

**Evaluation and Expression of Indolicidins and Polyphemusin Variants
in Plants for Broad-spectrum Disease Resistance**

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ABSTRACT

The cationic antimicrobial peptides, indolicidins and polyphemusins, have shown activity against animal pathogenic viruses. The aim of this work was to evaluate the efficacy of these peptides for engineering plants with a broad-spectrum disease resistance, including viral diseases. TTC, Evan's blue and phytotoxicity assays showed low cytotoxicity of indolicidin and polyphemusin variants (10R, 11R and PV5) on tobacco leaf discs, protoplasts and plantlets. *In vitro* assays using these variants showed a broad-spectrum activity against plant pathogenic bacteria, fungi and viruses. An assessment of *in planta* activity was performed by expressing these cationic peptides in transgenic tobacco (*Nicotiana tabacum* var Xanthi). The disease assays using detached leaves from transgenic tobacco plants showed a broad-spectrum disease resistance against bacteria, fungi and TMV. Further work is needed on optimization of expression of these peptides and their combinations for successful application of this approach under greenhouse and field conditions.

Supervisor: Dr. Santosh Misra (Department of Biochemistry and Microbiology)

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LIST OF ABBREVIATIONS

10R, synthetic indolicidin variant

11R, synthetic indolicidin variant

AMP, antimicrobial peptide

Amino acids,

A: alanine

C: cysteine

E: glutamic acid

G: glycine

I: isoleucine

L: leucine

P: proline

R: arginine

T: threonine

W: tryptophan

M: methionine

D: aspartic acid

F: phenylalanine

H: histidine

K: lysine

N: asparagine

Q: glutamine

S: serine

V: valine

Y: tyrosine

AU-PAGE, acetic acid urea polyacrylamide gel

BAP, benzylaminopurine

bp, base pair(s)

cDNA, complementary DNA

DAS-ELISA, double antibody sandwich immunosorbent assay

dH₂O, distilled water

DNA, deoxyribose nucleic acid

EDTA, ethylenediamine-tetra-acetic acid disodium salt

ER, endoplasmic reticulum

GA, gibberellic acid

GRP, glycine rich protein

GUS, β -glucuronidase

gDNA, genomic DNA

h, hours

OD, optical density

JA, jasmonic acid

kb, kilobase(s) or 1000 bp

LB, Luria Bertani medium

M, molar

MS, Murashige and Skoog medium
mM, millimolar
min, minutes
 μ l, microlitre
mRNA, messenger RNA
NaHClO₃, sodium hypochlorite
NAA, naphthalene acetic acid
nm, nanometer
PAGE, polyacrylamide gel electrophoresis
PCR, polymerase chain reaction
PDA, potato dextrose agar
PDR, pathogen-derived resistance
PEG, polyethylene glycol
PmBiP, Douglas-fir BiP
PV5, synthetic polyphemusin variant
PV8, synthetic polyphemusin variant
PVX, potato virus X
PVY, potato virus Y
rRNA, ribosomal RNA
RNA, ribose nucleic acid
RT, room temperature
SDS, sodium dodecyl sulfate
SSC, sodium chloride-sodium citrate
TMV, tobacco mosaic virus
Tris, tris (hydroxymethyl)-methylamine
TTC, 2,3,5-triphenyltetrazolium chloride
ZR, zeatin riboside

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DEDICATION

I would like to dedicate this thesis to my father. He left this world 18 years ago but I still feel that he is always with me; providing support and care in times I need him the most.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Genetic engineering is an effective strategy for developing disease resistant germplasm that increases yield, reduces losses and eliminates or reduces the use of pesticides. This technique has proved effective in controlling diseases in plants. Cationic peptides are important components of innate immunity and have been isolated from a wide variety of organisms. These widespread natural products vary greatly in their properties and spectrum of biological activities. Different peptides and their synthetic derivatives have found applications as antibacterial, antifungal and therapeutic agents. Their expression has resulted in the generation of disease-resistant transgenic plants. A major focus of research in the past was on the antibacterial and antifungal aspects of these cationic peptides on plant pathogens. Polyphemusin and indolicidin are two important cationic peptides, which have demonstrated *in vitro* antibacterial, antifungal and antiviral activities against animal pathogens. In addition to the assessment of antibacterial and antifungal activity, the following research examines the antiviral activity of these peptides against plant pathogens. This research is significant in that it not only evaluates the cytotoxicity and *in vitro* antimicrobial activity, but also shows *in planta* disease resistance conferred due to expression of these cationic peptides.

1.1. Literature Review

1.1.1. Disease management in plants

Plant diseases are responsible for enormous losses (\$30-\$50 billion annually) worldwide in cultivated and stored crops, and thus, are a major impediment to effective food

production and distribution (Baker et al., 1997). Worldwide, phytopathogens reduce crop productivity by 12% (Food and Agriculture organization (FAO), 1993), making effective and sustained control one of the most important issues of agriculture today. Broadly, approaches used to produce disease resistant plants can be divided into two main categories: conventional and non-conventional strategies.

1.1.1.1. Conventional strategies

In the past, containment of plant pathogens has relied on chemical pesticides. Millions of tons of pesticides costing \$26 billion dollars annually are applied to manage losses due to pest. Heavy reliance on chemical pesticides has far reaching implications not only on the environment but also on human health through residual toxicity (Barker, 1996). In addition, pesticides are becoming less effective because of increasing insecticide resistance in aphid populations. Long-term climatic changes leading to changes in vector populations, concern about the environmental effects of pesticides, as well as consumer concern about pesticide residues in food, have led to increased interest in finding alternative means of controlling phytopathogens.

Traditionally, plant-breeding strategies have been successfully used to produce a large number of resistant varieties. However, the increasing intensity of crop management has been accompanied by a growing number of diseases and a large number of infecting strains that have outpaced the development of new resistant plant varieties using conventional plant breeding strategies (Solomon-Blackburn and Barker, 2001). Unwanted effects like reduction in yield and fertility are often observed in the transfer of the

dominant resistance genes. Transfer of resistance genes into high-yielding crops is a time-consuming process.

1.1.1.2. Non-conventional strategies

The incorporation of specific disease resistant traits in plants through genetic engineering offers a means to prevent disease-associated losses without damaging the environment (Gadani et al., 1990). Non-conventional strategies for the production of disease-resistant crop plants have exploited gene transfer technology for molecular resistance breeding (Marcos et al., 1995; Punja, 2001). Such strategies have included expression of genes of plant defense response pathway components (Cao et al., 1998); expression of genes encoding plant, fungal or bacterial hydrolytic enzymes (Lorito et al., 1998; Mourgues et al., 1998); and expression of genes encoding elicitors of defense response (Keller et al., 1999) and small peptides (Cary et al., 2000; Li et al., 2001). Expression of foreign genes in plants, derived not only from plant sources but also from insects and mammals, is a promising strategy that can be exploited to promote disease resistance in plants (Chong and Langridge, 2000).

The main focus of this work was to provide broad-spectrum viral disease resistance in plants. Resistance to plant viruses can be conferred either by expressing part of the viral genome or virally associated sequences, popularly known as pathogen-derived resistance (PDR). Strategies for PDR are divided into those that require the production of protein and those that only require the accumulation of the viral nucleic acid sequences. According to (Beachy, 1997), the former confer resistance to a broad range of viral strains and viruses, whereas the latter provides high levels of specific virus resistance.

The concept of PDR states that certain key pathogen gene products present in the plant in a dysfunctional form, in excess, or at an inappropriate stage during viral replication cycle, could disrupt infection by the invading pathogen. An increasing number of reports on the use of viral genes for PDR has revealed a lack of correlation between the expression level of the transgenic protein and the level of resistance (Golemboski et al., 1990). The capsid protein, antisense RNA and truncated replicase protein have been used for the generation of transgenic plants for resistance to viruses (Golemboski et al., 1990; Noris et al., 1996; Bendahmane and Gronenborn, 1997). Many of these attempts were successful and some have led to the development of virus-resistant plants for commercial application (Lawson et al., 1990). Use of RNA-based resistance such as antisense RNA, satellite RNA and defective-interfering RNA are promising methods that have been used previously (Powell et al., 1989; Wang and Metzloff, 2005). Transgenic expression of dysfunctional viral Movement Proteins (MP) is also an effective strategy for developing resistance against viruses in plants (Malysenko et al., 1993; Cooper et al., 1995). Expression of a functional MP had either no effect on virus infection or caused increased susceptibility (Ziegler-Graff et al., 1991). Antiviral proteins for expression in plants may be derived from different sources, including naturally occurring peptides or synthetically modified derivatives of these peptides

1.1.2. Cationic antimicrobial peptides

Cationic antimicrobial peptides have been found in a variety of sources, from prokaryotes to higher eukaryotes (Hancock et al., 1995; Vizioli and Salzet, 2002). In the past 25 years, more than 800 cationic, gene-encoded antimicrobial peptides have been described.

The majority of peptides (96%) have a net positive charge but some have a net negative charge. In recent years, it has become clear that these endogenous peptide antibiotics constitute part of the first line of host defense (Boman, 1991). Cationic peptides, already present in first line of defense of all living organisms, can be induced and synthesized much more rapidly than immunoglobulin upon infection, before the adaptive immune system is activated, and can function without the high specificity and memory of immunoglobulin or immune cells (Boman, 1995; Nissen-Meyer and Nes, 1997). In mammals, antimicrobial peptides are present at high concentrations in phagocytes (e.g. macrophages, neutrophils, NK cells) and mucosal epithelial cells (e.g. Paneth cells). In lower life forms, such as invertebrates, which have no adaptive immunity, cationic peptides are the major defensive system against infection (Boman, 1995). Insects produce cationic peptides in their fat bodies and hemolymph, where they are induced upon bacterial challenge (Hoffmann and Hetru, 1992; Boman, 1995). Cationic peptides also function to keep the natural microflora at a steady state in a variety of different niches such as the skin, mouth, and intestine (Boman, 1995). They are active, not only against bacteria but also against fungi, viruses and even parasites (Vizioli and Salzet, 2002). The natural cationic peptides of animals and plants are synthesized as precursor peptides, then processed into their mature forms by cleavage of a signal peptide and a pro-sequence (Hancock, 1997).

The earliest peptide antibiotics used extensively in human medicine were the gramicidins and polymyxins. The lantibiotic, nisin is currently used as a food preservative (Delves-Broughton et al., 1996). MSI-78, a 22 residue magainin analogue has completed human Phase III clinical trials, showing equivalent efficacy to oral ofloxacin on polymicrobial

infections of individuals with diabetic foot ulcers (Hancock, 1997). IB-367 is a synthetic protegrin-like cationic peptide that has shown efficacy in early clinical trials against oral mucositis and the sterilization of central venous catheters. It is currently proceeding through Phase III clinical trials. In addition, the cationic protein rBPI 21 has recently completed Phase III clinical trials for meningococemia (Hancock, 2000). Another promising prospect for cationic peptides is plant and fish biotechnology where cationic peptides can be engineered into host organisms to permit enhanced disease resistance (Hancock and Lehrer, 1998). The ability of cationic peptides to bind to lipopolysaccharides, their anti-endotoxin properties (Gough et al., 1996), and their ability to act synergistically with conventional antibiotics as enhancers, are few of the features that make them attractive and potentially novel antibiotics. Furthermore, cationic peptides are gene-coded, and synthesized as precursors that undergo posttranslational modifications to become active, their production by gene engineering becomes possible and resistance against these antimicrobial peptides does not develop easily.

The discovery and characterization of novel anti-bacterial, anti-viral, anti-parasitic and anti-fungal peptides from natural sources as well as their synthetic and more potent variants, is a promising strategy to develop new pharmaceuticals against these microorganisms.

1.1.2.1. Structural features and categories

Cationic peptides show significant diversity in size, sequence and structure. They range from 12 to 46 amino acids long with diverse amino acid compositions (Hancock, 1997). Despite their diversity, cationic antimicrobial peptides have a net charge of at least +2 at

neutral pH, usually because of the presence of arginine or lysine residues in their amino acid sequence (Hancock, 1997). Their secondary structures often contain a hydrophobic domain and a hydrophilic domain. The basicity and amphipathicity of cationic peptides are essential for their antimicrobial activities (Hancock, 1997). The hydrophilic (positively charged) surface facilitates the interaction of the peptides with the negatively charged bacterial surface, e.g. lipopolysaccharide on the outer membrane of Gram-negative bacteria, teichoic acid on the Gram-positive bacteria, or negatively charged head groups of the phospholipids in the lipid bilayer (Kagan and Sokolov, 1994; Piers et al., 1994).

Nuclear magnetic resonance (NMR) has emerged as a useful technique for studying structural details of most of the known antimicrobial peptides. Analysis of the three-dimensional structure of these peptides has resulted in a better understanding of their function. Because a majority of these peptides are small in length, their three-dimensional structures can be obtained by NMR methods (Wuthrich, 1986). Based on the NMR structures of known peptides along with sequence analysis, antimicrobial peptides are broadly classified into five groups: helical, cysteine rich, sheet, antimicrobial peptides rich in regular amino acids and antimicrobial peptides with rare amino acids.

1.1.2.1.1. Helical antimicrobial peptides

Peptides in this category are highly amphipathic helices with hydrophobic and charged cationic surfaces. A well-identified and characterized helical cationic peptide is cecropin-A from the moth, *Hyalophora cecropia*. Magainins, another group of well characterized helical peptides, isolated from the skin of the African clawed frog, *Xenopus laevis* are

composed of 23 residues (Matsuzaki, 1999). NMR studies showed that both cecropins and magainins form amphipathic helical structures (Marion et al., 1986).

1.1.2.1.2. Cysteine-rich antimicrobial peptides

This group consists of peptides that are rich in cysteine residues and are present in a wide variety of organisms. The human neutrophil peptides HNP-1, -2 and -3 were the first cysteine-rich peptides isolated from human neutrophil granules (Ganz et al., 1985). Most of these molecules harbor a consensus motif of six cysteine residues forming three intramolecular disulfide bonds. Drosomycin, isolated from *Drosophila*, contains four disulfide bonds and three antiparallel strands with a helix between the first two strands (Landon et al., 1997) and represents another example of a cysteine-rich peptide.

1.1.2.1.3. Sheet antimicrobial peptides

A few of the known antimicrobial peptides of this class are approximately 20 residues long and contain one or two disulfide linkages that form a single hairpin structure. Horseshoe crab peptides, tachyplesins and polyphemusin, share a hairpin motif stabilized by two disulfide bonds (Kawano et al., 1990; Tamamura et al., 1993). These molecules form antiparallel β -sheets connected to a turn and contain two disulfide bridges (Tamamura et al., 1993). NMR studies with thanatin, a 21-residue defense peptide isolated from the hemipteran insect *P. maculiventris*, showed results similar to that of tachyplesin. NMR studies have shown that lactoferricin B, a 25 amino acid proteolytic derivative of lactoferrin adopts a sheet structure stabilized by a single disulfide bond when in solution (Hwang et al., 1998).

1.1.2.1.4. Antimicrobial peptides rich in regular amino acids

Some antimicrobial peptides are composed of a high proportion of regular amino acids. The structural conformation of such peptides differs from the regular helical or sheet peptides. Histatin, a peptide isolated from human saliva is rich in histidine residues and is active against *C. albicans* (Xu et al., 1991). Cathelicidins are proline rich peptides, while indolicidins (Selsted et al., 1992) and tritrypticin (Lawyer et al., 1996) are tryptophan rich. Bactenecins-Bac-5 and Bac-7, like cathelicidins, are proline-rich (Gennaro et al., 1989). In contrast, peptide PR-39, is rich in arginine residues (Agerberth et al., 1991).

1.1.2.1.5. Antimicrobial peptides with rare modified amino acids

Several peptides are unusual in being composed of rare modified amino acids. Examples of such peptides are those produced by lactic acid bacteria. Nisin, a lantibiotic, is produced by *Lactococcus lactis* and is composed of rare amino acids like lanthionine, 3-methylanthionine, dehydroalanine and dehydrobutyrine (De Vos et al., 1993). Another peptide, leucocin A, a 37-residue antimicrobial peptide isolated from *Leuconostoc gelidum*, has been shown to form an amphiphilic conformation (Gibbs et al., 1998).

1.1.2.2. Mechanism of peptide action

The mode of action of cationic peptides is not completely known, however specificity with regards to the pathogen as well as with the peptide has been demonstrated. The action of these peptides on bacteria, fungi and viruses is discussed in detail below.

1.1.2.2.1. Antibacterial action

Cationic peptides function by disrupting the cytoplasmic membrane of bacteria (Hancock and Lehrer, 1998). This action is proposed to involve three steps: (1) binding to the cell surface; (2) permeabilization of the outer membrane (in gram negative bacteria) and then the cytoplasmic membrane and (3) loss of cell viability as a result of cell lysis and DNA damage (Lichtenstein et al., 1988). Cell lysis is supposed to be initiated by the electrostatic interaction of cationic peptides with the negatively charged cell surface. For Gram-negative bacteria, the positively charged domain of the cationic peptides binds to the divalent cation binding sites of lipopolysaccharide (Sawyer et al., 1988; Piers et al., 1994). The displacement of the native cations Ca^{2+} and Mg^{2+} disrupts the structures of the outer membrane, due to the bulky size of the cationic peptides. This disruption subsequently results in the self-promoted uptake of cationic peptides (Hancock et al., 1995). For Gram-positive bacteria, the cell wall contains covalently bound, negatively charged teichuronic acid and carboxyl groups in the peptidoglycan, and these are probably the initial binding sites for the cationic peptides. The interaction between the peptides and the cytoplasmic membrane is thought to be determined by factors such as the anionic lipid composition of the bacterial membrane, and the presence of an electrochemical potential across the membrane. After positively charged cationic peptides bind to the negatively charged lipid head groups under the influence of a trans-membrane potential (oriented internal negative), the peptides insert into the membrane and undergo conformational changes. They then aggregate to form multimers, which allow them to form channels or pores with their hydrophobic faces positioned toward the membrane and their hydrophilic faces oriented towards the interior of these channels or pores. This

results in leakage of protons causing dissipation of the membrane potential and leakage of other small compounds causing cell death. After membrane permeability is altered, the simultaneous loss of the proton motive force, cessation of biosynthesis of macromolecules like DNA, RNA and protein, and leakage of intracellular contents, are responsible for eventual cell death (Fidai et al., 1997; Hancock, 1997).

Membrane selectivity

The same factors that are responsible for cell death also seem to regulate the selectivity of cationic peptides for bacterial membranes over eukaryotic cell membranes. The composition of the eukaryotic membrane is quite different from that of bacterial membranes that predominantly contain negatively charged lipids such as phosphatidylglycerol and cardiolipin, whereas the eukaryotic cell membrane is largely composed of zwitterionic lipids such as phosphatidylcholine and sphingomyelin. Eukaryotic cell membranes are rich in cholesterol, which may inhibit membrane insertion. Bacterial cells have large, trans-membrane potentials of around -140 mV, whereas eukaryotic plasma membranes have membrane potentials of only -20 mV (Yeaman and Yount, 2003). All of these factors contribute to the membrane selectivity of cationic peptides between prokaryotic and eukaryotic cells.

1.1.2.2.2. Antifungal action

The modes of action of antifungal peptides have been studied extensively (De Lucca and Walsh, 1999; Matsuzaki, 1999). Peptides that interact specifically with the lipid components of cell membranes often causing formation of pores or ion channels that

result in leakage of essential cellular minerals or metabolites or dissipate ion gradients in cell membranes. Other peptides have been shown to inhibit chitin synthase or β -D-glucan synthase. The synthetic peptide D4E1 complexes with ergosterol, a sterol present in the germinating conidia of a several fungal species, suggests a lytic mode of action (De Lucca and Walsh, 1999). Research is in progress to elucidate the antifungal action of cationic peptides at the molecular level.

1.1.2.2.3. Antiviral action

Not much is known about the mechanisms involved in the antiviral activity of antimicrobial peptides; however, based on the available literature, the antiviral mechanism can be broadly divided into four mechanisms: direct inactivation, virus-host cell interaction, suppression of viral gene expression and enhancement of the host's immunomodulatory properties.

Direct inactivation

Direct inactivation of the herpes virus by magainins (Egal et al., 1999), α -defensins (Lehrer et al., 1985; Daher et al., 1986), modelin I (Aboudy et al., 1994) and melittin (Baghian et al., 1997), HIV virus by tachyplesin (Murakami et al., 1991) and indolicidin (Robinson et al., 1998), stomatitis virus by tachyplesin (Murakami et al., 1991) and channel catfish virus (CCV) by esculentin (Chinchar et al., 2001) have been reported. The net cationic charge and ability to form amphipathic structures may enable these peptides to interact with the membranes of the enveloped viruses, which are composed of anionic phospholipids, and disrupt membrane structure. Here the disruption of membrane

integrity occurs because of the interaction between antimicrobial peptides and the virion (Lehrer et al., 1985; Daher et al., 1986). Recently, it was shown that dermaseptin S4 (DS4), which displays a broad-spectrum of activity against bacteria, yeast, filamentous fungi and herpes simplex virus I, also inhibits HIV-1 by disrupting virion integrity (Lorin et al., 2005). Antimicrobial peptides like esculentin not only lyse the viral envelope, but also affect the stability of the nucleocapsid (Chinchar et al., 2001). This can also be an effective mechanism in inactivating non-enveloped plant viruses.

Virus-host cell interaction

Interference with virus and host cell surface interactions is another mode of action adopted by antiviral peptides. Antiviral activity of dermaseptins against Herpes Simplex Virus (Belaid et al., 2002) is an example of this mechanism. Dermaseptin S4 showed an inhibitory effect only when applied to the virus before or during virus adsorption to the target cells, suggesting that the activity of this dermaseptin was exerted at a very early stage of virus proliferation, most likely at the virus cell interface (Belaid et al., 2002). In enveloped viruses, inhibition of viral-cellular membrane fusion has been observed. Examples include human immunodeficiency virus (HIV) by tachyplesin (Morimoto et al., 1991), and polyphemusin (Nakashima et al., 1992; Otaka et al., 1994) and herpes simplex virus (HSV) by apolipoprotein (Srinivas et al., 1990). T₂₂, a tachyplesin synthetic derivative, interferes with the process after HIV binding but before transcription of the HIV genome (Nakashima et al., 1992). In these cases, the antimicrobial peptides exerted a more profound effect on the cell fusion process than on virus penetration as seen by the inhibition of complete cell fusion by peptide treatment *in vitro* (Srinivas et al., 1990).

Antiviral activity shown by a number of α -helical synthetic cationic peptides was due to inhibition of virus entry in the cells (Jenssen et al., 2004). Defensins protect cells from infection by HSV by inhibiting viral adhesion and entry (Yasin et al., 2004).

Suppression of viral gene expression

Inhibition of viral gene expression is an effective mode of action of antimicrobial peptides against both non-enveloped and enveloped viruses. Melittin adopts this mechanism against both the plant virus TMV (Marcos et al., 1995) and enveloped HIV virus (Wachinger et al., 1998). Inhibition of HIV by melittin is mediated by the amphipathic α -helical part of the peptide and is a result of intracellular impairment of HIV protein production rather than a membrane effect (Wachinger et al., 1998). With TMV, melittin analogs require elicitation of the peptide along with the virus and binding causes a conformational change in the structure of the RNA (Marcos et al., 1995). Thus these antimicrobial peptides have effects at the level of gene expression.

Enhancement of immuno-modulatory properties

There is an enhancement of immuno-modulatory properties in response to some peptides (Hancock and Diamond, 2000; Chernysh et al., 2002). Antimicrobial peptides have been reported to be involved in many aspects of innate host defenses. They are associated with acute inflammation by acting as chemotoxins for monocytes, recruitment of T-cells through chemotaxis, enhancement of chemokine production and the proliferative response of T-helper cells (Hancock and Diamond, 2000). Synthetic alloferon has been shown to stimulate the natural cytotoxicity of human peripheral blood lymphocytes,

induce interferon synthesis in mouse and human models and enhance antiviral and anti-tumor resistance in mice (Chernysh et al., 2002). Corticostatin (Lehrer et al., 1985) acts by competing with the basic amino acid residues of adrenocorticotrophic hormone for its binding site (Zhu and Solomon, 1992). Despite the number of successful examples, the molecular basis of protein-mediated virus resistance in most cases is not understood.

1.1.2.3. *In vitro* activity of cationic peptides

Cationic peptides exhibit potent *in vitro* activities against a broad spectrum of microorganisms including bacteria, fungi, enveloped viruses, and tumor cells. For example, defensins are active against Gram-positive and Gram-negative bacteria (including spirochetes and mycobacteria), fungi and enveloped viruses (Lehrer et al., 1993). Magainins also exhibit anti-fungal and antiviral activities in addition to their anti-bacterial activities (Bevins and Zasloff, 1990). Defensins (Lichtenstein et al., 1988) (Barker, 1996) and maganin have also been shown to be active against tumor cells. Most interestingly, cationic peptides (e.g. tachyplesin) inhibit not only the growth of normally susceptible bacteria but also inhibit clinically important antibiotic-resistant strains, for example, methicillin-resistant *S. aureus* (Ohta et al., 1992).

1.1.2.4. Synthetic antimicrobial peptides

Most of the antimicrobial peptides are cationic and form an amphipathic secondary structure upon interaction with the surface of the cell membrane, resulting in the formation of ion channels and subsequently in cell lysis and death of the pathogen. These two properties have led to the design and synthesis of novel peptides with antimicrobial

activity. It was shown that antimicrobial activity could be separated from hemolytic activity through certain nucleotide sequence deletions or substitutions (Blondelle and Houghten, 1991). It has been found that some of the synthetic smaller peptides, in the absence of an amphipathic helical structure, have high levels of antimicrobial activity. Putative cationic amphipathic structures of naturally found proteins have been identified and engineered to display a broad-spectrum pathogen activities (Zhong et al., 1994). The modification of cationic antimicrobial peptides to determine structure-function relationships and/or to produce less toxic molecules with increased activity has been performed primarily on α -helical and β -structured peptides (Andreu et al., 1985; Bessalle et al., 1992). The important factors in the activity of synthetic antimicrobial cationic peptides are the position and nature of positively charged residues, the formation of specific secondary structures, and the creation of a hydrophobic face on the molecule. These factors are being exploited to design novel and effective drugs.

1.1.2.5. Cationic antimicrobial peptides from plants

A number of small peptides that display the ability to inhibit the growth of fungi, viruses and bacteria have been isolated from plants (Broekaert et al). Thionins were the first antimicrobial peptides to be isolated from plants (Broekaert et al., 1992). They act on both gram-positive and gram-negative bacteria, fungi, yeast, and various mammalian cell types. Other antimicrobial peptides were found to be structurally related to insect and mammalian defensins and were named "plant defensins". Whereas most antimicrobial peptides from animals and bacteria have high antibacterial activity, plant defensins have a high antifungal activity (Broekaert et al., 1992; Terras et al., 1992), reflecting the relative

importance of fungal pathogens in the plant world. These peptides are 50 to 100 amino acids in length and have broad-spectrum antimicrobial property (Boman, 1991; Cammue et al., 1992; Bessalle et al., 1993). These plant-expressed antimicrobial peptides are a promising source of natural and safe alternatives to antibiotics, thus there is intense interest in these plant-derived peptides.

1.1.2.6. Cationic peptides and disease resistance in plants

In the past few years it has become apparent that plants expressing cationic peptides exhibit broad-spectrum disease resistance including the ability to kill bacteria, fungi, protozoa, and, to some extent, viruses (Florack et al., 1994; Florack et al., 1995; Allefs et al., 1996; Hancock and Diamond, 2000; Ponti et al., 2003). The antibacterial, antifungal and antiviral activity shown by transgenic plants expressing cationic peptides is discussed below.

1.1.2.6.1. Antimicrobial peptide/ protein expression and bacterial resistance in plants

Many different genetic strategies have been proposed to engineer plant resistance to bacterial diseases, including inhibiting bacterial pathogenicity or virulence factors, enhancing natural plant defenses, artificially inducing programmed cell death at the site of infection and producing antibacterial proteins of non-plant origin (Mourgues et al., 1998). Genes encoding antibacterial proteins have been cloned and expressed in plants in an attempt to confer resistance to bacterial diseases. Antimicrobial amphipathic peptides, like cecropins and their synthetic analogs Shiva-1 and SB-37, have been expressed in transgenic potato and tobacco plants (Destefano-Beltran et al., 1991). Transgenic tobacco

plants expressing the Shiva-1 gene showed delayed symptoms and reduced mortality following inoculation with *Ralstonia solanacearum* and *Pseudomonas syringae* (Jaynes et al., 1993). However, as a result of the degradation of cecropins by plant proteases (Florack et al., 1995), no resistance to *R. solanacearum* or *P. syringae* pv. *tabaci* was found in transgenic plants. A stable cecropin analog (MB39) has been expressed in transgenic tobacco and plants showed no necrosis after leaf infiltration with *P. syringae* (Huang et al., 1997; Evans and Greenland, 1998; Jaynes et al., 1993). The expression of native cecropin in tobacco did not confer resistance to *P. syringae* or *P. solanacearum*, presumably due to degradation of the peptides by host proteases (Hightower et al., 1994). Expression of tachyplesin along with a signal sequence in plants has been shown to confer resistance against *Erwinia* soft rot (Allefs et al., 1996). The attacins, isolated from the giant silk moth, introduced into apple plants, have also shown a reduced susceptibility to *Erwinia amylovora* (Norelli et al., 1994; Norelli et al., 1998). Three different lysozyme genes (egg-white, T4-bacteriophage and human lysozyme) have been expressed in plants. Extracellular extracts from transgenic tobacco plants producing hen-egg-lysozyme inhibited the growth of several species of bacteria, but the susceptibility of the transgenic lines to bacterial diseases has not yet been reported (Trudel. J., 1995). Greenhouse and *in vitro* experiments indicated a partial resistance to *Erwinia carotovora* in transgenic potato plants producing the T4-bacteriophage lysozyme (During, 1996) and a slight decrease in the symptoms caused by *P. syringae* pv. *tabaci* in tobacco plants producing a human lysozyme (Nakajima H., 1997). The expression of a human lactoferrin gene in tobacco delayed the onset of symptoms caused by *R. solanacearum* (Zhang et al., 1998). Esculentin, from frog skin conferred resistance against *Pseudomans aeruginosa* when

expressed in tobacco (Ponti et al., 2003). The efficiency of these strategies has been improved by the modifications of the peptide genes and construction of synthetic molecules with enhanced expression and stability in plant tissues (Gao et al., 2000; Osusky et al., 2000; Osusky et al., 2004). Synthetic peptide D4E1 (Cary et al., 2000) and a plant defensin, alfAFP from *Medicago sativa* (Gao et al., 2000) have been successfully expressed in tobacco and potato for bacterial and fungal resistance. Similarly, a synthetic cecropin–melittin chimeric peptide and modified temporin provided resistance against *E. carotovora* in potato (Osusky et al., 2000; Osusky et al., 2004). Combined expression of several peptide genes, allowing synergistic effects, is a promising strategy to provide broad-spectrum disease resistance in plants.

1.1.2.6.2. Antimicrobial peptide/protein expression and fungal resistance in plants

Genes encoding hydrolytic enzymes such as chitinase and glucanase, which can degrade fungal cell-wall components, are attractive candidates for the antifungal genetic engineering approach, and are preferentially used for the production of fungal disease-resistant plants (Broglie et al., 1991; Jach et al., 1995). Cecropin A-derived peptides have been shown to be potent inhibitors of fungal plant pathogens (Cavallarin et al., 1998). The over-expression of defensins and thionins in transgenic plants reduced development of several fungal pathogens like *Alternaria sp.*, *Fusarium sp.*, and *Plasmodiophora sp.* and provided resistance to *Verticillium sp.* under field conditions (Gao et al., 2000). Expression of barley seed ribosome-inactivating proteins reduced development of *Rhizoctonia solani* in transgenic tobacco (Logemann et al., 1992). Combined expression of chitinase and ribosome-inactivating proteins in transgenic tobacco had an inhibitory

effect on *Rhizoctonia solani* development (Jach et al., 1995). Human lysozyme expression in transgenic carrot and tobacco, enhanced resistance to several pathogens, including *Erysiphe* and *Alternaria sp.* (Nakajima H., 1997; Takaichi and Oeda, 2000). Pokeweed (*Pytolacca americana*) antiviral protein expression in transgenic tobacco reduced *Rhizoctonia solani* infection (Wang et al., 1998). MSI-99, and a magainin analogue, imparted enhanced disease resistance in transgenic tobacco and banana against *F. oxysporum* and *Mycosphaerella musicola* (Chakrabarti et al., 2003). The same magainin analogue expressed via the chloroplast genome to obtain high levels of expression in transgenic tobacco showed inhibition of growth against spores of three fungal species: *Aspergillus flavus*, *Fusarium moniliforme*, and *Verticillium dahliae* (DeGray et al., 2001). Heliomicin and drosomycin expressed in transgenic tobacco conferred a minor but statistically significant enhanced resistance to the fungi *Cercospora nicotianae* (Banzet et al., 2002). Cecropin A-melittin hybrid and Cecropin A-derived peptides, were synthesized and tested for their ability to inhibit growth of *Phytophthora infestans* and other pathogens *in vitro* (Cavallarin et al., 1998). These and other synthetic cationic peptides (e.g. cecropin, melittin, temporin and their modified variants) with *in vitro* broad-spectrum antimicrobial activity expression in transgenic potato and tobacco, have provided enhanced resistance against a number of plant fungal pathogens, including *Colletotrichum*, *Fusarium*, *Verticillium sp.* and *Phytophthora sp.* (Cavallarin et al., 1998; Osusky et al., 2000; Osusky et al., 2004).

1.1.2.6.3. Antimicrobial peptide/protein expression and viral resistance in plants

A relatively small number of antiviral peptides have been described in antimicrobial peptide database (Wang and Wang, 2004). Virus resistance in plants has been obtained by expressing specific proteins. Examples of protein-mediated virus resistance mainly include the expression of viral coat proteins (Lomonossoff, 1995), but cases of protein-dependent pathogen-derived resistance due to the expression of viral movement proteins or replicases are also known (Beck et al., 1994; Tacke et al., 1996). In some instances, resistance is based on the expression of intact, functional proteins; in others the expression of the intact protein leads only to weak resistance or even to enhanced susceptibility. In contrast, expression of a dysfunctional protein may lead to strong resistance (Cooper et al., 1995; Tacke et al., 1996). A new strategy for engineering virus-resistant plants by transgenic expression of a dominant interfering peptide was shown by Rudolph et al. (2003). Transgenic *Nicotiana benthamiana* lines expressing the peptide fused to a carrier protein showed strong resistance against tomato spotted wilt virus, tomato chlorotic spot virus, groundnut ring spot virus, and chrysanthemum stem necrosis virus. This presents a promising strategy for expressing small peptides in plants. The advantage of using short sequences to engineer resistance is to minimize unpredictable or even deleterious effects observed in several cases after the expression of functional viral proteins (Prins et al., 1997). Expression of only a short peptide or artificial peptides not only minimizes the potential deleterious effect on the plant cell, but also prevents other undesirable consequences. One such concern is the evolution of new viruses by recombination with the transgene or by transcapsidation, which can virtually be excluded when an interfering but non-homologous and nonfunctional molecule is expressed in the

plant. Thus expressing cationic peptides with *in vitro* antiviral activities is a promising strategy to provide broad-spectrum disease resistance in plants.

1.1.2.6.4. Drawbacks of peptide expression in plants

Despite benefits gained from genetic engineering for disease resistance, this technology has some drawback and research for solutions is in progress. In addition to transgene integration, disease resistance depends on the peptide expression level in plants, which can, in some cases, be affected by homology-dependent gene silencing (Meyer, 1996). This is a serious threat for strategies using homologous sequences. Furthermore, in the case of proteins/peptides, these must be synthesized, exported from the cell, and transported to their desired location without undergoing major modification during the process, and remain stable at their destination, avoiding degradation by plant proteases (Hancock and Lehrer, 1998). *In vitro* testing of the leaf extracts from the plants expressing cationic peptides shows that expressed peptides are unstable or degraded by proteases (Cavallarin et al., 1998; Hancock and Lehrer, 1998; Cary et al., 2000; Li et al., 2001).

1.1.2.7. Cationic peptides and modulation of the innate defense components in transgenic plants.

Mounting evidence suggests that the innate immune system of plants shares many parallels with that of vertebrates and insects (Cohn et al., 2001), and relies on similar downstream signaling components. In both plants and animals, innate immune responses include the ability to recognize a pathogen and activate an effective defense, often

followed by ion fluxes, the production of reactive oxygen species, nitric oxide, and increased expression of defense-associated genes, including those encoding cationic peptides. An enhancement of immune responses by some cationic peptides has been noticed in animals (Hancock and Diamond, 2000; Chernysh et al., 2002). The cationic peptides are reported to be involved in many aspects of innate host defenses; acute inflammation, recruitment of T-cells, stimulation of chemokine production and the proliferative response of T-helper cells (Falla et al., 1996). For example, synthetic alloferon stimulated natural cytotoxicity of human peripheral blood lymphocytes and induction of interferon synthesis in mouse and human models (Chernysh et al., 2002). LL-37 and several other defensins, isolated from neutrophils, show chemokine-like activities and recruitment of immune cells (Yang et al., 1999; Bowdish et al., 2004). Indolicidin and other cationic peptides obtained from neutrophils and other sources may also play a similar role in innate immune responses in animals.

The exact mechanism or pathway for the resistance in plants expressing cationic peptides remains unknown. Recent studies have shown that MAP kinases are a critical component of defense signaling in plants (Asai et al., 2002). Based on the observations that LL-37 induces activation of extracellular signal-regulated kinase pathways in monocytes (Salzet, 2002), similar effects of cationic peptides on defense signaling pathways and other components of innate immunity in transgenic plants cannot be ruled out.

The structural and functional properties of the potential antiviral cationic peptides, polyphemusin and indolicidin, used in this work are discussed below.

1.1.2.8. Antiviral cationic peptide 'Polyphemusin'

Polyphemusins I and II, and their isopeptides (tachyplesins I to III), are cationic peptides found in the hemolymph of the Japanese horseshoe crab species *Limulus polyphemus* and *Tachyplesus tridentatus* respectively. They form antiparallel β -sheets, stabilized by disulphide bridges (Miyata et al., 1989), and have displayed activity against both fungi and viruses, including vesicular stomatitis virus, Influenza A virus, and Human Immunodeficiency Virus (HIV)-1 (Miyata et al., 1989; Murakami et al., 1991; Nakashima et al., 1992; Allefs et al., 1996; Murakami et al., 1999). Therefore, these arthropod peptides are of special pharmaceutical interest as potential therapeutic agents. The amino acid sequence of polyphemusin is RRYCYRKCYKGYCYRKCR. Among the arthropod peptides, only the secondary structure of tachyplesin I has been determined by NMR spectroscopy (Kawano et al., 1990). It was found to have a fairly rigid planar conformation consisting of an antiparallel, β -sheet structure, constrained by two disulfide bridges and connected by a type II, β -turn. In this planar conformation, five bulky hydrophobic side chains are located on one side of the plane, and six cationic side groups are distributed at the "tail" of the molecule. Although the NMR structure was not determined for polyphemusins, they have been proposed to form a similar structure to tachyplesin I with a similar distribution of positively charged residues due to their extensive sequence identity (Kawano et al., 1990). Like naturally occurring antimicrobial peptides, polyphemusins and tachyplesins are polycationic and amphipathic with an amidated C-terminus. These properties have been implicated in the mode of action and toxicity of tachyplesin I (Park et al., 1992).

Numerous studies of the antiviral action of this group of peptides against HIV-1 have

been carried out (Katsu et al., 1993; Tamamura et al., 1993; Tamamura et al., 1998; Arakaki et al., 1999). However, few studies have focused on the antimicrobial mechanism. Limited data have indicated that, at high concentrations (>100-fold the inhibitory concentration), these peptides cause morphological and permeability changes of bacterial cells and human erythrocytes and increase the K^+ permeability of bacterial cells, thereby reducing cell viability (Ohta et al., 1992; Katsu et al., 1993).

Development and characterization of polyphemusin analogs on the basis of rational design and their mechanism of action have been described (Zhong et al., 1994; Zhang et al., 2000). Three structural variants (PV5, PV7, and PV8) of polyphemusin I were designed with improved amphipathic profiles (Zhang et al., 2000). Circular dichroism analysis indicated that polyphemusin I and variants displayed the spectrum of a type II, β -turn-rich structure, but, like polyphemusin I, all three variants adopted a typical β sheet structure in an anionic lipid environment. Both polyphemusin I and variants are potent broad-spectrum antimicrobials and are bactericidal at their minimal inhibitory concentrations. All the variants show similar abilities to bind to bacterial lipopolysaccharide (LPS) and permeabilize bacterial outer membranes, while the hemolytic activity of the variants is 2-to 4-fold less than the parent molecule. The increase of amphipathicity of polyphemusin I generally resulted in variants with decreased activity on membranes and improved activity against bacteria (Zhang et al., 2000).

1.1.2.9. Antiviral cationic peptide ' Indolicidin'

Indolicidin, a 13-amino acid long cationic peptide rich in tryptophan and proline, (Schibli

et al., 2002) is present in the cytoplasmic granules of bovine neutrophils (Selsted et al., 1992). As a naturally occurring peptide, indolicidin, has a unique composition consisting of 39% tryptophan and 23% proline, with a generalized amino sequence of ILPWKWPWWPWR, and is naturally amidated at the carboxy terminus. Indolicidin is the smallest of the known naturally occurring linear antimicrobial peptides, contains the highest percentage tryptophan of any known protein, contains a high percentage of hydrophobic residues, and consists of only six different amino acids. Due to the distribution of proline and tryptophan residues throughout the indolicidin sequence, it may assume a structure distinct from the well-described helical peptides. It has a secondary structure of a poly-L-proline II-like (extended) helix when inserted into membranes. Indolicidin has shown *in vitro* inhibitory activity against Gram-negative and Gram-positive bacteria (Selsted et al., 1992; Falla et al., 1996; Friedrich et al., 2001), protozoa (Aley et al., 1994), fungi (Ahmad et al., 1995) and viruses (Hancock, R.E.W, personal communication; Robinson et al., 1998; Yasin et al., 2000). In addition, the peptide is cytotoxic to rat and human T-lymphocytes (Schluesener et al., 1993) and lyses erythrocytes (Ahmad et al., 1995).

The mechanism of action of indolicidin against Gram-negative bacteria has been established (Falla et al., 1996). The peptide binds to surface lipopolysaccharide (LPS) with a high affinity, resulting in self-promoted uptake across the outer membrane and subsequent channel formation in the cytoplasmic membrane, causing cell death. Due to its relatively small size and broad spectrum of antimicrobial activity, indolicidin has been suggested as a candidate for therapeutic use and has been used successfully in a mouse antifungal infection model (Ahmad et al., 1995).

Modification of indolicidin to increase the factors involved in its mechanism of action, led to the development of molecules with increased antimicrobial activity. The development and characterization of indolicidin analogues developed on the basis of rational design, mechanism of action and reduced toxicity were performed (Falla and Hancock, 1997). Modifications to increase the overall charge and amphipathic character of indolicidin resulted in a molecule, CP-11, which exhibited reduced toxicity to erythrocytes and broad-spectrum antimicrobial activity with increased activity against gram-negative bacteria (Subbalakshmi and Sitaram, 1998; Friedrich et al., 2001). 10R and 11R, two indolicidin variants used in this study were also developed earlier with increased antimicrobial activity (R.E.W Hancock, personal communication).

1.1.3. Cationic antimicrobial peptide and cytotoxicity

Plant genetic engineering raises the questions of environmental impact of transgenic plants and their potential toxicity or allergenic risks to humans and animals when consumed. For strategies involving the expression of peptides not normally belonging to the human or animal diet or not derived from plants, a thorough evaluation of toxicity is necessary (Franck-Oberaspach S. L., 1997). The main question concerning environmental impact is the potential effect on the natural micro-flora (e.g. the endophytic and epiphytic bacterial populations, and rhizosphere micro-flora and plants themselves), which may be affected by the expression of the transgene. This is particularly relevant for strategies with a wide spectrum of activities against different pathogens, such as those using antimicrobial peptides. Cytotoxicity of known and characterized cationic peptides has been tested against animal cells but new methods need to be developed to test their

cytotoxicity in plants. Also, before antimicrobial peptides can be introduced and expressed in plants, it is necessary to first evaluate their biosafety and cytotoxic effects, if any, on individual plant cells, tissues and even on whole plants. Also their *in vitro* activity spectrum against plant pathogens should be known. To measure the cytotoxicity of compounds against living cells during host-pathogen interaction or stress conditions, cell viability or cell death is an important variable to monitor, as mentioned above. Available methods can be modified to assess the effects of peptides against plants and plant pathogens.

1.1.3.1. Cytotoxicity assessment of peptides against plants and pathogens

Colorimetric assays have been designed for plants to identify cell viability under stress and in response to treatment with different compounds (Steponkus and Lanphear, 1967; Towill and Mazur, 1974; Mosmann, 1983; Borenfreund and Puerner, 1985; Baker and Mock, 1994; Able et al., 1998; Capasso et al., 2003). Cell viability assays are based either on the physical properties of cells (such as cytoplasmic streaming and membrane integrity), or on biochemical characteristics (such as DNA, RNA or protein synthesis) (Duncan and Widholm, 1990). Membrane integrity assays rely on the active uptake of dyes, such as neutral red or fluorescein by viable cells, or the passive staining of the contents of dead cells by dyes such as Evans blue (Baker and Mock, 1994) and trypan blue (Konopka et al., 1996) that leak through ruptured cells. Assays depending on the activity of the electron transport chain, in which a compound accepts an electron from the chain and in the process becomes reduced, are commonly used in animal systems. Colorless, tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

tetrazolium bromide (MTT), and 2,3,5-triphenyltetrazolium chloride (TTC), are widely used to assess viability of mammalian and plant cells (Lakon, 1949; Parker, 1953; Steponkus and Lanphear, 1967; Towill and Mazur, 1974; Duncan and Widholm, 1990; Caldwell, 1993; Badini et al., 1994; Khatun and Flowers, 1995; Voytik-Harbin et al., 1998; Duncan and Widholm, 2004). Reduction of TTC measures the overall oxidative metabolic processes of the cell. Evans blue has been used to determine cell death in microscopic studies (Taylor, 1980; Smith et al., 1982) as well as in leaf discs (Baker and Mock, 1994). Neutral red is a intracellular pH indicator useful for testing the overall viability of plant protoplasts (Fernandez et al., 2000).

The high sensitivity of fluorometric methods has resulted in the use of fluorogenic substrates, such as 4-methylumbelliferiyl heptanoate (MUH), to estimate proliferation and cytotoxicity in animal cells (Nociari et al., 1998). However, MUH has drawbacks including background fluorescence and insolubility. AlamarBlue™ is a tetrazolium-based dye, incorporating resazurin and resorufin as oxidation–reduction indicators that yield colorimetric changes and a fluorescent signal in response to metabolic activity; that occurs intracellularly involving reductases and the mitochondrial electron transport (Page et al., 1993). The blue non-fluorescent oxidized form becomes pink and fluorescent upon reduction (De Baun and De Stevens, 1951; Page et al., 1993). In the past, the AlamarBlue™ assay has found application in the analysis of viability and/or proliferation of bacteria (Baker and Mock, 1994), fungi (Pfaller et al., 1994; Tiballi et al., 1995; Farnia et al., 2004) and several normal and cancerous cell lines (Aley et al., 1994; Ahmad et al., 1995; Nakayama et al., 1997), including, corneal endothelial cells (Larson et al., 1997), neuronal cells (White et al., 1996) and oligodendrocytes (Back et al., 1999).

AlamarBlue™ has been used for specialized purposes such as the screening of anti-bacterial drugs (Alley et al., 1988; Collins and Franzblau, 1997) and cell-mediated cytotoxicity (Nociari et al., 1998). These colorimetric and fluometric assays can be modified and can be used to determine the cytotoxicity of the cationic peptides on plant pathogens and plant cells.

1. 2. Rationale and objectives

Expression of different cationic peptides in plants has resulted in the generation of disease-resistant transgenic plants. Polyphemusin and indolicidin are two important cationic peptides. These peptides and their structural variants have demonstrated *in vitro* antibacterial and antiviral activities against animal pathogens. These peptide(s) and their variants were selected as the model peptides in this study. The long-term goal is to tap into and exploit the potential of these cationic peptides to incorporate broad-spectrum pathogen resistance in plants. The intent of this project is to:

- (i) Test *in vitro* antimicrobial activities of polyphemusin and indolicidin variants against plant pathogens.
- (ii) Evaluate any cytotoxic effect of these peptides variants on plants.
- (iii) To express these peptides, in tobacco for resistance against microbes, including viruses and in *Arabidopsis* to study the role of cationic peptides in disease resistance in transgenic plants.

This investigation will help in developing effective environmentally friendly strategies to combat the growing threat of plant pathogens.

CHAPTER 2: MATERIALS AND METHODS

2.1. Peptides

The peptides used in this study were the synthetic indolicidin derivatives, 10R (MRRPWKWPWWPWR) and 11R (MRWKKWPWWPWRK), and modified synthetic derivative of polyphemusin, PV5 (MRRYCYRKCYKGYCYRKCR). Peptides 10R and 11R were synthesized using a model 430A peptide synthesizer (Applied Biosystems Inc, Foster City, CA) with the 0.25 mmol scale FastMoc chemistry software at the University of Victoria Genome BC Proteomics centre. PV5 was a gift from Dr. R.E.W Hancock, University of British Columbia, Vancouver, Canada. Lyophilized peptide powders (> 90% purity) were reconstituted in sterile, deionized water to a stock concentration of 1 mg/ml, filtered through a 0.2 μm Millipore filter and stored at $-20\text{ }^{\circ}\text{C}$ until further use.

2.2. Plant material

Tobacco (*Nicotiana tabacum* var. Xanthi) seeds were surface sterilized in 50% industrial bleach (6% hypochlorite) for 20 minutes at room temperature, followed by 3 rinses in sterile dH_2O . Seeds were placed on MS medium at an 18 h, 28°C day and 6 h, 22°C night cycle with $150\ \mu\text{E}/\text{m}^2/\text{s}$ of light. Seedlings were transferred to magenta boxes (Sigma: V-8505) with MS medium without any hormones. Leaves of non-transgenic plants were used for transformation. Non-transgenic and transgenic plants were transferred to the greenhouse, where they were planted in pots with moistened Sunshine mix # 2 soil (Sun Gro Horticulture, Bellevue, Washington, USA).

Arabidopsis thaliana (L.) Heynh seeds of ecotype Columbia were placed on a moistened Sunshine mix # 3 soil (Sun Gro Horticulture, Bellevue, Washington, USA) on 10 cm diameter pots, covered with nylon screen and then with Saran wrap secured with an elastic band. Pots were placed at 4°C for 2 days to promote uniform germination and then transferred to a growth chamber with an 18 h 24°C day and 6 hour 22°C night cycle with 150 $\mu\text{E}/\text{m}^2/\text{s}$ of light. Saran wrap was removed when plants began to push against its surface.

T₂ Seeds were collected from both non-transgenic and transgenic plants (tobacco and *Arabidopsis*), surface sterilized as described above and selected on MS medium containing kanamycin (50 $\mu\text{g}/\text{ml}$).

2.3. Pathogen cultures

Bacterial strains used in this study were the laboratory isolate of *Erwinia carotovora* and clinical isolates of *Proteus vulgaris*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* (ATCC 10145) and *Escherichia coli* (W 3110) obtained from the microbiology student laboratory, University of Victoria. Fungal pathogens used were *Phytophthora infestans* (isolate US 11 A1), *Botrytis cinerea*, *Fusarium oxysporum* obtained from Simon Fraser University, Vancouver and *Fusarium graminearum* (isolates: M12-04-1), obtained from the Canadian Grain Commission, Winnipeg. The plant viruses used were Tobacco mosaic virus (TMV) (ATCC 135 PV) obtained from American Type Culture Collection, Atlanta, United States, and TMV, PVX and PVY used for green house studies were common isolates used at AAFC, New Brunswick, Canada.

2.4. Protoplast isolation

Tobacco protoplasts were isolated from the mesophyll tissues of the non-transgenic tobacco plant leaves grown in magenta boxes as described by Medgyesy et al. (1980). Leaves were detached, excised with a sterile surgical blade and resuspended in maceration solution of 0.2% (w/v) Cellulase (Sigma, USA), 0.1% (w/v) Driselase (Sigma, USA) in solution I (0.4 M sucrose, 0.1 M glycine, 10 mM CaCl₂, and 10 mM MES (pH 5.6)). After incubation at 24°C for 12 h when over 90% of the cells became round-shaped, the protoplasts were filtered through a 40 µm nylon mesh, washed twice and resuspended in solution I to a density of 2×10^6 protoplasts/ml. After 3 days of cultivation at 24°C in low light ($5 \mu\text{E}/\text{m}^2/\text{s}$), viable protoplasts were quantified using a hemocytometer and adjusted to 5×10^3 /ml with the cultivation medium. Protoplasts were incubated with different concentrations of cationic peptides in a 24 well plate and were maintained at 25°C in dark.

2.5. In vitro antimicrobial activity of the peptides

2.5.1. In vitro antibacterial activity of the peptides

Optimal growth times (when all the bacterial strains were in log phase or stationary phase) were determined for each bacterial strain by incubating 80 µl of bacterial culture (1:25 diluted in LB) with 20 µl of LB in 96 well microtitre plates and measuring A_{630} (Beckman DU 60) for different intervals (0-24 h). To determine antibacterial activity of peptides, reconstituted peptides (40 µg/ml) in a final volume of 20 µl were mixed with a bacterial suspension (80 µl) (grown overnight in Luria-Bertani (LB) broth and diluted 1:25 times) in microtitre plates. Plates were incubated at RT with shaking for 8h. The

inhibitory effect was measured as the change in A_{630} (Beckman DU 60), and the inhibitory effect of peptides was calculated as a function of decrease in absorbance.

2.5.1.1. Minimum bacterial inhibitory concentration of peptides

The minimum inhibitory concentration of peptide derivatives against the plant pathogenic bacteria, *Erwinia carotovora*, was determined by inoculating 2×10^5 bacteria/ml with different peptide concentrations. After 4 h, this mixture of peptide and bacterial culture was plated on LB agar plates and was incubated overnight at 28°C. Bacterial survival was assayed by colony counts for different concentrations of peptide.

2.5.2. In vitro antifungal activity of peptides

2.5.2.1. Fungal growth zone inhibition assay

For assessing *in vitro* antifungal activity, a fungal growth zone inhibition assay was performed. For each culture, an agar plug (11mm) containing fungus was cut from the fungal culture plate and placed in the centre of a Petri dish with potato dextrose agar medium (PDA). Sterile paper discs were placed around the plug and different amounts of peptides were applied. Plates were incubated for 36 h at RT and inhibition was determined by measuring the zone of inhibition of each amount. Distilled H₂O was used as a control.

2.5.2.2. AlamarBlue™ reduction assay

The inhibitory concentration of the peptides against different fungi was determined using an AlamarBlue™ reduction assay and measuring cell proliferation. The fungal cells under

study were grown on PDA (potato dextrose agar) medium at RT until the surface of the agar was covered with the fungus. A sporangia suspension was then prepared by flooding the Petri dish with 5 ml Potato Dextrose Broth (PDB). The sporangia were collected using a sterile pipette and the concentration was adjusted to 1×10^4 spores/ml. To test the susceptibility of fungal pathogens to antimicrobial peptides, 80 μ l of spore cultures were added to different concentrations of peptides to make a final volume of 100 μ l in microtitre plates. Spores were incubated for 24-48 h at RT. To the mixture of the culture and peptide, 25 μ l of AlamarBlue™ (Biosource International, Camarillo, CA, USA) was added and reduction of the dye was assessed by measuring the fluorescence by exciting at 530-560 nm and measuring emission at 590 nm and data was expressed as fluorescence emission intensity units as a function of time of incubation and finally as percentage viability. Controls included were cultures to which no peptide was added, and wells with only saline and AlamarBlue™ and no culture.

2.6. Viral studies

2.6.1. Purification of Tobacco Mosaic Virus

The TMV culture obtained from ATCC was homogenized in 0.5 M phosphate buffer. Leaves of the 1-month-old non-transgenic tobacco plants, grown in the greenhouse were dusted with carborundum powder (600 grit) and the homogenate aliquot applied to the surface of the leaf. Symptoms of TMV infection (lesion and mosaic pattern) were observed within 10 days of inoculation. Leaves were collected from the infected plants 3 weeks post-inoculation and stored at 4°C. Tobacco mosaic virus (TMV) was purified from collected infected tobacco leaves. Twenty grams of leaf tissue was homogenized in

a mortar and pestle with a few grains of acid washed sand. Sixty ml of virion extraction buffer (0.5 M Phosphate buffer (Na_2HPO_4) and 1% (v/v) 2-mercaptoethanol) was added and mixed. The homogenate was filtered through a double layer of cheesecloth, and 0.8 ml of butanol (per 10 ml of filtrate) was added slowly. This was incubated for 15 min at RT and the supernatant was then clarified by low speed centrifugation at 10,000 x g for 30 min. Lightly pigmented aqueous phase was collected and transferred to a fresh tube. PEG 8000 (20%) was added and the tubes were mixed by inversion. The tubes were incubated on ice for 15 min. Virus was pelleted at 10,000 x g for 15 min at 4°C. Six ml of phosphate buffer was added to re-dissolve the pellet. Centrifugation was again performed at 10,000 x g for 15 min and supernatant was transferred to a new tube. PEG (20%) and NaCl were added to the supernatant at a final concentration of 4% (w/v) and stirred well until completely dissolved, and incubated on ice for 15 min. The virus pellet was collected by centrifugation at 10,000 x g for 15 min and resuspended in 3 ml of 10 mM phosphate buffer. The virus was then diluted and optical density readings were taken at 260 nm and 280 nm to determine virus purity and concentration.

2.6.2. Lesion assay for TMV

For assessing the *in vitro* antiviral activity of cationic peptides, the leaves of 4 week-old, non-transgenic tobacco plants grown in the green house, were detached. Half-leaves were randomly selected in each assay trial. Different amount of cationic peptides were mixed with 5 µg TMV and control (only virus and 0.05 M phosphate buffer, pH=8). Samples and control were applied to each half of the detached leaves. Leaves were rinsed with water and placed on dH₂O soaked filter paper in 6" Petri dishes. The samples were then

moved to a growth chamber (photoperiod of 12 h, 25°C) where they were monitored daily for appearance of symptoms. Lesions of 1-2 mm in diameter were counted after 3 days and compared with the control. Infected leaf halves and non-infected halves (control for each sample) were collected after 4 days of inoculation and each half (sample and control) leaf was homogenized to extract leaf sap (as described in next section) and stored at -20°C for ELISA and dot blot assays.

For comparing the antiviral activity of the transgenic tobacco plants with non-transgenic plants, leaves were detached from the green house grown tobacco plants and the lesion assay was performed as described above. The number of lesions was counted on transgenic plants and compared with the lesions on non-transgenic plants (control). Each half leaf was homogenized to extract leaf sap and stored at -20°C as above.

2.6.3. TMV RNA Isolation

The purified TMV preparation was diluted to 10 mg/ml with 10 mM phosphate buffer. Two hundred ml of 5X RNA extraction buffer (0.5M sodium chloride, 5 mM EDTA, 5% (w/v) SDS, 0.1M Tris -HCl) (pH to 8.0) was added to 0.8 ml of diluted virus preparation. One ml of phenol: chloroform (1:1) was added, vortexed briefly until an emulsion formed and then centrifuged at 13,000 x g for 5 min at RT. The upper aqueous phase was collected and transferred to a new tube. This extraction with phenol: chloroform was repeated twice. An equal amount of chloroform was added to the collected aqueous phase, the samples were vortexed, and again the aqueous phase was transferred to a new tube. To each tube, 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol were added. Samples were incubated at -20°C for 15 min. RNA was pelleted by

microcentrifugation at 13,000 x g for 15 min at 4°C. The pellet was dried and dissolved in 0.2 ml of DEPC-water. The yield was measured by measuring absorbance at A_{280} and A_{260} .

2.6.4. TMV Movement protein cDNA synthesis and cloning

Movement protein specific cDNA was synthesized from TMV RNA using a 26-nucleotide primer, designated TMV-RT (5-CTGACCGATCATTACTACTATTTTTC-3) that is complementary to nucleotide residues 5596-5571 in TMV genome. Reverse transcription was performed using superscript II RNase H⁻ Reverse Transcriptase from Gibco (Burlington, Ontario, Canada). PCR amplification of the cDNA was performed using a pair of PCR primers to amplify the movement protein gene of TMV. Primers amplifying nucleotide residues 4635-5445 in the TMV genome [(CATCACG ACAGAGGATGC (+ sense) and CACGTTTGTAATCTTCTC (- sense)] were used. The expected product size was 811 bp. The amplified band was excised and extracted from an agarose gel using a commercial gel purification kit (QIAquick[®] gel extraction kit, Qiagen, Mississauga, ON, Canada). The purified fragment was cloned using the Topo TA Cloning[®] Kit with pCR[®] Vector (Invitrogen, CA, USA). Positive clones were selected and sequenced at the sequencing facility, University of Victoria. Sequence data was matched with known sequence using the NCBI BLASTn search tool, which showed 100% identity with the TMV movement protein gene sequence.

2.6.5. Preparation of tissue extracts for dot blots and ELISA

Leaf extracts from systemically infected, collected and stored (-20°C) tobacco leaf halves (control and infected) from the lesion experiments were obtained by grinding leaf halves in liquid nitrogen. The powder was incubated on ice for 10 min and leaf sap collected by microcentrifugation at 13,000 x g for 25 min. Supernatants were collected in a fresh tube and frozen at -80°C until further use.

2.6.6. Double antibody sandwiched ELISA for TMV

Viral infection quantitation between control and infected samples was performed by double antibody sandwich ELISA with a commercial TMV detection kit (Agdia Inc., Indiana, USA). Sap was diluted 10 times and 100 µl of this diluted sap was used for ELISA. Tests were performed according to the manufacturer's instructions. A_{405} was determined with the aid of an automated microplate ELISA reader (Bio-Tek, Burlington, VT). A reaction was considered positive if the absorbance was high in the positive control provided by the manufacturer.

2.6.7. Dot Blot hybridization for TMV quantitation

A piece of Biodyne B nylon membrane (Pall) was cut and marked into 1 x 1 cm squares with a pencil. Five µl of the leaf extracts from samples and control were spotted onto the marked squares. Membranes were then baked at 80°C under vacuum for 1.5 h. Movement protein cDNA, cloned earlier was used as a probe. The DNA was labeled with [³²P]-dCTP using the random-primed procedure (Invitrogen) and used at 4x10⁶ cpm of purified probe in 1 ml of hybridization solution. Pre-hybridization and hybridization steps were

carried out at 65°C for 2h and 16h, respectively in PerfectHyb™ Plus buffer (Sigma) according to the manufacturer's protocol. Blots were then incubated in hybridization bottles containing 2 x SSC and 0.1% SDS solution for 20 min at 65°C followed by final wash in 0.1 x SSC, 0.1% SDS at 65°C for 10 min, and then exposed to X-ray film (Kodak BioMax) overnight at -80°C using an intensifying screen.

Dot blot signal quantification was done by measuring CPM (counts per minute) for each dot. Each square was cut and was suspended in scintillation cocktail, and radioactivity measured in scintillation spectrometry (Beckman LS 5000CE).

2.6.8. Greenhouse studies on transgenic plants for virus resistance

Greenhouse antiviral activity testing on the transgenic tobacco plants against TMV, PVX and PVY was performed at AAFC, Fredericton NB by Dr. R. Singh. Four plants of each transgenic line of each construct and non-transgenic plants (control) were inoculated with each virus. Each plant was inoculated with virus containing sap on three fully developed leaves and virus symptoms were compared. For TMV, development of lesions on susceptible leaves, for PVX, ring spot lesions and for PVY, strong vein clearing and necrotic veins symptoms in susceptible tobacco plants were observed.

2.6.9. Electron microscopy

TMV (5µg) was either incubated with 100 µg of peptide overnight at RT or only with dH₂O. Ten µl of preparations were added to Formvar (0.5%) coated 400-mesh copper grids, and negatively stained in 1% aqueous uranyl acetate (UA) or 0.5% phosphotungstic acid (pH 7.0). Grids were observed at 75kV on transmission electron microscope

(Hitachi-7000) at the EM facility, University of Victoria.

2.7. Cytotoxicity Assays

2.7.1. 2,3,5-Triphenyltetrazolium Chloride Reduction (TTC) assay

Leaf discs 11 mm in diameter were cut with a cork borer from the second and third expanded leaves and washed with dH₂O. Each disc was weighed, washed twice with dH₂O and then placed in different amount of peptides ranging from 0-100 µg, in dH₂O (control), Round-up™ (positive control) or carbenicillin (-ve control), in 24 well plates. The TTC assay for leaf discs and plantlets was performed according to Chang et al. (1999) with some modifications. Leaf discs or plantlets were placed in test tubes and 2 ml of TTC reagent [0.6% (w/v) TTC (Sigma T-8877), 0.05% (w/v) Tween 80, 50 mM sodium phosphate, pH=7.4] were added and infiltrated under vacuum, then incubated at 28 °C in darkness for 12 h. Four ml of 95% ethanol was added and boiled for 5 min to extract the formazan. The samples were centrifuged at 1000 x g and A₅₃₀ measured in Beckman DU-65 spectrophotometer. Viability was expressed as A₅₃₀. For the TTC assay for protoplasts, 1 ml of TTC reagent was added to 1 ml of protoplast preparation (5000 protoplasts) and infiltrated under vacuum for 1.5 h. Tubes were then incubated at 28 °C in the dark. Ethanol (2 ml) was added to each tube after incubation and samples were boiled to extract the formazan formed. Tubes were centrifuged at 1000 x g and 100 µl of the supernatant was transferred to a 96 well plate for measurement of A₄₉₀ using a plate reader (Elx808 Ultra Microplate Reader, Bio-Tek Instruments Inc.). Viability was expressed as A₄₉₀ / 5000 protoplasts.

2.7.2. Evan's Blue assay

The Evans blue assay was performed on leaf discs as described by Baker and Mock (1994). Leaf discs were incubated in different amount of peptides, distilled water or controls. Evan's blue solution (0.25% aqueous solution) was added to the incubated discs and these were ground using a glass pestle. Samples were centrifuged at 13,000 x g for 15 min. Supernatants were collected and absorbance was measured at 600 nm (Beckman DU-65).

2.7.3. Neutral Red assay

Protoplast viability was measured by staining protoplasts with neutral red (100 mg of neutral red dissolved in 100 ml of dH₂O, 200 µl of 1% acetic acid, and filtered). Two µl of neutral red were added to 20 µl of protoplast culture (5×10^3 protoplasts/ml) and treated either with peptide or with dH₂O. After incubation for 20 min at RT, viable protoplasts were counted by microscopy using a hemocytometer. The stained fractions of living protoplasts were calculated and percentage viability expressed accordingly.

2.7.4. Phytotoxicity assay

To assay the phytotoxicity of peptides on plantlets, axillary buds of tobacco plants were grown in the presence of peptides and assayed according to Ali and Reddy (2000), with some modifications. Different amount of each peptide were mixed with 1 ml of Murashige-Skoog (MS) medium with 0.6 % agarose and poured in 1.5 ml sterile Eppendorf tubes. As controls, peptides were replaced by either different amount of carbenicillin (Invitrogen), Round-up™ (glyphosate isopropylamine: Monsanto Canada

Inc, Mississauga, ON, Canada; amount of glyphosate was calculated as μg and was used in the assays) or dH_2O water. One axillary bud from each 2 week-old tobacco plant grown in magenta boxes, was weighed, measured in height and placed in Eppendorf tubes with the appropriate concentration of peptide or controls (water, carbenicillin (-ve control) and Round-up™ (positive control)). These Eppendorf tubes were then placed in 0.8% agar-containing culture tubes (100 x 2.5cm), and placed at 25°C. The plantlets were grown for 4 weeks and used for the TTC assay. The phytotoxicity of peptides on intact plantlets was also tested by observing growth parameters like shoot length and fresh weight.

2.7.5. Chlorophyll and carotenoids estimation

Leaf tissue (weighed for fresh weight) was pulverized in ice-cold 80% acetone with a mortar and pestle and extracted twice with acetone. The liquid was collected in a test tube. The extraction was repeated three times until the pellet was colorless. The combined acetone extracts were cleared by centrifugation and the volume was adjusted to 5 ml. Absorbance at 663 nm, 646 nm and 470 nm was determined for each sample (Beckman DU-60) and used for calculation of the total contents of Chlorophyll *a*, *b* and carotenoids according to the method of Lichtenthaler and Wellburn (1983). Concentrations were correlated first with the volume extracted then with the fresh weight of the leaf samples taken. The equations used for final calculations were: Chlorophyll-a = $12.21 (A_{663}) - 2.81 (A_{646}) \mu\text{g/ml}$; Chlorophyll b = $20.13 (A_{646}) - 5.03 (A_{663}) \mu\text{g/ml}$; Chlorophyll-total = Chlorophyll-a + Chlorophyll-b $\mu\text{g/ml}$ and Carotenoids = $(1000 (A_{470}) - (3.27 \text{ Chlorophyll-a} + 104 \text{ Chlorophyll-b}))/198 \mu\text{g/ml}$.

2.8. Expression of cationic peptides in plants

2.8.1. Construction of cationic peptide genes and DNA manipulation

Gene specific overlapping synthetic oligonucleotides were designed for the construction of PV5, PV8, 10R and 11R genes. These were based on the nucleotide sequences of the four peptides, determined by reverse-translation of the amino acid sequences using plant specific codons and modifying them by adding specific restriction enzyme sites for DNA cloning and expression. The genes were assembled by PCR using oligonucleotides shown in Figure 16.

DNA manipulations were carried out as described. To place the cationic peptide genes under a highly expressed promoter (2X35S) and leader sequence, their genes were first cloned into the vector pBI524. Further, to make the genes ready for transformation using *Agrobacterium tumefaciens*, the promoter and gene were cloned into pBI121 (work carried out by Dr. Benjamin Forward and completed by Dr. Milan Osusky, University of Victoria, BC Canada). The synthetic genes encoding PV5 and PV8 had NcoI and BamHI sites at their 5' and 3' ends respectively. Similarly, genes for 10R and 11R had XbaI and BamHI sites at 5' and 3' ends. These were first cloned in vector pBI524 and later digested with HindIII and EcoRI enzymes [pBI524 contains a duplicated enhancer cauliflower mosaic virus (CaMV) 35S promoter and an untranslated leader sequence from alfalfa mosaic virus (AMV) (RNA4) that acts as a cis active "translational enhancer"]. The HindIII-EcoRI fragment, containing a 2x35S promoter with an AMV RNA4 translation-enhancing element and PV5/PV8/10R/11R with a NOS terminator (NOS-ter) was ligated in to HindIII-EcoRI digested pBI121 vector (thus replacing the 35S promoter and the β -glucuronidase (GUS) gene with the NOS-terminator), yielding

the final sequence of PV5, PV8, 10R and 11R.

The *msrA3* gene used for *Arabidopsis* transformation was constructed previously as described (Osusky et al., 2004). Transformed *Agrobacterium* culture was used for *Arabidopsis* transformation.

2.8.2. Plant transformation, regeneration and selection of transgenic lines

2.8.2.1. Tobacco

Each of the recombinant plasmids were transferred from *E. coli* DH5 α cells into *Agrobacterium tumefaciens* MP90 by the freeze-thaw method (Holsters et al., 1978).

Young leaves of tobacco plants were surface-sterilized in 1% (v/v) NaHClO₃ for 2-5 min. and rinsed well with sterile water. An *Agrobacterium tumefaciens* MP90 culture harboring each of the vectors was grown for 1–2 days at 26–28 °C on a rotary shaker at 225 rpm to mid-log phase ($A_{600}=1$) in 15 ml liquid LB medium (Saambrook et al., 1989) supplemented with 100 mg/l kanamycin, and 10 mg/l gentamycin. Leaf discs were co-cultivated with this *A. tumefaciens*, blotted with sterile filter paper to remove excess bacteria, and placed horizontally on Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) and incubated for 2 d at 25°C. The co-cultivated leaf discs were then transferred to a shoot-inducing medium (MS medium containing 0.01 μ g/ml NAA, 1 μ g/ml BAP, 100 μ g/ml kanamycin and 500 μ g/ml carbenicillin). Regenerated calli were obtained after this selection. Young shoots were transferred to a root-inducing medium (MS medium containing 100 μ g/ml kanamycin and 200 μ g/ml carbenicillin). Regenerated plants were tested for the presence of chimeric gene constructs using PCR amplification of genomic DNA. The transgenic tobacco plants were then transferred to soil and

developing T₂ seeds were collected.

2.8.2.2. *Arabidopsis*

Arabidopsis thaliana plants were transformed according to the method of Clough and Bent (1998). *Agrobacterium tumefaciens* strain MP90 carrying the plasmid CaMV35S::MsrA3, was grown to stationary phase in liquid culture at 28°C, 250 rpm, in sterile LB broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.0) containing 50 µg/ml kanamycin and 10 µg/ml gentamycin. Cells were harvested by centrifugation for 20 min at RT at 5500 x g then suspended in infiltration medium [5.0% sucrose and 0.05% Silwet L-77 (Lehle Seeds, Round Rock, Texas, U.S.A.)] to a final A₆₀₀, of approximately 0.8 prior to use. The above ground portions of plants were dipped in infiltration medium containing *Agrobacterium* for 10 s with gentle swirling 2 days after removal of the primary bolt. One subsequent dip was made 7 days later. Following each dip, the plants were covered with a plastic bag for 24 h to retain moisture. Plants were no longer watered once seed-pods began turning brown. When plants were fully dried they were placed in a brown paper bag for 1 week prior to collecting seeds. Seeds were collected by rubbing plants and pods and filtering through sieve several times until seeds were free of debris. Seeds were sterilized using vapor phase sterilization as follows (Clough and Bent, 1998): Seeds were placed in 15 ml conical tubes (2-3 ml seeds per tube) with lids attached loosely. Tubes were placed in a rack inside a plastic vacuum desiccator (Bel-art #42025, 240 mm internal diameter) containing a 250 ml glass beaker with 150 ml bleach (5.25% NaHClO₃). Five ml of concentrated HCl was placed in a 10 ml glass beaker and floated on top of the bleach solution. The lid was placed on the desiccator and a slight vacuum applied. The desiccator was shaken slightly to spill the concentrated HCl into the bleach

and liberate chlorine gas for overnight sterilization. Sterile seeds were sprinkled on 150 x 15 mm selection plates (1/2 MS media, 0.8% agar, 1% sucrose, 50 g/ml kanamycin, 100 µg/ml ampicillin) and placed in dark at 4°C for 2 days. Plates were removed and placed in a growth chamber with 16 h light / 8 h dark at 22°C for 2 weeks. Healthy green transformants were selected and placed in moist soil in a growth chamber with an 18 h 24°C light/ 6 h dark covered with Saran wrap for the first 2 days of growth.

2.8.3. Molecular analysis

2.8.3.1. Genomic DNA and Total RNA extraction

Total Genomic DNA was extracted from the leaves of transformed and wild type plants using a GenElute™ Plant Genomic kit (Sigma). One hundred mg of leaves were ground in liquid N₂ and DNA was isolated according to the manufacturers instructions. Total leaf RNA was prepared using TRIzol (GIBCO BRL, Rockville, MD, USA) following the manufacturer's protocol.

2.8.3.2. Polymerase Chain reaction (PCR)

2.8.3.2.1. Tobacco

PCR was carried out in 50 µl reaction mixtures containing 200 ng of plant DNA, Taq PCR Master Mix (Qiagen, Mississauga, Ontario, Canada) and specific primers, with manual hot start and the following parameters: 94°C for 3 min, then 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s, followed by a final 10 min incubation at 72°C were used. Primers for plants with the PV5, PV8, 10R and 11R constructs that amplified the appropriate genes were: forward primer, the primer with sequence from base 746-765

in CaMV35S promoter 5'-CCTTCGCAAGACCCTTCCTC-3', 20-mer and reverse primers were gene specific primers 5'-GGATCCTTTATCTGCACTTTC-3', 21-mer for PV5 and PV8; 5'-GGATCCTTTATCTTCTCC-3', 18-mer for 10R and 5'-GGATCCTTTACTTTCTTC-3', 18-mer for 11R.

2.8.3.2.2. Arabidopsis

The primers used in PCR amplification of the *msrA3* gene were gene specific primers: forward primer 5'-ATGTTTCTGCCCTAATCGGGAGGGTTCTCTCGGGAATCC TGTA-3', 45-mer and reverse primers 5' CATCGCAAGACCGGCAACAGA-3', 21-mer, 51 nucleotides downstream from *SacI* site in pBI 121. The expected product was 143 bp (Osusky et al., 2004). Parameters used in PCR cycles were similar used in tobacco PCR as described above for tobacco.

2.8.3.3. Reverse Transcription PCR

2.8.3.3.1. Tobacco

PV5, PV8, 10R and 11R mRNA expression was determined by RT-PCR from the total RNA isolated from control and transgenic plants. Primers used in PCR amplification of PV5 and PV8 gene were gene specific primers with forward primer 5'-CCATGGCTCTTGCAGCTTTC-3', 20-mer and reverse primer 5'-GGATCCTTTATCTGCACTTTC-3', 20-mer; of 10R were forward primer 5'-ATGAGAAGACCTTGGAAGTG-3', 20-mer and reverse primer 5' CTTTATCTTCTCCAAGGCC-3', 19-mer; and of 11R forward primer were 5'-ATGAGATGGAGAAGATGGC-3', 19-mer and reverse primer 5' CTTTACTTTCTTCTCCAAGG-3', 20-mer. The PCR was performed using the *Taq* PCR Master Mix kit from Qiagen (Mississauga, Ontario,

Canada). Reverse transcription was performed using SuperScript II RNase H⁻ Reverse Transcriptase from Gibco (Burlington, Ontario, Canada) with oligo dT from Boehringer Mannheim (Mannheim, Germany) as a primer.

2.8.3.3.2. Arabidopsis

MsrA3 mRNA expression was determined by RT-PCR from the total RNA isolated from control and transgenic plants. Primers used in PCR amplification of gene *msrA3* were gene specific with forward primer 5'-ATGTTTCTGCCCCTAATCGGGAGGGTTCTCTCGGGAATCCTGTAA-3', 45-mer and reverse primers 5' CATCGC AAGACCGGCAACAGA-3', 21-mer, 51 nucleotides downstream from *SacI* site in pBI 121. Expected product was 143 bp. The PCR was performed using the *Taq* PCR Master Mix kit from Qiagen (Mississauga, Ontario, Canada). Reverse transcription was performed using SuperScript II RNase H⁻ Reverse Transcriptase from Gibco (Burlington, Ontario, Canada) with oligo dT from Boehringer Mannheim (Mannheim, Germany) as a primer.

2.8.3.4. Southern analysis

Four µg of tobacco DNA from each line were digested with 200 U *EcoRI* restriction enzyme for 26 h in a 200 µl reaction mix, concentrated and electrophoresed on a 1% (w/v) agarose gel. The gel was incubated at RT with shaking in denaturing solution (0.5 M NaOH, 1 M NaCl) for 30 min and rinsed in distilled water for 30 min. The gel was transferred to a Biodyne B nylon membrane (Pall) by the alkaline DNA transfer method using alkaline transfer buffer (0.4 N NaOH) for 18 h. The membrane was dried on a

vacuum gel drier or 30 min. Probes were labeled with [³²P]-dCTP using the random-primed procedure (Invitrogen) and used at 2x10⁶ CPM of purified probe in 1 ml of hybridization solution. Pre-hybridization and hybridization steps were carried out at 65°C for 2 h and 16 h, respectively in PerfectHyb™ Plus buffer (Sigma) according to the manufacturer's protocol. Blots were then incubated in hybridization bottles containing 2 x SSC and 0.1% SDS solution for 20 min at 65°C followed by final wash in 0.1 x SSC, 0.1% SDS at 65°C for 10 min, and then exposed to X-ray film (Kodak BioMax) overnight at -80°C using an intensifying screen. The membrane was also analyzed with a PhosphorImager Screen (Molecular Dynamics, USA).

2.8.3.5. Northern analysis

Thirty µg of total RNA was subjected to electrophoresis on 1% agarose-formaldehyde gels. The gel was neutralized by soaking in 2 x SSC for 10 min. The gel was transferred onto Biotodyne B nylon membrane (Pall) using the capillary method and 20 x SSC as transfer buffer. RNA was fixed by baking at 80°C in a vacuum drier for 30 min. Probes (gene specific) were labeled with [³²P]-dCTP using the random-primed procedure (Invitrogen). Pre-hybridization and hybridization steps were carried out at 65°C for 2 h and 18 h, respectively in PerfectHyb™ Plus buffer (Sigma) according to the manufacturer's protocol. Blots were then incubated in hybridization bottles containing 2 x SSC and 0.1% SDS solution for 20 min at 65°C followed by final wash in 0.1% SSC, 0.1% SDS at 65 °C for 10 min, the membrane was exposed to a PhosphorImager Screen (Molecular Dynamics).

2.9. *In planta* disease assays

2.9.1. Antibacterial activity

2.9.1.1. Antibacterial activity of plant extracts

Leaf extracts from transgenic and non-transgenic (control) tobacco leaves were obtained by grinding in liquid nitrogen. The powder was incubated on ice for 10 min, leaf sap was collected by microcentrifugation at 13,000 x g for 25 min, and the supernatant collected in a fresh tube. Plant extracts (20 μ l) were mixed with a bacterial suspension (80 μ l) (grown overnight in Luria-Bertani (LB) broth and diluted 1: 25 times) in 96-well plates and incubated at RT for different time periods (0-24 h). The inhibitory effect was measured as change in A_{630} (Beckman DU-65) and the survival was calculated.

2.9.1.1.1. Antibacterial activity of plant extracts with protease inhibitor

Leaf extracts from transgenic and nontransgenic tobacco leaves were weighed and extraction buffer was added in a ratio 1:5 (w/v). The extraction buffer either had a protease inhibitor cocktail [10 μ l of protease inhibitor cocktail (Sigma; P 9599) in 100 μ l of 0.05M phosphate buffer as per manufacturer's recommendation], or no protease inhibitor cocktail (control). The final extract was collected by microcentrifugation at 13,000 x g for 25 min. The supernatant was collected in a fresh tube. Extracts (50 μ l) were mixed with a bacterial suspension (50 μ l) (grown overnight in Luria-Bertani (LB) broth and diluted 1: 25 times) in 96-well plates and incubated at RT for 4 h. The inhibitory effect was measured as the decrease A_{630} and compared with the samples with protease inhibitor and samples without protease inhibitor as a control.

2.9.1.2. Antibacterial activity of detached leaves from transgenic plants

An overnight culture of bacteria was diluted to a concentration of about 1×10^7 cells/ml. An aliquot of 10 μ l of this suspension was applied onto a tobacco leaf surface followed by piercing with a needle as a way of inoculating bacteria into the leaf. The inoculated leaves were kept at RT. After 7 days of incubation, chlorotic haloes had formed around the inoculated points. Comparison of the area of the chlorotic haloes was the criterion used to evaluate disease resistance in transgenic plants versus non-transgenic plants (control). Pictures were taken by digital camera (Nikon Coolpix 990).

2.9.2. Fungal studies

2.9.2.1. Antifungal activity of detached leaves from transgenic plants

Transgenic and non-transgenic tobacco plants were grown in a greenhouse at 26°C under natural and artificial light (15/9 h photoperiod, 200 mE/m²/s). Leaves were collected from 2-month-old (6-8 leaves developed) plants. Similarly positioned leaves (counted from the bottom) from same age plants were detached and placed in Petri plates with moist filter paper discs. There were two inoculation trials with two replicates arranged in a randomized manner for each trial. Fungal strains were cultured on PDA medium. Plugs of inoculum were prepared by cutting fungal mycelium with a cork borer from a culture plates. The plugs were placed mycelial side down on adaxial side of each plant in the centre on the mid vein. The inoculated leaves were then placed in diffused light at RT. The extent of lesion was recorded at the appropriate time, and the difference in the non-transgenic and transgenic plants was observed. Pictures were taken with a digital camera (Nikon Coolpix 900).

2.10. Cationic fractions from transgenic plants

2.10.1. Purification of cationic fractions from plants

Cationic proteins/peptide fractions were isolated from non-transgenic (control) and transgenic plants. The total tobacco cationic protein preparations were obtained by extraction with 0.5N HCl and acetone. Young leaves (250 mg) were frozen in liquid N₂ and 0.5 ml of 0.5N HCl was added and homogenized. The mixture was centrifuged at 13000 x g for 10 min and the supernatant was aliquoted as 200 µl samples in Eppendorf tubes. Acetone (1.2 ml) was added to each Eppendorf, mixed by inversion and incubated overnight at -20°C. Samples were centrifuged at 13000 x g for 10 min, the supernatant was discarded and the pellet was dried in a vacuum. One hundred µl of urea/formic acid mixture (2.5 M urea, 30 mM formic acid) was added to each tube with pellet, mixed with vortexing and centrifuged at 13000 x g. Supernatants were combined, filtered through a column filter and analyzed by acetic acid-urea (AU) 15% PAGE. Two ml of filtered supernatant was concentrated using a speed vacuum drier to a final volume of 500 µl.

2.10.2. High Performance Liquid Chromatography of isolated cationic fractions

The cationic filtered and concentrated supernatants from both transgenic and non-transgenic plants were fractionated by reverse phase high performance liquid chromatography (RP-HPLC) using a Vydac C₁₈ column (2.6 x 25 cm; Vydac Separations Group, USA). The separation was performed on Beckman System Gold equipped with chromatograph, programmable solvent pump module 126 and diode array detector

module 168. Proteins were eluted with a linear gradient of 0-60% solvent B (0.1% (v/v) TFA (trifluoroacetic acid) in 100% acetonitrile) in solvent A (0.1% TFA) at a flow rate of 1 ml/min for 110 min and collected every min. The peaks were detected by reading the absorbance at 230 nm. The hydrophilic fractions were collected manually, lyophilized, redissolved in dH₂O and analyzed on UA gel.

2.10.3. Characterization of peptide fractions

Masses and purity of the fractions of interest were checked by mass spectrometry. One hundred μ l of the HPLC fraction was dried by speed vacuum and sent for amino acid analysis, MALDI-TOF mass spectrometry and N terminal sequencing by standard Edman degradation at Advanced Protein Technology Centre (APTC), Hospital for Sick Children, University of Toronto.

2.11. Statistical analysis

Statistical analysis of the data was performed with SPSS software (version 12; SPSS Inc, Chicago, IL, USA). ANOVA was used to test for significant differences between different treatments and control using Tukey's test, antimicrobial activity of transgenic and non-transgenic plants with a rejection limit of $P > 0.05$.

CHAPTER 3: RESULTS

3.1. *In vitro* antimicrobial activity of polyphemusin and indolicidin derivatives

Antimicrobial activity of 10R, 11R and PV5 peptides against various animal pathogens have been shown previously, however, there is no data available on the antibacterial, antifungal or antiviral activity of these peptides against plant pathogens. To determine the potential antimicrobial activity of these peptides against plant pathogens, the synthetic peptides were tested against a selection of bacteria, fungi and a virus.

3.1.1. Antibacterial activity of 10R, 11R and PV5

All the bacterial strains were in stationary phase at 8 hour interval (data not shown). *In vitro* bactericidal activities of peptides at the concentration of 40 µg/ml and incubated with peptide for 8 hours was tested against different bacteria. Figure 1 shows significant inhibition of growth of *Erwinia carotovora*, *Staphylococcus epidermidis* and *Bacillus subtilis* by 10R, 11R and PV5 as well as of *Escherichia coli* by PV5.

The minimal inhibitory concentration (MIC) of 10R, 11R and PV5 for *Erwinia carotovora* was calculated and defined as the lowest peptide concentration that reduced 2×10^5 bacteria by at least 95 %. PV5, 11R and 10R showed complete bactericidal activity at concentrations of 10 µg/ml, 10µg/ml and 40 µg/ml respectively in the log phase of their growth (4 hours) (Figure 2). These results show that PV5 is the most potent antibacterial agent among the candidate peptides tested. Also, 11R showed bactericidal activity at a much lower concentration than 10R. These results also show a broad antibacterial spectrum of 10R, 11R and PV5.

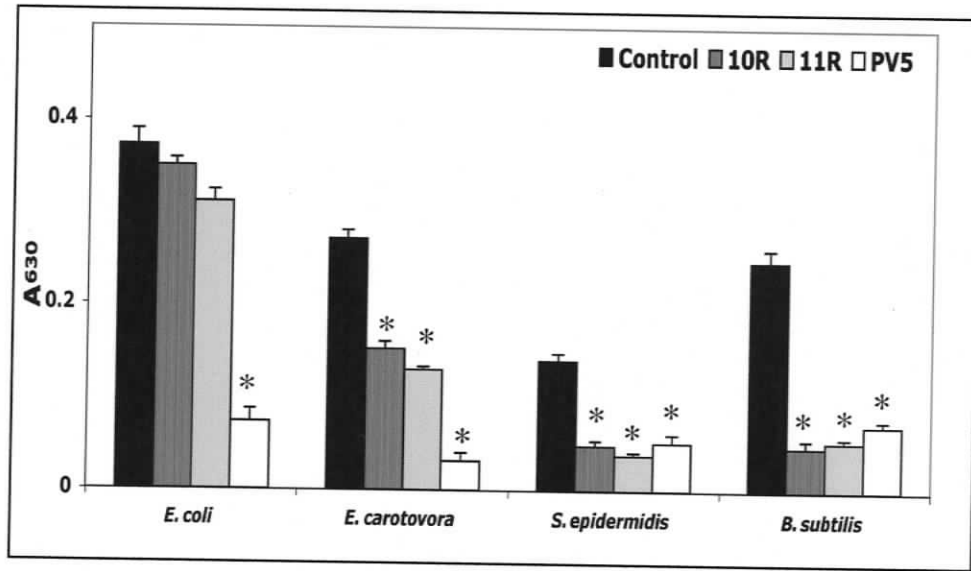


Figure 1. *In vitro* bactericidal activities of 10R, 11R and PV5

E. coli, *E. carotovora*, *S. epidermidis* and *B. subtilis* were incubated with the indicated peptides at 40 $\mu\text{g/ml}$ for 8 h as described in "Materials and Methods" and growth was scored by measuring A_{630} . The asterisk denotes the significant reduction in growth relative to the control at $p < 0.05$ level (Tukey's test), $n=3$.

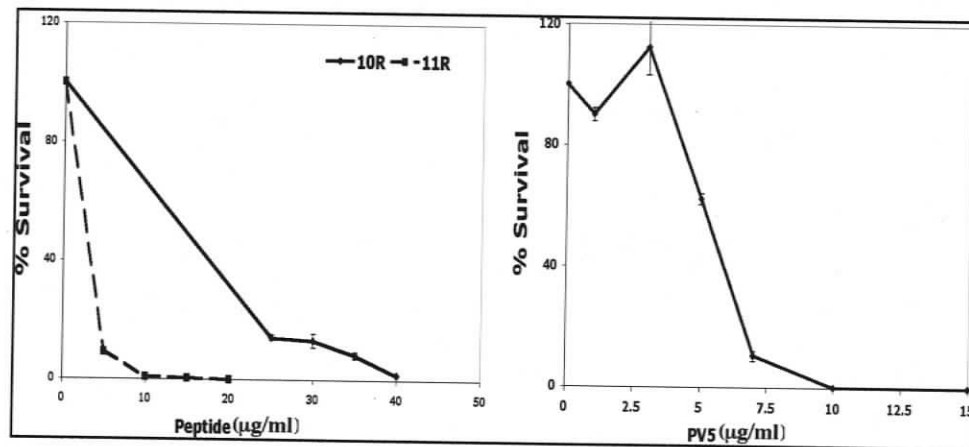


Figure 2. Minimum inhibitory concentration of 10R, 11R and PV5 on *Erwinia carotovora*

E. carotovora was incubated with (A) 10R and 11R (B) PV5 (at indicated concentrations) for 4 h, plated, viability assayed and survival was scored. Results represents the average and Standard Error of Means (SEM) of three independent experiments.

3.1.2. Antifungal activity of 10R, 11R and PV5

Fusarium oxysporum and *Botrytis cineria* were used to test the ability of PV5, 10R and 11R to inhibit fungal growth using a fungal growth zone inhibition assay (Osusky et al., 2005). There was no retardation in the growth observed around the discs (Figure 3).

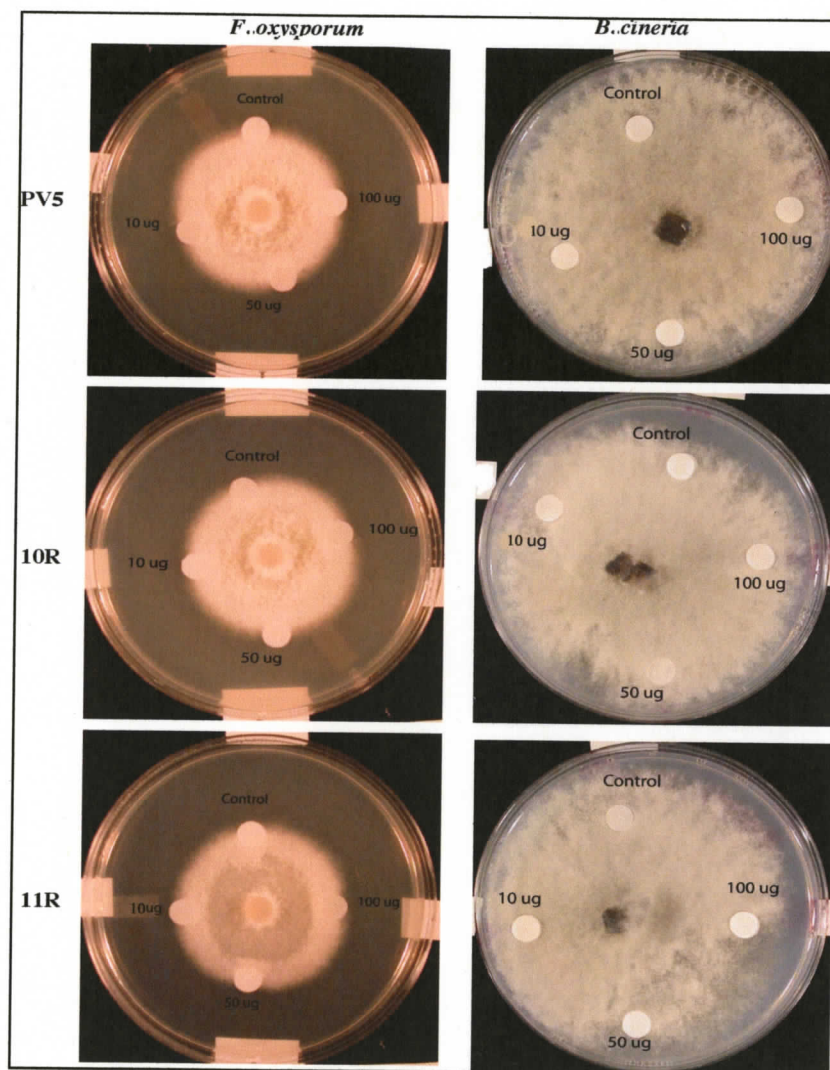


Figure 3. *In vitro* antifungal activities of PV5, 10R and 11R

F. oxysporum and *B. cineria* were incubated with the indicated synthetic peptides (μg) in a total volume of 15 μl on sterile paper discs. Pictures were taken after 24 h (*Fusarium*) or 48 h (*Botrytis*) of incubation at RT. Sterile distilled water was used as a control. Experiment was repeated 3 times.

The AlamarBlue™ assay has been shown to be more sensitive than the zone inhibition assay (Osuska et. al, unpublished data). To test the antifungal activity of these peptides against fungal strains, “the Alamar blue reaction” and measurement of fluorescence was used in this assay. Two representative fungi, *Fusarium graminearum* and *Verticillium sp* were used.

Tables (1- 3) show the percentage fungal viability using 10R, 11R and PV5 respectively. There was no antifungal activity against *Verticillium* for all three peptides. 10R and 11R showed significant reduction in viability of *Fusarium graminearum* (Table 1 and 2) even at 10 µg/ml and were potent anti-*Fusarium* agents (~95% reduction in growth) at 100 µg/ml. PV5 also showed significant *Fusarium* inhibition but only at 50 µg/ml and 100 µg/ml (Table 3). There was significant increase in viability observed relative to control (no peptide) in samples with *Verticillium sp.* for 10R and 11R at all concentrations tested and also for both *Fusarium* and *Verticillium*, when treated with 10 µg/ml of PV5.

Table 1. Antifungal activity of 10R determined using AlamarBlue™

The conidia (2×10^5 spores/ml) were incubated with the indicated concentrations of 10R. Fluorescence was scored and viability determined in relation to the control as described in Materials and Methods. Results show the values from three independent experiments and values after \pm are the SEM of % values (n=3). The asterisk denotes the significant decrease and cross represents significant increase from control at $p < 0.05$ (Tukey’s test).

Fungal Species	Fungal Strain	Fungal Viability (%)			
		10R-Peptide concentration (µg/ml)			
		10	25	50	100
<i>Fusarium graminearum</i>	M12-04-1	41.1 \pm 7.61 *	21.2 \pm 4.94 *	14.84 \pm 4.43*	4.59 \pm 0.71*
<i>Verticillium sp.</i>		138.2 \pm 4.16†	131.5 \pm 4.88†	131.95 \pm 5.25†	136.93 \pm 5.86†

Table 2. Antifungal activity of 11R determined using AlamarBlue™

The conidia (2×10^5 spores/ml) were incubated with the indicated concentrations of 11R. Fluorescence was scored and viability determined in relation to the control as described in Materials and Methods. Results show the values from three independent experiments and values after \pm are the SEM of % values ($n=3$). The asterisk denotes the significant decrease and cross represents significant increase from control at $p<0.05$ (Tukey's test).

Fungal Species	Fungal Strain	Fungal Viability (%)			
		11R-Peptide concentration ($\mu\text{g/ml}$)			
		10	25	50	100
<i>Fusarium</i>					
<i>graminearum</i>	M12-04-1	59.6 \pm 13.28*	27.3 \pm 5.80*	10.40 \pm 1.90*	4.09 \pm 0.74*
<i>Verticillium</i>					
sp.		147.8 \pm 6.72†	158.3 \pm 6.39†	163.98 \pm 5.23†	150.59 \pm 5.49†

Table 3. Antifungal activity of PV5 determined using AlamarBlue™

The conidia (2×10^5 spores/ml) were incubated with the indicated concentrations of PV5. Fluorescence was scored and viability determined in relation to the control as described in Materials and Methods. Results show the values obtained from three independent experiments and values after \pm are the SEM of % values ($n=3$). The asterisk denotes the significant decrease and cross represents significant increase from control at $p<0.05$ (Tukey's test).

Fungal Species	Fungal Strain	Fungal Viability (%)			
		PV5-Peptide concentration ($\mu\text{g/ml}$)			
		10	25	50	100
<i>Fusarium</i>					
<i>graminearum</i>	M12-04-1	190.9 \pm 12.59†	80.2 \pm 6.21	25.01 \pm 3.17*	22.03 \pm 2.69*
<i>Verticillium</i>					
sp.		122.6 \pm 13.62†	101.2 \pm 2.64	103.2 \pm 0.27	91.47 \pm 1.16

3.1.3. Antiviral activity of 10R, 11R and PV5

Indolicidins and polyphemusin have shown *in vitro* antiviral activity against many animal viruses (Miyata et al., 1989; Murakami et al., 1991; Nakashima et al., 1992; Allefs et al., 1996; Murakami et al., 1999). However, there was no report of testing these peptides and their variants on plant viruses. Therefore activity of these peptides on tobacco mosaic virus (TMV) was determined.

TMV was purified from the inoculated tobacco leaves and its purity was confirmed at $A_{260/280}$ using a UV spectrophotometer and by negative staining under the electron microscope (data not shown). *N. tabacum* plants developed local lesions when inoculated with both peptides treated or non-treated (control) TMV on one half of the leaves within 6 days (Figure 4A and 4B). The lesion number was counted and an ANOVA analysis was performed to determine the significant differences from the control. Figure 5 shows the lesions formed in two replicates compared to the control for indolicidin treated TMV. Only carborundum was applied on the other half and was used as a negative control. The results show the significant *in vitro* inhibition in TMV propagation by 10R and 11R at 10 μg (Figure 4A). No further reduction in lesions was observed when 100 μg peptide was used for virus treatment (Figure 4A). PV5 was not statistically significant relative to the control as determined by ANOVA analysis (Tukey's test) (Figure 4B).

Figure 5 shows the effect of treatment by 10R or 11R on TMV infectivity. Infected tobacco leaves showed lesion formation after 2 days, which expanded during the course of the experiment. Differences in lesion number were observed in control (non-treated TMV) and peptide treated infected leaf samples.

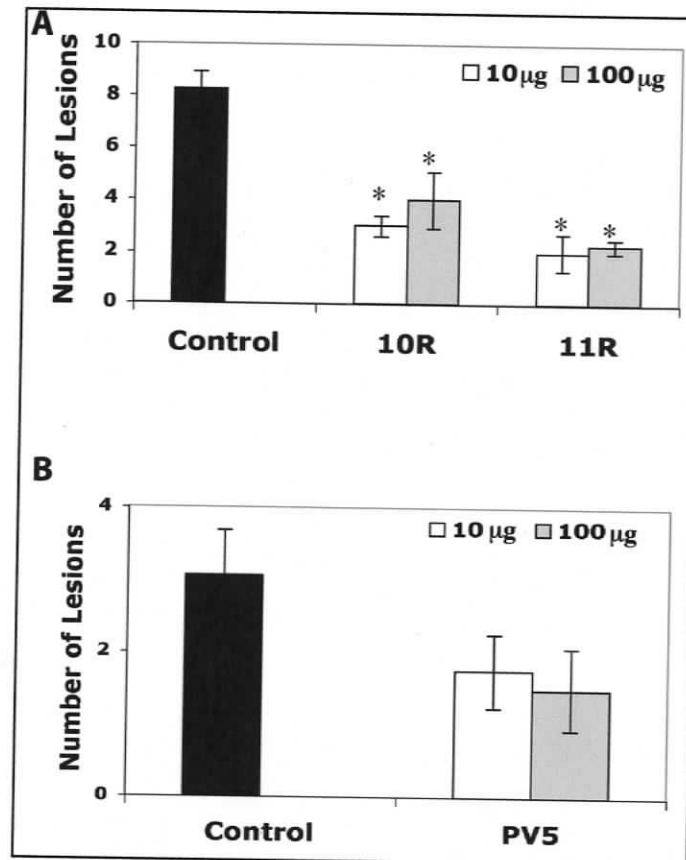


Figure 4. Effect of 10R, 11R and PV5 on infectivity of TMV

TMV was mixed with 10 µg and 100 µg of (A) 10R and 11R (B) PV5, incubated for 24 h at RT and inoculated onto 3 or 4 tobacco leaves. Results shown are the average of three independent experiments. Error bars indicate standard error of means (SEM). The asterisk denotes significant reduction in growth relative to the control at $p < 0.05$ (Tukey's test).

DAS-ELISA is a sensitive method to detect and quantitate virus infection in plants.

Figure 6 shows the reduction in TMV infectivity in tobacco plants after peptide treatment as determined by DAS-ELISA.

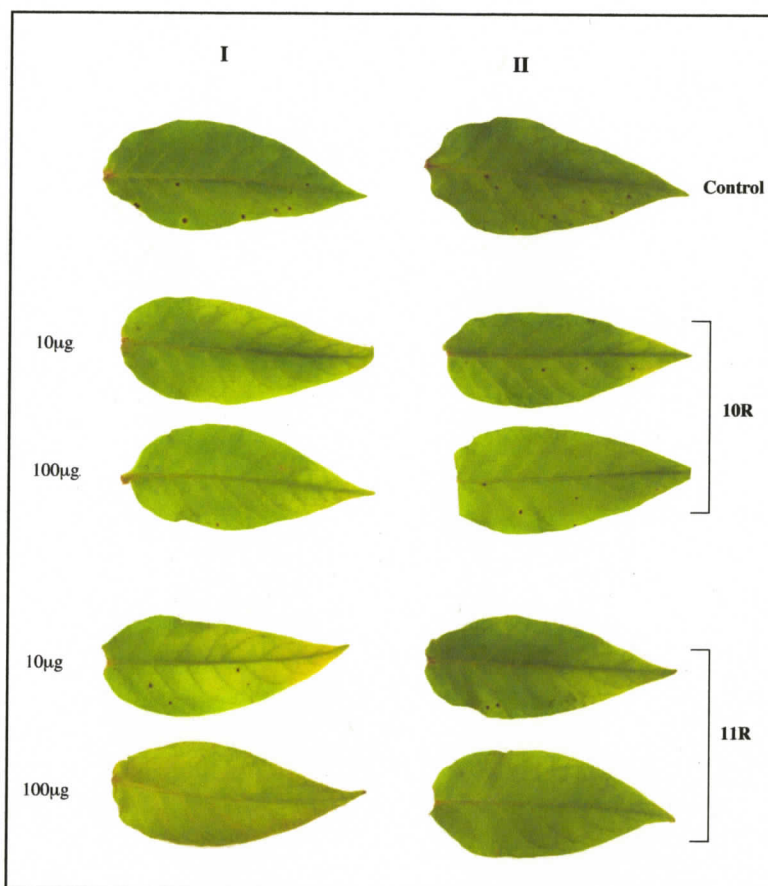


Figure 5. Effect of 10R and 11R on infectivity of TMV and lesion formation

TMV, treated with the indicated amounts of peptides, was applied on one half of the non-transgenic tobacco leaf and incubated at 25 C for 6 days. The observed reduction in the number of lesions relative to the control indicates the antiviral activity of 10R and 11R. I and II indicate the 2 replicates.

The DAS-ELISA performed with the TMV infected leaf halves showed a significant reduction in virus accumulation in peptide-treated virus infected leaves than in non-treated leaves. However, for 11R, the 10 µg treated virus infected leaf-half showed no significant difference compared to the control (Figure 6). There was no significant difference between control and PV5 treated samples (Figure 6B).

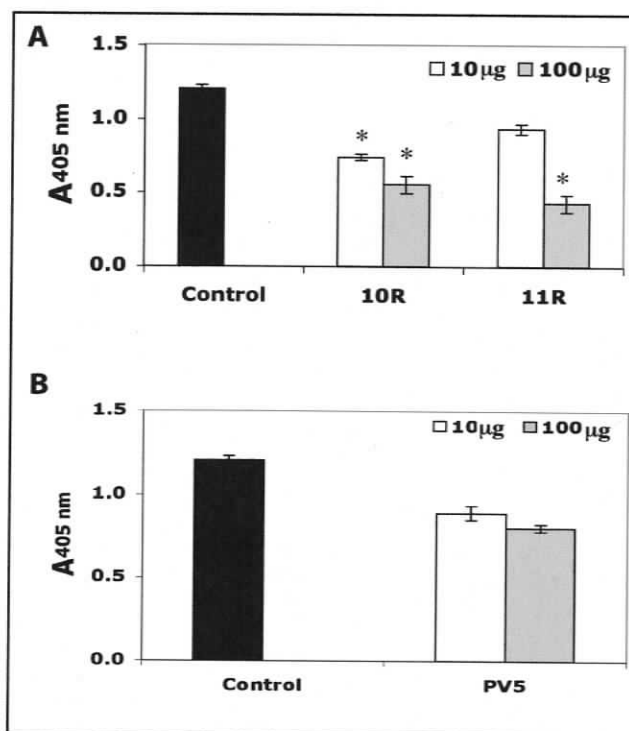


Figure 6. DAS-ELISA and *in vitro* anti-TMV activity of 10R, 11R and PV5

TMV was detected in leaf extracts of TMV infected leaf halves by DAS-ELISA. Leaves were either infected with (A) 10R and 11R treated (B) PV5 treated TMV or non-treated (control) and incubated at 25°C for 5 days. One hundred µl of 1:10 diluted leaf extract was used for TMV detection. The results show the average of 3 independent experiments. The asterisk denotes significant reduction in growth relative to the control at $p < 0.05$ (Tukey's test).

Dot Blot hybridization is also a very sensitive technique to quantify the extent of virus infection in plants (Rosenberg, 1985). The TMV movement protein cDNA probe was cloned, the sequence was confirmed (data not shown) and it was used as a probe. The results obtained from dot blot hybridization with the TMV movement protein cDNA probe are in agreement with the ELISA results (Figure 7A and 7B). In this case, instead of antibodies, a ^{32}P -labelled cDNA probe was used to assess the extent of TMV infection and the amount of TMV RNA. To further confirm the reliability of this hybridization and extent of infection, the dots were excised and the radioactivity was assayed for each

sample. Figure 7A and 7B show the level of radioactivity (CPM) present in 10R, 11R and PV5 samples respectively and corresponds to the extent of hybridization and amount of TMV in the sample. Figure 7 demonstrates the significant reduction in the hybridization signal and radioactivity in the samples treated with either 10 μ g or 100 μ g of 10R and 11R. The results for the polyphemusin variant PV5 were not statistically significant. The Lesion assay, DAS-ELISA and dot blot hybridization together show antiviral activity of 10R and 11R peptides against TMV, but do not show significant *in vitro* antiviral activity of polyphemusin derivative against TMV.

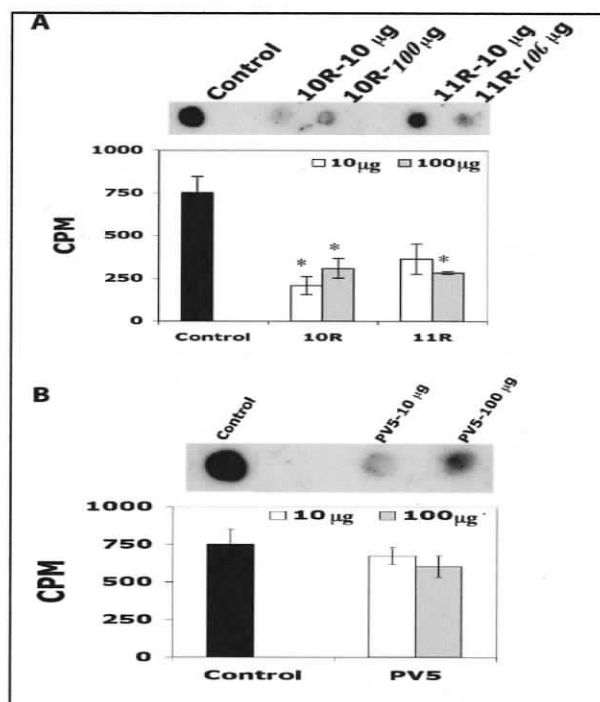


Figure 7. Dot-blot hybridization and *in vitro* anti-TMV activity of 10R, 11R and PV5 (A) 10R, 11R (B) and PV5 treated TMV, inoculated on tobacco and probed with TMV-MP-cDNA as probe. Five μ l of 1:10 diluted leaf extract were used for TMV detection. The extent of hybridization was confirmed by 32 P radioactivity assay. The CPM results shown are the average of three independent experiments. The asterisk denotes significant reduction in growth relative to the control at $p < 0.05$ (Tukey's test).

3.2. Cytotoxic assessment of PV5, 10R and 11R on leaf discs, protoplasts and plantlets

The toxic effect of different amounts (20 μg , 60 μg and 100 μg) of cationic peptides (10R, 11R and PV5) on tobacco protoplasts, leaf discs and plantlets was determined and the feasibility for plant expression of these peptides was evaluated.

3.2.1. Optimization of colorimetric assays for tobacco

A time course study was performed to determine the optimal incubation time for TTC, Evan's blue and neutral red assays for both leaf discs and protoplasts. Based on the incubation of leaf discs in dH_2O for different time periods (4, 8, 12, 24 and 48 h), formazan formation in the TTC assay was detected at maximal level at 12 h (Figure 8A). Leaf discs started to lose viability in standard conditions in dH_2O after 12 h of incubation. The leakage of formazan was seen, reducing the A_{530} at 24 h and 48 h of incubation (Figure 8A). According to this result, 12 h was the appropriate incubation time for the TTC assay to assess the effect of peptides on viability. Maximum absorbance in Evan's blue assay was seen at 12 h in dH_2O (Figure 8B). As the Evan's blue assay measures cell death, a sudden increase and then decrease at 24 hours in A_{600} shows that after 12 h of incubation leaf discs start losing their viability, *in vitro*. Thus, the appropriate time to measure the cytotoxic effect (cell death) was 12 h, when the discs are viable and A_{600} remains stable (Figure 8B). At this time period even a small effect of the peptides can be determined by an increase in A_{600} . At extended periods of incubation time (48 h), less Evan's blue was retained in the leaf discs. This may be due to the leakage of the Evan's blue stain after the dead cells of the leaf discs began desiccating. All leaf discs stained with Evan's blue developed a thin dark-blue ring around the perimeter of the discs due to

mechanical wounding. Since this was common in both control and treated samples, it did not interfere with the assay.

For the protoplasts TTC assay, the maximum absorbance A_{490} was observed in samples that were incubated in dH_2O for 24 h (Figure 8C). Being an isolated cell, this difference in protoplast from leaf disc was expected. Leakage of formazan were seen at 48 h and thus a decrease in absorbance was observed (Figure 8C). This was similar to leaf discs, therefore, for later experiments, protoplast samples were incubated in the dark for 24 h after TTC addition.

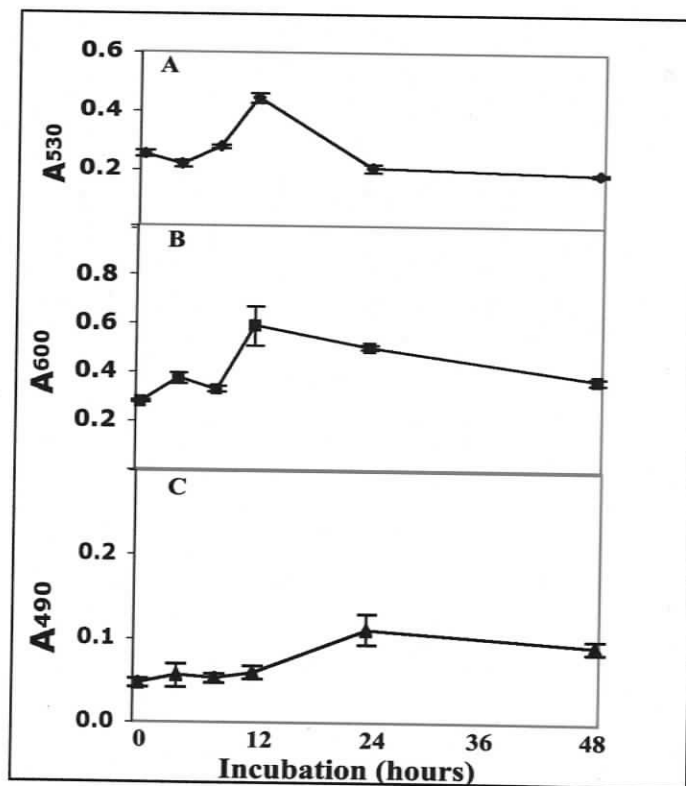


Figure 8. Effect of incubation times on viability of leaf discs and protoplasts

(A) Changes in viability (A_{530} /g fresh weight) of leaf disc as measured by TTC reduction activity in tobacco leaf discs (B) Changes in uptake of Evan's blue in tobacco leaf discs (C) Changes in viability of tobacco protoplasts (Absorbance at 490 nm/5000 protoplasts) as measured by TTC reduction activity. Means \pm S.E.M (n=3) are indicated.

3.2.2. Leaf discs and TTC

There was no significant difference in the viability of the leaf disc samples incubated for 12 h in 20 μg of peptides 10R, 11R and PV5 as compared to control (dH₂O treated samples)(Figure 9). In 10R treated samples significant reduction in the viability was seen with 100 μg treatments. This trend was similar to that seen with carbenicillin, which was used as negative control. PV5 showed significant reduction in absorbance at 60 μg and 100 μg of peptide. 11R treated samples did not show any toxicity even at 100 μg . The significant reduction in viability relative to control (non-treated) in boiled samples and Round-up™ treated leaf discs show the reliability of this assay for assessment of viability of plant leaf discs.

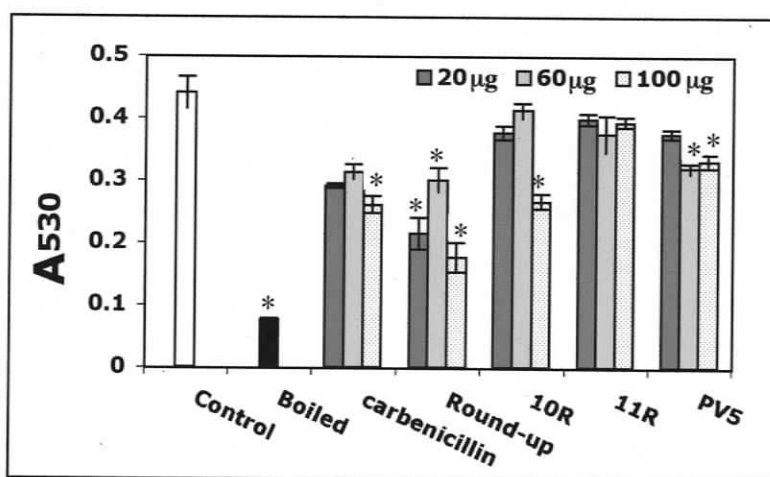


Figure 9. Effect of different amounts of peptides on tobacco leaf disc viability as shown by TTC assay

The sample treated with dH₂O only was used as a control. Round-up™ was used as a positive control. Carbenicillin was used as a negative control. Means \pm S.E.M (n=3) are indicated. The asterisk denotes the significant difference from control at $p < 0.05$ (Tukey's test).

3.2.3. Leaf discs and Evan's blue

In this assay, absorbance is directly proportional to the toxicity of the compound. Figure

10 shows a significant increase in absorbance relative to control with Round-up™ and boiled samples (shown by asterisk) and illustrates the reliability of the method to assess the cytotoxicity of compounds on leaf discs.

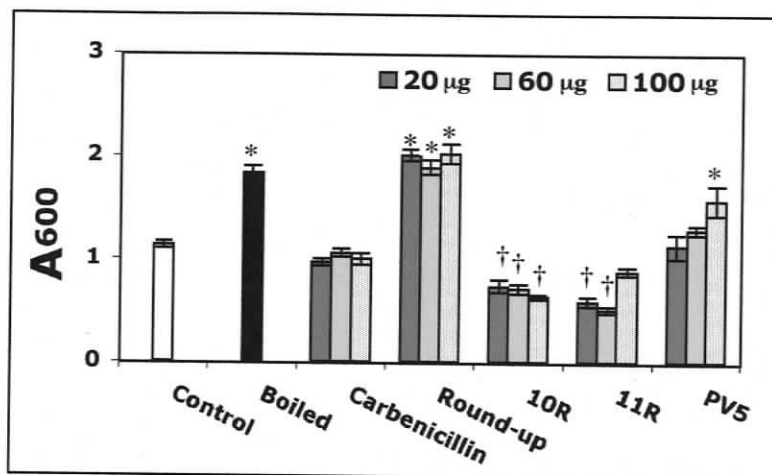


Figure 10. Effect of different amounts of peptides on tobacco leaf disc viability as shown by Evan's blue assay

The sample treated with dH₂O only was used as a control. Round up™ was used as a positive control. Carbenicillin was used as a negative control. Means \pm S.E.M (n=3) are indicated. The asterisk denotes the significant increase and cross represents significant decrease from control at $p < 0.05$ (Tukey's test).

In PV5 treated leaf discs at 100 µg of peptide, there was a significant increase in absorbance showing cytotoxicity at this high peptide amount. In carbenicillin treated samples, there was no cytotoxic effect observed even at 100 µg in contrast to that seen in TTC assay in Figure 9.

In 10R and 11R treated leaf discs there was a significant decrease in absorbance observed relative to control indicating the high viability of leaf discs and lack of cytotoxic effect of these peptides on plants. Leaf discs treated with 100 µg of 11R showed no significant decrease or increase relative to control, thus indicating the low cytotoxicity of this

peptide even at this high amount. Results obtained with TTC assay (Figure 9) showing no cytotoxicity of 11R treatments were confirmed by Evan's blue assay (Figure 10).

3.2.4. Protoplasts and TTC

The TTC assay performed with tobacco protoplasts and different concentrations of the peptides and carbenicillin, showed no significant reduction in viability relative to control (dH₂O) for the protoplasts treated with 10R and 11R cationic peptides except for samples treated with 100 µg of PV5 and carbenicillin (Figure 11).

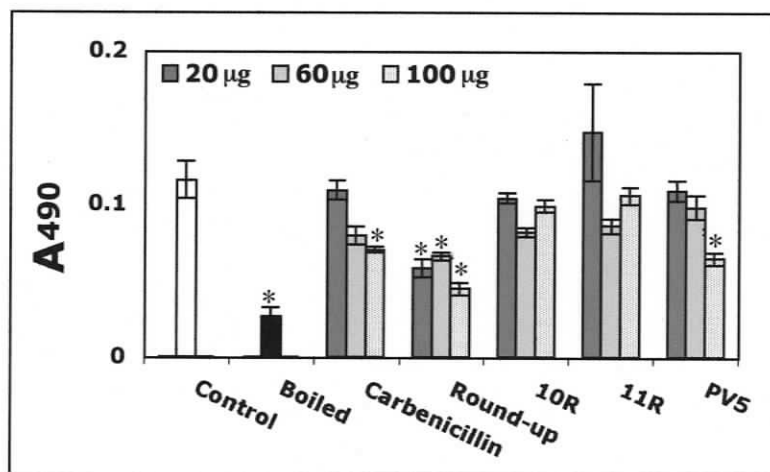


Figure 11. Effects of different amounts of peptides on tobacco protoplasts as shown by TTC assay

The sample treated with dH₂O only was used as a control. Viability = $A_{490}/5000$ protoplasts. Round-up™ was used as a positive control. Carbenicillin was used as a negative control. Means ± S.E.M (n=3) are indicated. The asterisk denotes the significant difference from control at $p < 0.05$ (Tukey test).

Reduction in viability was observed in protoplasts treated with 60 µg and 100 µg of the PV5 (Figure 11). This trend was almost similar to carbenicillin (Figure 11), which was used as a negative control. Results seen in Figure 11 are similar to those seen in leaf disc TTC assay and Evan's blue assay (Figures 9 and 10).

Round-up™ and boiled samples showed a lower absorbance and thus lower viability. Protoplast assay because of differences in protoplast number, fragility and lack of cell wall makes it less sensitive as compared to leaf disc assays.

3.2.5. Protoplasts and Neutral red

There was no significant reduction in viability relative to the control in 11R as observed by neutral red assay. However, protoplasts have lower viability in PV5 and 10R treatments even at low peptide amount (Figure 12). However, there was no difference seen in viability relative to dH₂O treated discs for 60 µg treatments in 10R and PV5 but toxicity at 10 µg and 100 µg.

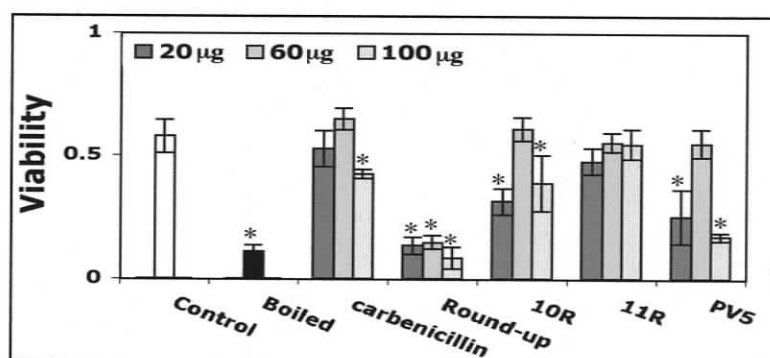


Figure 12. Effects of different amounts of peptides on tobacco protoplasts viability as assessed by the neutral red assay.

Viability is defined here as the fraction of living protoplasts/fraction of total protoplasts as observed under light microscope. The sample treated with dH₂O only was used as a control. Round-up™ was used as a positive control. Carbenicillin was used as a negative control. Means \pm S.E.M (n=3) are indicated. The asterisk denotes the significant difference from control at $p < 0.05$ (Tukey test).

The PV5 treatment showed a cytotoxic effect at 100 μg of peptide (Figure 13), indicating the toxicity of this peptide. This result was a confirmation of the cytotoxic effect seen for 100 μg of PV5 by TTC and neutral red assays (Figures 11 and 12).

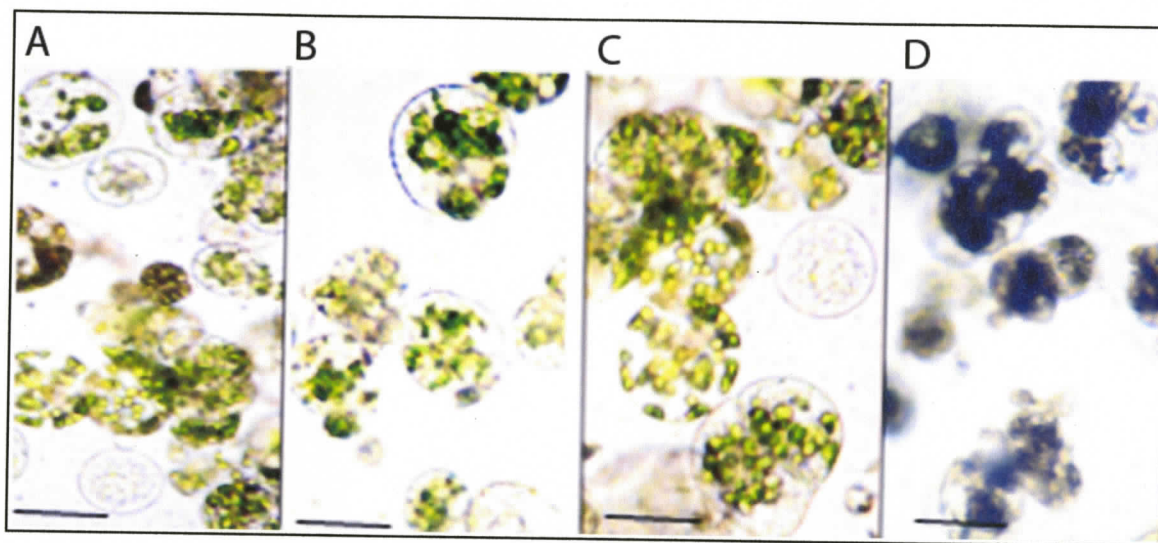


Figure 13. Effect of PV5 on the viability of tobacco protoplasts *in vitro*

Tobacco mesophyll protoplasts were cultivated for 5 days before addition of the peptide. Photographs were taken 24 h after incubation of plant protoplasts (A) control (B) 10 μg (C) 40 μg and (D) 100 μg of PV5. Experiment was repeated 3 times. Bar: 50 μm .

3.2.6. Phytotoxicity assays

Table 4 shows the results of the phytotoxicity assay. No significant differences in shoot length and fresh weight relative to control were seen except when plantlets were grown with 20 μg and 100 μg of PV5 and all amounts of Round-up™. In the positive control (Round-up™ grown plants), most of the plantlets were dead (Figure 14) and the total chlorophyll and carotenoid levels were significantly low. Total chlorophyll levels in the plantlets grown in presence of 11R (20-100 μg), showed no significant differences

relative to the control. In plantlets grown at 10 µg of PV5, 100µg of 11R, 60 µg of 10R, 10 µg and 60 µg of carbenicillin showed a significant increase in chlorophyll or carotenoids as compared to the control. Viability, as assessed by TTC assay for the plantlets grown in the different amounts of peptides, was similar to the control except at 100 µg of PV5 and 10 µg of carbenicillin, where a significant reduction was observed.

Table 4. Phytotoxicity assay of the plantlets grown in different amounts of peptides and controls

Peptides	Weight (gm)	Height (cm)	Chlorophyll (mg/gFW)	Carotenoids (mg/gFW)	Viability ^d
Control ^a	0.246± 0.011	2.633± 0.033	0.285± 0.009	0.037± 0.005	2.758± 0.097
Carbenicillin ^b	20µg	0.325± 0.008	2.000± 0.231	0.411± 0.023*	1.980± 0.112*
	60µg	0.259± 0.015	2.300± 0.115	0.409± 0.011*	2.470± 0.056
	100µg	0.218± 0.024	2.767± 0.145	0.316± 0.018	2.421± 0.201
Round-up ^c	20µg	0.064± 0.004*	0.900± 0.058*	0.167± 0.002*	1.482± 0.074*
	60µg	0.064± 0.009*	0.900± 0.058*	0.163± 0.006*	1.438± 0.070*
	100µg	0.052± 0.008*	1.000± 0.058*	0.136± 0.008*	1.337± 0.057*
PF 10R	20µg	0.210± 0.022	1.967± 0.285	0.246± 0.006	2.867± 0.032
	60µg	0.237± 0.008	2.400± 0.153	0.384± 0.009*	2.722± 0.079
	100µg	0.245± 0.016	1.900± 0.100	0.276± 0.006	2.414± 0.113
PF 11R	20µg	0.218± 0.025	2.067± 0.176	0.236± 0.013	3.015± 0.117
	60µg	0.233± 0.015	2.267± 0.088	0.304± 0.004	2.718± 0.125
	100µg	0.235± 0.004	2.167± 0.088	0.242± 0.016	2.605± 0.138
PV5	20µg	0.249± 0.027	1.533± 0.233*	0.405± 0.008*	2.359± 0.049
	60µg	0.254± 0.018	1.967± 0.120	0.338± 0.010	2.083± 0.101
	100µg	0.174± 0.012	1.533± 0.145*	0.262± 0.011	1.840± 0.063*

^aControl is the treatment with distilled water.
^bCarbenicillin was used as negative control. Round-up was used as positive control
Values after by ± signs are standard error of means
Means followed by * are significantly different from control at P<0.05 (Tukey's test)
^dViability was measured by TTC assay as describe in materials and methods.

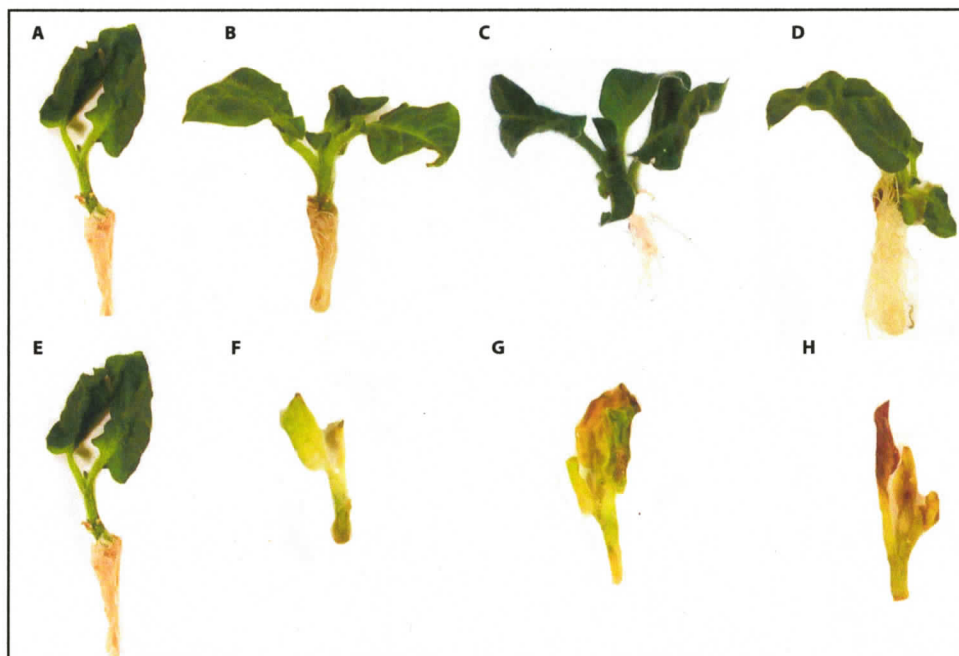


Figure 14. Phytotoxicity and morphological differences in plantlets

(B-D) 11R and (F-H) Round-up™. B and F indicates plantlets grown in 10µg of peptide and round-up respectively, C and G indicates plantlets grown in 60µg of 11R and Round-up™ respectively and D and H indicates plantlets grown in 100µg of 11R and Round-up respectively.

3.3. Construction of plant expression vectors

Inhibition of bacterial, fungal and viral growth combined with low cytotoxicity to plant tissues, protoplasts and plantlets by PV5, 10R and 11R paved the way for the introduction of these peptides into plants to test their efficacies. Cationic peptide nucleotide sequences (PV5, PV8, 10R and 11R) were obtained through reverse translation of known amino acid sequences using potato specific codons (Figure 16). The genes for cationic peptides were assembled using the polymerase chain reaction (PCR) (Figure 16: arrows represent forward and reverse overlapping primers used for gene synthesis) and the expression vectors were constructed as described in “Materials and Methods”.

An additional signal peptide from *Pseudotsuga menziesii* BiP (the endoplasmic reticulum (ER) molecular chaperone) was added in the polyphemusin constructs of PV5 and PV8 constructs (Figure 16). The vectors (pPV5, pPV8, p10R and p11R carrying 2x35S-AMV-PV5, 2x35S-AMV-PV8, 2x35S-AMV-10R, and 2x35S-AMV-11R) were used for plant transformation (Figure 15). The constructs were transferred to *Agrobacterium tumefaciens* and used for leaf disc transformation of tobacco.

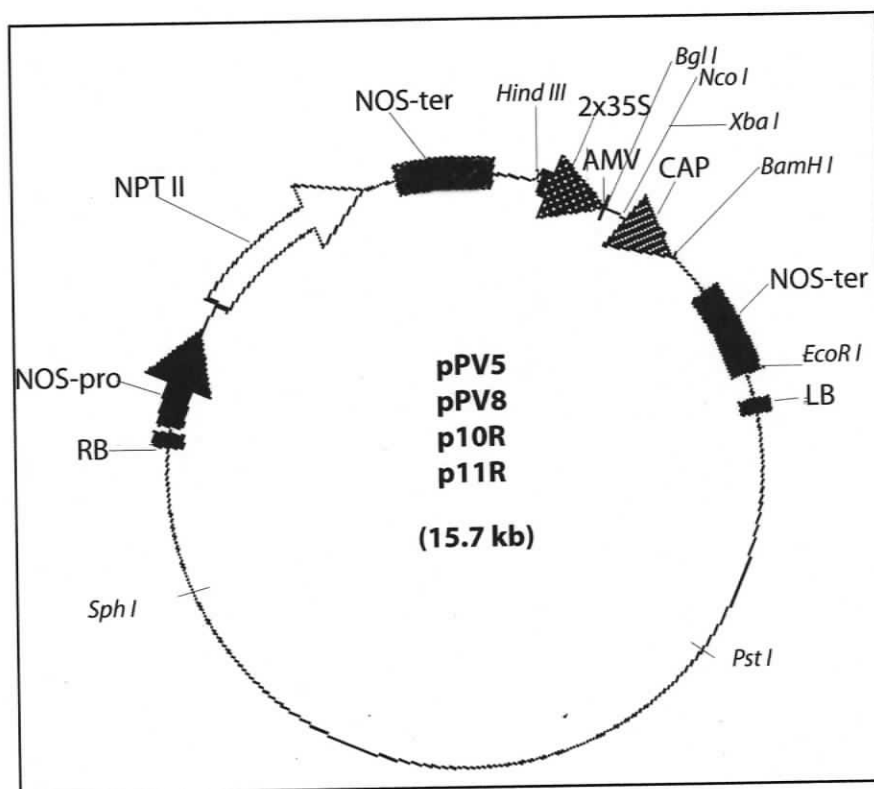


Figure 15. The schematic outline of a plant expression vectors for cationic peptide cDNA

The abbreviations are as follows: RB and LB, the right and left border regions of the Ti plasmid; NOS-pro and NOS-ter, promoter and terminator respectively, of the nopaline synthase gene; NPT II, neomycin phosphotransferase II; 2x35S, duplicated enhancer CaMV 35S promoter; AMV, leader sequence from alfalfa mosaic virus RNA4; CAP, protein coding sequence of either 10R, 11R, PV5 or PV8.

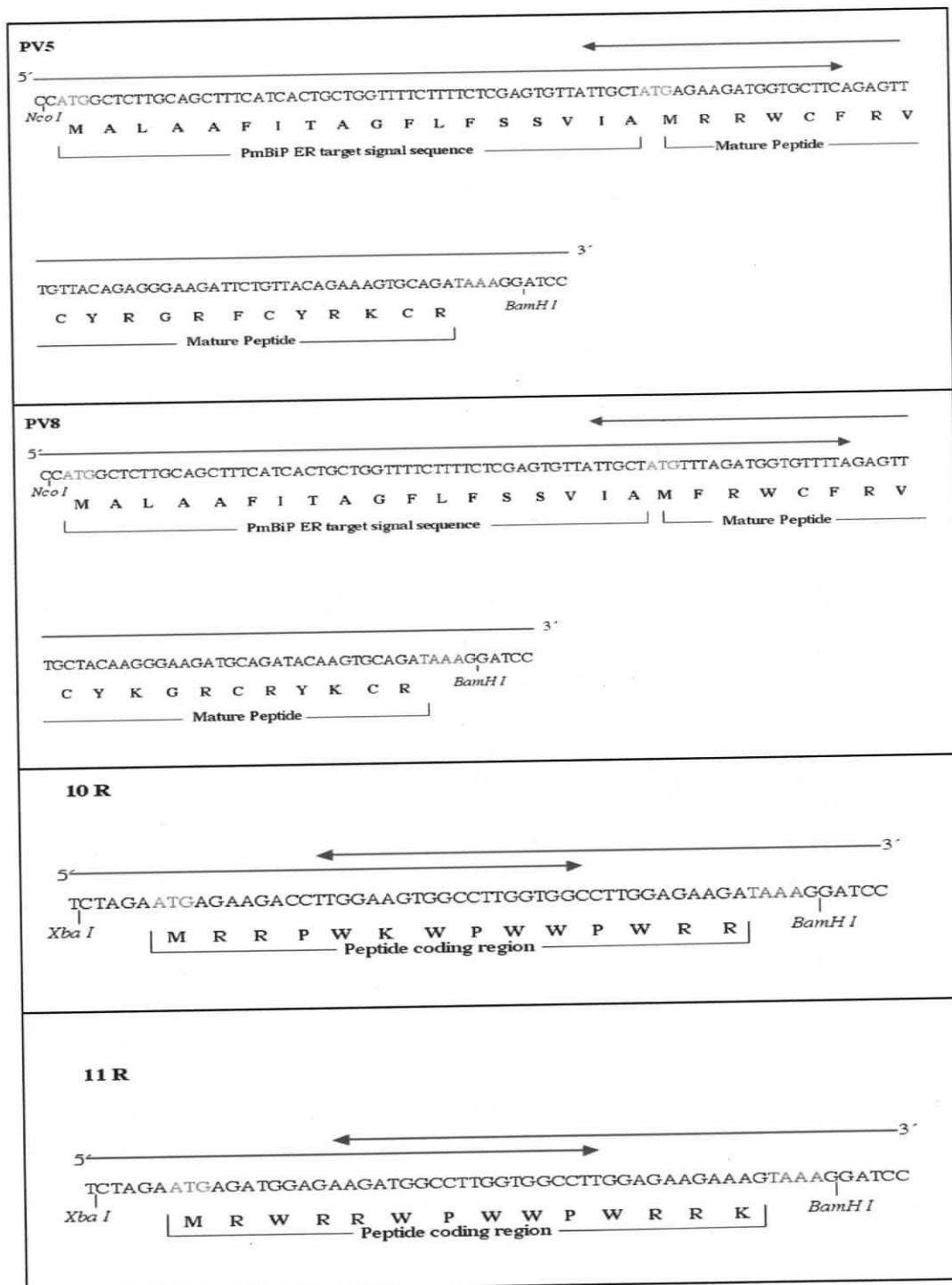


Figure 16. Nucleotide and amino acid sequences of the cationic peptides

Restriction enzymes sites are indicated in italics. Arrows indicate the positions for the overlapping PCR primers used for gene synthesis. ER target signal peptide is shown for PV5 and PV8 gene synthesis.

3.4. Production of transgenic plants and molecular analysis

N. tabacum var Xanthi was transformed by *Agrobacterium*-mediated transformation as described in “Materials and Methods”. High regeneration with multiple shoots per explant was achieved on the selective medium (Figure 17A). Putative transgenic shoots were randomly selected and rooted in the presence of selective agent. All plants retained the normal morphology (Figure 17B and 17C) with no indication of cytotoxicity due to the expression of PV5, PV8, 10R or 11R.

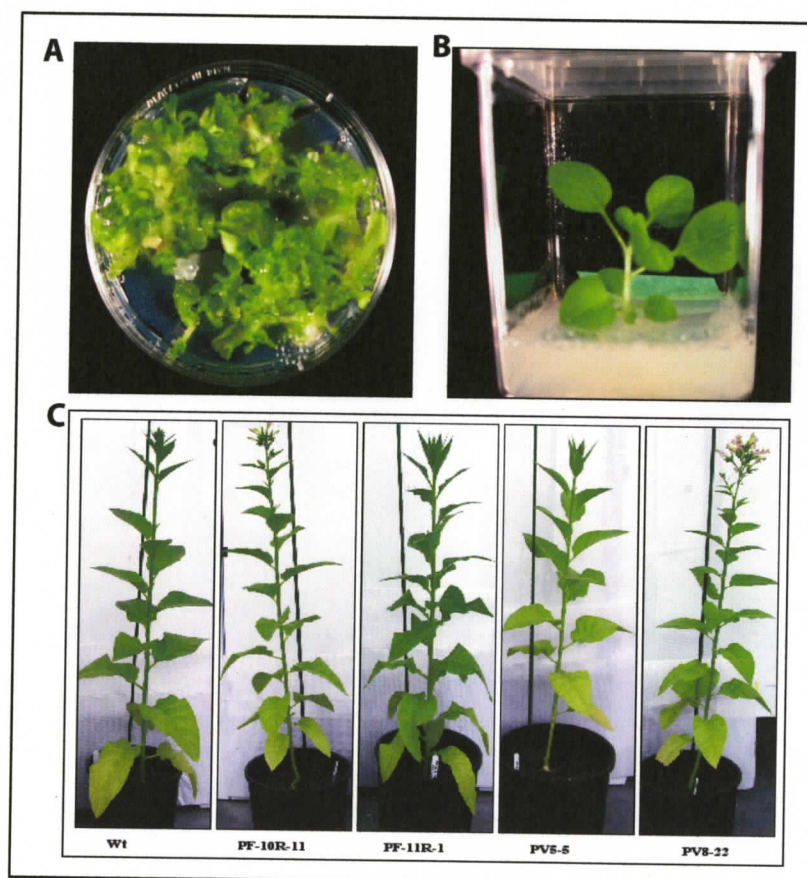


Figure 17. Tobacco transformation and morphology

(A) Multiple shoot regeneration from tobacco leaves and petioles after 30 days on selection medium. (B) Transgenic plant regeneration in tissue culture. (C) Morphological characteristics of transgenic tobacco plants grown in green house.

PCR analyses using upstream primers specific for the CaMV 35S-promoter and downstream primers specific to either the cationic peptide gene (10R and 11R) or the PmBiP signal sequence (PV5 and PV8) were performed. DNA from an untransformed plant of similar age was used as a negative control (wt). In all transgenic tobacco plants, bands of predicted sizes were observed. In 10R and 11R plants, a band of the expected size of 60 bp was observed [Figure 19 (i) A and (ii) A] and 110 bp was seen for PV5 and PV8 [Figure 18 (i) A and (ii) A]. Southern analyses of transgenic tobacco plants were performed to determine the stable transgene integration and copy number while Northern blot analyses were performed to confirm gene expression.

Southern analysis indicated that one to five copies of the transgene were maintained in the plant genome [Figure 18 (i) B]. A single copy of PV5 was present in line # 5, #6, #7 and #19. Multiple copies of genes were integrated in line # 8 and #11. The expression of the transgene in the transformed plants could not be determined by RT-PCR (data not shown). The accumulation of PV5 transcripts was determined by Northern blot analysis of leaf RNA. The result showed PV5 expression in lines # 1, # 5, #6, #7, # 9 and #19. Lines # 8 and # 11 with multiple gene copies showed no expression. This may be the result of gene silencing or could be due to the positional effect, which often occurs when higher gene copy numbers are found in the genome. PV8 lines # 17, # 22 and #25 showed a single gene copy in the genome. However, only line # 17 and # 22 showed high-level expression (Figure 18). Line # 32 had multiple gene copies but showed good expression while line # 25 had single gene copy and showed poor expression, thus illustrating that the correlation between high copy number and gene silencing is not consistent.

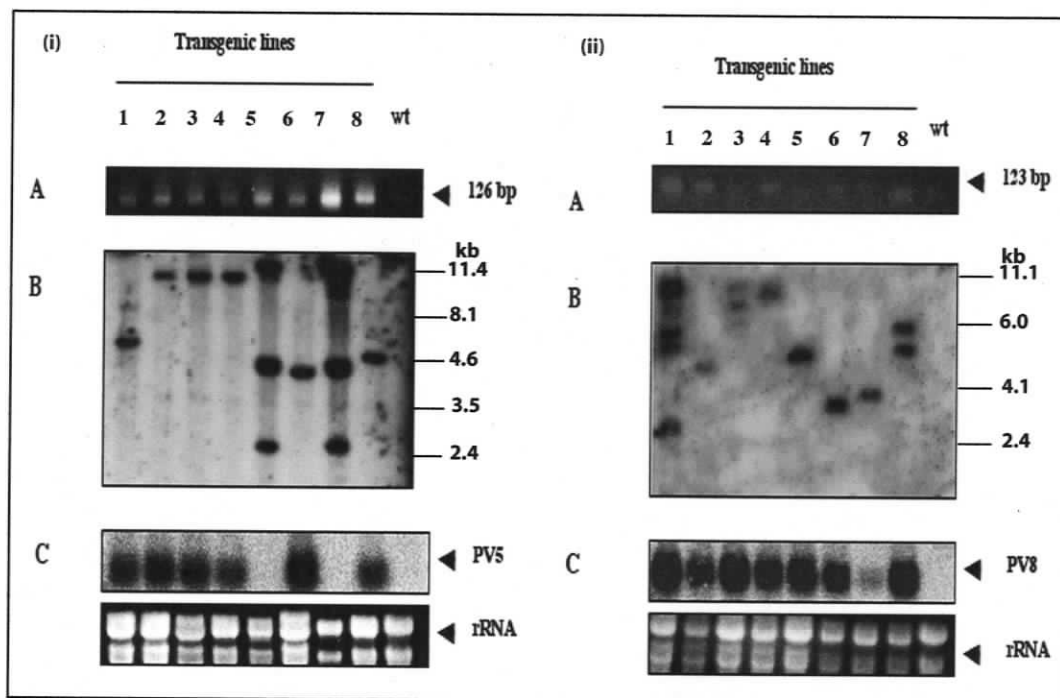


Figure 18. PV5 and PV8 gene integration and expression

(i) PV5 and (ii) PV8. (A) The cationic peptide coding sequence was PCR amplified from DNA isolated from control (non-transformed, lane wt) and transgenic (lanes 1-8: transgenic tobacco lines # 1, # 5, # 6, # 7, # 8, # 9, # 11 and # 19 for PV5 and transgenic tobacco lines # 1, # 2, # 13, # 15, # 17, # 22, # 25 and # 32 for PV8 respectively). (B) Southern blot analysis of transgenic plants. Tobacco leaf DNA was digested with *EcoR I*, electrophoresed and hybridized with a ^{32}P -labelled PV5 or PV8 probe. The number of bands reflects the number of transgene insertions. For lane description see (A). (C) Northern blot analysis of PV5 (i) PV8 (ii) mRNA accumulation in tobacco. Total RNA was prepared from leaves of transgenic plants. RNA samples (30 μg each) were separated by denaturing formaldehyde agarose gel electrophoresis, blotted and hybridized with a ^{32}P -labelled PV5 or PV8 probe. Ethidium bromide stained ribosomal RNA bands (rRNA) are shown as loading control.

The presence of 10R in the tobacco genome was confirmed by PCR and integration by Southern analysis (Figure 19). A single copy of 10R was detected in lines # 4 and # 21 (confirmed later with repeating the blot with these 2 lines separately, data not shown). Multiple copies were detected in Line # 11, # 19 and # 20. The expression was low in # 4 and # 20 again showing no correlation between gene copy integration and expression. The presence of the 11R transgene in transformed plants was confirmed by PCR and

Southern blot (Figure 19 (ii)). Lines # 1 and # 16 showed single gene copy and showed high 11R expression. Lines # 7, # 12, # 13 and # 17 showed multiple gene copy integration and high expression. Line # 7, # 12, # 13 and # 17 in 11R (Figure 19 (ii) B) show similar pattern in gene integration. This could be the result of selecting lines from same callus and thus are clones of each other.

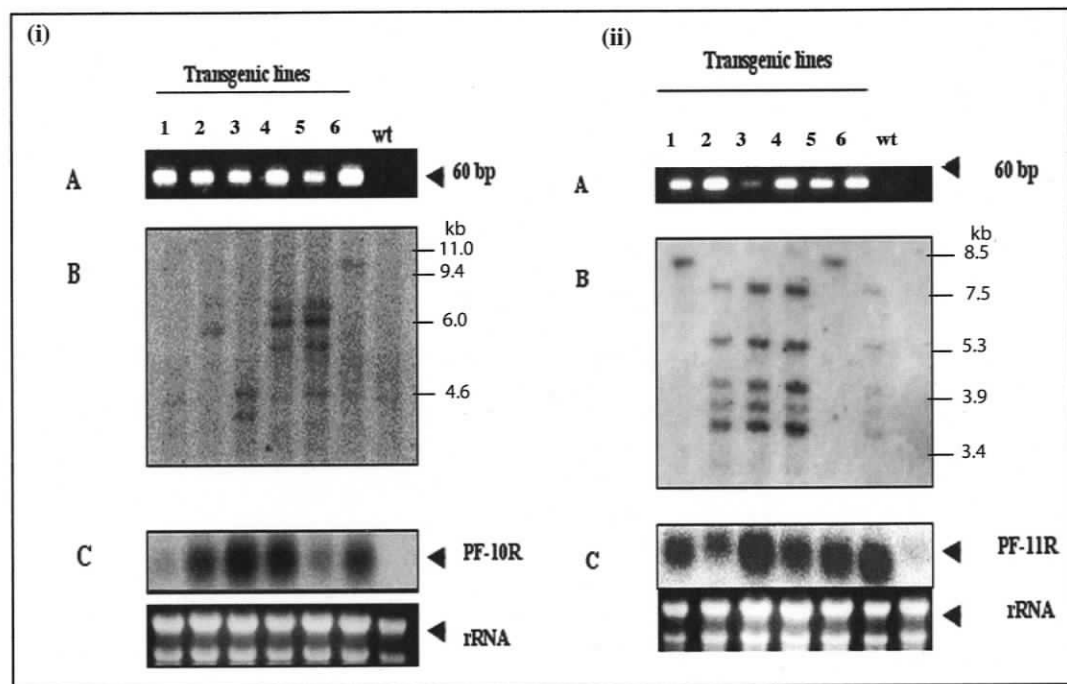


Figure 19. 10R and 11R gene integration and expression

(i) 10R and (ii) 11R. (A) The cationic peptide coding sequence was PCR amplified from DNA isolated from control (non-transformed, lane wt) and transgenic (lanes 1-6: transgenic tobacco lines # 4, # 6, # 11, # 19, # 20 and # 21 for 10R and transgenic tobacco lines # 1, # 7, # 12, # 13, # 16 and # 17 for 11R respectively). (B) Southern blot analysis of transgenic plants. Tobacco leaf DNA was digested with EcoR I, electrophoresed and hybridized with a ^{32}P -labelled 10R or 11R probe. The number of bands reflects the number of transgene insertions. For lane description see (A). (C) Northern blot analysis of 10R (i) or 11R (ii) mRNA accumulation in tobacco. Total RNA was prepared from leaves of transgenic plants; RNA samples (30 μg each) were separated by denaturing formaldehyde agarose gel electrophoresis, blotted and hybridized with a ^{32}P -labelled 10R or 11R probe. Ethidium bromide stained ribosomal RNA bands (rRNA) are shown as loading control.

The T₀ plants from all constructs were transferred to the greenhouse for seed development and subsequent selection of the T₁ generation for further analysis.

3.5. Bacterial disease resistance of transgenic plants

3.5.1. Detached leaf assay

To investigate the ability of transgenic plants to resist bacterial infection, detached leaves from soil grown transgenic and control (non-transgenic and GUS), 4-6 weeks old plants, were placed in Petri dishes with wet filter paper. Leaves were wounded and inoculated with 10 µl (~5x10⁵cfu) of an *E. carotovora* suspension. All of the transgenic lines shown in Figure 20 had a single gene copy but high expression levels. After 1 day at RT, water soaked areas were visible around the inoculated points of the control and transgenic plants. The size of these areas in control leaves increased with time. After 6 days, the extent of decay was much higher in the control plants as compared to the transgenic plants. Figure 20 shows that transgenic plants expressing 11R (line # 16) were more resistant to *Erwinia* than plants expressing 10R (line # 6). The infected leaf in this case remained green and showed less decay. Similarly, PV5 (line # 5) expressing plants were more resistant to *Erwinia* than plants expressing PV8 (line # 22) (Figure 20). There were three independent experiments performed and all the transgenic- bacterial-infected leaves showed resistance in the similar pattern as shown in Figure 20.

3.5.2. Transgenic leaf extracts assay

Leaf extracts from transgenic and control plants were tested against *Erwinia carotovora* to confirm the antibacterial activity shown by the leaves of transgenic plants (Table 5).

Extracts were also tested with the Gram-positive bacterium *Staphylococcus epidermidis* to compare the activity of these peptides shown *in vitro* with the expressed peptides in leaves.

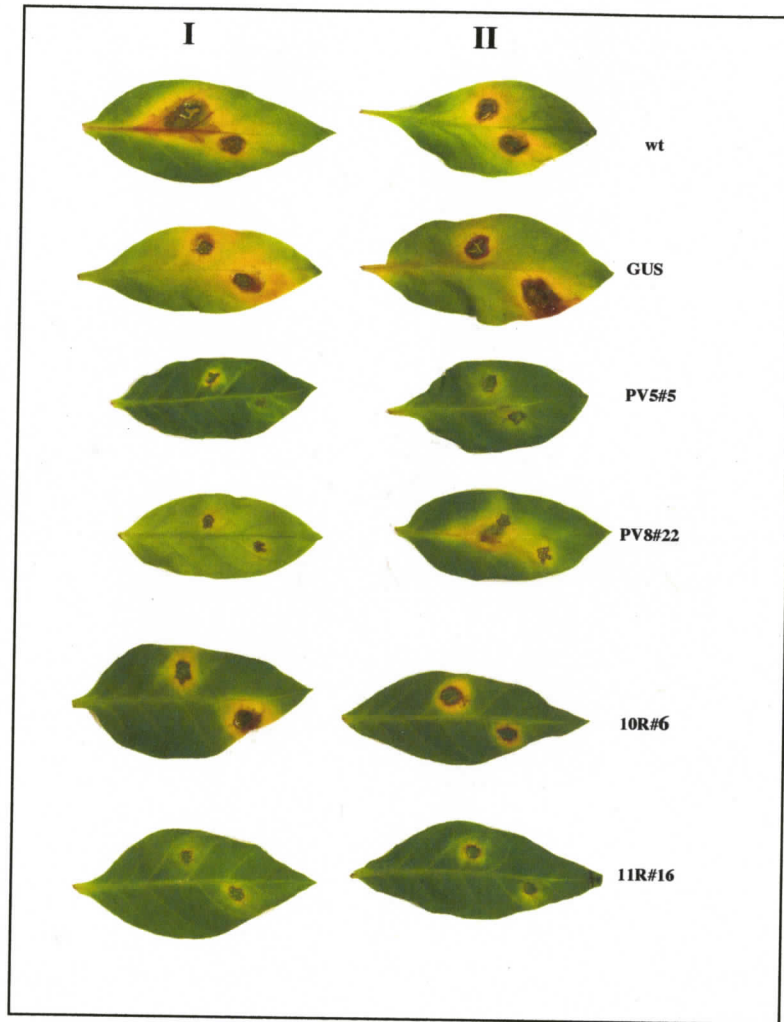


Figure 20. Control and transgenic tobacco challenged with the bacterial pathogen *E. carotovora*.

Detached leaves from 4 to 6-week-old control (wt), transgenic control (GUS), PV5 #5 transgenic, PV8 #22 transgenic 10R#6 transgenic and 11R#16 transgenic tobacco plants were wounded and infected with $\sim 5 \times 10^5$ cfu of *E. carotovora* and incubated at RT. Pictures were taken 5 days after infection. I and II represent replicates. Pictures shown are the representatives of the best results from 3 independent experiments.

Table 5. Antibacterial activity of the tobacco leaf extracts expressing PV5, PV8, 10R and 11R.

Leaf extract preparation and antibacterial assays were performed as described in "Materials and methods".

	Absorbance at 630 nm	
	<i>E.carotovora</i>	<i>S. epidermidis</i>
wt	0.505±0.015	0.405±0.028
Gus	0.444±0.013	0.343±0.022
PV5-1	0.402±0.009 ^a	0.309±0.022
PV5-5	0.393±0.013 ^a	0.301±0.022
PV5-6	0.386±0.009 ^{a,b}	0.298±0.020
PV5-7	0.396±0.010 ^a	0.323±0.015
PV5-9	0.395±0.010 ^a	0.312±0.023
PV5-19	0.395±0.010 ^a	0.304±0.025
PV8-1	0.366±0.020 ^a	0.314±0.024
PV8-13	0.423±0.005	0.325±0.021
PV8-15	0.394±0.012 ^a	0.312±0.028
PV8-17	0.396±0.023 ^a	0.315±0.023
PV8-22	0.380±0.033 ^a	0.283±0.028
PV8-32	0.385±0.021 ^a	0.313±0.029
10R-6	0.363±0.012 ^{a,b}	0.308±0.025
10R-11	0.425±0.015 ^a	0.313±0.024
10R-19	0.407±0.019 ^a	0.292±0.025
10R-21	0.390±0.020 ^a	0.314±0.033
11R-1	0.390±0.030 ^a	0.304±0.023
11R-7	0.405±0.018 ^a	0.312±0.021
11R-12	0.406±0.022 ^a	0.320±0.021
11R-13	0.413±0.017	0.323±0.023
11R-16	0.398±0.020 ^a	0.320±0.025
11R-17	0.389±0.036 ^a	0.317±0.025

^a shows the significant differences between mean values of non-transgenic tobacco plants and transgenic tobacco plants and ^b between GUS and transgenic plants at P< 0.05 and n=3. values after ± sign are standard error of means

Plants extracts from PV5 and PV8-expressing leaves were tested and most of the transgenic lines showed significant reduction in bacterial growth relative to non-

transgenic (wt) plants except PV8 #13. Extracts from plants expressing GUS also showed antibacterial activity as compared to non-transgenic plants. No significant reduction in transgenic leaf extracts relative to GUS was observed in any polyphemusin-expressing sample (Table 5) except in sample PV5 #6. Transgenic plant extracts from 10R and 11R constructs showed similar results as PV5 and PV8 with significant resistance relative to non-transgenic and GUS seen only in 10R #6. Most of the transgenic lines showed significant differences from non-transgenic plants (Table 5). Plant extracts when tested with *Staphylococcus epidermidis* showed no significant reduction in growth relative to either non-transgenic or GUS or to both. There was no significant difference between wt and GUS in activity as determined by ANOVA analysis (Tukey's test), despite of some antibacterial activity seen for GUS.

3.6. Fungal disease resistance of transgenic plants

To study the response of 10R-, 11R-, PV5- and PV8-expressing tobacco plants to fungal infection, detached leaf fungal assays were performed. Detached leaves from 8-10 week old, greenhouse grown plants from various lines of different constructs, were placed in Petri dishes with wet filter paper and inoculated with agar plugs containing actively growing strains of fungi. Figures 21-23 shows the results of the fungal infection assays on control (non-transgenic and transgenic GUS control) and 3 transgenic lines, one with a single copy of the gene (10R # 6, 11R # 16, PV5 # 5 and PV8 # 22), and two lines of each construct with multiple gene insertions (10R # 11 and 10R # 19, 11R # 17 and 11R # 13, PV5 # 19 and PV5 # 16 and PV8 # 32 and PV8 # 11).

Table 6. Antifungal activity of transgenic tobacco leaves expressing 10R, 11R, PV5 and PV8.

Construct	Line #	<i>Botrytis cineria</i>				<i>Verticillium sp.</i>				<i>Fusarium oxysporum.</i>			
		Leaf				Leaf				Leaf			
		1	2	3	4	1	2	3	4	1	2	3	4
wt		-	-	-	-	-	-	-	-	-	-	-	-
GUS		-	-	-	-	-	+/-	-	+/-	+/-	+	+/-	+/-
PV5	#5	+	+	+	+	+	+	+/-	+/-	+	+	+	+/-
	#19	+	+	+	+/-	+	+/-	+/-	+/-	+	+/-	-	-
	#6	+	+	+/-	+/-	+	+/-	+/-	+/-	-	-	-	-
PV8	#22	+	+	+	+	+	+	+	+/-	+/-	-	-	-
	#32	+	+	+	+/-	+	+/-	+/-	+/-	+/-	+/-	-	-
	#1	+	+/-	+/-	+/-	+	+	+	+/-	+/-	+/-	+/-	-
10R	#6	+	+/-	+/-	+/-	+	+	+	+/-	+/-	-	-	-
	#11	+/-	+/-	+/-	+/-	+	+/-	+/-	+/-	-	-	-	-
	#19	-	-	-	-	+	+/-	+/-	-	-	-	-	-
11R	#16	+	+	+/-	+/-	+	+	+	+	-	-	-	-
	#17	+	+	+	+/-	+	+	+/-	+/-	+	+/-	-	-
	#13	+	+/-	+/-	-	+	+	+	+/-	+/-	-	-	-

+ indicates the resistance in detached leaf against indicated fungal strain.
 +/- indicates the partial resistance in detached leaf against indicated fungal strain.
 - indicates no resistance.
 Results showed are from 2 independent experiments and with 2 replicates each (4 leaves of each construct, GUS and non-transgenic (wt) plants).

Botrytis cineria (Figure 21), *Verticillium sp.* (Figure 22) and *Fusarium oxysporum* (Figure 23) were inoculated on the detached leaves. Six to twenty days after infection the leaves from the non-transgenic tobacco (wt) and transgenic control (GUS) were heavily infected by *Botrytis* and *Verticillium* infection. In contrast, infected areas on the transgenic leaves, independent of the gene copy number, were much smaller and limited to the area around the site of contact with the fungus containing agar plugs (Figure 21-23). Similar patterns of resistance were seen in PV5 and PV8 transgenic lines. Table 6 shows the extent of resistance shown by different transgenic plants in two independent experiments with 2 replicates of each (total of 4 leaves).

Detached leaves of GUS show partial resistance against *Verticillium* and *Fusarium*. PV5 #6 did not show any resistance against *Fusarium* but PV #5 was resistance and PV5 #19

was partially resistant to this fungus. Most of the leaves of PV5 expressing lines were partially resistant to *Verticillium* and showed complete resistance towards *Botrytis*. Partial resistance was observed in PV8 #1 and PV8 # 32 against *Fusarium* but not in PV8 #22. However, all PV8 lines were resistant to *Botrytis* and *Verticillium*.

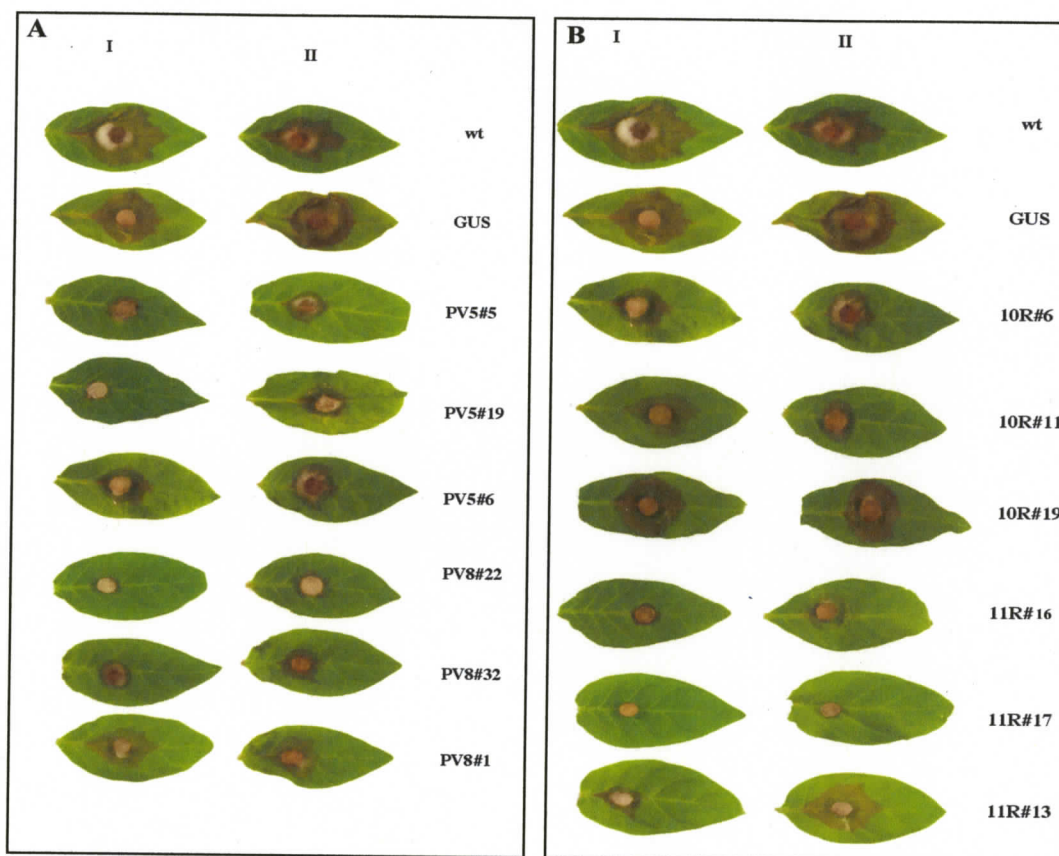


Figure 21. Resistance of transgenic tobacco expressing PV5, PV8, 10R and 11R to *Botrytis cineria*

Detached leaves from 4-8 weeks, greenhouse grown, non-transgenic tobacco control, transgenic GUS control (A) PV5 and PV8 (B) 10R and 11R expressing transgenic plants (with indicated lines) were infected with agar plugs containing growing culture of *Botrytis cineria* and incubated at RT. Pictures were taken at 6 days after infection. Pictures shown are the representatives of the best results from 2 independent experiments. with 3 replicates of each.

10R-expressing plants (10R #6 and 10R #11) showed partial resistance to *Botrytis* but line 10R #19 did not show any resistance to this fungus. Also, 11R expressing plant lines showed resistance to *Verticillium*. 11R # 16 and 11R # 17 showed resistance to *Botrytis*. None of the 11R expressing lines had resistance to *Fusarium*.

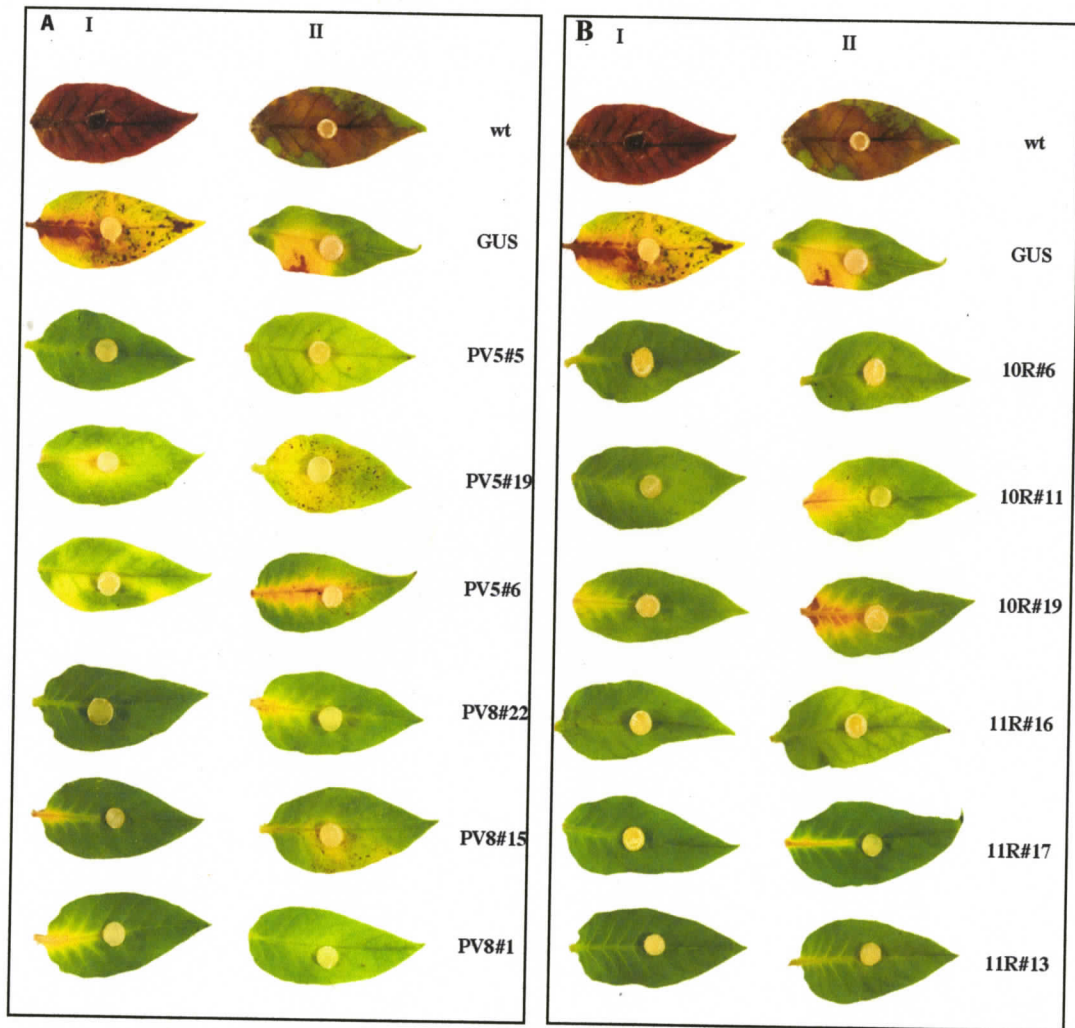


Figure 22. Resistance of transgenic tobacco expressing PV5, PV8, 10R and 11R to *Verticillium sp.*

Detached leaves from 4-8 weeks, greenhouse grown, non-transgenic tobacco control, transgenic GUS control (A) PV5 and PV8 (B) 10R and 11R expressing transgenic plants (with indicated lines) were infected with agar plugs containing growing culture of *Verticillium sp.* and incubated at RT. Pictures were taken at 20 days after infection. Pictures shown are the representatives of the best results from 2 independent experiments with 3 replicates of each.

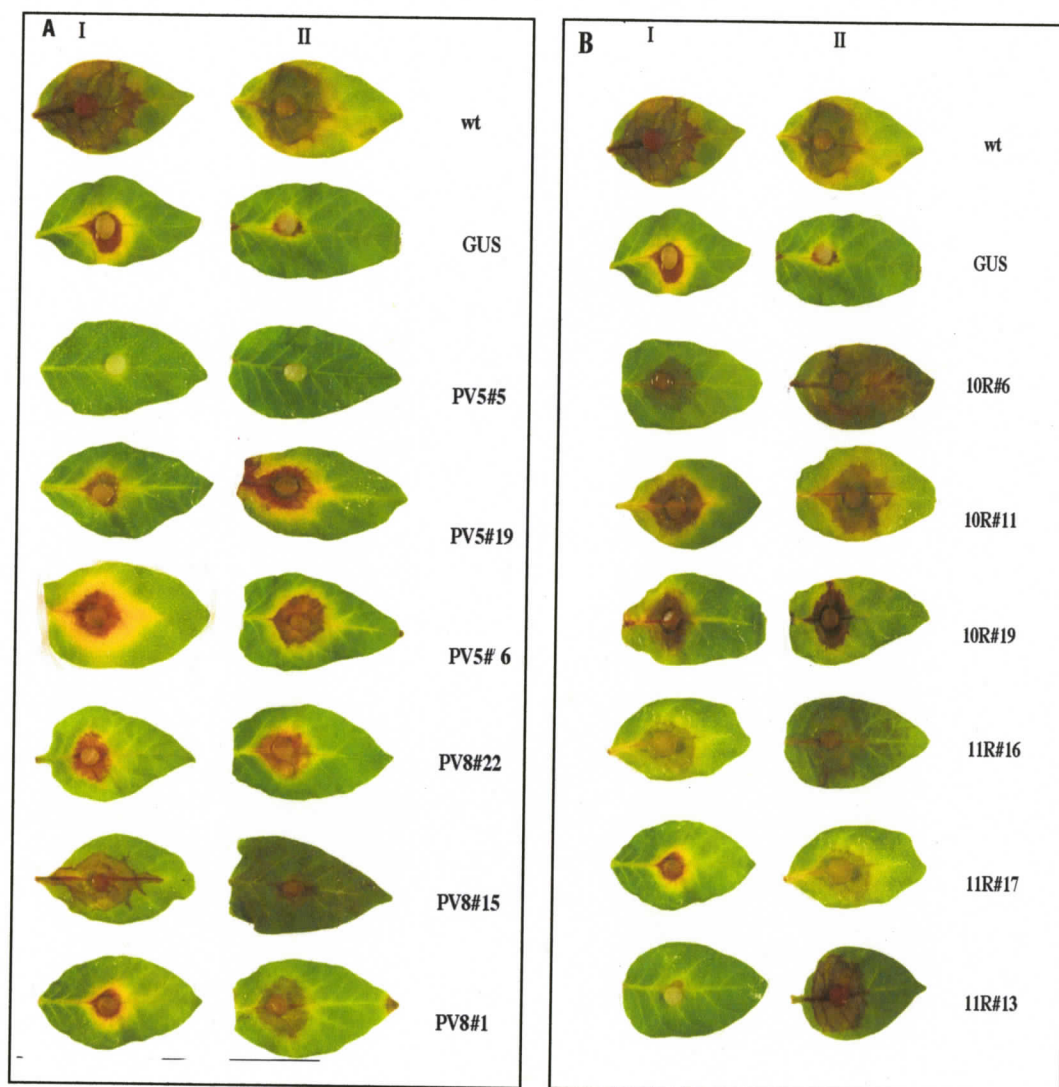


Figure 23. Resistance of transgenic tobacco expressing PV5, PV8, 10R and 11R to *Fusarium oxysporum*.

Detached leaves from 4-8 weeks, greenhouse grown, non-transgenic tobacco control, transgenic GUS control, (A) PV5 and PV8 (B) 10R and 11R expressing transgenic plants with (indicated line) were infected with agar plugs containing growing culture of *Fusarium oxysporum* and incubated at RT. Pictures were taken at 14 days after infection. Pictures shown are the representatives of the best results from 2 independent experiments with 3 replicates of each.

3.7. Viral disease resistance of transgenic plants

3.7.1. Detached leaf assays

Transgenic plants expressing polyphemusin and indolicidins were tested for resistance to Tobacco Mosaic Virus (TMV). The transgenic lines were selected on the basis of their performance of antibacterial and antifungal resistance as well and gene copy number. A single gene copy line was selected for each construct (10R #6, 11R #16, PV5 #5 and PV8 #22). One line of each construct with multiple gene insertion was also examined. There was a significant reduction in number of lesions in transgenic plants tested (Figure 24) relative to the lesions formed on non-transgenic leaves

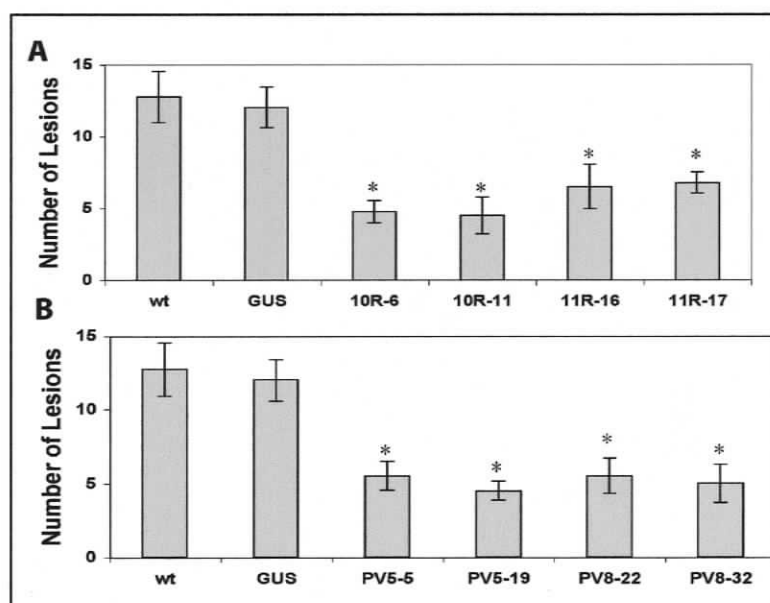


Figure 24. Effect of expression of 10R, 11R, PV5 and PV8 in transgenic tobacco plants on infectivity of TMV

(A) 10R and 11R expressing tobacco and (B) PV5 and PV8 expressing tobacco plants. TMV was inoculated on one half of each leaf. The number of necrotic local lesions elicited by TMV was counted and compared with non-transgenic (wt) and transgenic control (GUS). Values are the average of four independent experiments and error bars show SEM, n=4. Significant differences between mean values of controls (non-transgenic and Gus) and transgenic tobacco plants are indicated by * at $p < 0.05$ (Tukey test).

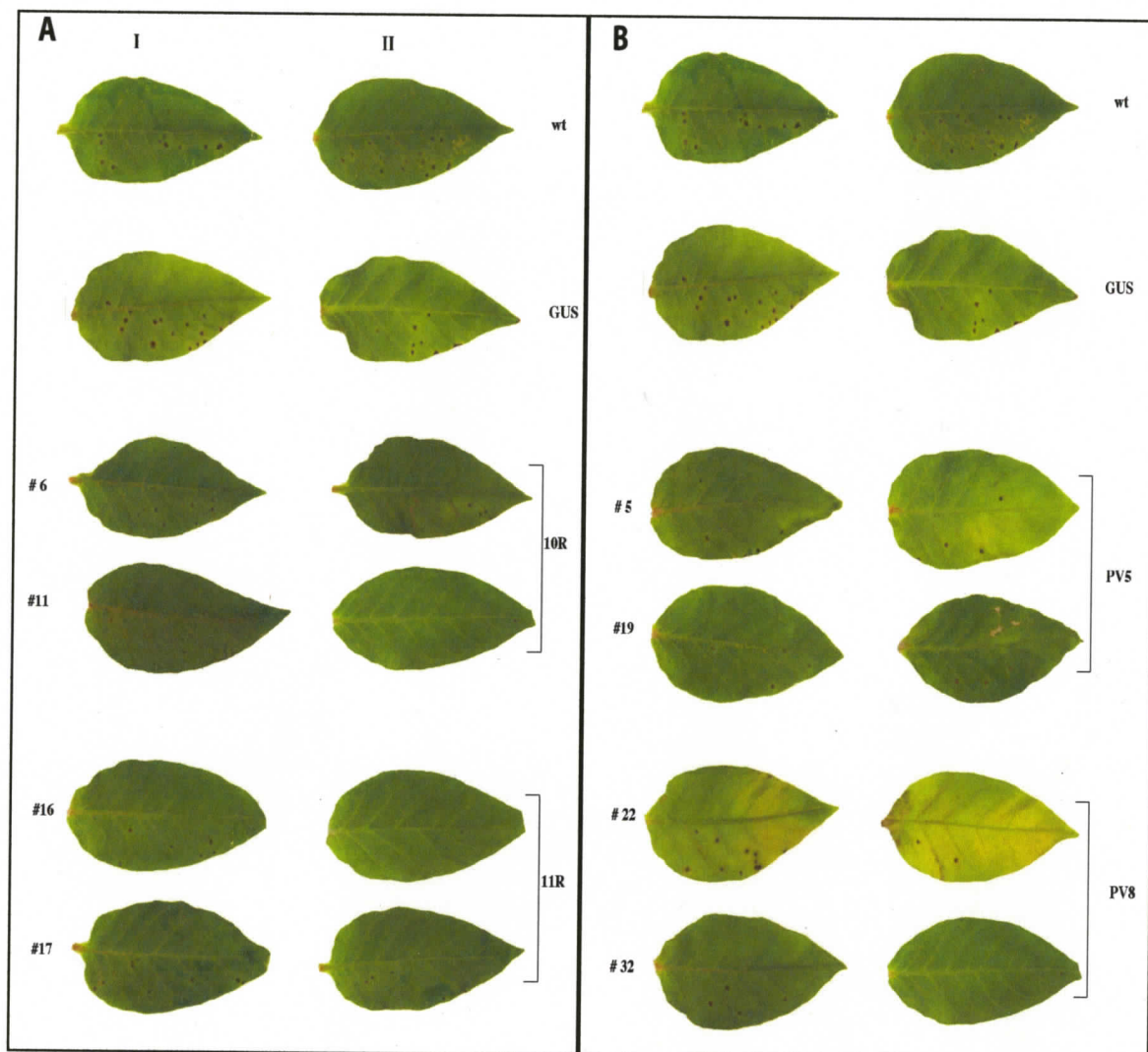


Figure 25. Necrotic local lesions elicited by TMV inoculation.

(A) 10R- and 11R- and (B) PV5- and PV8- expressing tobacco leaves compared to non-transgenic (wt) and transgenic controls (GUS).

Lesions on transgenic leaves were counted and compared with the controls (non-transgenic plants) (Figure 25). Significant reduction in the number of lesions in transgenic lines (For 10R, 11R, PV5 and PV8), independent of the gene copy number was observed (Figure 24

3.7.1.1. DAS-ELISA

Results seen by DAS-ELISA of leaf extracts were similar to that seen in the lesion forming leaves (Figure 26A and 26B) except for PV5 #19 leaves, which did not show a significant reduction in TMV infectivity as seen by the lesion assay (Figure 26B).

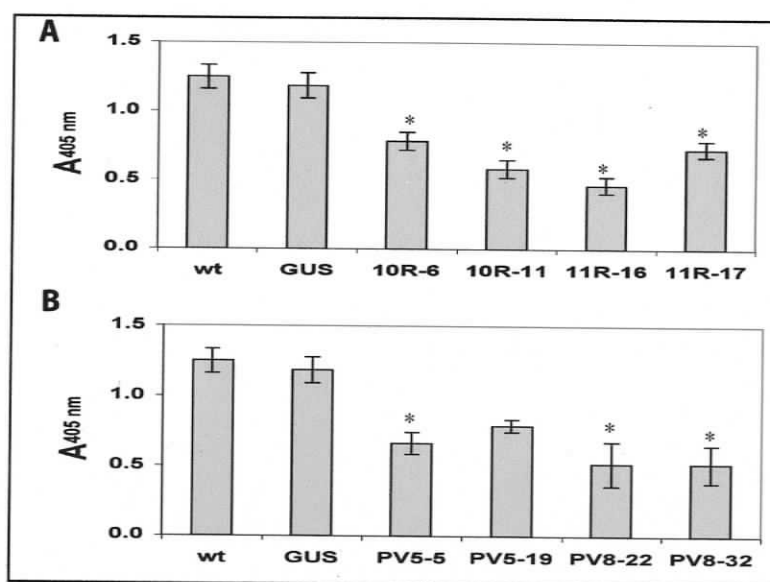


Figure 26. DAS-ELISA based TMV resistance shown by transgenic tobacco plants

(A) Tobacco plants expressing 10R and 11R and (B) plants expressing PV5 and PV8. Detached leaves were infected with TMV. ELISA was performed as described in "Materials and Methods". A_{405} represents the DAS-ELISA absorbance values. Error bars show SEM, $n=4$. Significant differences between mean values of controls (non-transgenic and GUS) and transgenic tobacco plants are indicated by * at $p < 0.05$ (Tukey's test).

3.7.1.2. Dot Blot hybridization

Figure 27A (a) and 27B (a) show the dot blot hybridization with the TMV movement protein cDNA. Further quantification of the ^{32}P radioactivity counts of hybridization signal was performed [Figure 27A (b) and 27B (b)]. Sample 11R #17 (Figure 27A) did not show a significant reduction in TMV propagation. Earlier results from lesion counts

and DAS-ELISA (Figure 24 and Figure 26) showed significant virus resistance in this sample.

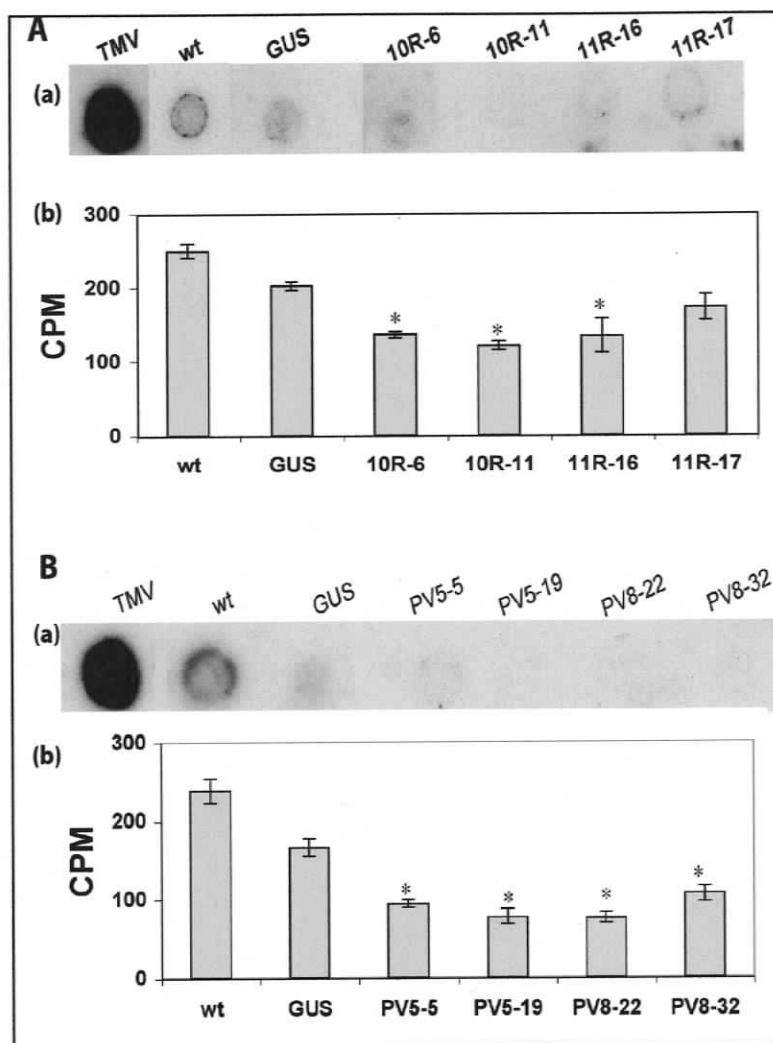


Figure 27. Quantitative dot-blot hybridization with transgenic tobacco plant extracts.

Hybridization was performed with Detached leaves were infected with TMV movement protein (MP)-cDNA probe and TMV infected 10R and 11R (A) and PV5 and PV8 (B) expressing transgenic tobacco plant extracts. Dot-blot was performed as described in "Materials and Methods". Confirmation of extent of hybridization was done by ^{32}P radioactivity determination (b). CPM results show the average of 3 independent experiments. Significant differences between mean values of controls (non-transgenic and Gus) and transgenic tobacco plants are indicated by * at $p < 0.05$ (Tukey's test).

The results for virus-testing using detached leaves, the transgenic plants expressing 10R, 11R, PV5 and PV8 showed reduction in TMV. There was no difference in the antiviral activity in transgenic lines either with single or multiple gene copies.

3.7.2. Greenhouse studies and viral resistance

Table 7. Susceptibility of transgenic tobacco plants expressing 10R, 11R, PV5 and PV8 to infection by TMV, PVX and PVY

Line	PVY	PVX	TMV
1. Non transgenic	+ve, more severe	+ve	numerous lesions
2. Gus	+ve, more severe	+ve	+ve
3. PV5 #1	+ve more severe	+ve	+ve
4. PV5 #5	+ve	+ve	1/4 *
5. PV8 #1	+ve	+ve	+ve bunchy*
6. PV8 #22	+ve	+ve	+ve
7. 10R #6	+ve	+ve	+ve
8. 10R #19	+ve	+ve	numerous lesions
9. 11R #12	+ve	+ve	+ve
10. 11R #13	+ve	+ve	+ve

*= These samples show systemic spread of TMV, while the samples 1 and 2, which are controls (as non-transgenic or containing GUS gene) behave as hypersensitive host.

Table 7 shows the data for visual symptoms of *Nicotiana tabacum* var. Xanthi inoculated with PVY, PVX, or TMV. Four plants of each line were inoculated with each virus except for line 10 (11R #13), where only one plant was inoculated with each virus.

There is no difference in any of the tobacco lines relative to control for the viruses tested. The tobacco cultivar used was hypersensitive to TMV, thus inoculated leaves develop necrotic local lesions. Each plant was inoculated with virus containing sap on three fully

developed leaves. Each leaf, irrespective of the transgene, developed over 300 lesions, indicating a high susceptibility to TMV. Similarly, the PVX strain used in this study caused ring spot lesions on most tobacco plants. Similar symptoms were seen on all tested tobacco lines with lesion numbers similar to those seen in TMV infection. PVY necrotic strain was used for inoculation on leaves. All plants developed strong vein clearing and necrotic vein symptoms, typical of this strain, thus, these lines did not show any resistance to these viruses, though symptoms were more severe in the non-transgenic and GUS plants.

3.8. Partial cationic fraction characterization from tobacco plants

3.8.1. Cationic fraction isolation

Figure 28 shows the AU-PAGE with total cationic fractions isolated from transgenic (PV5 # 6 and 11R # 13) and non-transgenic tobacco plants. This gel separates fractions based on the charge with highly positively charged fractions moving farther. The bands seen on the upper part of the gel are histone fractions from the plants (Dr. J. Ausio, personal communication). Preliminary results in this study have indicated antibacterial activity of the purified tobacco histone fractions (data not shown).

The bands for cationic fractions were faint and showed that the total fractions on filtration retain part of cationic fractions and that some is lost in the process of filtration. For the cationic fractions, interestingly, a smear was observed in electrophoretic path instead of a discrete band in non-transgenic and transgenic fractions (Figure 28A and 28B). The smears were especially more intense in the transgenic plants fractions when same amount of fractions were loaded.

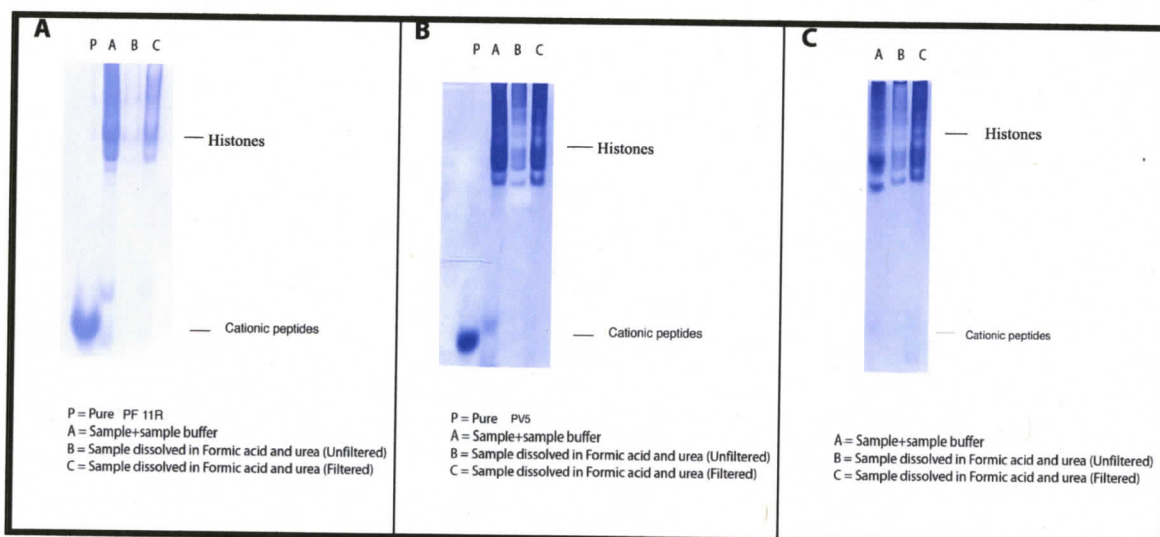


Figure 28. Acetic-acid urea polyacrylamide gels with total cationic fractions

Fractions from transgenic plants expressing 11R (A), PV5 (B) and non-transgenic plants (C) Cationic fractions were isolated as described in “Materials and Methods” and were electrophoresed. Different lanes correspond to the samples as indicated. Non-labeled smears (top) indicate tobacco histones.

3.8.2. Cationic fraction partial purification

Figure 29 shows the reverse-HPLC data for 11R (Figure 29A), PV5 (Figure 29B) and non-transgenic plant cationic fractions (Figure 29C). Peaks marked by #25, #26, #18, #19, #20 and #21 correspond to the positively charged and hydrophilic fractions that eluted first and may correspond to cationic peptide fractions. The other peaks in Figure 29 correspond to the fractions rich in plant histones (Dr. J. Ausio personal communication). There was increase in the intensity of the reverse-HPLC peaks in the transgenic plant fractions [peak # 18, # 19 (Figure 29 A) and # 25, # 26 (Figure 29B)] relative to the one seen in non-transgenic plant fractions (peak # 20 and 21) (Figure 29 C). In AU-PAGE for these fractions, smears seen were at a slightly higher level than the pure peptides in transgenic fractions [Figures 29A (inset) and 29B (inset)]. These RP-HPLC fractions were used for further analysis.

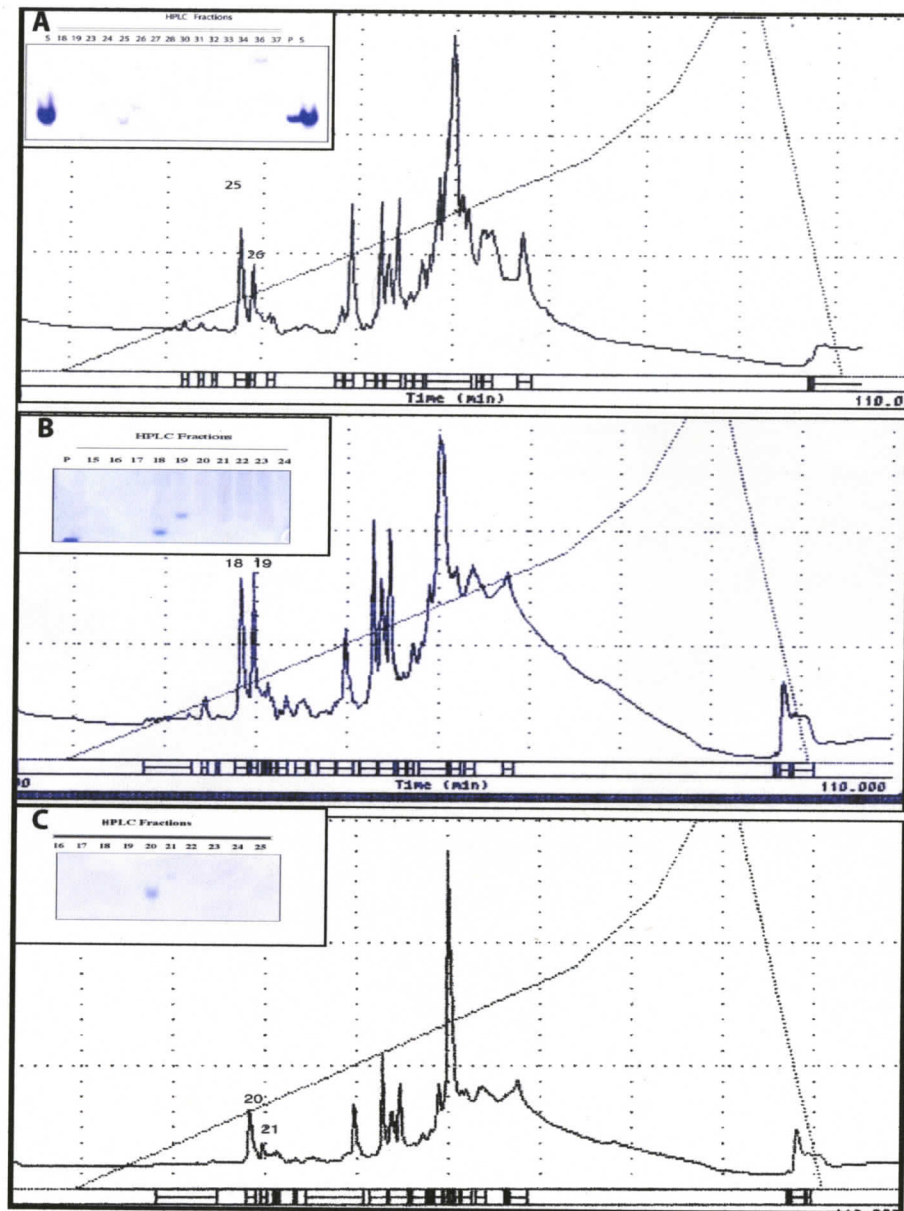


Figure 29. Reverse-phase high performance liquid chromatography of the cationic fractions

(A) 11R (B) PV5 and (C) non-transgenic fractions. Filtered cationic fractions were subjected to RP-HPLC on a Vydac C18 column. Elution was achieved at a flow rate of 1 ml/min with an acetonitrile gradient and other parameters as described in "Materials and Methods". The inset in each figure shows the numbered fractions, which were vacuum dried, dissolved in, distilled water and analyzed with AU-PAGE. Lane P corresponds to the pure peptide used as a marker.

3.8.3. Cationic fraction characterization

Further characterization of the fractions rich in cationic peptide was done by amino acid analysis (data not shown). All fractions were found to be rich in glycine, arginine, tyrosine and lysine residues. Determination of mass for HPLC-purified cationic fractions was done using MALDI-TOF mass spectrometry (data not shown) and results showed that each fraction has many peptides or peptide fragments. However, in fractions from PV5 (# 19) and 11R (#26) plants there was a peak observed at m/z 2763.99 Da and m/z 2283.15 Da respectively. These peaks were not seen in the non-transgenic fraction (#21) as expected.

PV5 and 11R variants expressing lines (with multiple copy gene insertion and high expression) were chosen for this study as the representative of the transgenic tobacco plants. The study would be extended to 10R and PV8 expressing plants in future studies.

CHAPTER 4: DISCUSSION

Commercial agricultural practices are commonly threatened by recurring bacterial, fungal and viral infections. Pesticides and insecticides have been commonly used to contain phytopathogens, however, their extensive use has contributed to the chemical contamination of the environment. Attempts have been made to bolster plant defenses against microorganisms by genetically engineering plants to express cationic peptides (Gao et al., 2000; Osusky et al., 2000; Osusky et al., 2004; Osusky et al., 2005; Yevtushenko et al., 2005). This approach has proven effective for combating bacterial and fungal diseases in plants. Because the primary target of the cationic peptides is the cell membrane and not a specific receptor or substrate, these peptides confer their activity against a broad-spectrum of pathogens and there is less probability of resistance arising by variation of their metabolic pathways. There is no report of tapping the antiviral property of these cationic peptides, shown *in vitro* against animal viruses, for providing viral resistance in plants. Therefore, the focus of this study was on antiviral cationic peptides.

To confer broad-spectrum resistance against plant pathogens including viruses, indolicidin(s) and polyphemusin(s) variants were screened for their *in vitro* antimicrobial activity and their cytotoxicity on plant tissues and protoplasts. Indolicidin and polyphemusin variants were expressed in plants by genetic engineering and assessment of disease resistance of the transgenic plants was performed.

4.1. 10R, 11R and PV5 show broad-spectrum *in vitro* activity

Erwinia carotovora was previously shown to be highly sensitive to cationic peptides (Osusky et al., 2000; Osusky et al., 2004; Osusky et al., 2005) and similar activity was demonstrated by 10R, 11R and PV5 in this work. Some strains of *Bacillus* are resistant to cecropins and other cationic peptides (Hultmark et al., 1982; Casteels et al., 1989). However, 10R, 11R and PV5 showed strong antibacterial activity against *Bacillus subtilis* (Figure 1) exhibiting the wide-range antibacterial nature of these peptides. The weak Gram-negative bactericidal activity of 10R against normally sensitive, LPS-deficient strains of *E. coli* suggested that the low positive charge of indolicidins may not interact well with the negative charge of the outer membrane (Mangoni et al., 2000; Osusky et al., 2004). During the evaluation of antibacterial activity, the concentration of salts and nutrients above diluent threshold values can inhibit the effect of antimicrobial peptides on bacteria or on the germination of fungal conidia (Jacobi et al., 2000); therefore, all the inhibition assays were performed in LB medium which was 25-fold diluted. The minimum inhibitory concentrations (MIC) of PV5 and 11R for *E. carotovora* were 2-4 times higher than that of other peptides described earlier (Osusky et al., 2000; Osusky et al., 2004; Osusky et al., 2005). The MIC of 10R was similar to MsrA1 (Osusky et al., 2000).

The *in vitro* efficacy of 10R, 11R and PV5 against plant pathogenic fungi differed tremendously. The antifungal activity of 10R and 11R seemed to be cultivar-specific but this needs to be further explored. 10R, 11R and PV5 showed increased inhibition of *Fusarium graminearum* with increase in the concentration (Table 1-3). In case of *Verticillium sp.*, using the AlamarBlue™ assay for determining antifungal activity of 10R and 11R, there was a significant increase in fluorescence and hence viability (Table 1-2).

PV5, when tested with *Verticillium*, showed significant increase only in 10 µg/ml (Table 3). AlamarBlue™ reaction is dependent on the number of spores used, species of the fungi and the incubation time (Amanda Berg, personal communication). The anomaly seen in the viability relative to control in testing *Verticillium sp.* (Table 1-4) could be due to these factors or can result due to differences in the cell wall composition. Further studies are required to confirm the trend observed

Earlier reports showed PV5 to be more antibacterial and antiviral (Murakami et al., 1991; Arakaki et al., 1999; Zhang et al., 2000) than antifungal in nature. In this study, PV5 showed significant reduction in *Fusarium graminearum* and thus this peptide variant had antifungal activity in addition to antibacterial and antiviral activities. PV8 show antibacterial and antifungal activity previously against animal pathogens (Zhang et al., 2000).

There are no reports describing the *in vitro* antiviral activity of a polyphemusin or an indolicidin against plant viruses. 10R, 11R and PV5 possess antiviral activity against animal viruses (Murakami et al., 1991; Robinson et al., 1998; Arakaki et al., 1999; Murakami et al., 1999). Indolicidin variants, 10R and 11R showed *in vitro* antiviral activity in this study towards TMV but polyphemusin variant PV5 did not. Tobacco Mosaic Virus was used because it represents the best-studied plant virus and is a specific tobacco pathogen. *In vitro* testing of 10R and 11R peptides against TMV on tobacco showed a reduction in viral infection as demonstrated by a reduction in the number of lesions, and subsequently by ELISA and dot blot hybridization. This is the first report of antiviral activity of indolicidin peptides against a plant virus. Cationic antimicrobial peptides have shown to form pores in membranes, leading to cell lysis. Polyphemusin

had no *in vitro* anti-TMV activity but have shown earlier to have antiviral activity against membrane bound animal viruses (Murakami et al., 1991; Arakaki et al., 1999; Murakami et al., 1999). This suggests the specificity of polyphemusin activity on pathogen membranes.

Although indolicidin is presumed to act on bacteria and fungi by disrupting membranes (Falla, et al. 1996), its mechanism of action remains to be established. Interestingly, indolicidin has also been shown to permeabilize membranes of bacteria, but the membrane permeabilization does not lead to lysis (Falla, et al. 1996). Indolicidin mainly reduces synthesis of DNA of the pathogen (Sitaram, et al. 2003; Falla, et al. 1996). Recently, indolicidin was also shown to bind to calmodulin (Sitaram, et al. 2003), showing the changes in cytoplasmic permeability and the transport of the peptide across the membrane. Similar effect by indolicidin variants (10R and 11R) is possible on TMV RNA and hence resulting in virus inhibition.

4.2. 10R, 11R and PV5 have low cytotoxicity and Phytotoxicity

In vitro cytotoxicities and phytotoxicities of 10R, 11R and PV5 synthetic peptides were assessed and compared in order to evaluate their potential usefulness in augmenting the natural defense systems in plants. Synthetic derivatives of polyphemusin and indolicidin peptides have been shown to have low cytotoxicity to animal cell lines (Falla and Hancock, 1997) and higher toxicity to microorganisms (Nakashima et al., 1992; Selsted et al., 1992; Yasin et al., 2000), which makes them good candidates for expression in plants. Low cytotoxic effect of indolicidin derivatives (10R and 11R) up to 60 μ g and polyphemusin derivative (PV5) up to 10 μ g except in neutral red assay was found in plant

cells as determined by several cytotoxicity assays in this study. For the TTC assay with leaf discs, formazan formed in tissues was measured at 530 nm rather than at 490 nm to avoid interference with pigments such as chlorophyll (Steponkus and Lanphear, 1967).

The optimization of incubation time for colorimetric assays is necessary and differs among plant tissues (Parker, 1953), algae (Chang et al., 1999; Capasso et al., 2003) and suspension tissue cultures (Towill and Mazur, 1974). Earlier, the intensity and extent of TTC staining were successfully employed to predict the germinability of seeds and viability of plant tissues exposed to stressful conditions and injury (Steponkus and Lanphear, 1967; Towill and Mazur, 1974; Able et al., 1998; Chang et al., 1999). In addition to the TTC reduction method, the dyes neutral red, fluorescein diacetate and erythrosine are used to assess the viability of animal cells (Borenfreund et al., 1988), plant protoplasts (Oprisko et al., 1990) and green microalgae (Saga et al., 1989). Neutral red is commonly used for testing the viability of human cell lines (Borenfreund et al., 1988) but is less commonly used for plants (Fernandez et al., 2000). In this study, all of these colorimetric assays were used on plant protoplasts and/or leaf discs. There was no similarity in the results was observed between the TTC and neutral red assay in tobacco protoplasts and hence the reliability of protoplast-TTC method also remains questionable due to the physical nature of the protoplasts.

At 100 μg , PV5 exhibited cytotoxicity when observed by light microscopy (Figure 13). Similar cytotoxicity at high concentrations was reported earlier with cecropin and melittin (Mills and Hammerschlag, 1993; Yevtushenko et al., 2005). The high sensitivity of the protoplasts may be associated with the lack of cell walls.

The parallel in the relative changes between TTC reduction activity in the leaf discs and Evan's blue staining, suggest that these assays could be used to determine the cytotoxic effects of peptides and other compounds against plant leaf tissues. The results indicated that synthetic 10R, 11R and PV5 have low cytotoxicity toward tobacco leaf discs, protoplasts and plantlets up to 10 μg of peptide. Together these tests provide a set of indicators of cytotoxicity and can readily be adapted for mass screening of peptide(s) and their variants, particularly in association with combinatorial methods.

Earlier, it had been proposed that abiotic or biotic stress in plants results in the generation of reactive oxygen species and hence an increase in antioxidant molecules (Young and Britton, 1990; Slooten et al., 1995; Kummerova and Kmentova, 2004). The increase in carotenoids observed in plantlets grown in 100 μg of 11R and 60 μg 10R (Table 4) can be attributed to the abiotic stress caused by the peptides, which in turn causes an increase in carotenoids.

Cationic peptides may show different specificities in their activity against pathogens as well as on mammalian or plant cells (Blondelle and Houghten, 1991). The microbicidal range of action of most antimicrobial peptides is 2-40 $\mu\text{g}/\text{ml}$ (Hancock, 1997). The plant tissues and protoplasts were tested at higher amount of cationic peptides (20, 60 and 100 μg) than their range of action on pathogens as shown in this work (Figure 2). Cytotoxic assays showed that at 20 μg , 10R, 11R and PV5, have no discernible toxic effects. This tested amount (20 μg) is much higher than the amount likely to be expressed in transgenic plants (Carmona et al., 1993; Osusky et al., 2000; Osusky et al., 2005). Earlier it has been shown that plants with 2x35S CaMV promoter express cationic peptides, in the range of 1 $\mu\text{g}/\text{g}$ – 5 $\mu\text{g}/\text{g}$ of fresh tissue weight (Osusky et al., 2000; Osusky et al.,

2005). The lowest amount tested in this study was 20 μg on 20 mg of fresh leaf disc. No cytotoxic effect was seen at this test amount, for any of the peptides. PV5 showed phytotoxic effect on plants at 100 μg , leaf discs at 60 μg and on protoplasts at 20 μg (Table 4, Figure 9-13). These are also a very high amount and it is highly unlikely that PV5 would reach this amount in transgenic plants.

Use of carbenicillin as the negative control was based on the hypothesis that there would be no effect of this antibiotic against plant leaf discs, protoplasts as well as plantlets. Carbenicillin has been extensively used at high concentrations up to 500 $\mu\text{g}/\text{ml}$ in plant tissue culture and have been found to be safe on plant calli and shoots during transformation and selection processes. Results (Figures 9, 11, 12 and Table 4) show toxicity of this antibiotic on plantlets, leaf discs and protoplasts at 100 μg under the conditions of the experiments in this work. This indicates the use of other compound as negative control other than potent antibiotics like carbenicillin in future studies.

The colorimetric and phytotoxicity assays used for determining the effects of peptides on leaf discs, protoplasts and plantlets provided reliable, quick and efficient methods that could easily be adapted for screening peptides and other compounds to assess their safety in plants. Based on the data, the safety of these cationic peptides, supports their credibility and application as environmentally friendly alternatives to current antimicrobial approaches.

4.3. Transgenic plants expressing 10R, 11R, PV5 and PV8 are resistant to plant pathogens including TMV

In planta studies showed that tobacco plants expressing 10R, 11R, PV5 and PV8 are resistant to plant pathogenic virus, bacteria and fungi. These results are similar to recent works using synthetic variants and chimeras of other cationic peptides for conferring broad-spectrum bacterial and fungal resistance in plants (Gao et al., 2000; Yevtushenko et al., 2005).

Earlier studies showed that the use of the 2x35S CaMV promoter leads to a significant level of cationic peptide transcripts in plants (Osusky et al., 2000; Osusky et al., 2004; Osusky et al., 2005). A similar level of expression was achieved for 10R, 11R, PV5 and PV8 in this work when 2x35S CaMV was used to drive the expression of peptide(s). The signal peptide from *Pseudotsuga menziesii* BiP sequence (the endoplasmic reticulum (ER) molecular chaperone) functions to retain BiP in the ER and subsequently help in folding and assembly of the newly synthesized peptide (Forward and Misra, 2000). This BiP signal sequence was included in polyphemusin constructs of PV5 and PV8 (Figure 15) in order to enhance the yield of the peptide. However, the effectiveness of this strategy was not established in this study.

The effects of genomic position and copy number on transgene expression have been reported in plants (Hobbs et al., 1993; Allen et al., 2000). Because transgenes integrate at random sites during transformation, some integration may occur in transcriptionally active chromatin environments, or in transcriptionally inert chromatin regions (Mengiste et al., 1999). It is believed that transgenes in heterochromatic areas are prone to silencing and give rise to reduced and/or variable expression (Mengiste et al., 1999; Allen et al., 2000). Figure 18 (i) shows no expression in lane 5 and lane 7, which could reflect this positional insertion effect. The other reason for a lack of expression could be gene

silencing. Plants with multiple copies of the transgene and/or high levels of transgene transcription are more likely to exhibit gene silencing than plants with a single copy and low-level transcription (Hart et al., 1992; Flavell, 1994; Meyer, 1996; Meyer and Saedler, 1996). There are conflicting reports about the relationship between copy number and expression level (Hobbs et al., 1993). The two variables have been shown to be negatively correlated (Cervera et al., 2000), not correlated (Bauer et al., 1998), or positively correlated (Hobbs et al., 1993). This inconsistency was observed in this work (Figure 18 and 19).

Experiments with detached leaves from transgenic tobacco plants demonstrated bacterial resistance when tested with *E. carotovora* - the primary cause of bacterial soft rot in plants. Similar results have been shown earlier with *E. carotovora* and expression of other cationic peptides in plants (Allefs et al., 1996; Osusky et al., 2004; Osusky et al., 2005). This *in planta* antibacterial activity is in agreement with the strong *in vitro* antibacterial activity seen for the synthetic derivatives of the expressed peptides (Figure 1 and 2). Leaf extracts from the transgenic plants showed significant reduction in *Erwinia* growth relative to non-transgenic plants (Table 5) but not to GUS plant extracts, except two lines. This leaves the scope to question the effect of simple transformation event. However, earlier reports with the expression of cationic peptides in tobacco have shown the activity of proteases at very early stage of post-translational events (Cavallarin et al., 1998; Hancock and Lehrer, 1998; Cary et al., 2000). This fact was also supported by the negative effect of transgenic plant extracts on *S. epidermidis*. *In vitro* studies showed a significant bactericidal effect of 10R, 11R and PV5 on *S. epidermidis* (Figure 1). Also, to exclude the possibility that the increased resistance of transgenic plants is triggered

simply by the transformation event, control plants transformed with the vector pBI 121 (expressing GUS) were included in all disease assays. Because the increased resistance (except in testing with *Fusarium oxysporum*) was observed only in plants expressing these peptides, the results strongly suggest that the resistance of transgenic tobacco plants is caused by the expression of the 10R, 11R, PV5 or PV8.

Plants expressing 10R, 11R and PV8 showed resistance to *Botrytis* and *Verticillium* but not to *Fusarium oxysporum*, indicating species specificity of the expressed peptides. *Fusarium* species are highly virulent fungi and have been shown to overcome the *in planta* resistance provided by cationic peptides (Cavallarin et al., 1998). Transgenic plants except PV5 #5 did not show any resistance against *Fusarium*. However, earlier studies with synthetic 10R, 11R and PV5 showed *in vitro* resistance against *Fusarium graminearum* (Table 1-3). The fungal resistance seen for *Botrytis* and *Verticillium* by some lines in PV5, 10R and 11R expressing plants is contradictory to the *in vitro* results of peptides on these fungal strains (Figure 4 and Tables 1- 3). PV5 showed some resistance against *Fusarium* when expressed *in planta*, but not in *in vitro* studies.

In addition to broad-spectrum bacterial and fungal resistance, transgenic plants expressing 10R, 11R and PV5 are resistant to TMV in lab conditions. This is the first report of viral resistance in plants expressing cationic peptides. A new strategy for engineering virus-resistant plants by transgenic expression of a dominant interfering peptide was shown by Rudolph et al. (2003) and presented a promising strategy for expressing small peptides in plants. Expression of only a short peptide or artificial peptides minimizes the potential deleterious effects on the plant cells. Greenhouse and field studies differed from the laboratory set-up in that the viral inocula greatly exceed

those used in the laboratory. The high TMV inocula used in the greenhouse studies might have surpassed the viral resistance of cationic peptide expressing plants, which was seen in the detached leaf assays. Also, it has been reported that, in contrast to greenhouse conditions, detached leaves of plants grown in the laboratory have an extremely different profile of expression of defense modulators and additional wounding causes pronounced differences in expression of defensive genes (Steinite and Levinish, 2002).

It is clear from this work that although these antimicrobial peptides are broad-spectrum peptides, their activity does show specificity for certain strains of fungi and bacteria. Similar specificity against other plant viruses is expected. Also, the data obtained in laboratory assays differ from that obtained in greenhouse studies. The lack of viral resistance in transgenic plants, tested in the greenhouse (Table 7), suggests the need for further work to develop strategies to enhance the expression of peptides.

If successful, this approach could provide an environmentally friendly and effective approach to combat plant diseases.

4.4. Partial purification of 11R and PV5 from transgenic tobacco

In order to examine the expression and the amount of 11R and PV5 in transgenic tobacco, partial purification and characterization of the cationic fraction using amino acid analysis, RP-HPLC and MALDI-TOF was performed. The identities of the expressed peptides could not be confirmed by amino acid analysis or RP-HPLC. However, MALDI-TOF analysis showed the presence of expressed PV5 and 11R in oxidized forms. The presence of modified forms of plant expressed cationic peptides has been shown earlier

(Banzet et al., 2002). Confirmation and further characterization of these tobacco-expressed peptides by proteomics and Western blots is needed.

The tobacco histones seen in Figure 28 were partially purified by RP-HPLC (data not shown). The presence of helical peptides showing homology to cleaved-forms of histones and their activity against bacteria and fungi (Park et al., 1998) has been reported. It is suggested that histones act as ubiquitous components of host defenses (Hirsch, 1958). Future studies are required to obtain more information about the antimicrobial properties of the isolated tobacco histone fractions in this work.

CHAPTER 5: FUTURE STUDIES AND CONCLUSION

5.1. Future Studies

The best strategy for providing enhanced and broad-spectrum resistance using cationic peptides is the co-expression of different molecules with complementary modes of action that act at different stages of disease development. There are reports of synergistic action of antimicrobial peptides. Combinations of 10R, 11R, PV5 and PV8 and other potential peptides may be a successful strategy for generating broad-spectrum disease resistance including resistance against viruses, in plants. Efforts have been made to express defense molecules in plants using specific promoters (Rao et al., 1998; Nagadhara et al., 2003; Yevtushenko et al., 2005). Strategies for a regulated and/or inducible, and tissue-specific expression of peptides may prove to be effective for better performance in the greenhouse as well as in the field.

The heterogeneity observed in the samples when separated by RP-HPLC indicated that these chromatographic techniques were not sufficient to isolate the target peptides from transgenic plants. The yields of a peptide from transgenic plants depend on several factors including synthesis, processing, export and proteolytic degradation. A combination of selective protease inhibitors and subsequent purification steps are required to isolate the peptides from the transgenic plants. Proteomics studies will be useful to characterize the oxidized form of peptides observed in this work. To further quantify the expressed peptides in transgenic plants, one strategy would be to generate antibodies, and perform Western blotting (Osusky et al., 2005).

Future characterization of unknown cationic peptides/proteins (Fraction #20, Figure 31) can reveal their function and may provide novel tools for genetic engineering in plants either as a signaling molecule or as a plant-derived antimicrobial agents. Initial results with tobacco histone fractions demonstrated their antibacterial activity and warrant further study of plant derived antimicrobial peptides.

In addition to direct antimicrobial activity, there are reports of the role of cationic peptides in the regulation of innate immunity and defense responses in animals (Hancock and Diamond, 2000; Chernysh et al., 2002). Some antimicrobial peptides may upregulate the production of interleukins (Welling et al., 1998) in animals. Plant hormones, which are involved in defense signaling in plants (Imanishi et al., 1997; Pieterse and van Loon, 1999; Thomma et al., 1999) are the homologues of interleukins in plants and may be regulated in the similar fashion by the antimicrobial peptides (AMPs). There is evidence of antimicrobial protein's up-regulation by microbial elicitors through proteolytic cascade in plants (Subramaniam et al., 1997; Garcia-Olmedo et al., 1998), and of an extensive cross talk among different defense components in plants, including peptides (Dangl and Jones, 2001; Taylor et al., 2004; Taylor and McAinsh, 2004). In the present and previous studies, there was an indication of enhancement of disease resistance in transgenic plants against the pathogens, despite a poor *in vitro* inhibition by cationic peptides. Therefore, it is possible that the expression of the cationic peptides in plants modulates defense gene expression or provides cross talk with other defense signaling pathways. A suggested model of the disease resistance signaling initiated by expression of the cationic peptides in the plants is shown here (Figure 30)

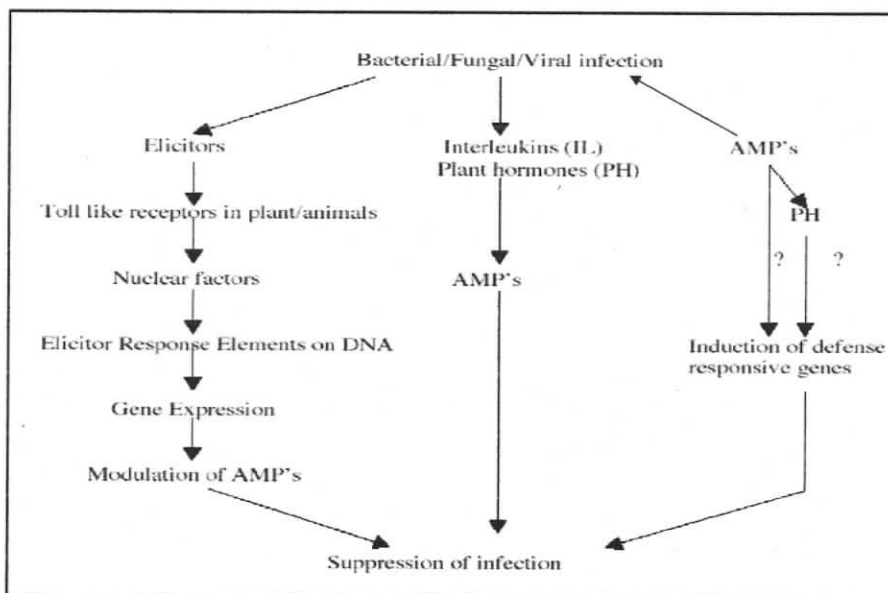


Figure 30. Simplified model depicting the possible role of antimicrobial peptides in disease resistance in plants

Question marks denote the proposed induction of defense genes by AMPs when expressed in plants.

Future work may determine whether other components of the immune signaling pathways are activated in response to the expression of cationic peptides in transgenic plants. To examine the role of cationic peptides in immune responses in plants, MsrA3, an N-terminally modified temporin A, showing broad-spectrum antimicrobial activity in plants (Osusky et al., 2004), was introduced in *Arabidopsis thaliana* (data not shown). The complete genome sequence of *Arabidopsis* has permitted the investigation of gene profiling in response to various cues (Schaffer et al., 2000; Schenk et al., 2000; Zhu, 2003). Future microarray analysis and transcript profiling of transgenic *Arabidopsis* plants will provide useful information on the effects of heterologous peptide expression in transgenic plants.

5.2. Conclusion

Ethical concerns have often been expressed about the production of genetically modified plants and animals, including humans. However, organisms naturally produce many cationic peptides as part of their innate defenses against infection. The results on the low cytotoxicity of 10R, 11R and PV5 synthetic peptides in plants are promising. The incorporation of cationic peptides into plants through genetic engineering offers a means to prevent disease-associated losses as well as to protect the environment.

Successful application of a transgenic approach using these peptides to control plant diseases, particularly viruses, will likely help eradicate plant diseases, reduce the environmental impact of intensive agriculture, especially use of pesticides and improve the quality of our food.

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