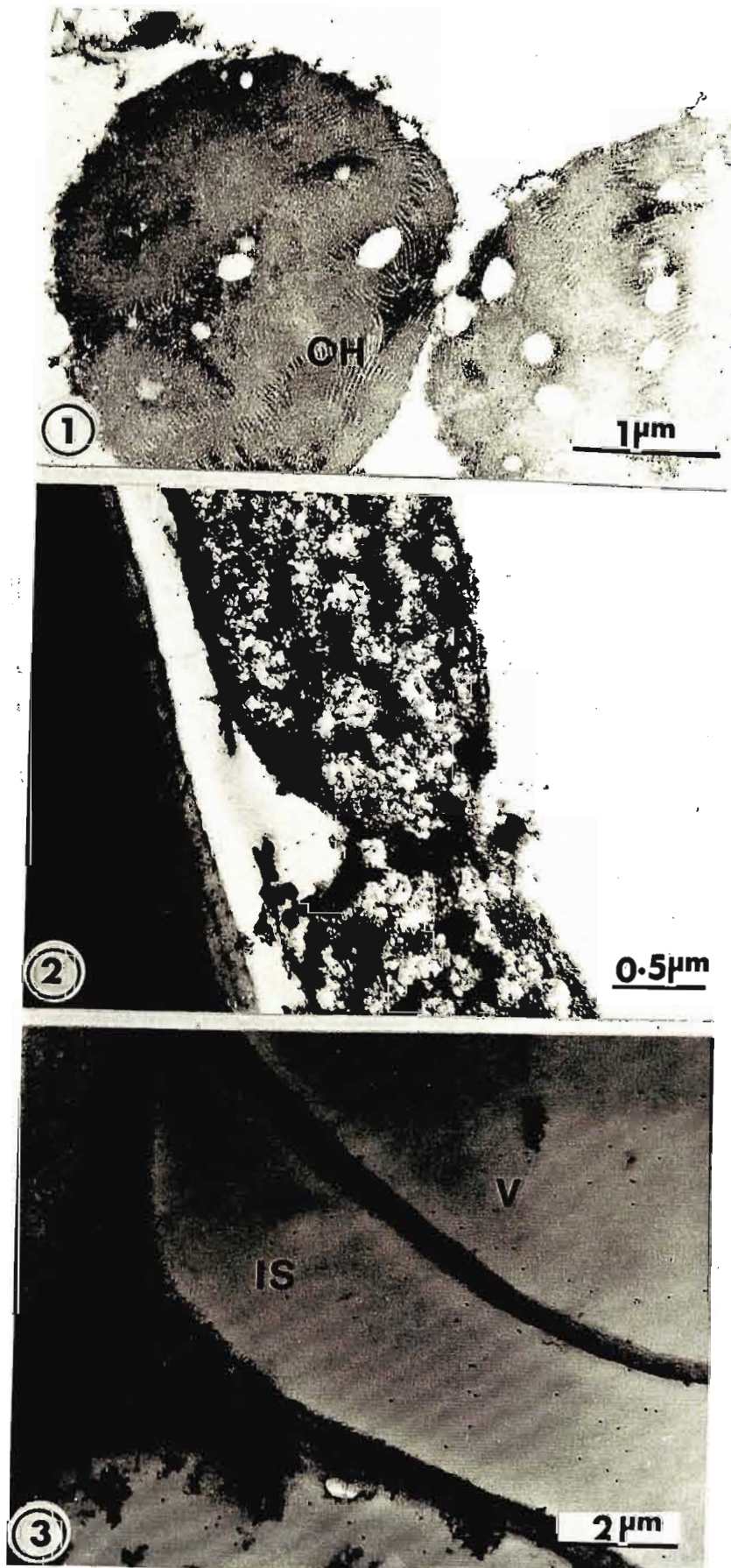


Figure 3.6: Electron micrograph of organelles in transgenic sugarcane indicating the presence of pleurocidin. The gold probes were concentrated within the cell wall and intercellular spaces (1), small amounts of peptide were also detected in the chloroplast (2) and the nucleus (3). N: Nucleus; IS: Intercellular Space, CW: Cell Wall, CH: Chloroplast; V: Vacuole

CHAPTER FOUR

CONCLUSION

The research described in this study is directed towards a better understanding of the genetic engineering of microbial disease resistance in plants, and, specifically refers to two aspects of antimicrobial peptide expression in transgenic plant systems. Firstly, a study was undertaken to assess the stability of antimicrobial peptides in plant systems. Degradation is one of several factors that may affect the level of accumulation of transgene products in plants. In plants engineered to secrete antimicrobial proteins into the intercellular compartment of leaves, the degradative activity of proteases present in leaf intercellular fluid is critical to achieving desired transgene function. Two cationic antimicrobial peptides ESF1-GR7 and pleurocidin were assessed for susceptibility to degradation in vitro by ICF extracted from various crops. Stability of peptides was observed using biotoxicity assays, reverse-phase HPLC and gel electrophoresis techniques. Proteolytic degradation of peptides by plant extracts was observed as a reduction in biotoxicity of peptides following exposure to intercellular fluid extracts. This was in line with observations made by other researchers (Hightower *et al.*, 1994; Mills *et al.*, 1994; Owens and Huette, 1997). The fact that the peptide was degraded over time could suggest that a steady state balance between excretion of the peptide intercellular spaces and its subsequent degradation by proteases could be achieved. As cleavage of the peptides appeared to be a precise process (Owens and Huette, 1997), it may be beneficial to alter the amino acid sequences at the weak cleavage points without adversely compromising bioactivity. These findings also pointed to the possibility of tailoring antimicrobial peptide genes to reduce the rate of degradation in target crops.

The antimicrobial peptide pleurocidin which is found in the skin mucous secretions of *Pleurocentes americanus* was used as the target for overexpression in hybrid *Saccharum* species. The generated transgenic sugarcane plants had low levels of pleurocidin protein in leaf tissue. Poor protein levels have been attributed by other authors to a number of factors including poor translation, inefficient post-translational modification, protein instability or a combination of these factors (Florack *et al.*, 1995; Hightower *et al.*, 1994, and Salmon *et al.*, 1998).

An attempt was made to localize the expression of transgenic pleurocidin in three month old sugarcane plant leaves. For this, immunocytochemistry studies were used in conjunction with transmission electron microscopy. Pleurocidin was predominantly found in the intercellular spaces and cell walls of the transgenic plants. This result was consistent with that observed by Howosaka and Ohashi (1988), and Ohashi *et al.*, (1992). The expression system used to clone pleurocidin into sugarcane included genes for extracellular secretion of the gene product. Therefore, pleurocidin located in the cell wall and cytoplasm may be attributed to the secretory process underway. Howosaka and Ohashi (1988), identified a similar trend when they assessed the expression of pathogenesis-related proteins in tobacco leaves. The gene for pleurocidin was introduced into transgenic sugarcane for constitutive expression. Constitutive expression of any potential bactericidal peptide would be advantageous for inhibiting the early step of pathogen infection via stomata (Okamoto *et al.*, 1998) . This is an important step for improving the resistance of plants grown in fields. As pathogenic bacteria easily penetrate plants from wounded sites, the combination of constitutive and inducible production systems will be ideal for plant improvement. Localization of pleurocidin in within the intercellular spaces and the cell wall would therefore be beneficial to the plant's innate immune system.

The two inter-related studies provide insight into the potential of cationic peptides in transgenic disease control systems. When considered together, it may be seen that while pleurocidin was vulnerable to *in vitro* proteolytic degradation in the presence of intercellular fluid from sugarcane leaves, the peptide was also readily detected in the transgenic sugarcane plant. Although care was taken to prevent injury of the cells during extraction of ICF , there may still have been some cell damage and so protease was clearly evident. *In vivo*, it appears that a significant amount of peptide is being expressed and is reaching the desired locale. Engineering of pleurocidin to resist protease degradation could perhaps enhance its stability in the transgenic sugarcane.

Future studies could concentrate on two main areas. Firstly, research can be carried out on further enhancing the activity of the antimicrobial peptides ensure that the peptide is guarded

from proteolytic degradation once expressed in a foreign environment.

The preliminary study carried out on localization of pleurocidin in transgenic sugarcane plants provided a brief insight into transgenic expression. Immunochemical time-course analysis studies would need to be done on the transgenic plants in order to determine whether transgenic protein expression is continuous or dependent on stage of development of the plant.

In this study, pleurocidin was only detected in the leaf tissue, further analysis could still be done on stem and root tissues to determine if expression pattern are the same.

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