Expression of BMAP18 in Transgenic Potato for Production and Enhanced Disease Resistance

by

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B.Sc., University of Victoria, 2004

This Thesis is Submitted in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

Supervisory Committee

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Abstract

Cationic Antimicrobial Peptides (CAPs) exhibit broad-spectrum activity against a variety of microbial pathogens at concentrations that are non-toxic to higher eukaryotes. These properties make them excellent candidates for both pharmaceutical and agricultural applications. Here BMAP18, a CAP with an especially high charge to mass ratio, is evaluated for efficient production of the peptide and enhancement of disease resistance in transgenic potato. *In vitro* analyses indicated that BMAP18 had potent activity against a variety of clinically and agriculturally relevant pathogens, including the protozoan parasite *Trypanosoma brucei*. *In planta* activity was assessed by transformation of potato (*Solanum tuberosum* L.) plants with a synthetic BMAP18 gene under control of the enhanced CaMV 35S promoter or the Douglas-fir luminal Binding Protein (PmBiP) promoter. Stable transformants expressing the transgene were shown to accumulate the peptide and exhibited enhanced resistance to *Fusarium* wilt and bacterial soft rot caused by *Erwinia carotovora*.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

[α-^{32}P]dCTP	radiolabeled deoxycytidine triphosphate
°C	degrees Celsius
A	absorbance
AMV	alfalfa mosaic virus
bp	base pairs
BSA	bovine serum albumin
BSF	bloodstream form
CaMV	cauliflower mosaic virus
CAP	cationic antimicrobial peptide
cm	centimeters
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FBS	fetal bovine serum
Fv	variable fragment
g	gram
GUS	β-glucuronidase
HPLC	high performance liquid chromatography
hr	hour
HT	human transferrin
IgA	immunoglobulin A
IgG/IgM	immunoglobulin G/immunoglobulin M
kDa	kiloDaltons
KLH	keyhole limpet hemocyanin
L	litre
LB	Luria-Bertani medium

LePAL	tomato phenylalanine ammonium lyase
LPS	lipopolysaccharide
M	molar
mAbs	monoclonal antibodies
mg,	milligram
MIC	minimum inhibitory concentration
min	minute(s)
mL	millilitre
mM	millimolar
mm	millimeter
MS	Murashige and Skoog medium
MW	molecular weight
nm	nanometer
NosT	nopaline synthase terminator
NPTII	neomycin phosphotransferase II
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCF	procyclic culture form
PetM	petiole medium
PmBiPPro	Douglas fir BiP promoter
SDS	sodium dodecylsulphate
sec	second(s)
SSC	saline sodium citrate
T-DNA	transfer DNA
TTP	thymidine triphosphate
U	units
UTR	untranslated region
UV	ultraviolet
UVic	University of Victoria
V	Volts

*win*3.12T	truncated wound-inducible Poplar promoter 3.12
µg	microgram
µL	microlitre
µM	micromolar

Acknowledgements

I thank all those who supported me throughout the course of this degree. In particular, I am grateful to my supervisor Dr. S. Misra for encouraging me to pursue graduate studies and for her constant faith. Thanks to Milan Osusky for construction of the synthetic BMAP18 gene and to Brad Binges for assistance in the greenhouse. I would like to recognize and thank all members of the Pearson lab for their collaboration regarding antibody production and the kind gift of trypanosomes and culture medium. Also, I want acknowledge my lab mates, past and present, especially Dr. Dmytro Yevtushenko, Apurv Bhargava and Mariana Vetrici, for their constant support, guidance and camaraderie. Also, I thank my friends in the Biochemistry and Microbiology Department and the Centre for Forest Biology whose scientific conversation and companionship made the whole graduate school experience so enjoyable. In particular, many thanks to Cory Brooks for his patience and support through the highs and lows of this past year. Finally, I must thank my parents, Gino and Cathie, and brothers, David and Paul, for their love, and encouragement in all I undertake.

Chapter 1: Literature Review

This study examines the feasibility of recombinant expression of BMAP18 in transgenic potato for pharmaceutical and veterinary purposes as well as enhancement of disease resistance in plants. BMAP18 is a highly cationic antimicrobial peptide with proven activity against a variety of bacteria and fungi as well as the protozoan parasite, *Trypanosoma brucei* (Skerlavaj *et al.,* 1996; Haines *et al.,* 2002).

1.1 Cationic Antimicrobial Peptides

1.1.1 Origins and Structures

Cationic antimicrobial peptides (CAPs) are a diverse group of small, positively charged peptides that are involved in the innate immune defenses of all complex organisms. In mammals, CAPs are found primarily in tissues exposed to pathogen threat, such as the skin and mucosal epithelia, where they are released as an antimicrobial cocktail (Zasloff, 2002). However, they are also found in circulation in the leukocytes of vertebrates and in the hemolymph of insects and crustaceans (Kurata *et al.,* 2006). In insects, circulating hemocytes are recruited to the site of injury to release their peptide arsenals (Brown et al., 2006).

Through selective pressure, CAPs have evolved into a diverse group of peptides that share a single unifying trait: the ability to adopt a secondary structure in which clusters of hydrophobic and cationic amino acids are spatially organized to discrete sectors of the molecule creating an amphipathic character (Zasloff, 2002). Similarities in secondary

structure, amino acid composition, genetic structure and tissue of origin have been used to group the peptides into families. For example, dermaseptins are isolated from frogs and other amphibians, while cecropins and abaecins are isolated from insects. Defensins are characterized by a disulphide bridged β-sheet motif, and are further classified based on species of origin. Cathelicidins share a genetic structure in which the CAP is translated with a conserved N-terminal proregion (Hancock and Diamond, 2000). Some CAPs are rich in rare amino acids, such as the proline-rich bactenectins, and cysteine-rich thionins. To date, databases (http://www.bbcm.univ.trieste.it/) catalogue over 800 naturally occurring CAPs in plants and animals alone, and still more are being discovered. In addition, CAPs are being synthetically designed to perform specific functions based on trends observed for the activities of the naturally occurring peptides (Hilpert *et al.*, 2005).

1.1.2 Mode of Action

The presence of CAPs in tissues and cells involved in host defense supports the hypothesis that these peptides evolved as elements of innate immunity (Brogden, 2005). In fact, CAP inhibition assays indicate that their antimicrobial activity is required to maintain the balance of commensal bacteria and prevent the onset of disease caused by opportunistic pathogens (Boman, 1995). The mechanisms by which CAPs function in host defense are as diverse as the peptides themselves. They exhibit direct antimicrobial activity by creating ion-permeable pores in target cell membranes or by translocating across the membrane to intracellular targets. In addition, some CAPs act indirectly by recruiting effector cells of innate immunity, such as macrophages and T cells, to the site of infection or by inactivating endotoxins to prevent sepsis.

1.1.2.1 Direct antimicrobial activity

CAPs act directly on microbial membranes causing cell death through one of the following mechanisms: 1) insertion into the membrane forming a pore through which ions and other cell contents may leak, 2) depolarization of the membrane by forming a peptide carpet on the surface, 3) disruption of membrane composition and function by shuffling of the phospholipids between the planes of the bilayer, and 4) induction of apoptosis through interaction with intracellular components (Zasloff, 2002). Regardless of the mode of killing, three steps must occur for a CAP to lyse a cell: attraction, attachment and permeabilization (Brogden, 2005). Attraction of a peptide to the surface of its target occurs through electrostatic bonding of the positively charged peptide with the negatively charged surface of its target. Attachment of a CAP to a cell membrane occurs when the concentration of peptide is low in relation to the concentration of phospholipids. Peptides adsorb to the membrane through a receptor-independent mechanism and are embedded into the lipid head group layer in a parallel orientation (Brogden, 2005). At this point, the peptides must fold into amphipathic structure in order to insert their hydrophobic face into the fatty acyl core of the lipid membrane, while their hydrophilic face interacts with the polar phospholipid head groups (Oren and Shai, 1999). Insertion into the membrane occurs once the relative concentration of CAP to lipid increases. Accumulation of CAPs on the membrane surface induces a positive curvature that destabilizes the parallel orientation of the peptide with the membrane (Kobayashi *et al.*, 2004). The peptides rotate to become perpendicular to the plane of the membrane and either form stable membrane pores, or are translocated to an intracellular target. The

exact mechanism by which CAPs cause cell death is dependent on the cell type and surface structure.

1.1.2.2 Antibacterial activity

The outer surfaces of bacteria are negatively charged due to the presence of lipopolysaccharides on Gram-negative or lipoteichoic acids on Gram-positive bacteria on the outer membranes (Bucki and Janmey, 2006). Upon interaction of a CAP with the outer membrane of Gram-negative bacteria, divalent cations stabilizing the LPS are displaced by the positively charged amino acids of the peptides (Tossi *et al.*, 2000). This allows CAPs access to the periplasmic space and the inner membrane. Once in the periplasm, CAPs may be attracted to the negative charge of the carboxyl groups on the amino acids of peptidoglycan, facilitating passage of the peptide through this barrier (Tossi *et al.*, 2000) and access to the acidic phospholipids of the inner membrane (Shai, 2002). It remains to be explained how CAPs traverse surface structures such as the polysaccharide coat of encapsulated bacteria (Brogden, 2005).

1.1.2.3 Antifungal activity

In comparison to prokaryotes, the membranes of eukaryotes contain a greater proportion of zwitterionic phospholipids and sterols (McPhee and Hancock, 2005). The slight negative charge due to the transmembrane potential generated by active respiration is required to attract CAPs to the surface of eukaryotic cells. For example, the activity of histatin 5 against *Candida albicans* is inhibited by the addition of sodium azide, a potent metabolic inhibitor (Helmerhorst *et al.*, 1999). Also, Lee *et al.* (2002, 2002a) reported an

energy-dependency for antifungal activity of both SMAP-29 and an HP-magainin hybrid peptide.

Once attracted to the surface, antifungal peptides cause cell lysis through interaction with sterols to form membrane pores, or through an unknown membrane surface receptor-specific mechanism. The cecropin variant, D4E1, a CAP with activity against a variety of fungal pathogens, has been shown to bind preferentially to ergosterol, a common lipid of fungal membranes (De Lucca *et al.,* 1998). Membrane association of [S^{35}]-labeled Dm-AMP1, a plant defensin, was shown to be saturable and inhibited by pre-incubation with unlabeled Dm-AMP1 or related peptides (Thevissen *et al.,* 2000). This evidence of a target-specific mode of action is corroborated by the existence of a Dm-AMP1 resistant yeast strain (Thevissen *et al.*, 1999).

The asymmetrical phospholipid distribution between the layers of higher eukaryotic cell membranes prevents the attraction and attachment of CAPs; however, as in fungi, enhanced metabolic activity can mediate activity of CAPs against mammalian cells. For example, magainin 2 was found to form pores and cause lysis in bladder cancer cells, but not in normal fibroblasts, indicating specificity for metabolically active cells (Lehmann *et al.,* 2006). Similarly, BMAP-28 is toxic to tumor cells and active, but not resting, lymphocytes (Risso *et al.,* 2002).

1.1.2.4 Anti-protozoan activity

CAPs active against parasitic protozoa have been isolated from their insect vectors. Anti-malarial defensins, cecropins and a novel peptide, gambicin, were isolated from the insect vector *Anopheles gambiae* (mosquito) (Arrighi *et al.*, 2002). Structure function analysis of these and other CAPs led to the design of novel synthetic CAPs that were tested for efficacy in reducing parasite prevalence in infected mosquitoes. It was found that peptides with flexible structures, such as random coils and turns, were more effective than α-helices or β-sheets (Arrighi *et al.*, 2002). Similarly, a glycine-rich peptide isolated from *Glossinia morsitans morsitans* exhibited potent activity (95% growth inhibition) against procyclic form (insect host) trypanosomes at only 5 μM, however, the same concentration reduced the viability of bloodstream form (mammalian host) parasites by only 30%. This may be due to differences in the surface structures of the two forms, which mediate attraction of the peptide to the membrane surface (Hu and Aksoy, 2005).

1.1.2.5 Membrane pore formation

Once attracted to the surface, membrane-active peptides form pores by either the barrel-stave, carpet, or toroidal pore mechanisms (Oren and Shai, 1999). In the barrel-stave model, CAPs aggregate on the membrane surface and insert into the bilayer such that their charged surfaces face a central pore while the hydrophobic surfaces stably interact with the acyl chains of membrane lipids. For this mechanism to occur, the CAP must be at least 22 amino acids long to span the membrane (Shai, 2002). Smaller peptides attached to the membrane surface can induce localized membrane thinning to form a toroidal pore. In a toroidal pore, the positive surfaces of the CAPs are interspaced with

the phosphate head groups of membrane lipids. This both stabilizes the pore by preventing repulsion of the positively charged peptides, and allows for shuffling of lipids from the outer layer of the membrane to inner leaflet. The carpet model describes a situation in which CAPs lying on the membrane surface cause severe distortion of the phospholipid packing resulting in invaginations that rupture the bilayer (Shai, 2002). In each mechanism, lethality results from loss of the proton-motive force and leakage of ions and metabolites from the cytosol (Friedrich *et al,* 2000).

1.1.2.6 Intracellular targets

Insertion of a CAP into a membrane may be a lethal event as described above, or it may be a transient event associated with transport of the peptide across the membrane surface. Comparison of the activities of six CAPs of diverse structure against Gram-positive bacteria indicated that permeabilization of the membrane does not directly correlate with antimicrobial activity. Friedrich *et al.* (2000) found that low levels of permeabilization are sufficient to allow transport of peptides to the cytosol where they may interact with intracellular anionic targets. Indeed, fluorescence studies of histatin 5 on the yeast *Candida albicans* indicated that association with the cell membrane was transient and the target was the mitochondrial membrane (Helmerhorst *et al.* 1999).

Translocated CAPs cause cell death by impeding cell wall synthesis, nucleic acid synthesis, protein synthesis, enzyme function, or formation of the cytoplasmic membrane septum (Zasloff, 2002) or by inducing apoptosis (Nagaoka *et al.,* 2006; Risso *et al.,* 2002). Detailed studies of the peptides buforin 2 and magainin 2 indicate that membrane

translocation occurs via formation of a transient toroidal pore. Once inserted into the membrane, repulsion of the positively charged surfaces of the peptide helices causes destabilization of the pore resulting in translocation of the peptide to the cytoplasm (Kobayashi *et al.*, 2004). Translocation of magainin 2 is accompanied by shuffling of membrane phospholipids between the bilayers causing further disruption of membrane function (Matsuzaki *et al.*, 1996).

Translocation of CAPs across the membrane can also occur without membrane disruption or other harmful effects. Antiviral peptides can translocate across mammalian membranes to act against viral targets without harming the host cell. CAPs designed for treatment of feline immunodeficiency virus (FIV) were shown to prevent binding and internalization of the virus, and also to inhibit virus assembly and release from chronically infected cat kidney cortex epithelial cells (Ma *et al.*, 2002). Similarly, anti-herpes simplex virus peptides, in addition to inhibiting viral infection, also prevent cell-to-cell transfer by an unknown mechanism (Andersen *et al.*, 2004).

1.1.3 Resistance to CAPs

At all stages of direct antimicrobial activity, CAPs interact in a general manner with the phospholipids of cell membranes, as opposed to a specific protein component. Because of this, development of resistance to CAP-mediated killing is uncommon as it requires restructuring of the cell membrane character. However, there is evidence of membrane alteration by species of *Morganella* and *Serratia* to remove acidic lipids that attract CAPs. Alterations to cell surfaces such as LPS can also prevent attraction of CAPs. For example, an inner membrane protein of *Salmonella typhimurium* and *E. coli*, PhoP,

activates a signaling cascade that results in modification of the lipid A component of LPS upon interaction with magainin 2 (Tran *et al.*, 2005; Shi *et al.*, 2004). Another approach to resistance was observed in *Porphyromonas gigivalis,* which developed secreted proteases that degrade CAPs before they can act; however, this is only effective against peptides that contain the epitopes necessary for proteolysis (Zasloff, 2002). Regardless, resistance to the direct activity of CAPs does not extend to their indirect activity.

1.1.4 Interactions with effector cells

Some CAPs prevent infection indirectly by recruiting effector cells to the site of infection. For example, human LL-37 and porcine PR-39 are chemotactic for peripheral blood leukocytes, such as monocytes, neutrophils, CD4+ T cells and mast cells (Zanetti, 2004). PR-39 also functions in wound repair through induction of cell surface heparin sulphate proteoglycans called syndecans. Syndecans are required for wound repair as they mediate cell proliferation, migration and allow interactions between cells. PR-39 is released by leukocytes at the wound site where it both kills bacteria, preventing further injury and promotes healing by inducing syndecan production (Gallo *et al.*, 1994).

CAPs may also modulate the innate host response by stimulating the cellular components of innate immunity. Alloferons, histidine/glycine-rich weakly basic peptides isolated from blow fly (*Calliphora vicina*), stimulate natural killer (NK) cells to produce interferon thereby protecting mice against infection with influenza virus A and B, and grafted tumor cells. Because NK cells are important in protection against viruses and cancer, alloferon-based therapy of Herpes Simplex Virus and Hepatitis B and C is currently under clinical study (Chernysh *et al.*, 2002). Also, a 10 amino acid long

lactoferrin derivative (peptide 2) activates neutrophils and macrophages, increasing phagocytic killing of *Candida albicans* 1.5-fold, and leading to an increase in NO production. Granulocyte-macrophage colony stimulating factor (GM-CSF) also stimulates macrophages to produce reactive oxygen species. Treatment of macrophages with both peptide 2 and GM-CSF exerted a synergistic effect on both peroxide generation and LPS-stimulated nitrite production (Tanida *et al.*, 2001). This resulted in acceleration of inflammation and led to elimination of the pathogen.

1.1.4.1 Modulation of the inflammatory response

While the inflammatory response is an important part of host defense, it may also cause sepsis and damage to host tissue through release of reactive oxygen species (ROS). CAPs prevent sepsis by inhibiting cytokine production and secretion, preventing production of reactive oxygen species (ROS), and binding of LPS. LL-37 binds LPS and reduces the production of ROS by inhibiting nitric oxide synthase (Zanetti, 2004, Ciornei *et al.*, 2005). BMAP-28 prevents sepsis by inducing apoptosis rather than lysis, thus preventing the release of LPS and other and bacterial agonists. Once translocated across the membrane, BMAP-28 interacts with the cation-gated mitochondrial permeability transition pore allowing the release of cytochrome c resulting in apoptosis (Risso *et al.*, 2002). Treatment of mice infected with heat-killed *Staphylococcus aureus* with BMAP-28 reduced lethality and decreased levels of tumor necrosis factor-α (TNF–α) and interleukin-6 (inflammatory cytokines) in plasma. Similarly, a modified defensin composed of only the β-defensin core region prevented LPS-induced TNF-α secretion and LPS-mediated mitogen-activated protein kinase induction in murine monocytes

(Motzkus *et al.,* 2006). Co-administration of CAPs and traditional antibiotics may prove an effective method of quickly clearing infection while preventing septic shock. Simultaneous administration of temporin L and beta-lactam antibiotics produced strong antimicrobial activity, and reduction of plasma LPS and TNF-α levels, resulting in high survival in a rat septic shock model (Giacometti *et al.,* 2006).

Taken together, CAPs are effective elements of host defense capable of rapid response to invasion by a variety of pathogens and induction of cell-mediated innate immune response.

1.1.5 Cathelicidins

The cathelicidin family of CAPs is a diverse group that displays many of the activities described above. They are characterized by a common genetic structure, in which each cathelicidin gene is composed of four exons. The first exon encodes a pre sequence that targets their accumulation to secretory granules. It is followed by two exons that encode a conserved pro-region that bears sequence similarity to the porcine leukocyte protein cathelin. The last exon encodes the CAP region of the peptide (Hancock and Diamond, 2000; Zanetti, 2004). While the N-terminal cathelin proregion of cathelicidins is highly conserved, the C-terminal CAP region is divergent in both sequence and structure. Cathelicidins may contain α-helices, extended helices, loop-structures, or β-sheets with two or three disulphide bridges (Ramanathan *et al.* 2002) and range in size from 12-100 amino acids (Gennaro and Zanetti, 2000).

Transcription of cathelicidin genes is induced by LPS, interleukin-6 and bacterial infection (Wu *et al.,* 2000). Upon translation, the prepropeptides accumulate in secretory granules until they are activated by proteolytic processing. The pre-region is cleaved by signal peptidase while the cathelin domain remains bound until activation of the CAP (Shinnar *et al.,* 2003). The cathelin domain and the directed accumulation within secretory granules serve to inactivate the peptide and protect it from proteases. Cathelin is negatively charged and interacts with the antimicrobial domain to neutralize its charge. Cleavage of the cathelin domain by elastase releases the active CAP. In addition, the free cathelin domain may also exhibit antimicrobial activity. For example, the cathelin domain of hCAP-18 is active against certain strains of bacteria that are resistant to the CAP domain, LL-37 (Zaiou *et al.*, 2003).

Of the cathelicidins identified, the majority adopt a linear, α-helical conformation upon interaction with membranes (Tossi *et al.*, 2000). This folding occurs in such a way that the helix formed is amphipathic, with one face bearing the positively charged residues and the other the hydrophobic amino acids. A common feature of linear α-helical cathelicidins (human LL-37, bovine BMAP-27 and BMAP-28, SMAP-29, chicken fowlicidin-1, and guinea pig CAP11) is the presence of a hydrophobic tail at either the N- or C-terminus that functions in both hemolytic activity and LPS binding. Removal of this tail has a negligible effect on the antimicrobial activity, but greatly reduces the peptide's cytotoxic properties. In general, cathelicidins exhibit a rapid, potent, broad-spectrum antimicrobial effect and neutralize endotoxin to prevent sepsis. This activity makes them excellent candidates for the development of novel antimicrobial agents (Gennaro and

Zanetti, 2000). Therefore, this study seeks to produce the cathelicidin variant BMAP18 for such therapeutic use.

1.1.5.1 BMAP18

BMAP18 (bovine myeloid antimicrobial peptide) is an 18 amino acid long, linear, α-helical cathelicidin with a net charge of +10 at neutral pH. It was derived from BMAP-27, originally isolated from bovine neutrophils, by deletion of the C-terminal hydrophobic tail. BMAP-27 is both highly antimicrobial and hemolytic, however the hemolytic activity is localized within the hydrophobic C-terminus (Benincasa *et al.*, 2003, Risso *et al.*, 1998, Skerlavaj *et al.*, 1996). Removing the C-terminal tail reduced the lytic effects of the peptide on human neutrophils from over 90% for BMAP-27 to 11% for BMAP18 at 30 μM (Skerlavaj *et al.*, 1996). The higher than average cationic nature of BMAP18 favors interaction with highly negatively charged membrane surfaces. Evidence of this selectivity includes lower MIC values for Gram-negative bacteria over Gram-positive (Benincasa *et al.*, 2003) due to the presence of LPS. Also, BMAP18 has potent activity against the protozoan parasite *Trypanosoma brucei* (Haines *et al.*, unpublished).

Trypanosoma brucei is the causative agent of African Sleeping Sickness or African Trypanosomiasis, one of the most devastating diseases affecting sub-Saharan Africa. Current estimates indicate that 60 million people in 37 countries are at risk of infection and as many as 500,000 people are currently infected. This protozoan parasite is vectored by the tsetse fly, which transfers the infection to both humans and cattle to which it is

pathogenic and may even be fatal. While residing in its insect host, the parasite is covered with a set of proteins called the procyclins that impart a highly negative character that attracts BMAP18. In a mammalian host, the surface switches to a variable surface antigen glycoprotein coat. However, this form of the trypanosomes is equally susceptible to BMAP18 (Haines *et al.* Unpublished). It is not known how the peptide is able to traverse this glycoprotein coat of trypanosomes (Brogden, 2005). Because cattle are a crucial part of existence in sub-saharan africa, trypanosomiasis is of great concern not only to human health but is also of economic importance. Economic losses of approximately US $4 billion are incurred annually through loss of livestock to the disease. All currently available medications are costly and difficult to administer as they require injection and some require hospitalization for weeks due to their inherent toxicity. These difficulties are compounded by the lack of medical facilities in the regions where trypanosomiasis is endemic. Therefore, the development of a new anti-trypanosomal therapeutic agent that is inexpensive, safe and easy to deliver is absolutely needed. Production of BMAP18 may provide a safe and effective novel treatment for African trypanosomiasis, if produced efficiently.

1.2 Recombinant Protein Production Platforms

To exploit the anti-trypanosomal activity of BMAP18 for the treatment of humans and cattle infected with *Trypanosoma brucei,* large amounts of the peptide must be produced in an inexpensive, efficient manner that can meet fluctuating demand in a largely rural, under-developed region. Chemical synthesis of the peptide by *de novo* synthetic peptide chemistry is not feasible due to the prohibitively high cost. Alternatively, the peptide may be produced through recombinant technology in bioreactors of bacterial or eukaryotic

(yeast, insect or mammalian) cell culture, or within transgenic animals or plants. When contemplating how best to produce a recombinant peptide, one must consider a) in which biological systems it may be safely expressed, b) whether eukaryotic post-translational modification machinery is required for proper function and c) which system would yield the most peptide at the lowest cost in the shortest amount of time.

1.2.1 Bacteria

Bacteria offer the most rapid system for cloning and expression of a recombinant gene. However, the inherent toxicity of CAPs to bacterial cells prevents the direct production of the peptides in bacteria. To circumvent this toxicity, CAPs must be expressed as fusion proteins to neutralize their positive charge or otherwise inactivate their antibacterial activity (Pazgier and Lubkowski, 2006; Xu *et al.*, 2006). One example is the expression of the CAP SMAP-29 in *E. coli* through fusion to intein (Morassutti *et al.* 2005). Inteins are self-cleaving protein splicing elements that may be activated simply by the addition of a nucleophilic agent such as dithiothreitol. This method allowed simple processing of the fusion product to release the CAP. However, only a modest yield of 0.1-0.2 mg of pure peptide per litre of culture was attained. In order to increase yield, tandem repeats of CAPs fused to anionic peptides have been expressed in *E. coli* as a single translational unit. The peptide monomers are easily released from each other and from their fusion partners by chemical cleavage at their N-terminal methionine residues by cyanogen bromide (Morin *et al.*, 2006, Kim *et al.*, 2006). Therefore, each transcript yields multiple peptide products. Using this method Kim *et al.* (2006), produced approximately 60 mg of pure recombinant lactoferricin from one litre of *E. coli* culture.

1.2.2 Yeast

Yeasts, as eukaryotes, are generally less sensitive to the antimicrobial properties of CAPs than bacteria and can, therefore, be engineered to produce unmodified CAPs without inhibiting growth. For example, as a consequence of limited success producing the CAP CM4 as a fusion protein in *E. coli*, Zhang *et al.* (2006) transformed *Pichia pastoris* with the CM4 gene under control of the tightly regulated aldehyde oxidase 1 (AOX1) promoter. This promoter is inhibited by the presence of glucose and glycerol and induced by the addition of methanol. Upon induction, up to 40 mg of recombinant CM4 were secreted into 1 L of medium, yielding 15 mg of 98% pure peptide (Zhang *et al.*, 2006). It is interesting to note that CM4 is active against a variety of fungi with an MIC of 2-32 μM, yet its production in *P. pastoris* did not effect growth. The same group has also expressed a cecropin-melittin hybrid using this method and achieved 22 mg of peptide per litre of fermentation culture after HPLC purification (Jin *et al.*, 2006).

1.2.3 Insect Cells

Expression of recombinant proteins in cultured insect cells allows for high yield production of properly folded eukaryotic proteins. For example, expression of an anti-trypanosomal CAP originally isolated from the tsetse fly, attacin, in *Drosophila* S2 cells produced biologically active peptide. A His-tag and V5 epitope fused to the C-terminus facilitated purification and identification of the recombinant product without greatly affecting anti-trypanosomal activity (Hu and Aksoy, 2005).

1.2.4 Plants

Plants offer several advantages for production of recombinant CAPs over bioreactors. As higher eukaryotes, they are capable of most post-translational modifications that may be required to produce functional peptides including proper folding, glycosylation and proteolytic cleavage. When compared to bacterial fermentation systems, transgenic plants are capable of producing the same yield at 2-10% of the cost (Twyman, 2003). Production of recombinant proteins in bioreactors requires highly specialized facilities, media, and reagents and highly trained personnel, which are costly. When compared to transgenic animals, transgenic plants can be produced more rapidly and there is no risk of contamination of the recombinant product with mammalian viruses or prions. A variety of therapeutics, vaccines and medical diagnostic tools have been produced in plants using gene transfer technology: fully assembled and functional secretory IgA (Ma *et al.*1995) and many other antibodies; vaccines against hepatitis B (Kong *et al.*, 2001; Thanavala *et al.* 2005), human immunodeficiency virus (Horn *et al.*, 2004), canine parvovirus (Gil *et al.* 2001), to mention a few; and human epidermal growth factor (Wirth, 2004), erythropoietin (Cheon *et al.*, 2004) and hemoglobin (Dieryck *et al.*, 1997).

There is only one example, however, in which a CAP has been recombinantly expressed in a transgenic plant for the purpose of production and purification. SMAP-29 was expressed in transgenic tobacco using an intein fusion similar to that used for its expression in *E. coli* (Morasutti *et al.*, 2002). In addition, a chitin-binding domain was translationaly fused to the peptide downstream of intein for affinity purification. This allowed for simple purification of the product on a chitin column followed by on-column

self-mediated cleavage of intein and the affinity tag (Morasutti *et al.*, 2002). Though functionally active pure SMAP-29 was produced and purified from the transgenic plants, the yield was not quantified. Other studies, focusing on expression CAPs in transgenic plants to confer disease resistance, have reported excellent yields. Osusky and colleagues produced a cecropin-melittin hybrid (ECEMA) in potato tubers at 3-4 μg per gram fresh weight (Osusky *et al.*, 2000) and a dermaseptin b1 variant (MsrA2) in potato leaves to 1-5 μg per gram fresh weight (Osusky *et al.*, 2005). A slightly higher yield of 6-7 μg of peptide per gram of fresh leaf tissue was achieved by using a wound-inducible promoter from poplar to direct expression of MsrA2 in transgenic tobacco (Yevtushenko *et al.*, 2005). Expression of a CAP isolated from *Dahlia merckii,* Dm-AMP1, in transgenic eggplant accumulated the peptide to 0.66% of the total soluble protein content (Turrini *et al.*, 2004). In addition to producing CAPs for pharmaceutical purposes, expression of CAPs in transgenic plants can enhance plant defenses against biotic stresses.

1.3 Engineering Disease Resistance in Transgenic Plants

Engineering of crop plants resistant to a variety of plant pests and pathogens is a rapidly expanding field. Enhancement of germ-line disease resistance reduces the need for chemical pesticides that are damaging to both human health and the environment. To this effect, transgenic plants have been produced that express proteins that either have direct antimicrobial activity, stimulate endogenous defense systems, or prevent disease by evading or neutralizing toxins.

Examples of proteins that have been expressed for direct activity against pathogens include CAPs, lysozymes, antibodies, transferrins, ribosome inactivating proteins (RIPs),

enzymes involved in synthesis of phytoalexins, and other PR (pathogenesis-related) proteins. Schaefer *et al.* (2005) compared several of these elements in transgenic tomato lines for conferral of resistance to early blight caused by *Alternaria solani*. Transgenic lines expressing an iris RIP, a maize β-glucanase, or a *Mirabilis jalapa* CAP were infected with the fungal pathogen and assessed for lesion development over a three week period. Of the lines tested, the CAP- and β-glucanase-expressing lines showed greater resistance than non-transgenic control and RIP-expressing plants, with less than 5% of leaf area covered with early blight lesions, while control leaves were up to 50% consumed by blight. However, none of the lines generated were 100% protected from infection and no one plant was without lesions (Schaefer *et al.*, 2005). It would be interesting to cross the resistant transgenic lines to determine whether co-expression of the defense transgenes would have a combinatorial effect on disease resistance.

Enhancement of endogenous defense systems of plants may be achieved through over-expression of endogenous defense genes or introduction of heterologous plant defense genes such as resistance (R) genes and their *avr* gene (pathogen avirulence gene) counterparts, signaling hormone biosynthesis proteins (salicylic acid, ethylene, hydrogen peroxide, jasmonic acid), proteins modulating cell wall synthesis, and elicitors of the hypersensitive response. A good example is the conferral of resistance to *Xanthomonas capestris* pv. *Vesicatoria* (*Xcv*) in transgenic tomato through expression of the pepper *Bs2* R gene (Tai *et al.*, 1999). Expression of *Bs2* inhibited growth of *Xcv* encoding the corresponding virulence gene *avrBs2,* and prevented the emergence of chlorotic symptoms when a detached leaf was challenged with the bacterial pathogen. Unlike some

avr genes that may easily mutate to avoid R gene activity, *avrBs2* is highly conserved thus is likely required for fitness of *Xcv.* Therefore, expression of its corresponding R gene *Bs2* may provide durable resistance. In another example, a potent elicitor derived from *Phytophthora cryptogea*, cryptogein, was expressed under the control of a native pathogen-inducible promoter in transgenic tobacco. This provided resistance to a variety of unrelated fungal pathogens through induction of the hypersensitive response, which localized the infection preventing dissemination through the plant (Keller *et al.,* 1999). This approach is advantageous as it provides systemic resistance upon pathogen-challenge and does not consume nutritional resources in the absence of stimulus.

Another approach to enhance resistance is to inhibit disease by expressing proteins that can neutralize pathogen toxins or effectors: toxin inhibitors, target proteins that are insensitive to toxins, antibodies, and even RNAi (Gurr and Rushton, 2005). Resistance to two pathovars of *Pseudomonas syringae* was achieved by evading their respective toxins. Expression of *ttr*, a tabtoxin resistance gene that codes for an acetyltransferase, in transgenic tobacco eliminated all chlorosis caused by *P. syringae* pv. *tabaci* infection when compared to control plants (Anzai *et al.*, 1989). However, because the transgene is directly targeted against the toxin, mutations easily arising in the pathogen toxin may allow it to evade the inactivating acetyltransferase. Expression of the toxin-insensitive ornithine carbamoyltransferase encoded by the pathogen *P. syringae* pv. *phaseolicola* in transgenic tobacco (de al Fuente-Martinez *et al.*, 1992) and bean (de al Fuente-Martinez *et al.*, unpublished) eliminated the formation of watery lesions and chlorosis observed in control plants when infected with the pathogen. By using the pathogen's own resistance

mechanism to protect transgenic plants, the bacterium must mutate both the toxin and the target in order to restore pathogenesis; thus, the resistance achieved is more durable than that achieved by inhibiting the toxin directly.

While each of these strategies has its advantages and uses (for an extensive review see Gurr and Rushton, 2005), only the expression of CAPs allows for durable resistance against a broad spectrum of pathogens.

1.3.1 CAPs in Transgenic Plants

The potential for CAPs to enhance the natural pathogen defenses of plants has been investigated in numerous studies (*see* Table 1). To date, a variety of CAPs have been recombinantly expressed in pear (Puterka, *et al.*, 2002), wine grape, cotton (Rajasekarn *et al.*, 2005), eggplant (Turrini *et al.*, 2004), potato, rice, tobacco, tomato, canola (Kazan *et al.*, 2002) and banana. In each case, different genetic tools have been employed in order to achieve a high level of peptide production to ensure the greatest degree of disease resistance. These tools include: strong constitutively expressed promoters, such as the enhanced CaMV 35S RNA promoter with the alfalfa mosaic virus RNA4 translational enhancer, or the *Arabidposis thaliana* ubiquitin 3 (Atubq3) promoter; wound-inducible promoters, like the tomato LePAL promoter and *win*3.12T promoter from *Populus tremuloides*; signal peptides to direct accumulation of the peptide product to a particular sub-cellular location; and fusion peptides to improve stability.

Table 1: Expression of CAPs in transgenic plants. Examples of strategies for CAPs expression to enhance disease resistance.

Expression Strategy	Peptide	Size (a.a.)	Net Charge	Plant Host	Engineered Resistance	Reference
Constitutive expression:						
Enhanced CaMV 35S promoter, AMV translational enhancer	MsrA2	32	2	potato	*Phytophthora infestans, Rhizoctonia solani, Pythium paraecandrum, P. splendens, Erwinia carotovora, Phytophthora erythroseptica*	Osusky *et al.*, 2005
Enhanced CaMV 35S promoter, AMV translational enhancer	MsrA3	19	2	potato	*Erwinia carotovora, Phytophthora infestans, Phytophthora erythroseptica*	Osusky, *et al.* (2004)
Enhanced CaMV 35S promoter, AMV translational enhancer	MsrA1	34	6	potato	*Phytophthora cactorum, Fusarium solani, Erwinia carotovora*	Osusky *et al.*, 2000
Pathogen-induced expression:						
LePAL promoter	Shiva-1			potato	*Erwinia carotovora*	Yi, *et al.*, (2004)
*win*3.12T promoter	CEMA	29	7	tobacco	*Fusarium solani*	Yevtushenko, *et al.* (2005)

Table 1: *continued from previous page*

Expression Strategy	Peptide	Size (a.a.)	Net Charge	Plant Host	Engineered Resistance	Reference
Extracellular accumulation:						
PR-1b[a] signal peptide	Myp30	24	3	tobacco	*Peronospora tabacina, Erwinia carotovora*	Li *et al.*, 2001
Enhanced CaMV 35S promoter, pea vicillin secretory signal	MSI-99	38	5	tomato	*Pseudomonas syringae,* not *Alternaria alternata, Phytophthora infestans*	Alan, *et al.* (2004)
Atubq3[b] promoter, pea vicillin secretory signal	MSI-99, *mag2*	22, 23	6,3	Chardonnay	Not tested	Vidal, *et al.* (2003)
Atubq3 promoter, pea vicillin secretory signal	MSI-99	38	5	tobacco and banana	*Sclerotinia sclerotorium, Alternaria alternata, Botrytis cinerea,* and *Fusarium oxysporum, Mycosphaertella musicola*	Chakrabarti, *et al.* (2003)
Insect signal peptide Plant-derived signal peptide	cecropin B	60	8	tobacco	Not resistant to pathogens tested	Florack, *et al.* (1995)
CaMV 35S promoter, Endopolygalacturonase-inhibiting protein leader sequence	Esculentin-1	46	6	tobacco	*Pseudomonas syringae, P. aeruginosa, Phytophthora nicotianae*	Ponti, *et al.* (2003)

[a]PR-1b = *Nicotiana tabacum* pathogenesis-related protein 1b, [b]Atubq3 = *Arabidopsis thaliana* ubiquitin 3

Table 1: *continued from previous page*

Expression Strategy	Peptide	Size (a.a.)	Net Charge	Plant Host	Engineered Resistance	Reference
ER retention:						
Zmubq1[c] promoter, tobacco PR-1a[d] secretion signal fusion	cecropin A	59	8	rice	*Magnaporthe grisea*	Coca, *et al.* (2006)
Translational fusions:						
Enhanced CaMV 35S promoter, TMV[e] Ω translational enhancer, , GUS fusions	Sarcotoxin IA	**39**	5	tobacco	Not tested	Okamoto, *et al.* (1998)
CaMV 35S promoter, β-conglycinin transit peptide at N-terminus, intein-CBD[f] at C-terminus	SMAP-29	**31**	9	tobacco	Not tested	Morassutti, *et al.* (2002)
Potato virus X promoter, viral coat protein-fusion	Killer peptide (KP)	**10**	1	tobacco	*Pseudomonas syringae* pv. *tabaci*	Donini, *et al.* (2005)
CaMV 35S promoter, prosystemin fusion	Pep11	**11**	4	tomato	*Phytophthora infestans*	Jones, RW, *et al.* (2004)
Chloroplast transformation:						
16S rRNA promoter	MSI-99	22	6	tobacco	*Pseudomonas syringae, Aspergillus flavus, Fusarium moniliforme, Verticillium dahliae, Colletotrichum destructivum*	DeGray *et al.*, 2001

[c]Zmubq1 = *Zea mays* ubiquitin 1, [d]PR-1a = *Nicotiana tabacum* pathogenesis-related protein 1a, [e]TMV = tobacco mosaic virus, [f] CBD = cellulose binding domain

1.3.1.1 Promoter selection:

Promoter choice is an important aspect of recombinant protein production. While the CaMV 35S and Atubq-1 promoters are excellent for use in pilot studies, promoters that are able to direct expression either spatially, temporally or both are required for commercial applications. Constitutive promoters provide high level transgene expression in all tissues at all developmental stages and allow researchers to rapidly determine whether their product of interest may be produced within their chosen host plant. However, inducible or tissue-specific expression of transgenes is desirable for engineering resistance to pathogens, especially in crop plants that are intended for human consumption. Yi *et al.* (2004) compared the efficacy of expression of a cecropin B analog driven by either the enhanced CaMV 35S promoter or the tomato phenylalanine ammonium lyase (LePAL) promoter in potato. Upon comparison of several lines of each construct with similar levels of the selection marker neomycin phosphotransferase II (NPTII), it was found that the pathogen-inducible promoter led to more reliable resistance to *Erwinia carotovora* (Yi *et al.*, 2004). While the reasons behind this finding are not entirely understood, it was proposed that over-expression of a transgene during early developmental stages may lead to a form of transgene silencing.

1.3.1.2 Sub-cellular localization:

When recombinantly expressed in plants, CAPs accumulate within the cytosol (Turrini *et al.*, 2004). Alternatively, to improve CAP expression, stability, and function, they may be directed to a particular sub-cellular location by the incorporation of a translationally fused signal peptide sequence. Accumulation in the intercellular spaces may also prevent

the phytotoxic effects of CAP expression, and allow contact of the peptides with invading pathogens before they enter the cell (Alan *et al.*, 2004). Levels of cecropin B transcript in transgenic tobacco indicated that the incorporation of the peptide's native N-terminal secretory signal increased transcription by four- to five-fold. However, substitution of the insect secretory signal with a plant-derived sequence increased cecropin B transcripts 10- to 12-fold (Florack *et al.*, 1995). Based on these findings, later studies employed plant-derived signals for secretion of CAPs into the apoplast. Commonly used plant secretory signals include those derived from pea vicillin protein and the tobacco PR-1a and PR-1b proteins.

Secretion of esculentin-1 directed by the signal sequence of endopolygalacturonase inhibiting protein imparted resistance to the bacterial pathogens *Pseudomonas syringae* pv. *tabaci* and *P. aeruginosa* and greatly enhanced tolerance to the fungal pathogen *Phytophthora nicotianae* (Ponti *et al.*, 2003). Comparison of disease resistance of transgenic tobacco expressing a cytosolic and extracellular magainin II analog, Myp30, indicated that though CAP secretion is advantageous for protection against fungal pathogens, it does not benefit defenses against the bacterial pathogen *E. carotovora* (Li *et al.*, 2001). This difference in susceptibility to fungal and bacterial pathogens may be due to the lifestyles of the pathogens tested. Fungal mycelia growing in the intercellular spaces would not encounter cytosolic CAPs; thus, secretion is necessary to confer resistance to fungal pathogens. The bacteria *E. carotovora* is a necrotic pathogen that secretes cell wall-degrading enzymes. Loss of the plant cell wall induces lysis enabling

the release of CAPs into the apoplast where their antibacterial activity is realized (Li *et al.*, 2001).

Proteolytic degradation is one of the biggest challenges facing stable accumulation of CAPs in transgenic plants. While modifications to the primary structure of magainin II reduced its susceptibility to a variety of proteases, the protection was not complete and the peptide could not be consistently detected in transgenic tobacco plants in which it was expressed (Li *et al.*, 2001). In an attempt to evade endogenous proteases, Coca *et al.* (2006) compared recombinant production of cecropin B in transgenic rice through secretion into the apoplast and accumulation within the endoplasmic reticulum by directing accumulation to the secretory pathway and adding a KDEL retention signal to the C-terminus. Recombinant cecropin B was successfully isolated using both approaches, but transgenic lines retaining the CAP in the ER exhibited the greatest level of resistance against the rice blast fungus. In addition, secretion of the CAP into the apoplast caused decreased fertility. Therefore, ER retention of recombinant CAP may prevent phytotoxicity or other aberrant physiological effects. Similarly, expression of the single chain anti-oxazolone Fv antibodies under control of the CaMV 35S promoter and retention in the endoplasmic reticulum through addition of a KDEL sequence yielded 2% total soluble protein in the tubers (Artsaenko *et al.*, 1998).

1.4 Rationale and Objectives

The hypotheses of this thesis are 1) the highly cationic antimicrobial peptide BMAP18 can be produced in transgenic potato, and 2) production of biologically active BMAP18 in transgenic potato will enhance disease resistance.

1.4.1 Construct Rationale

To evaluate the feasibility of stable accumulation of BMAP18 in transgenic potato and determine its effect on disease resistance to plant pathogens, three transformation vectors were prepared. Each construct contained a different promoter to either provide strong constitutive expression, or spatio-temporal direction of peptide production. The nopaline synthase 3' terminator region (*Nos*T) is included in all three constructs to satisfy the requirement of the transcript for a 3' transcriptional termination signal including a polyadenylation signal to prevent run-on transcription, which could lead to gene silencing. As part of the T-DNA region of the *Agrobacterium tumefaciens* Ti plasmid, the *Nos*T is well adapted to function in plants.

The first construct prepared utilized the duplicated-enhancer CaMV 35S promoter (2x35S) with the AMV RNA4 5' leader translational activator for high-level production. This enhanced 35S promoter, developed by Kay *et al.* (1987), exhibits ten times the transcriptional activating properties of the wild type 35S promoter, while the AMV translational enhancer increases translational efficiency by up to four times (Datla *et al.*, 1993).

Two additional constructs were prepared for this study using the Douglas-fir (*Pseudotsuga menziesii*) luminal binding protein (BiP) promoter (Forward *et al.*, 2002) to drive transcription of the BMAP18 gene. Because BiP is thought to be involved in the unfolded protein response of the ER following stress (Oh *et al.*, 2003), its promoter ought to positively regulate expression in response to wounding as well as other abiotic stresses.

Indeed, *in silico* analysis of the PmBiP promoter identified numerous elements relating to biotic and abiotic stresses. Some of these cis-acting elements have been shown to play a role in tissue-specific regulation of BiP accumulation. Characterization of two soybean BiP promoters by GUS activity assays in mature transgenic tobacco plants indicated that the promoter directs expression in all parts of the roots, except the root cap, and in the vascular tissues of stems, especially those of the inner phloem tissues (Buzeli *et al.*, 2002). The PmBiP promoter was shown to be functional in *Arabidopsis* by histochemical GUS staining, with relatively high constitutive expression in most tissues and up to two-fold induction upon wounding. Strongest expression was observed in actively-dividing tissues such as expanding leaves and leaf primordia. The promoter's function in potato has also been characterized by GUS assays and has been shown to drive exceptionally high GUS production in potato tubers (Yevtushenko, D., *personal communication*). Because potato tubers are modified subterranean swollen stems, these findings may be explained through the high activity of the BiP promoter in rapidly dividing stem tissues. It is yet to be seen how wounding of tubers may affect the activity of the BiP promoter, but strong expression is expected. In this study, both the full-length (PmBiPPro1-1, -2242 to +16, 2,258 bp) and a truncated form (PmBiPPro1-3, -1243 to +16, 1,259 bp) are tested because, in *Arabidopsis*, the truncated form drove stronger expression in vascular tissues, lateral roots and root tips than the full-length promoter (Forward *et al.*, 2002).

1.4.2 Objectives

The objectives of this study were to:

(1) Characterize the *in vitro* antimicrobial activity of synthetic BMAP18 against trypanosomes, as well as a variety of human and plant pathogens.

(2) Characterize the toxicity of synthetic BMAP18 to potato.

(3) Construct BMAP18 plant expression cassettes.

(4) Transform potato, regenerate plants and select for transformants.

(5) Analyze transgenic plants for the presence of *BMAP18* transcripts and peptide.

(6) Produce antibodies against BMAP18 to aid in detection of the peptide in plant extracts.

(7) Assay transgenic plants for increased resistance to a bacterial (*Erwinia carotovora*) and a fungal (*Fusarium solani*) pathogen.

Chapter 2: Materials and Methods

2.1 In vitro Studies

2.1.1 Synthetic Peptides

Two peptides, BMAP18 (GRFKRFRKKFKKLFKKLS) and BMAP18C (GRFKRFRKKFKKLFKKLSGSGC), were synthesized using Fmoc (*N*- (9-fluorenyl) methoxycarbonyl) chemistry at the Nucleic Acid and Peptide Services Facility at the University of British Columbia. BMAP18 was prepared with an amidated C-terminus which is likely to be present when produced *in vivo*. The C-terminal four amino acids of BMAP18C facilitated the covalent linkage of the peptide to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) carriers, as well as POROS beads for affinity purification of immunized polyclonal antibodies. The peptides were purified by analytical high performance liquid chromatography (HPLC) to greater than 95% purity then lyophilized and stored at –20 °C.

Conjugation of BMAP18 with maleimide-activated KLH (as an adjuvant) and maleimide-activated BSA (as a control carrier) was performed as per the manufacturer's (Pierce, Rockford, Illinois) standard protocol with 1 mg of peptide: 1 mg of carrier protein. Coupling of BMAP18 to BSA resulted in precipitation of the protein, thus no purification steps were performed; however, BMAP18-KLH was purified using a D-Salt™ Desalting Column (Pierce, Rockford, Illinois). Quantitation was not done.

MsrA2 (MAMWKDVLKKIGTVALHAGKAALGAVADTISQ) was previously synthesized using the Fmoc method, by AnaSpec, Canada. This peptide was also purified to greater than 95% purity by HPLC and stored as a lyophilized powder at –20 °C.

2.1.2 Plant material

Potato plants (*Solanum tuberosum* L. cultivar Desiree) were grown and maintained aseptically in culture tubes containing MS medium (Murashige and Skoog basal salt mixture (Sigma, M5524), Gamborg's vitamins (Sigma, G1019) 400 - 900 mg MES-hydrate, 15 - 20 g/L sucrose and 6 g/L agar (Murashige and Skoog, 1962)) with a photoperiod of 16 hrs light: 8 hrs dark at 24 °C. To obtain tubers and mature leaf tissue, three plants of each line were transferred from tissue culture to a pot containing 2 parts topsoil: 1 part peat moss: 1 part composted steer manure and grown in a growth chamber with a 16 hrs light: 8 hrs dark photoperiod at 18 °C at the Bev Glover Greenhouse Research Facility, University of Victoria. Transgenic control plants transformed with PmBiPPro::GUS constructs were obtained from D. Yevtushenko.

2.1.3 Microbial cultures

2.1.3.1 Bacterial cultures

All bacterial strains used in this study, *Erwinia carotovora* ssp. *carotovora*, *Bacillus subtilis, Staphylococcus epidermidis, Proteus vulgaris, Pseudomonas aeruginosa* (ATCC 10145) and *Escherichia coli* (DH5α), were obtained from the microbiology student laboratory, University of Victoria, and maintained in Luria-Bertani medium containing no salt (10 g/L tryptone, 5 g/L yeast extract). All cultures were grown at 37 °C except *E. carotovora*, which was grown at 25-28 °C.

2.1.3.2 Fungal cultures

Fusarium solani was obtained from the lab of Dr. Punja (Simon Fraser University, Burnaby, B.C.), maintained on potato dextrose agar (Difco) or V8 agar (10% Campbell's V8 juice, 1.5% agar, 5 mM MES-hydrate, pH 6.4) plates and cultivated at room temperature under low light.

2.1.3.2 Trypanosome cultures

Procyclic form *Trypanosoma brucei* YTat were obtained from Dr. T Pearson, University of Victoria, and grown in PCF medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum with additives for procyclic culture form trypanosome growth as described by Fish *et al.* (1989)) at 27 °C with 5% CO_2.

2.1.4 AlamarBlue™ reduction assays

2.1.4.1 Antibacterial activity assays

To determine the minimal inhibitory concentration of BMAP18 against a number of bacterial pathogens, a series of 2-fold dilutions of peptide were prepared in a polypropylene 96-well microtitre plate (COSTAR/Corning Inc., Cat. No. 3790) and a defined amount of bacterial cells were added. Growth was measured through the addition of alamarBlue™, a dye that is non-specifically reduced from blue to pink by metabolic intermediates of actively growing cultures. The optimal concentration of cells to yield this reaction was species-specific and determined empirically. Ten-fold dilutions of overnight cultures in Mueller-Hinton broth were incubated with alamarBlue™ reagent for 4 hrs. The concentration that was 10-fold less than that which yielded a colour change was used for antibacterial assays. The peptide stock was prepared by dissolving 1 mg of

BMAP18 in 1 mL of 0.2% BSA/0.01% acetic acid. Peptide dilutions were prepared in phosphate buffered saline (PBS), pH 7.4 by serial dilution from 64 μg/mL in 10 μL volumes. Eighty microlitres of bacterial culture diluted in Mueller-Hinton broth were added and the plates were incubated at appropriate bacterial growth conditions for 24 hrs. After incubation, 10 μL of alamarBlue™ reagent (Biosource International) were added and the plates were incubated for 4 hrs to allow for reduction of the dye. The contents of each well were transferred to a flat-bottomed black/clear microtitre plate and fluorescence was measured using a Cytofluor 2300 microtitre plate reader with an excitation wavelength of 540 nm and emission wavelength of 590 nm. Media containing no peptide or bacterial culture were used as a zero growth control and culture with no peptide added was considered 100% growth. Each concentration of peptide was assayed in triplicate.

2.1.4.2 Anti-trypanosome activity assay

Anti-trypanosomal activity was determined similarly to antibacterial activity above except for the following differences. The concentration of trypanosomes in a healthy log phase culture was determined using a haemocytometer and adjusted to 2.0×10^4 trypanosomes/mL in fresh PCF medium. The peptide was diluted from lyophilized powder in 0.2% fetal bovine serum/PBS, pH 7.4. The trypanosomes were then incubated with the peptide for 66 hrs, before the alamarBlue™ reagent was added, and 6 hrs were allowed for dye reduction. Again, each concentration of peptide was assayed in triplicate. To determine the heat stability of the peptide activity, an aliquot of 1 mg/mL

BMAP18 was boiled for 5 min at 100 °C, then assayed as above alongside the untreated BMAP18.

2.1.5 Antifungal Assay

Conidia were released from a fresh culture of *Fusarium solani* grown on V8 agar by washing the surface of the plate with 6 mL of 1/25 diluted potato dextrose broth (Difco) and gently rubbing with a glass rod. The suspension of conidia was collected using a 10 mL pipette and filtered through two layers of Kimwipe tissues to separate conidia from mycelia. The concentration of conidia was measured using a haemocytometer and adjusted to 2 x 10^5 conidia/mL with 1/25 diluted potato dextrose broth. Two-fold dilutions of BMAP18 in sterile water were prepared in triplicate and 1 x 10^4 conidia were mixed with 50 μL of peptide dilution in a droplet on a 35 mm Petri plate, sealed with Parafilm, and incubated under low light at room temperature for 24 hrs. Photographs were taken using a Nikon Coolpix 900 digital camera attached to a confocal microscope at the UVic Advanced Imaging Lab. Each concentration was assayed in triplicate and the experiment was repeated twice.

2.1.6 Phytotoxicity Assays

2.1.6.1 Growth inhibition assay

Non-transgenic potato plantlets were grown in the presence of synthetic BMAP18 to determine if exogenous application of peptide has any toxic or growth inhibitory effects. The antibiotic carbenicillin which is generally regarded as safe for plants was used as a negative control, while Round-up (Monsanto Canada Inc.), a potent weed killer, was used as a positive control. Twenty, sixty and one hundred micrograms of BMAP18,

carbenicillin and Round-up were added to 1 mL of MS medium in an eppendorf tube. Apical buds taken from healthy 4 - 5 week old non-transgenic potato plants were propagated in these media under standard growth conditions for 28 days. The shoot lengths were measured and compared to those of plants grown in unaltered MS medium.

2.1.6.2 Protoplast isolation and challenge with BMAP18

Mesophyll protoplasts from healthy non-transgenic potato leaves were isolated by the method of Sidorov, *et al.* (1994). Healthy, dark-green, fully expanded leaves of 4 - 5 week old plantlets grown aseptically in tissue culture were sliced using a sharp scalpel into 1 mm wide strips and spread on top of 6 mL of digestion solution (0.5 M sucrose, 5.0 mM $CaCl_2 \cdot 2H_2O$, 10 mM MES-hydrate, 0.6% cellulase (Onozuka R-10) (Research Products International Corp., Cat. No. C3200), 0.6% Macerozyme R-10 (Research Products International Corp., Cat. No. M22010), 0.2% cellulysin (Calbiochem, Cat. No. 219466), pH 5.6 (Sidorov, *et al.* 1994)) in a 9 cm Petri plate. Digestion occurred overnight (16 hrs) in darkness at 25 °C. Three volumes of wash solution (0.5 M sucrose, 5.0 mM $CaCl_2 \cdot 2H_2O$) were added and the plate was swirled to resuspend leaf debris and release protoplasts. The protoplasts, suspended in wash solution, were collected from the plate through gentle pipeting and transferred to a sterile funnel with a sterile nylon mesh filter to remove leaf debris. The plate was then rinsed with a further three volumes of wash solution to collect remaining protoplasts. Filtered protoplasts were transferred to a sterile 40 mL ultracentrifuge tube and 1 mL of W5 solution (154 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$, 10.7 mM KCl, 5.55 mM glucose) was layered on top. Living protoplasts were separated by spinning at 375 x g for 8 min in a table-top swinging bucket centrifuge

(Beckman GS-15), which caused them to collect at the interface of the wash and W5 solutions. The protoplasts were carefully collected from this interface using a 10 mL pipette and transferred to a new sterile ultracentrifuge tube. The tube was topped-up with W5 solution and spun again at 60 x g for 4 min to pellet the protoplasts. The W5 solution was removed by careful aspiration and 2 mL of W-S-S cultivation medium (Sidorov, e*t al.*, 1987) were added. The protoplast pellet was resuspended by gentle "hand-vortexing", allowed to settle, and then resuspended again in the same manner. The concentration of cells was determined using a haemocytometer and adjusted to 2 x 10^5 cells/mL. To test cytotoxicity of BMAP18, 50 μL of cells (1 x 10^4 cells) were mixed with 50 μL of BMAP18 diluted in W-S-S medium at various concentrations (0 - 200 μg/mL) as a droplet on a 35 mm Petri plate, and incubated under standard plant growth conditions for 24 hrs. After incubation, 10 μL of Evan's Blue stain (0.5% w/v Evan's blue, 0.7 M sorbitol) was added to each droplet and allowed to diffuse for 1 hr. Evan's blue is able to penetrate dead cells, but does not stain living protoplasts. The droplets were viewed under a PhotoZoom™ Inverted Microscope (Bausch & Lomb) and photographed using a Nikon F-601 camera. This experiment was performed twice with similar results.

2.2 Transformation & Molecular Analysis of Transgenic Plants

2.2.1 Vector construction

The gene sequence for BMAP18 was derived by reverse translation of the amino acid sequence using codons optimized for expression in potato. Synthetic overlapping oligonucleotide primers were designed based on this sequence (ML1 5'-ATG GGA CGC TTC AAG AGA TTC CGC AAG AAG TTC AAG AAG CTC TTC AAG AAG CTC TCC TAA-3', and ML2 5'-TTA GGA GAG CTT CTT GAA GAG CTT CTT GAA CTT

CTT GCG GAA TCT CTT GAA GCG TCC CAT-3') and amplified by PCR along with additional primers (ML3 5'-GGA TCC GAG CTC TTA GGA GAG CTT CTT G-3', and ML5 5'-TCT AGA CCA TGG GAC GCT TCA AGA G3') that added restriction sites and the 5' and 3' ends of the gene to facilitate cloning. The synthetic gene was then inserted into the vector pBI221 using *Xba*I and *Sac*I digests of the PCR product and vector, placing the BMAP18 gene under the control of the CaMV 35S promoter with the nopaline synthase terminator at its 3' end. At this point correct synthesis of the gene was confirmed by DNA sequence analysis and the plasmid was called pTRT211. The gene and terminator sequences were then excised using *Nco*I and *Eco*RI and inserted into the vector pBI524 to place BMAP18 expression under control of the enhanced 35S promoter and AMV translational enhancer. Proper insertion of the gene into this vector, named pTRT524-3, was confirmed by restriction analysis. The expression cassette comprised of promoter, gene and terminator was then excised using *Hin*dIII and *Eco*RI and inserted into pBI121 in place of the *uidA* (GUS gene) cassette for cultivation in *Agrobacteria* for plant transformation. This final vector construct was confirmed by restriction analysis and called pTRT1211.

For spatio-temporal control of BMAP18 production, two constructs were prepared with the PmBiP promoter sequence: full-length PmBiPPro1-1, and truncated PmBiPPro1-3 (Forward, *et al.*, 2002). Plasmids derived from pBI121 containing these promoters and the GUS gene were prepared for ligation of the *BMAP18-Nos*T fragment of pTRT211 by excising the GUS gene and *Nos*T with *Xba*I and *Eco*RI. Successful ligation was confirmed by restriction analysis and DNA sequencing at the Centre for Biomedical

Research, University of Victoria. Primers used for sequencing corresponded to the 5' (BMAP18-F: CTAGACCATGGGACGCTTC) and 3' (BMAP18-R: GCTTCTTGAAGA GCTTCTTGAAG) ends of the BMAP18 gene.

All three constructs were transformed into *Agrobacterium tumefaciens* MP90 by the standard freeze-thaw method (Holsters, et al., 1978) and selected on LB plates containing 50 mg/L kanamycin and 10 mg/L gentamicin. Plasmids were isolated and characterized by restriction analysis to confirm stable transformation.

2.2.2 Transformation of potato

Petioles taken from 4 - 5 week old plants grown in sterile tissue culture were pre-cultivated in liquid PetM medium (4.3 g Murashige and Skoog basal salt mixture, Gamborg's vitamins, 40 mg adenine sulphate, 20 g glucose, 20 g mannitol, 900 mg MES-hydrate, 50 μg gibberellin A_3, 20 μg α-naphthaleneacetic acid, 2 mg trans-zeatin riboside/L, pH 5.7) at 24 °C under low light for 2 days. The explants were then transferred to fresh medium, and *Agrobacteria* carrying either pTRT1211, pBiPPro1-1::BMAP18 or pBiPPro1-3::BMAP18 were added to an OD_{600} of ~1.0. The bacteria were allowed to infect the explants for 1 - 2 hrs with slow shaking before they were removed and blotted on sterile filter paper to remove excess bacteria. The explants were then placed on solid PetM (containing 0.4% agarose) and cultivated at 24 °C under low light for 3 - 4 days. After infection, explants were washed twice for 1 hr in liquid PetM containing 1 g/L carbenicillin, then cultivated on a selective medium (solid PetM containing 100 mg/L kanamycin and 500 mg/L carbenicillin, and 3 g/L agarose) under standard plant growth conditions, transferring to fresh medium every two weeks until

shoots were generated from the calli. These shoots were excised and rooted in MS medium containing 20 g/L sucrose and 25 mg/L kanamycin to select for transformants.

2.2.3 Molecular analysis of transgenic plants

2.2.3.1 Southern and northern blot analyses

Genomic DNA was extracted from leaves of 4 - 5 week old aseptically grown transgenic and control plants using the Sigma GenElute Plant kit (Cat. No. G2N-70) and quantified using a Beckman DU-65 spectrophotometer. Three micrograms of DNA from each line were digested with 150 U of *Eco*RI over two days in a 200 μL reaction volume. Digested DNA was concentrated in a vacuum concentrator to 20 μL and loaded on a 1.0 % agarose gel in 1 x Tris-borate-EDTA buffer and run at 1 V/cm for 24 hrs. After electrophoresis, the gel was stained with ethidium bromide to visualize even digestion and loading of the genomic DNA. The DNA was then transferred to a Biodyne B nylon membrane (Pall Gelman Laboratory) according to the membrane manufacture's recommendations except the depurination step was omitted and QuickDraw Blotting Paper (Sigma, P6928) was used as the absorbent pad to maximize transfer efficiency. Complete transfer after two days was confirmed by re-staining the gel with ethidium bromide and visualizing under UV light. The membrane was placed between two pieces of filter paper and baked under vacuum at 80 ˚C for 40 min to fix DNA.

Total RNA was isolated from 4 - 5 week old aseptically grown plants using the TRIzol® (Invitrogen, Cat. No. 15596-026) method, including the optional addition of a high salt solution to remove polysaccharides and proteoglycans from the RNA pellet. The concentration of RNA isolated was quantified using a Beckman DU-65

spectrophotometer. A 1.2% formaldehyde agarose gel was prepared according to the recommendations of the QIAGEN RNeasy® Mini handbook, except 8 μg/mL ethidium bromide was added directly to the 5 x RNA loading buffer. Twenty-five micrograms of RNA from each line were loaded on the gel and electrophoresed at 3 V/cm for 3 hrs. The gel was imaged under UV light to confirm equal loading before transfer. Transfer to a Biodyne B nylon membrane (Pall Gelman Laboratories) was carried-out as per the recommendations of the manufacturer, except QuickDraw Blotting Paper (Sigma, P6928) was used as the absorbent pad to maximize transfer efficiency. Complete transfer after two days was confirmed by re-staining the gel with ethidium bromide and visualizing under UV light. The membrane was placed between two pieces of filter paper and baked under vacuum at 80 °C for 40 min to fix RNA.

2.2.3.2 Probe preparation

A ssDNA probe complementary to the coding strand of the BMAP18 gene was prepared for detection of both transgene insertion and mRNA transcripts by unidirectional PCR as follows. A restriction fragment comprising the BMAP18 gene and the nopaline synthase terminator was prepared by digestion of pTRT211 with *Xba*I and *Eco*RI and purified by gel extraction using the QIAquick® Gel Extraction Kit (QIAGEN, Cat. No. 28704). The primer ML3, used to synthesize the BMAP18 gene, directed polymerization of the antisense probe. The PCR reaction mixture was prepared as follows: 125 ng *BMAP18-Nos*T fragment, 5 μL 10x Strip-EZ PCR buffer (Ambion, Cat. No. 1475), 1 μM ML3 primer, 200 nM dATP/TTP/dGTP, 75 μCi [α-^{32}P]dCTP and 37.5 U of Taq polymerase (Invitrogen, Cat. No. 18038-018) in a 50 μL reaction volume. The reaction product was

amplified using the following cycling conditions: 94 °C for 3 min, then 40 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 30 sec, followed by a final elongation step of 72 °C for 10 min. The synthesized probe was separated from unincorporated nucleotides using a ProbeQuant™ G-50 Micro Column (Amersham Biosciences, Prod. No. 27-5335-01). The cpm/μL was determined by adding 1 μL of the reaction mixture or purified probe to 3 mL of ScintiVerse™ scintillation solution (Fisher Scientific) and measuring with a Beckman LS 5000CE scintillation counter. Incorporation of radiolabel into the probe was measured by comparing the cpm/μL of the probe before and after the column purification and taking the ratio of the two.

$$\% \text{ incorporation} = \frac{\text{cpm/μl purified probe}}{\text{cpm/μl PCR reaction}} \times 100\%$$

Greater than 50% incorporation was achieved each time the probe was prepared.

2.2.3.3 Hybridization

Both Southern and northern blots were hybridized in the same manner. Baked blots were rehydrated by soaking in 2 x SSC for 10 min before transfer to hybridization tubes. They were then prehybridized at 65 °C for at least 1 hr with Perfecthyb™ Plus (Sigma, H7033) hybridization buffer. The hybridization buffer was changed and the radiolabeled probe was added to a concentration of 2 x 10^6 cpm/mL of hybridization buffer. Hybridization was performed at 65 °C for 24 - 48 hrs. Excess probe was washed from the blots using low stringency washing steps. All wash solutions were added at room temperature and washing was performed in the hybridization oven at 65 °C for the Southern blots or 50 °C for the Northern blots. Each blot was washed twice with a solution of 2 x SSC/0.1% SDS for 5 min, and twice with a solution of 1 x SSC/0.1% SDS for 10 min. Washed blots

were blotted on a piece of clean filter paper and wrapped in plastic film prior to exposure. Exposure of Kodak BioMax MR film was conducted at –80 °C using an intensifying screen until a strong signal with minimal background was obtained. Exposure times for individual blots varied and are indicated in Results and the captions of the appropriate figures.

2.2.3.4 Protein isolation and analysis

Cationic proteins were isolated from the third leaflet pair of the fourth or fifth leaf of plants grown in a growth chamber as well as all healthy, green leaves of tissue-culture grown plants. To extract and solubilize cationic proteins, 200 - 250 mg of tissue were ground to a fine powder using liquid nitrogen and a mortar and pestle, transferred to a 2 mL microcentrifuge tube and mixed with two volumes (0.4 - 0.5 mL) of 0.5 M HCl. Each tube was vortexed to mix then kept on ice until all samples were processed (not more than 30 min). The samples were centrifuged for 10 min at 14,000 x g at 4 °C, and the supernatant was collected in a clean microcentrifuge tube. Two hundred microlitre aliquots of supernatant were mixed with 1.2 mL of ice-cold acetone in new 2 mL tubes, inverted to mix and incubated at –20 °C overnight. After precipitation, the samples were centrifuged for 10 min at 14,000 x g at 4 °C to pellet the protein. The supernatant was removed by aspiration followed by drying in a vacuum concentrator for about 20 min. The protein pellets were resuspended in a mixture of PBS, pH 7.4 and protease inhibitor cocktail (100:1) (Sigma, P9599) to give a final volume equal to half of the original weight of the fresh tissue. The concentration was estimated using UV absorbance and the

equation: [protein] (mg/mL) = $1.55A_{280}$-$0.76A_{260}$. Samples were stored at –20 °C until use.

Cationic protein samples were analyzed by Tricine-SDS-PAGE (Schägger, H., 2006). The gels were run at 26 V (0.43 V/cm) for 18 hrs and stained using the silver staining method of Tsai and Frasch (1981) with the following modifications. The gel was fixed for 1 hr in 40% ethanol/10% acetic acid, rinsed in 20% ethanol/5% acetic acid, and then soaked in 20% ethanol/5% acetic acid/0.7% (w/v) periodic acid. Developer was prepared in 125 mL of distilled water with 12.5 mg tripotassium citrate and 125 μL of 37% formaldehyde. Development was terminated by the addition of 10% acetic acid.

In order to enrich the samples for mass spectrometry analysis, affinity purification was performed using affinity purified polyclonal antibodies coupled to POROS 20G Protein G beads (Applied Biosystems, cat. No. 2-3111-00). The beads were resuspended by finger flicking and two 25 μL aliquots were taken into clean 0.6 mL microcentrifuge tubes. An equal volume (about 20-30 μg) of either wild type (control) or 2x35S::BMAP-34 (transgenic) cationic protein was added to each tube and mixed by flicking. The protein solution was allowed to incubate with the beads overnight at 4 °C (21.5 hrs) to ensure binding of BMAP18. The beads were then resuspended and washed with 0.5 mL of PBS/0.1% octyl β-D–thioglucopyranoside by gentle mixing followed by 5 min of centrifugation at 16 000 x g at 4°C and removal of the supernatant. The wash was repeated once taking care to remove all supernatant. Peptides bound to the antibodies were eluted by adding 100 μL 0.1% formic acid and flicking to mix. The elution was incubated for 15 min at 4 °C, resuspended, and then spun for 5 min at 16 000 x g at 4 °C.

The supernatant was carefully collected and submitted to the UVic Genome BC Proteomics Facility for analysis by MALDI-TOF MS.

2.3 Production of Polyclonal and Monoclonal Antibodies

2.3.1 Immunization:

Prior to immunization, test bleeds were taken of all animals to be immunized to serve as negative controls. Two female Balb/c mice were immunized by interperitoneal injection of 25 μg BMAP18-KLH conjugate in complete Freund's adjuvant, and boosted with the same amount of peptide in incomplete Freund's adjuvant after 3, 5, and 7 weeks before a final intravenous boost 11 weeks after the initial immunization. Two female New Zealand White rabbits were immunized by subcutaneous injection of 200 μg BMAP18-KLH in complete Freund's adjuvant, followed by 3 boosts of the same amount of peptide in incomplete Freund's adjuvant at two-week intervals. All animals were housed and cared for at the UVic Animal Care Facility. After immunization, polyclonal antisera from rabbits and mice was obtained by cardiac bleed and stored with 0.1% sodium azide at –20 °C. The polyclonal antisera titre was determined by ELISA against BMAP18-BSA.

2.3.2 Production of hybridoma cells:

Immunized mice were killed by cervical dislocation 3 days after boosting with BMAP18-KLH intravenously. Spleens of immunized mice were removed, punctured and flushed with serum-free DMEM (Gibco/BRL Cat. # 23700-057, containing high glucose, L-glutamine and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),

supplemented with 0.5 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 U/L penicillin/streptomycin, and 1 mM sodium pyruvate) to extract spleenocytes. Lymphocytes were isolated using a Ficoll-Paque gradient and counted to calculate the amount of SP2/0 cells needed to give a 1:5 ratio of spleen lymphocytes to SP2/0 cells for hybridization. The cells were washed to remove Ficoll-Paque, pelleted and combined. Following centrifugation and removal of residual DMEM, cells were incubated at 37 °C and 1 mL of warmed polyethylene glycol-1500 was slowly added, followed by the gradual addition of warmed serum-free DMEM to a final volume of 40 mL. Lastly, 10 mL of heat-inactivated FBS was added to aid in removal of residual polyethylene glycol from the fused cells. The cells were pelleted and resuspended in recovery medium and allowed to grow at 37 °C and 5% CO_2 over night. On the following day, fused cells were pelleted and added to semi-solid methocult selective medium for plating onto Petri dishes. Individual hybridoma clones were then picked and cultivated in DMEM/20% FBS for several days before being transferred to tissue culture flasks with DMEM/10% FBS medium. Following selection of monoclonal antibodies (mAbs) by ELISA and immunoblot analysis, hybridoma cultures were preserved in 90% FBS with 10% DMSO and stored in liquid nitrogen.

2.3.3 ELISA:

Isolated hybridoma clones were screened for anti-BMAP18 activity by ELISA. Ninety-six-well microtitre plates (Falcon 3915 Pro-bind) were coated with 500 ng of conjugated BMAP18-BSA or human transferrin (HT) and allowed to dry overnight at 37 °C. Wells were blocked by addition of 200 μL of 3% (w/v) skim milk powder/PBS for 1 hr at 37 °C, then rinsed with quick washes of PBS/0.05% Tween-20. One-hundred microlitres of

undiluted supernatant from each hybridoma culture were added, allowed to incubate for 90 min, and washed with PBS/0.05% Tween-20 to remove unbound antibodies. Bound mAbs were detected by addition of a goat-anti-mouse IgG/IgM conjugated with alkaline phosphatase (Caltag Laboratories). After removal of unbound secondary antibody and incubation with a *p*-nitrophenyl phosphate substrate in diethanolamine buffer overnight, absorbance was read at 405 nm. Clones were selected on the basis of strong signal with BMAP18-BSA and low cross-reactivity with HT. Altogether, twelve hybridoma clones were selected for immunoblot testing.

2.3.4 SDS-PAGE and immunoblotting:

Selected hybridoma clones were tested for suitability to immunoblot procedures. BMAP18-BSA was loaded in as many lanes as monoclonal antibodies (mAbs) were tested on a commercially prepared SDS-PAGE (Invitrogen, Cat. No. NP0321BOX). The gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Pall Corporation) by electrotransfer and then cut into strips, each strip representing one lane of the gel. These strips were blocked in blocking buffer (5% skim milk/0.1% Tween-20/PBS), then incubated for 1 hr at room temperature with secreted mAbs in the hybridoma culture supernatant diluted 1/2,000 in blocking buffer. The strips were then washed with 0.1% Tween-20/PBS and incubated with secondary antibody (goat anti-mouse IgG/IgM conjugated to horse radish peroxidase, Caltag Laboratories) at 1:50,000 dilution in blocking buffer for 1 hr. The strips were washed, the chemiluminescent substrate (Pierce Super Signal West Dura) was added, and detection was conducted according to the

manufacturer's recommendations. Kodak Biomax-MR film was exposed to the blot until a strong signal was observed (~1 min).

2.3.5 Dot blot analysis:

Dot blot analysis was performed to determine whether the prepared antibodies were capable of detecting free BMAP18. Five strips of PVDF membrane were prepared according to the manufacturers recommendations (Pall Corporation) before 500 ng of pure BMAP18, and 1 μg of BMAP18-BSA were directly applied to each strip. An additional strip was spotted with 500 ng of MsrA2 as a control for cationic peptide detection. The membrane strips were allowed to air dry and stored at 4 °C overnight. The strips were then re-hydrated and blocked with 5% skim milk powder/0.1% Tween-20/PBS for 1 hr at room temperature with shaking. Excess blocking buffer was removed with two 1 min rinses and one 5 min wash with PBS/0.1% Tween-20. Primary antibodies tested included prebleed sera from rabbits prior to immunization (negative control), polyclonal antisera of two immunized rabbits, affinity purified anti-BMAP18 rabbit antiserum and anti-MsrA2 rabbit antiserum (positive control). Each primary antibody was diluted 1/2000 in PBS/0.1% Tween-20 and incubated with one membrane strip for 1 hr at room temperature with shaking. Unbound antibodies were removed by the following series of washes with PBS/0.1% Tween-20: two rinses of 1 min each, 1 wash for 15 min, and three 5 min washes. The strips were then incubated with the secondary antibody (goat-anti-mouse IgG/IgM conjugated to horseradish peroxidase, Caltag Laboratories, Ltd.) diluted 1/150 000 in PBS/0.1% Tween-20 for 1 hr with shaking. Excess secondary antibody was removed using the same washing procedure as that for the primary antibodies. Bound secondary antibody was visualized by chemiluminescent

detection using the ECL plus Western Blotting Detection System (Amersham Biosciences) and Kodak Biomax MR film.

2.4 In vivo bioassays

2.4.1 Resistance of detached leaves to *Fusarium solani*

Leaves were collected from two-month-old transgenic and control plants grown in a growth chamber. For this assay, the second leaflet pair of the fourth or fifth leaf from the top of each plant was chosen. Care was taken to choose leaves that were of similar size and of good health. Because three plants of each line were grown and two leaflets taken from each plant, six leaflets of each line were assayed. The experiment was performed twice, giving a final sample size of 12.

Whole leaves were removed from the plants and transported to the lab where the second leaflet pair was removed and transferred to a 15 cm Petri plate containing three layers of 12.5 cm filter paper moistened with 5 mL of sterile distilled water. Three leaflets were placed on each plate (one from each leaf) and a 0.75 cm diameter plug of *Fusarium solani* taken from the perimeter of an actively growing culture on PDA was placed on each leaflet near, but not on, the midrib. The Petri plates were then sealed with Parafilm™ and incubated at room temperature at low light until symptoms were observed, 7 days after infection. Representative leaflets were photographed using a Nikon Coolpix 990. Lesion area was quantified using the "Histogram" function of Adobe® PhotoShop® 7.0.

2.4.2 Co-cultivation of transgenics and fungal pathogens

Control and BMAP18-expressing transgenic lines were grown in magenta boxes for three weeks before a 0.75 cm agar plug of fresh *F. solani* culture was placed on the medium approximately 1 cm from the stem of the plantlet. At this point, the magenta boxes were transferred to the laboratory bench to avoid contamination of the culture room. The fungus was allowed to grow over the medium and the date at which it contacted the base of the plant's stem was recorded as Day 0. Daily observations of *Fusarium* wilt symptoms were made until the plant was deemed dead. In the instance when a particular symptom did not develop throughout the experiment, the number of days until plant death was recorded. The experiment consisted of two to four plants of each line and was repeated twice.

2.4.3 Resistance of tubers to bacterial soft rot

Tubers grown from mature, wilted, transgenic and control potato plants were harvested, washed and stored at 4 °C in brown paper bags until use. To determine the effect of BMAP18 expression on resistance to soft rot disease caused by *Erwinia carotovora*, 1 - 3 month old tubers were warmed to room temperature, surface sterilized with 70% ethanol for 30 sec then 1/4 diluted commercial bleach (Javex^{TM}) for 8 min, rinsed in sterile distilled water and sliced into three to four 5 mm sections. The slices were placed on three layers of damp filter paper in a 9 cm Petri plate. An overnight culture of *E. carotovora* grown in Luria-Bertani medium without salt was diluted to 1 x 10^7 cfu/mL in PBS, pH 7.4 (to dilute the medium, while maintaining the osmotic balance) and 10 µL of diluted culture were applied to the centre of each potato slice. Because *E. carotovora* is

most active in warm moist environments and anaerobic conditions, the plates were sealed with Parafilm™ and incubated at 25 - 28 °C in darkness for six days. The tuber slices were scored as resistant or susceptible on the basis of the absence or presence of soft rot lesions and photographed using a Nikon Coolpix 990 digital camera. The experiment was performed four times with three slices of one tuber of each line and equivalent age in each experiment.

2.5 Statistical Analyses

Statistical analyses were performed using SPSS 15.0 for Windows ® (SPSS Inc, Chicago, IL, USA). For the phytotoxicity assay, an independent sample t-test was used to detect significant differences in growth of treatment samples compared to controls with a confidence level of 95%. For the detached leaf assay, the mean lesion areas of each line were compared by one-way analysis of variance (ANOVA) followed by Tukey's HSD test with a rejection limit of $p > 0.10$.

Chapter 3: Results

3.1 *In vitro* Studies

Previous studies have shown that BMAP18 has potent antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria, as well as the protozoan parasite *Trypanosoma brucei* (Skerlavaj, *et al.*, 1996; Haines, *unpublished data*). In order to confirm these findings and determine the toxicity of the peptide to plant-specific pathogens, *in vitro* assays were conducted with synthetic BMAP18. In addition, the phytotoxicity of BMAP18 was evaluated to determine whether its expression in transgenic plants may cause harmful effects. The minimum inhibitory concentration (MIC) values determined are defined as the concentration of BMAP18 required to inhibit greater than 90% of growth and are summarized in Table 2.

Table 2: MIC values determined for BMAP18.

	MIC	
Cell type	μg/mL	μM
Erwinia carotovora	2	0.854
Escherichia coli	8	3.41
Pseudomonas aeruginosa	8	3.41
Proteus vulgaris	> 64	> 27.3
Staphylococcus epidermidis	> 64	> 27.3
Bacillus subtilis	> 64	> 27.3
Fusarium solani	8	3.41
Trypanosoma brucei (PCF)	8	3.41
Solanum tuberosum protoplasts	75	32.0

3.1.1 Antibacterial activity assays

To determine the antibacterial activity of BMAP18, the alamarBlue™ reduction assay was performed with a variety of bacteria including Gram-negative, and Gram-positive strains that are pathogenic to humans or plants. In this assay, the dye reagent, alamarBlue™, is non-specifically reduced from blue to pink by metabolic intermediates of actively growing cells such that colour-change is directly related to viability.

Prior to assaying BMAP18 activity, the appropriate concentration of cells to use for each culture was optimized. Ten-fold serial dilutions of each bacterial strain were prepared and incubated with alamarBlue™ for 4 hours before measuring colour development. A 10-fold dilution of the minimal concentration required to produce a colour change was used in the antibacterial assay so that in the absence of growth no measurable colour-change would be observed while any growth that occurred during incubation with peptide would be readily apparent.

After optimizing growth conditions for alamarBlue™ reduction, the cultures were incubated with two-fold serial dilutions of synthetic BMAP18 from 64 μg/ml to 0.125 μg/ml in a 96-well microtitre plate overnight, prior to addition of the dye. This allowed adequate time for the peptide to act if present in sufficient quantities, but also allowed bacteria in those wells where the concentration of BMAP18 was below the MIC to multiply to a density capable of reducing the alamarBlue™ reagent. Controls included wells containing no peptide (100% growth) and wells containing no cells (background fluorescence of the media). Reduction of alamarBlue™ was measured using a spectrofluorimeter and percent growth inhibition was calculated as follows:

$$\% \text{ growth inhibition} = (F_{100\ \% \text{ growth}} - F_{[BMAP18]}) / F_{100\ \% \text{ growth}}) \times 100\% \qquad \text{(Equation 1)}$$

Figure 1 illustrates the dose-dependent antibacterial activity of BMAP18. These data indicate that this CAP has potent antimicrobial activity against Gram-negative bacteria. The MIC of the potato pathogen *Erwinia carotovora* was extremely low at 2 μg/mL (0.854 μM). *Escherichia coli* and *Pseudomonas aeruginosa,* were also highly susceptible with MICs of 8 μg/mL (3.41 μM), however, *Proteus vulgaris* was less susceptible (MIC > 64 μg/mL or 27.3 μM). Activity against Gram-positive was not observed at the concentrations of peptide tested; therefore, the MIC values for these strains are recorded as greater than 64 μg/mL (> 27.3 μM).

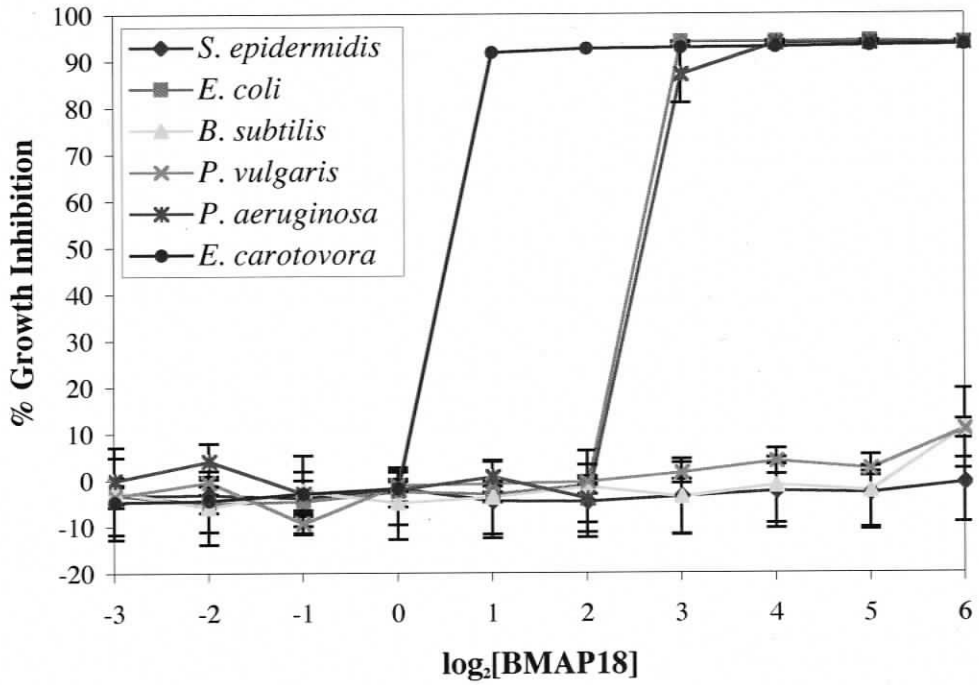


Figure 1: Antibacterial activity of BMAP18.

Two-fold serial dilutions of BMAP18 (indicated as the $\log_2$ of the concentration in μg/mL) were incubated with a variety of bacterial pathogens for 24 hrs before alamarBlue™ reagent was added. The % growth inhibition was determined using Equation 1. Each concentration of peptide was measured in triplicate and the bars represent the standard error of the mean.

3.1.2 Anti-trypanosomal activity assay

The activity of BMAP18 against both procyclic form (PCF) and bloodstream form (BSF) *Trypanosoma brucei* has been previously characterized by Haines *et al.* (*unpublished data*); therefore, the purpose of this experiment was to determine the effect of boiling on the anti-trypanosomal activity of BMAP18. Should the peptide accumulate to sufficient levels in transgenic potato tubers, they could serve as an edible treatment for trypanosomiasis, which would necessitate their boiling prior to consumption. Also, it is proposed that boiling of transgenic potato tubers may facilitate purification of the recombinant peptide from the bulk of endogenous heat-labile proteins. Figure 2 indicates that boiling of BMAP18 for 5 minutes prior to addition of trypanosomes did not significantly alter its activity. In addition, the MIC of BMAP18 against PCF trypanosomes was determined to be 3.41 μM.

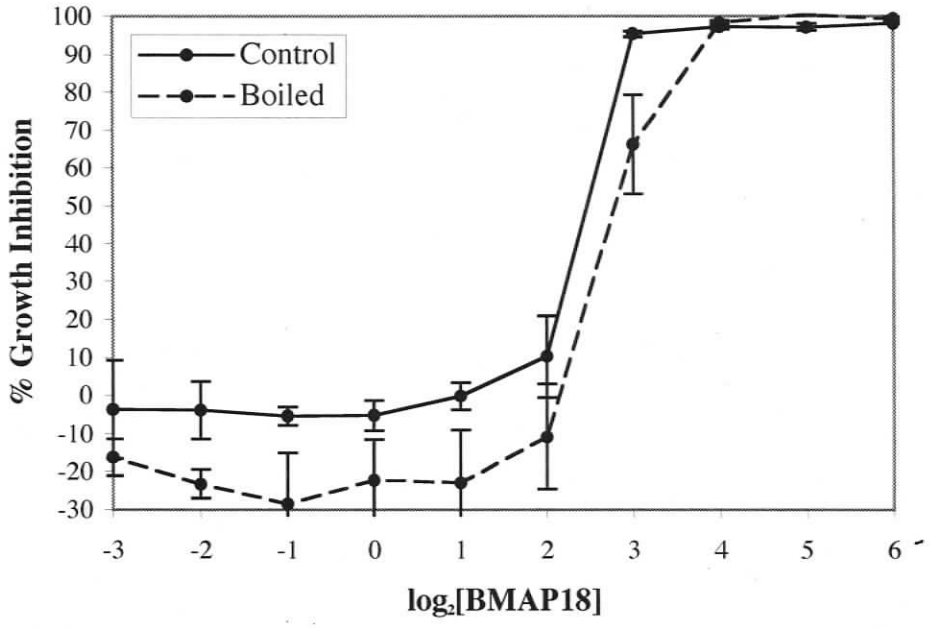


Figure 2: Effect of boiling on BMAP18 activity.

Two-fold serial dilutions of both untreated and boiled BMAP18 (indicated as the $\log_2$ of the concentration in μg/mL) were incubated with procyclic form trypanosomes for 66 hrs prior to alamarBlue™ reagent addition. As in Figure 1, Equation 1 was used to determine the % growth inhibition. Each concentration of peptide was measured in triplicate and the bars represent the standard error of the mean.

3.1.3 Anti-fungal activity assay

A comparative study of the relative antifungal activities of selected CAPs against a variety of agronomically important *Fusarium* species indicated that BMAP18 has potent antifungal activity (S. Misra, *unpublished data*). Some of the most important diseases affecting potato production and storage are caused by fungal pathogens. For example, *Fusarium solani* causes wilt of potato plants in the field and dry rot in stored tubers. Therefore, the MIC value of BMAP18 against this significant potato-specific pathogen was examined. Conidia collected from a healthy fresh culture of *F. solani* were incubated with two-fold serial dilutions of BMAP18 for 24 hrs. Observation under an

inverted light microscope (Figure 3) indicated germination was completely inhibited at 16 μg BMAP18/mL, while 90% of germination was inhibited by 8 μg/ml. Below the MIC, extensive hyphal growth was observed, indicating that the relationship between inhibition of germination and peptide concentration was not linear, and that a thresh-hold concentration is required for BMAP18 to exert anti-fungal activity. This experiment was performed twice and the results presented in Figure 3 are representative photos taken of the second trial.

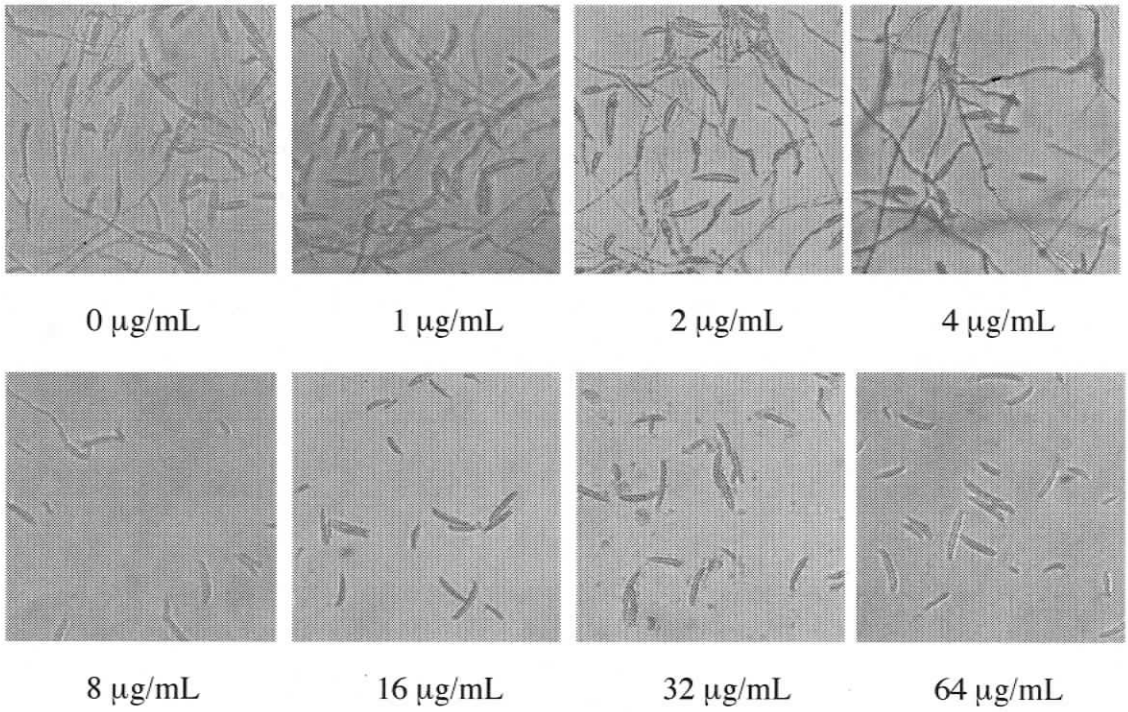


Figure 3: Inhibition of germination of *F. solani* conidia.

Conidia collected from a fresh *F. solani* culture growing on V8 agar were incubated with the indicated concentrations of BMAP18 for 24 hrs then observed under an inverted light microscope and photographed using a digital camera. The MIC is taken to be 8 μg/mL as few hyphae were observed at this concentration of peptide. Each concentration of peptide was assayed in triplicate and the experiment was performed twice with similar results.

3.1.4 Phytotoxocity Assays

The above data sufficed to confirm that BMAP18 has potent activity against a variety of microorganisms; however, to be a suitable candidate for production in transgenic plants, toxicity against plants cells must be low (less than that anticipated to be accumulated) and not adversely affect growth.

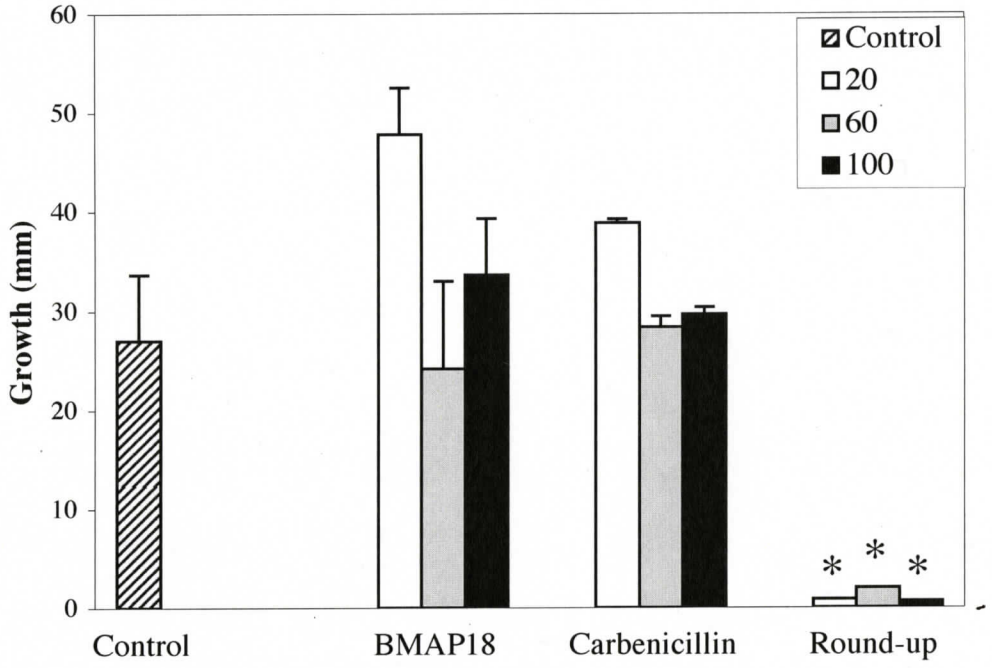
3.1.4.1 Plant growth inhibition assay

To determine the effect of exogenous BMAP18 on growth of potato plantlets, *in vitro* culture media was supplemented with either carbenicillin (negative control), Rσund-up® (positive control) or BMAP18 at 20, 60, and 100 μg/mL concentrations. The graph shown in Figure 4A indicates that cultivation of potato explants in media containing up to 100 μg/ml BMAP18 did not inhibit plantlet growth. However, the amount of root development does appear to be slightly diminished in the presence of BMAP18. Though this effect was neither quantified, nor thoroughly examined, a qualitative assessment of root development may be taken from Figure 4B, where photographs of control plants (unmodified MS medium) and plants grown in the presence of with BMAP18 are presented.

3.1.4.2 Protoplast isolation and challenge with BMAP18

As a more sensitive test of phytotoxocity (Yevtushenko *et al.,* 2005), protoplasts were isolated from healthy leaves of non-transgenic potato plants and incubated with two-fold dilutions of BMAP18 in protoplast cultivation medium. After 24 hrs, dead cells were stained with Evan's Blue dye, visualized under an inverted light microscope and photographed. Representative photos of the results are presented in Figure 5. BMAP18

A



B

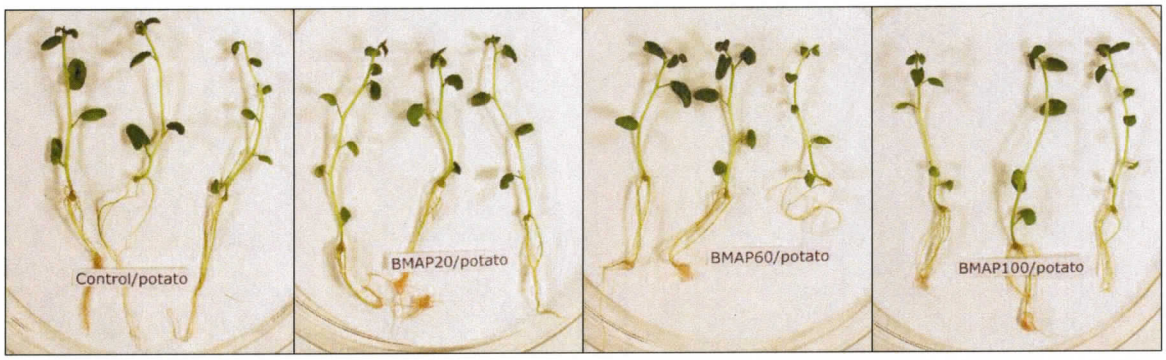


Figure 4: Phytotoxicity of BMAP18.

Apical buds of non-transgenic potato plants were propagated in media containing BMAP18, carbenicillin (negative control) or Round-up ™(positive control) at 20, 60 and 100 μg/mL, and cultivated for 28 days. A) Growth was determined by comparing the length of the shoot before and after cultivation. Results are presented as means and standard error of the means of six replicates from two trials of three replicates each. Asterisks indicate significant differences from control according to independent sample t-tests with $p < 0.05$. B) Photos taken of plants grown in control medium or medium containing BMAP18. The numbers indicate the concentration of BMAP18 in μg/mL.

was nontoxic to potato protoplasts at concentrations below 75 μg/mL. The images shown in Figure 5 are representatives of the results of two independent experiments, each performed in triplicate.

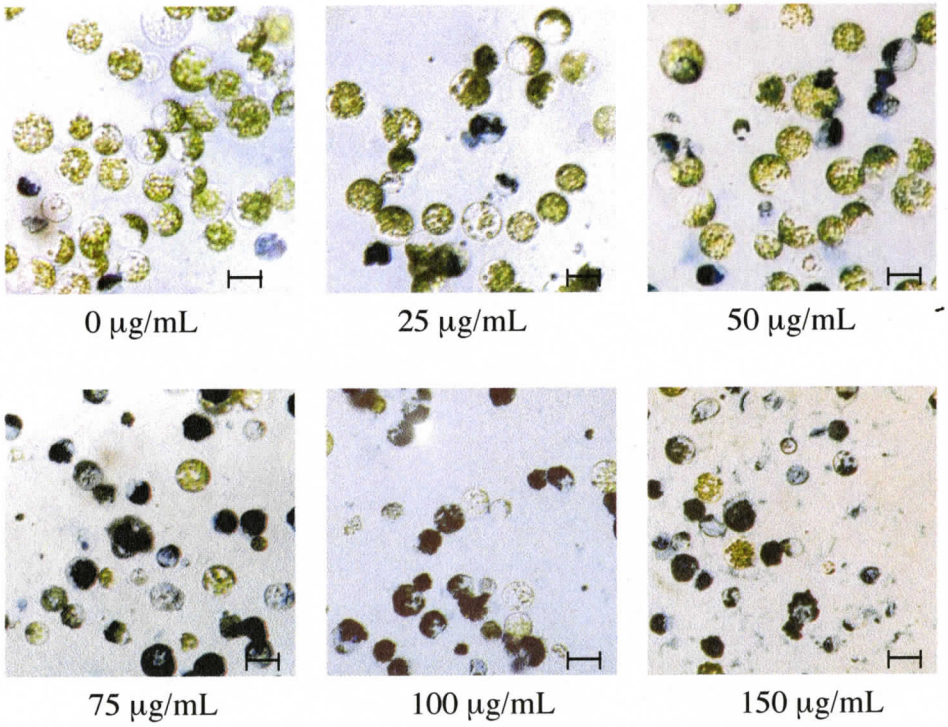


Figure 5: Toxicity of BMAP18 to potato protoplasts.

Freshly isolated, healthy protoplasts were incubated with the indicated concentrations of BMAP18 for 24 hrs then stained with Evan's Blue. Healthy, living protoplast remained green, while dead cells are stained blue. Each concentration of peptide was assayed in triplicate, and the experiment was repeated twice with similar results. Scale bar: 50 μm.

3.2 Transformation & Molecular Analysis of Transgenic Plants

The potent antimicrobial activity of BMAP18, combined with its low toxicity to potato plants and protoplasts make it an excellent candidate for expression in transgenic potato for high-level recombinant production and conferral of enhanced resistance to phytopathogens. To express the peptide in transgenic potato, three constructs were prepared as described below and shown in Figure 6.

3.2.1 Vector construction

For optimal expression in potato, the nucleotide sequence of the BMAP18 gene was prepared by reverse translation of the amino acid sequence using codons that are preferred for potato expression (see Figure 7A). Synthetic primers corresponding to the entire 54 nucleotide sequence were prepared and amplified in the presence of additional primers that added the required restriction sites to the 5' and 3' ends of the coding region of the synthetic BMAP18 gene (Figure 7B and C). After cloning into pBI221 and propagation in *E. coli*, the inserted synthetic gene was sequenced and analyzed by restriction digestion to ensure no errors had been introduced through PCR or cloning (Figure 8A). This vector was named pTRT211.

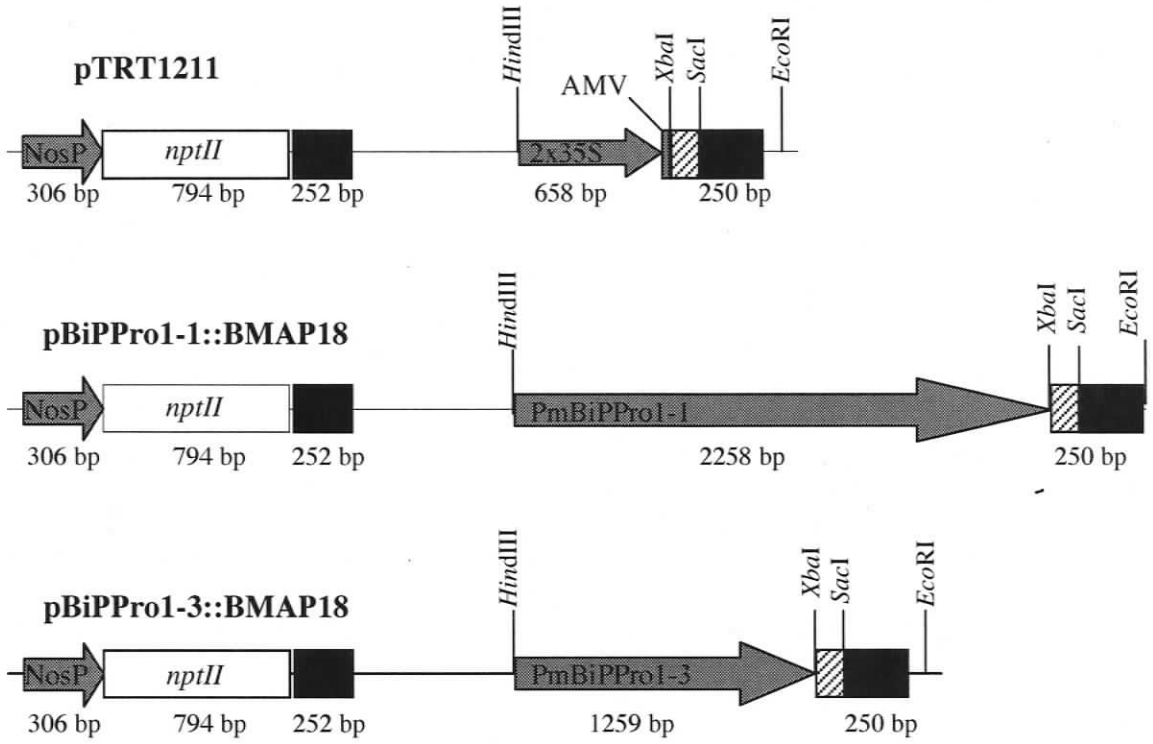


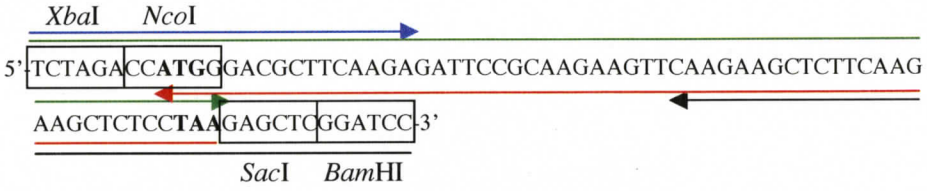
Figure 6: T-DNA regions of BMAP18 expression vectors.

Proportional representations of the T-DNA regions of transformation vectors prepared in this study. Promoters are depicted in grey, while the *Nos*T is represented in black. The *nptII* selection marker is shown in white and the 80 bp BMAP18 gene is represented by the cross-hatched box. All restriction enzymes used in DNA manipulations are indicated.

A

Gly-Arg-Phe-Lys-Arg-Phe-Arg-Lys-Lys-Phe-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Ser

B



C

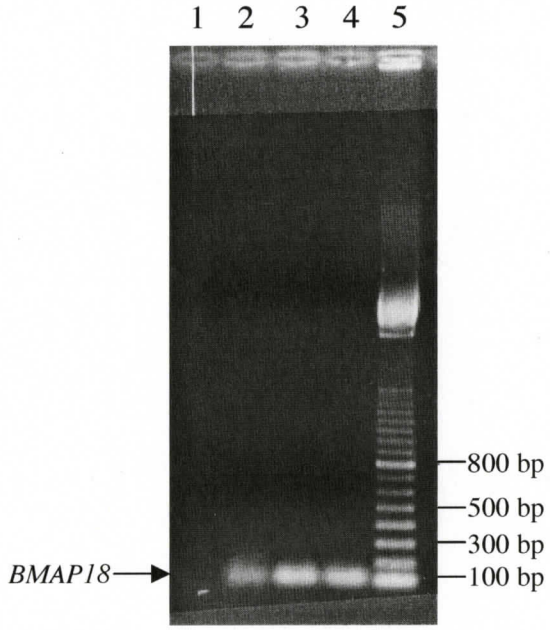


Figure 7: Synthesis of the BMAP18 gene.

A) The amino acid sequence of BMAP18. B) Nucleic acid sequence of the BMAP18 gene and the primers used for its synthesis. ML1= green, ML2 = red, ML3 = black, ML5 = blue. Boxes indicate restriction sites. Translational start and stop codons are highlighted in bold. C) Ethidium bromide stained agarose gel showing PCR synthesis of the BMAP18 gene. Lane 1: Control using only primers ML1 and ML2, Lane 2: Control using only primers ML3 and ML5, Lanes 3 and 4: all four primers, representing the BMAP18 gene with restriction sites at 5' and 3' ends, Lane 5: Size standard. The full synthetic BMAP18 gene is 80 bp long.

To facilitate ligation into pBI524, the BMAP18 gene was excised from pTRT211 along with the downstream *Nos*T, and inserted in place of the *uidA-Nos*T region of pBI524. This placed the BMAP18 gene downstream of the enhanced CaMV 35S promoter (2x35S) and AMV RNA4 translational enhancer for high-level constitutive expression (Datla *et al.*, 1993). The 2x35S::BMAP18 construct was excised and cloned into pBI121 in place of the CaMV 35S promoter and *uidA* for transformation into *Agrobacterium tumefaciens*. This vector contains the sequences necessary for maintenance of the plasmid in both *E. coli* and *A. tumefaciens* as well as a selectable marker in the T-DNA region *nptII* (*neomycin phosphotransferase II*), which confers plant resistance to kanamycin. The restriction analysis shown in Figure 8B confirmed proper insertion of the gene cassette into the transformation vector pTRT1211.

For spatio-temporal control of transgene expression, the BMAP18 gene and *Nos*T were excised from pTRT211 and inserted into pBiPPro1-1 and pBiPPro1-3 (Forward *et al.*, 2002) replacing the *uidA-Nos*T region. Proper insertion of the gene and terminator downstream of the full-length (PmBiPPro1-1) and 5'-truncated (PmBiPPro1-3) BiP promoters is shown in Figure 9 and was confirmed by DNA sequencing.

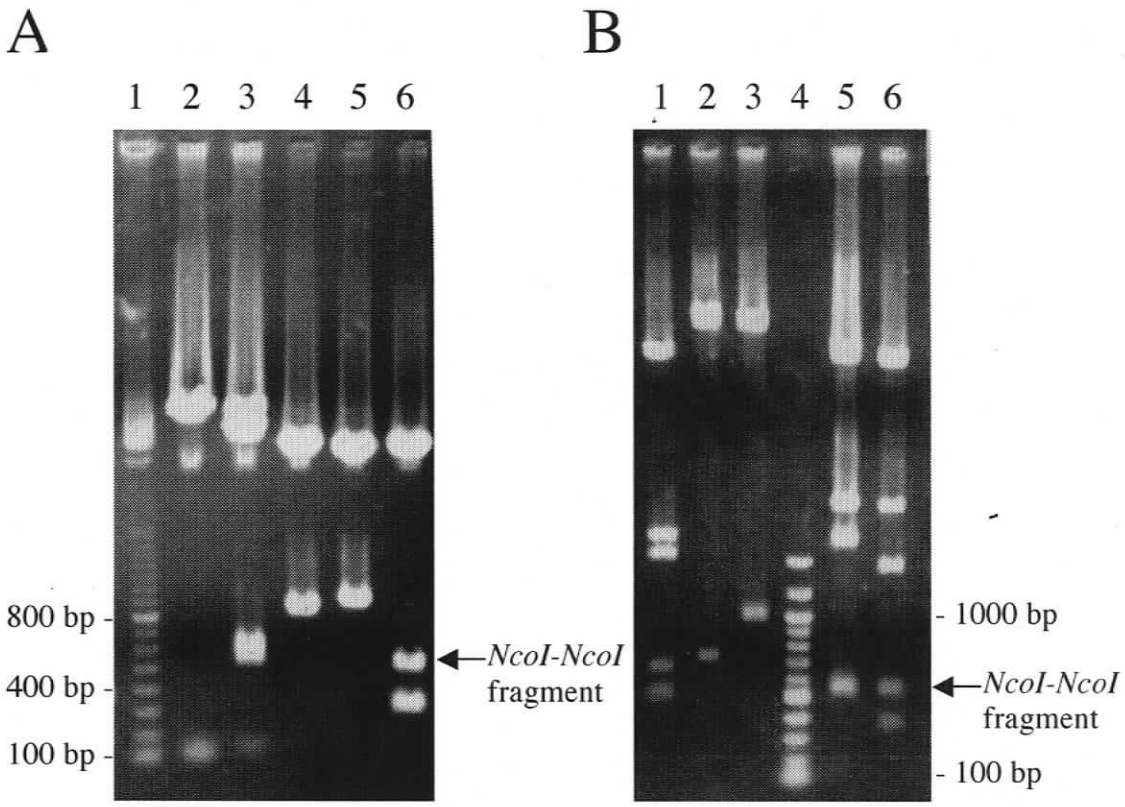


Figure 8: Restriction analyses of BMAP18 constructs.

A) pTRT211. Lane 1: Size standard, Lane 2: *Xba*I and *Sac*I (the BMAP18 gene, 80 bp), Lane3: *Nco*I and *Sac*I (the BMAP18 gene, 74bp, and the *Nco*I-*Nco*I fragment), Lane 4: *Hin*dIII and *Xba*I (the CaMV 35S promoter, 834 bp), Lane 5: *Hin*dIII and *Sac*I (35S::*BMAP18,* 914 bp), Lane 6: *Nco*I and *Eco*RI (*BMAP18-Nos*T, 432 bp, and the *Nco*I-*Nco*I fragment). B) pTRT1211. Lane 1: *Hin*dIII and *Nco*I (the enhanced CaMV 35S promoter-AMV, 658 bp), Lane 2: *Hin*dIII and *Sac*I (2x35S*::BMAP18,* 738 bp*),* Lane3: *Hin*dIII and *Eco*RI (2x35S*::BMAP18-Nos*T, 1090 bp), Lane 4: Size standard, Lane 5: *Nco*I and *Sac*I (*BMAP18,* 80 bp), Lane 6: *Nco*I and *Eco*RI (*BMAP18-Nos*T, 351 bp).

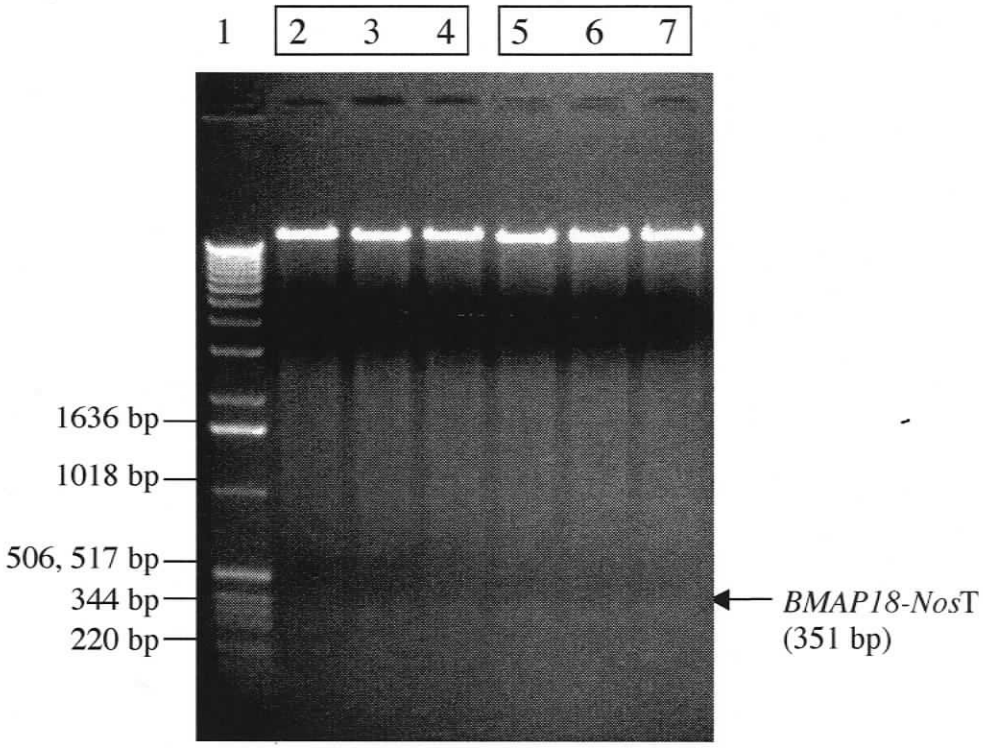


Figure 9: Restriction analysis of PmBiP promoter constructs.

Lane 1: Size standard, Lanes 2-4: *Xba*I-*Eco*RI digestion of pBiPPro1-1::BMAP18, Lanes 5-7: *Xba*I-*Eco*RI digestion of pBiPPro1-3::BMAP18.

3.2.2 Transformation of potato and molecular analysis

Stem, leaf, and petiole explants of healthy *Solanum tuberosum* cv. Desiree were transformed by co-incubation with *A. tumefaciens* harboring the above described transformation vector plasmids. Calli of successfully transformed tissues generated numerous shoots, which were rooted in MS medium containing 20 g/L sucrose and 50 mg/L kanamycin. Because kanamycin inhibits root formation in potato plants not expressing NPTII, only those shoots that generated healthy plantlets with fully formed roots were chosen for further analysis. As shown in Figure 10, no phenotypic differences were observed between non-transgenic and transgenic potato plants or tubers.

3.2.2.1 Southern analysis

Putative transgenic plantlets were screened for stable integration of the BMAP18 transgene by Southern hybridization. Of the shoots selected from transformed calli, the majority contained the BMAP18 gene cassette, with the exception of those generated from the first transformation attempt using pTRT1211.

Successfully transformed plantlets carrying one to six copies of the transgene cassette were obtained and propagated for further analysis. Of the lines transformed with pTRT1211 (2x35S::BMAP lines) (Figure 11A), seven stably transformed lines (2, 26, 27, 33, 34, 36, and 37) and two 'escapee' lines (9 and 23) were maintained for detection of BMAP18 transcript. Three bands representing the BMAP18 gene were detected in lines 2, 26 and 34, while a single band was detected in lines 27, 33, 36 and 37. The strong intensity of the single band in line 27 may indicate a tandem insertion event, in which

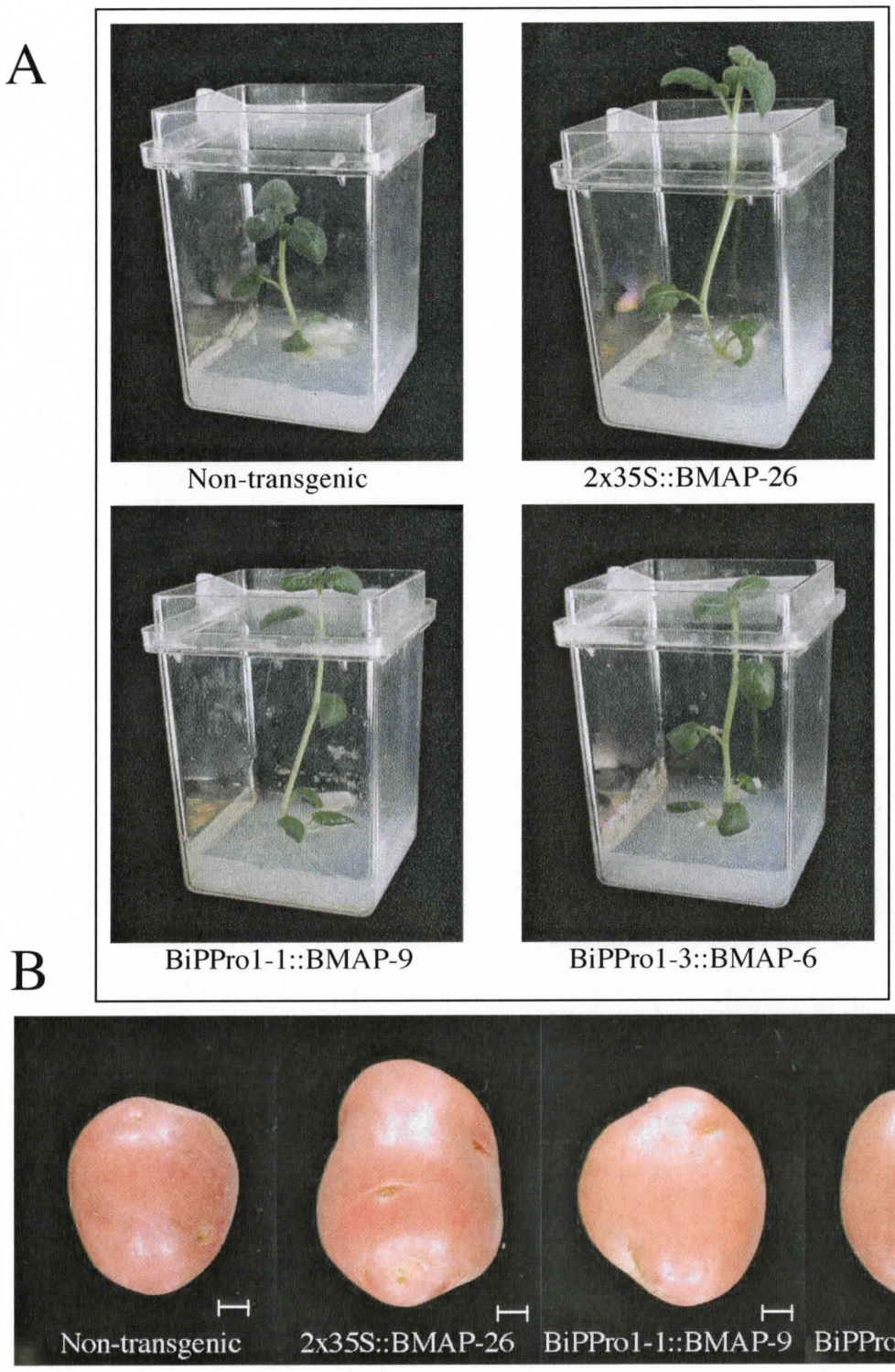


Figure 10: Comparison of control and transgenic potato phenotypes.

Representative transgenic and control potato A) plants and B) tubers are shown to demonstrate that transformation and expression of BMAP18 did not result in phenotypic abnormalities. Scale bar: 1.0 cm.

two copies of the gene were inserted directly adjacent to one another. Multiple copies and tandem insertions were also observed in the lines transformed with pBiPPro1-1::BMAP18 (Figure 12A) and pBiPPro1-3::BMAP18 (Figure 13A). Southern analysis showed one to six copies of the transgene in eight transgenic lines of BiPPro1-1::BMAP and eleven lines of BiPPro1-3::BMAP. Of these, seven BiPPro1-1::BMAP and six BiPPro1-3::BMAP lines of varying gene copy numbers were chosen for northern analysis. Line 13 of BiPPro1-1::BMAP was not stably transformed, but was also analyzed by northern as an 'escapee' control.

3.2.2.2 Northern analysis

Attempts to detect BMAP18 transcripts by reverse transcription - polymerase chain reaction (RT-PCR) were unsuccessful largely because the small size of the gene made the distinction between PCR product and primer dimers difficult (data not shown). Northern analyses of total RNA extracted from transgenic lines carrying the BMAP18 gene under control of the PmBiP promoters were initially performed using a probe prepared by random primer labeling of the *BMAP18-Nos*T fragment of pTRT211; however, this led to detection of a band corresponding to the *nptII* transcripts as well as a non-specific band at ~600 bp that was present in both transgenic and control lines and ran very near to the putative BMAP18 transcripts. Because the 5' portion of the *Nos*T is transcribed as a 3' UTR of the transgene, it is reasonable to expect that the *nptII* transcripts, which are also terminated by *Nos*T, would be detected. To improve the specificity of detection of *BMAP18* transcripts, an alternative probe was prepared using unidirectional PCR that was complementary to the *BMAP18* mRNA and did not contain any of the *Nos*T or

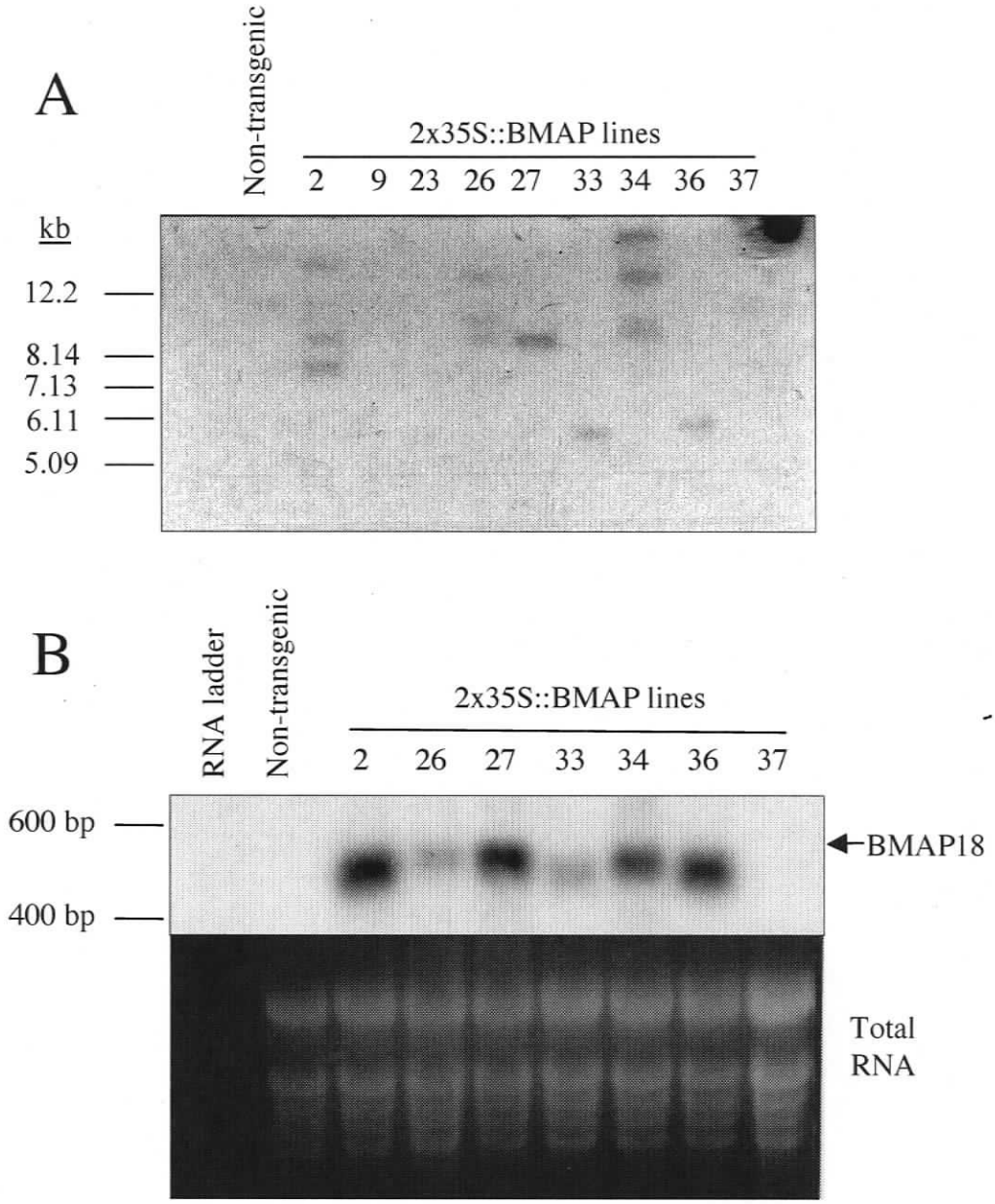


Figure 11: Molecular analysis of 2x35S::BMAP plants.

A) Southern analysis of genomic DNA (3 μg) isolated from leaves of putative transgenic plants digested with *Eco*RI, electrophoresed at 1 V/cm for 24 hrs, transferred to a Biodyne B nitrocellulose membrane, and hybridised with a ^{32}P-labeled ssDNA probe antisense to the BMAP18 gene B) Northern analysis of total RNA (25 μg) isolated from leaves of 4 week old plants separated on a 1.2% FA gel at 3 V/cm for 3 hrs and transferred to a nitrocellulose membrane. The blot was hybridized at 65 °C for 40 hrs with a ^{32}P-labeled ssDNA probe complementary to BMAP18 transcript, washed and left to expose Kodak MR film for 24 hrs prior to developing. The ethidium bromide stained ribosomal RNA bands indicate equal loading of total RNA.

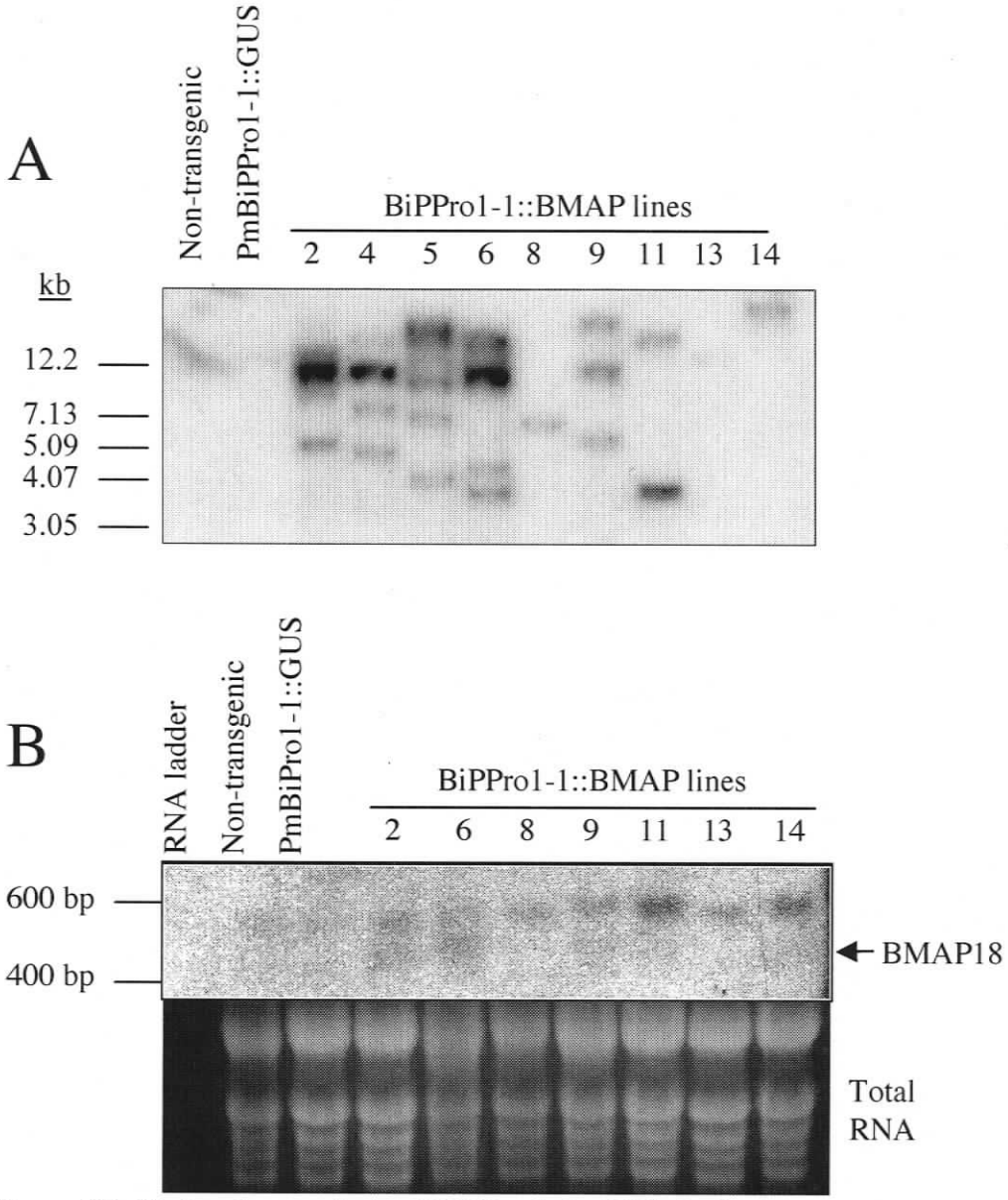


Figure 12: Molecular analysis of BiPPro1-1::BMAP plants.

A) Southern analysis of genomic DNA (3 μg) isolated from leaves of putative transgenic plants digested with *Eco*RI, electrophoresed at 1 V/cm for 24 hrs, transferred to a Biodyne B nitrocellulose membrane, and hybridised with a ^{32}P-labeled ssDNA probe antisense to the BMAP18 gene. B) Northern analysis of total RNA (25 μg) isolated from leaves of 4 week old plants electrophoresed on a 1.2% FA gel at 3 V/cm for 3 hrs and transferred to a nitrocellulose membrane. The ^{32}P-labelled probe was prepared through unidirectional PCR to yield a ssDNA sequence anti-sense to the BMAP18 mRNA. The blot was hybridized at 65 °C for 40 hrs, and exposed Kodak MR film for 14 days prior to developing.

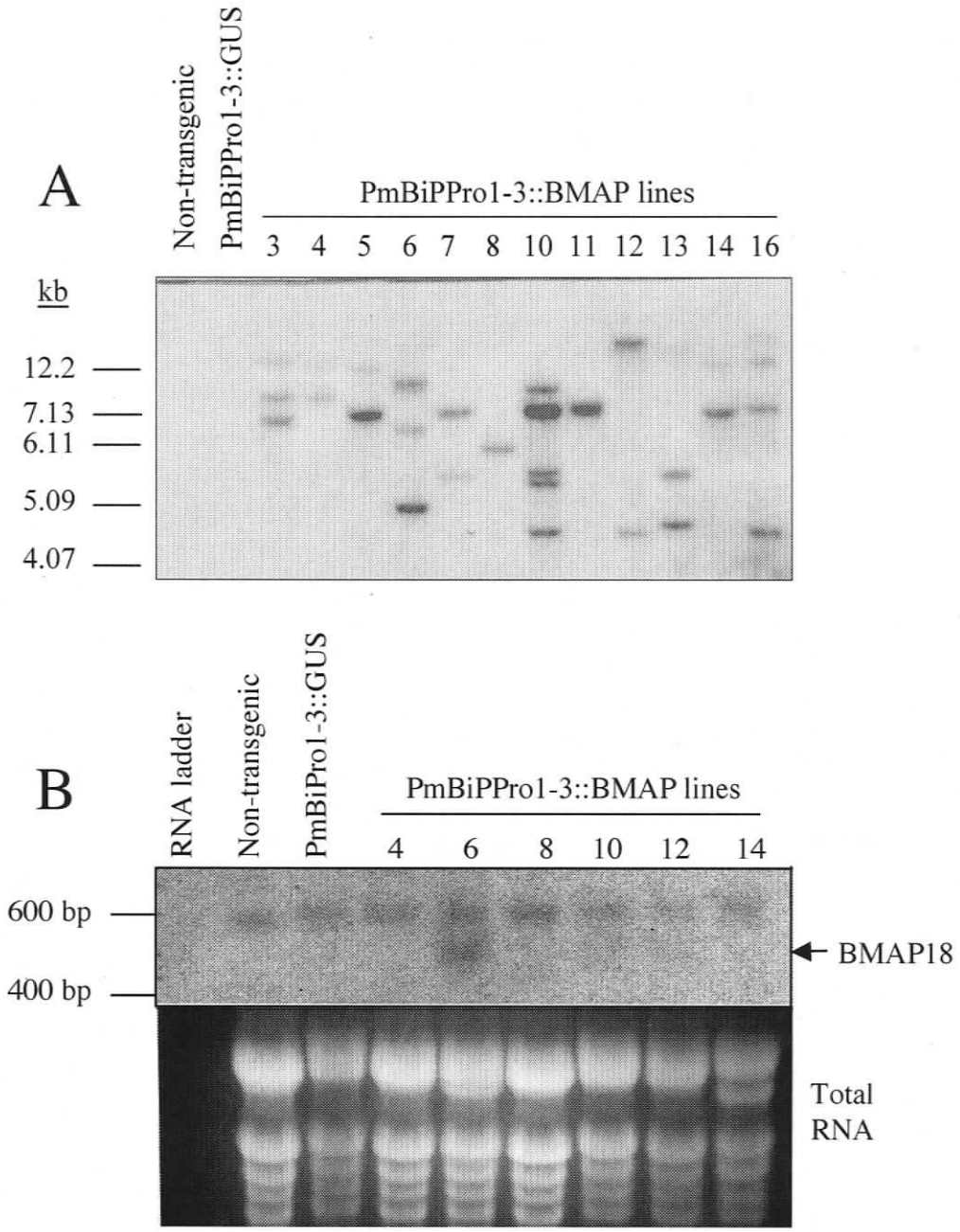


Figure 13: Molecular analysis of PmBiPPro1-3::BMAP plants.

A) Southern analysis of genomic DNA (3 μg) isolated from putative transgenic plants digested with *Eco*RI, electrophoresed at 1 V/cm for 24 hrs, transferred to a Biodyne B nitrocellulose membrane, and hybridized with a ^{32}P-labeled ssDNA probe antisense to the BMAP18 gene. B) Northern analysis of total RNA (25 μg) isolated from leaves of 4 week old plants electrophoresed on a 1.2% FA gel at 3 V/cm for 3 hrs and transferred to a nitrocellulose membrane. The ^{32}P-labelled probe was prepared through unidirectional PCR to yield a ssDNA sequence anti-sense to the BMAP18 mRNA. The blot was hybridized at 65 °C for 40 hrs, and exposed Kodak MR film for 14 days prior to developing.

promoter sequences. This probe did not hybridize to *nptII* transcripts, though it did detect the non-specific band at 600 bp. Nonetheless, a band unique to lines transformed with the BMAP18 gene was detected at ~450 bp using both the random primer labeled (not shown) and anti-sense probes (Figure 12B and 13B) after 1 - 2 weeks of exposure.

Detection of BMAP18 transcripts in 2x35S::BMAP lines was much more rapid, with strong signal in all lines except line 37 after 24 hours of exposure (Figure 11B). Exposure of up to two weeks did not detect transcripts in line 37, though the non-specific band at 600 bp was detected (not shown).

3.2.3 Protein analysis

3.2.3.1 Tricine SDS-PAGE

In general, detection of very small peptides by electrophoresis is difficult, as was observed by De Gray and colleagues (2001), who despite strong transcript level were unable to detect peptide below 14 kDa on a 16% glycine gel. Tricine-SDS-PAGE is specially adapted for resolution of peptides between 1 and 70 kDa. Therefore, cationic protein samples were isolated from control and 2x35S::BMAP lines that accumulated BMAP18 transcripts for analysis by Tricine-SDS-PAGE (Schägger, 2006). For comparison, synthetic BMAP18 and a non-transgenic control protein sample spiked with synthetic BMAP18 were used. As is seen in Figure 14, BMAP18 does not migrate according to its molecular weight of 2.3 kDA, but instead produces a smear between the major bands at ~7 kDa and 5 kDa with the majority of staining density at a distance corresponding to ~6 kDa (Figure 14). The anomalous migration of this 2.3 kDa peptide may be due to its

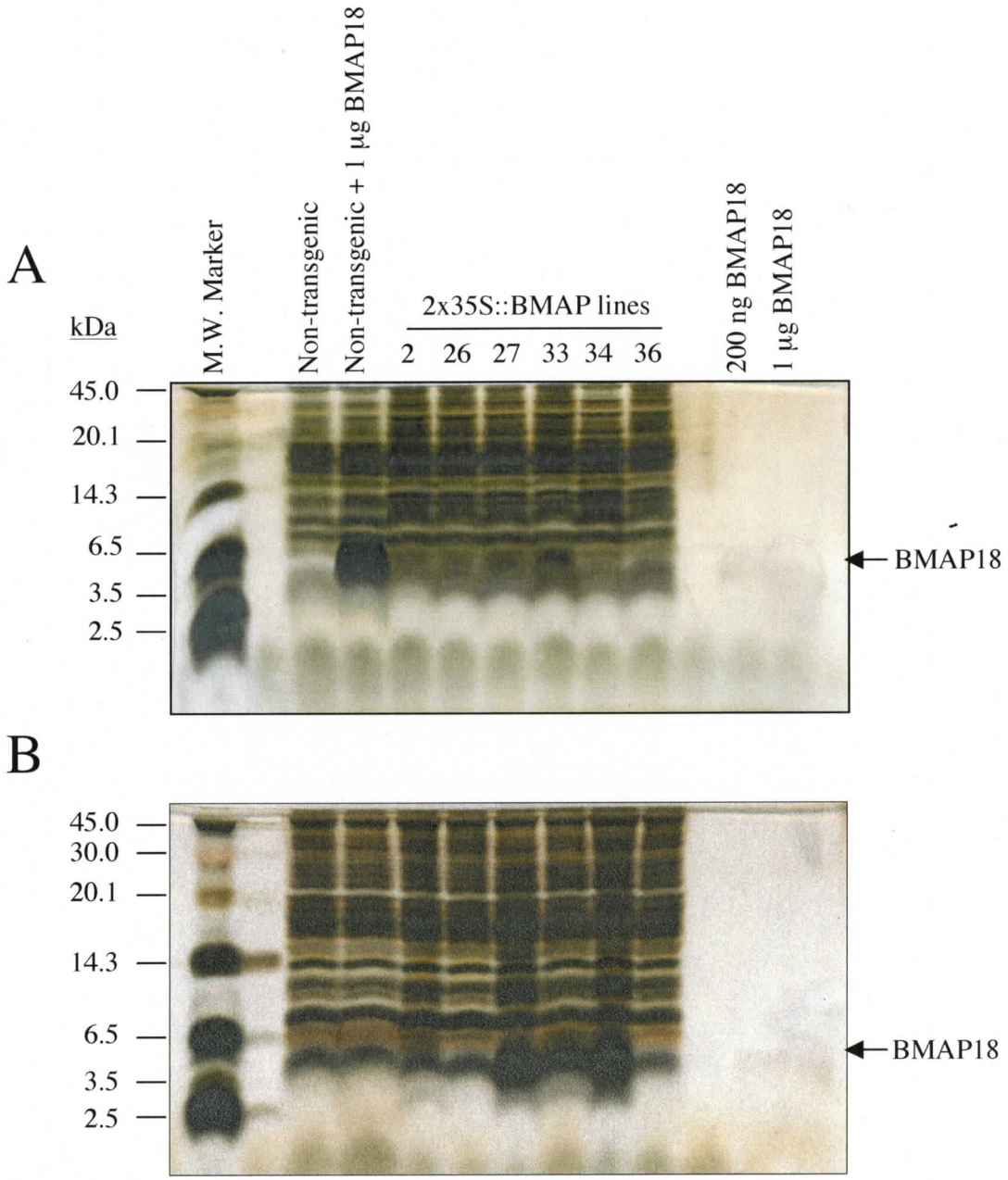


Figure 14: Tricine-SDS-PAGE analysis.

Acid-soluble, heat-stable proteins extracted from leaves of A) mature greenhouse-grown or B) young, tissue-culture grown BMAP18-expressing 2x35S::BMAP plants were analyzed by Tricine-SDS-PAGE. Protein loading in gel B was identical to that of gel A. Electrophoresis of synthetic BMAP18 indicated that the peptide migrates as smear from 7 to 5 kDa, as opposed to a sharp band at a distance corresponding to its molecular weight of 2.1 kDa.

highly positive charge to mass ratio. Because migration in an SDS-polyacrylamide gel is dependent on the association of SDS with the peptide bond imparting a uniform negative charge that is proportional to the size of the peptide, neutralization of this charge by the positive charges of BMAP18 may inhibit migration. Nonetheless, differences in the banding patterns of proteins isolated from transgenic and control plants in this region, indicate the presence of BMAP18 in lines 2x35S::BMAP-2, 26, 27, 33 and 34.

3.2.3.2 Mass Spectrometry

Initial attempts to extract protein from gel slices of the putative BMAP18 bands were unsuccessful. Therefore, to simplify the protein samples for mass spectrometry analysis, affinity purification was performed using affinity purified polyclonal antibodies coupled to POROS beads. The bound peptides were eluted and carefully collected for submission to the UVic Genome BC Proteomics Facility for Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis. In addition, total protein was isolated from leaves of tissue-culture grown control and transgenic plants (lines 2x35S::BMAP-33 and 2x35S::BMAP-34), and submitted for MALDI-TOF MS. The pure synthetic BMAP18 was identified with a mass of 2343.68 Da, however, a non-peptide contaminant present in all analyzed potato protein samples preferentially ionized and interfered with the detection and identification of BMAP18.

3.3 Production of Polyclonal and Monoclonal Antibodies

To detect the presence of BMAP18 in transgenic plant protein extracts by immunoblot analysis, polyclonal and monoclonal antibodies (mAbs) were prepared. Immune serum of rabbits injected with BMAP18 conjugated to the carrier adjuvant keyhole limpit hemocyanin (BMAP18-KLH) was collected and screened for affinity for BMAP18 coupled to the unrelated carrier protein bovine serum albumin (BMAP18-BSA). In addition, monoclonal antibodies were prepared by fusion of murine myeloma cells with spleenocytes of mice immunized against BMAP18-KLH.

3.3.1 Screening of hybridoma cells

Five hundred and seventy-six hybridoma clones selected from methocult plates and cultured in DMEM/10% FBS were screened for affinity for BMAP18-BSA and low cross-reactivity with human transferrin by enzyme-linked immunosorbent assay (ELISA) (Table 3). Based on these findings, eleven hybridoma clones were selected and maintained for immunoblot analysis.

Table 3: ELISA screening of anti-BMAP18 monoclonal antibodies.

Hybridoma culture supernatants were added to microtitre plates coated with either BMAP18-BSA or human transferrin. After washing, bound mAbs were detected using goat-anti-mouse IgG/IgM- horse radish peroxidase conjugated secondary antibodies with a chemiluminescent substrate. After overnight development the absorbance was read at 405 nm using a spectrophotometric plate reader. The signal obtained from the BMAP18-BSA plate (Signal) was divided by the signal from the human transferrin plate (Noise) to give the Signal to Noise ratio.

	Absorbance at 405 nm		
Clone ID	BMAP18-BSA	Human Transferrin	Signal : Noise
3B7	3.000	0.048	62.50
6H12	3.000	0.054	55.56
2C1	3.000	0.055	54.55
2F11	3.000	0.062	48.39
1B12	3.000	0.064	46.88
6B1	3.000	0.067	44.78
5D2	3.000	0.072	41.67
1C12	1.521	0.048	31.69
3A3	1.198	0.061	19.64
1D12	0.845	0.06	14.08
2C7	0.469	0.046	10.20

A_{405} values of 3.000 indicate the absorbance was greater than the spectrophotometer's detection limit.

3.3.2 Immunoblot analysis

Ten of the selected hybridoma clones were tested for suitability to immunoblot procedures. BMAP18-BSA was loaded on a SDS-PAGE and transferred to a PVDF membrane then cut into strips, each strip representing one lane of the gel. These strips were incubated with secreted mAbs in the hybridoma culture supernatant, washed, and incubated with secondary antibody for chemiluminescent detection. All mAbs tested were shown to detect BMAP18-BSA through immunoblotting (Figure 15A).

3.3.3 Detection of synthetic BMAP18

The ability of the prepared antibodies to detect free BMAP18 was investigated by dot blot analysis. Direct application of the peptide to the membrane prevented losses that may have arisen through acrylamide gel electrophoresis and transfer of the peptide onto the membrane. MsrA2 and BMAP18-BSA were included as positive controls. BMAP18-BSA controlled for activity of the antibodies as they had been previously shown to detect the conjugated peptide, while MsrA2 controlled for binding of a cationic peptide to the PVDF membrane and its detection by polyclonal antiserum. Figure 15B shows that conjugated BMAP18 may be detected on a PVDF membrane, but not the free peptide. It was concluded that either BMAP18 does not stably bind the PVDF membrane, or that binding of the peptide to the membrane induces a conformation that does not favor recognition of the peptide by the antibodies. Subsequent trials in which the membrane was stained with amido black indicated that BMAP18 does stably bind PVDF, and is not removed through the washing steps involved in immunodetection (not shown). Therefore, the latter case in which binding to the membrane obscures the immunogenic epitopes of BMAP18 is likely the reason the free peptide cannot be detected by immunoblotting.

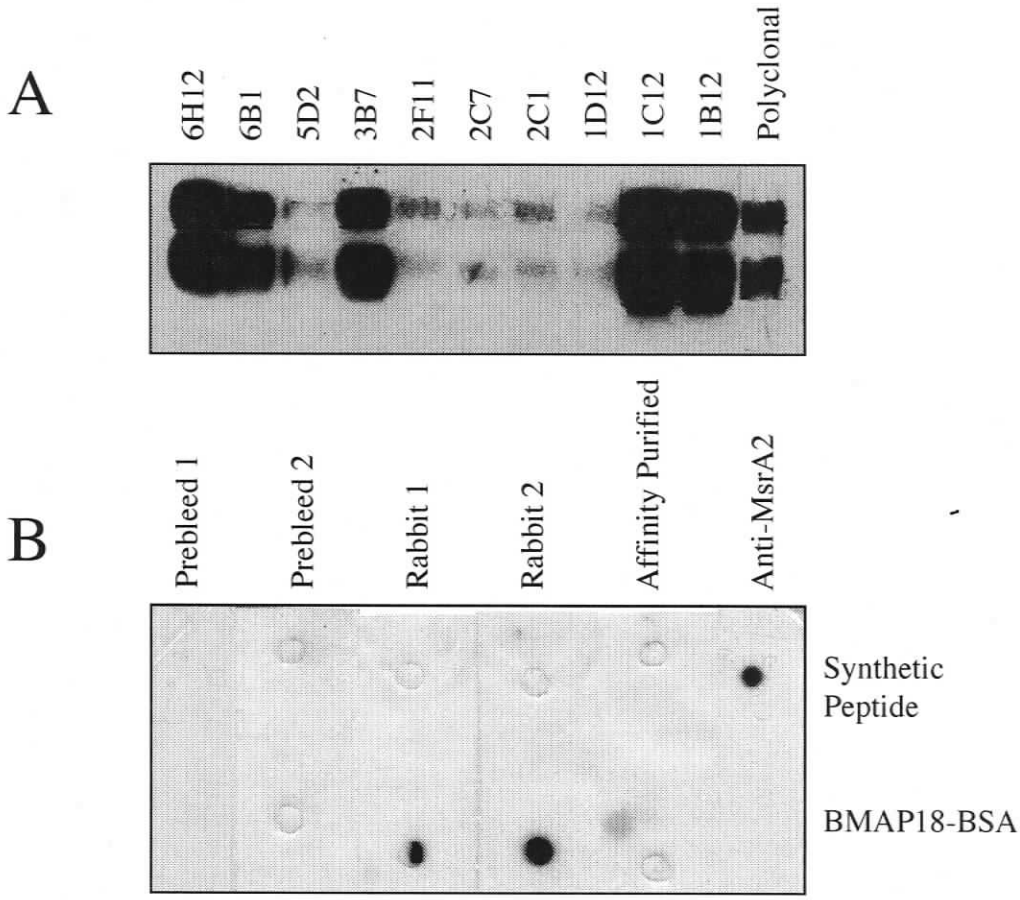


Figure 15: Immunodetection of BMAP18.

A) Immunoaffinity blot of BMAP18-BSA with indicated hybridoma culture supernatant as primary antibody. Bound antibodies were detected by goat-anti-mouse IgG/IgM antibody coupled to horseradish peroxidase and visualized using chemiluminescent detection. B) Dot blot analysis of BMAP18 using polyclonal antisera. One microgram of BMAP18-BSA or 500 ng of BMAP18 of MsrA2 were detected with the indicated antisera. Prebleed 1 and 2 are negative control sera taken from rabbits 1 and 2, respectively, prior to immunization. Rabbit 1 and 2 represent the polyclonal antisera from two rabbits inoculated with BMAP18-KLH. Affinity Purified is a preparation of these polyclonal antisera with enhanced specificity. Bound antibodies were detected by secondary antibodies (goat anti-mouse IgG/IgM) conjugated to horse radish peroxidase and chemiluminescence.

3.4 *In vivo* bioassays

Though the BMAP18 peptide could not be definitively detected in plant extracts, the high-level of expression of transcripts indicates that it may be present in levels sufficient to confer resistance to phytopathogens.

3.4.1 Resistance of detached leaves to *Fusarium solani*

In vitro analysis indicated that BMAP18 inhibited germination of *Fusarium solani* conidia at low micromolar concentrations. To investigate whether expression of the BMAP18 gene in transgenic potato may confer resistance to this pathogen, mature, healthy, fully expanded leaflets of soil-grown plants were infected with *F. solani*. Daily observations were made until a clear difference between lesion development on control leaves and BMAP18-expressing leaves could be made. After 7 days of infection, photos were taken and the areas of the lesions surrounding the agar plugs were quantified using imaging software. Figure 16 shows the results of three experiments with three to six replicates each. In each case, the lesion area of BMAP-18 expressing leaves was reduced compared to that of non-transgenic and GUS control leaves, with statistically significant reductions observed in lines 2x35S::BMAP-2, -27, -33, and BiPPro1-1::BMAP-9 and BiPPro1-3::BMAP-6. To better illustrate the difference in disease symptoms between control and BMAP18-expressing leaflets, representative leaves of the first trial are presented in Figure 17. It is noteworthy that the tissue surrounding the lesions of BMAP18-expressing leaves remains dark green; whereas, the tissue surrounding the lesions of control leaves is somewhat yellowed.

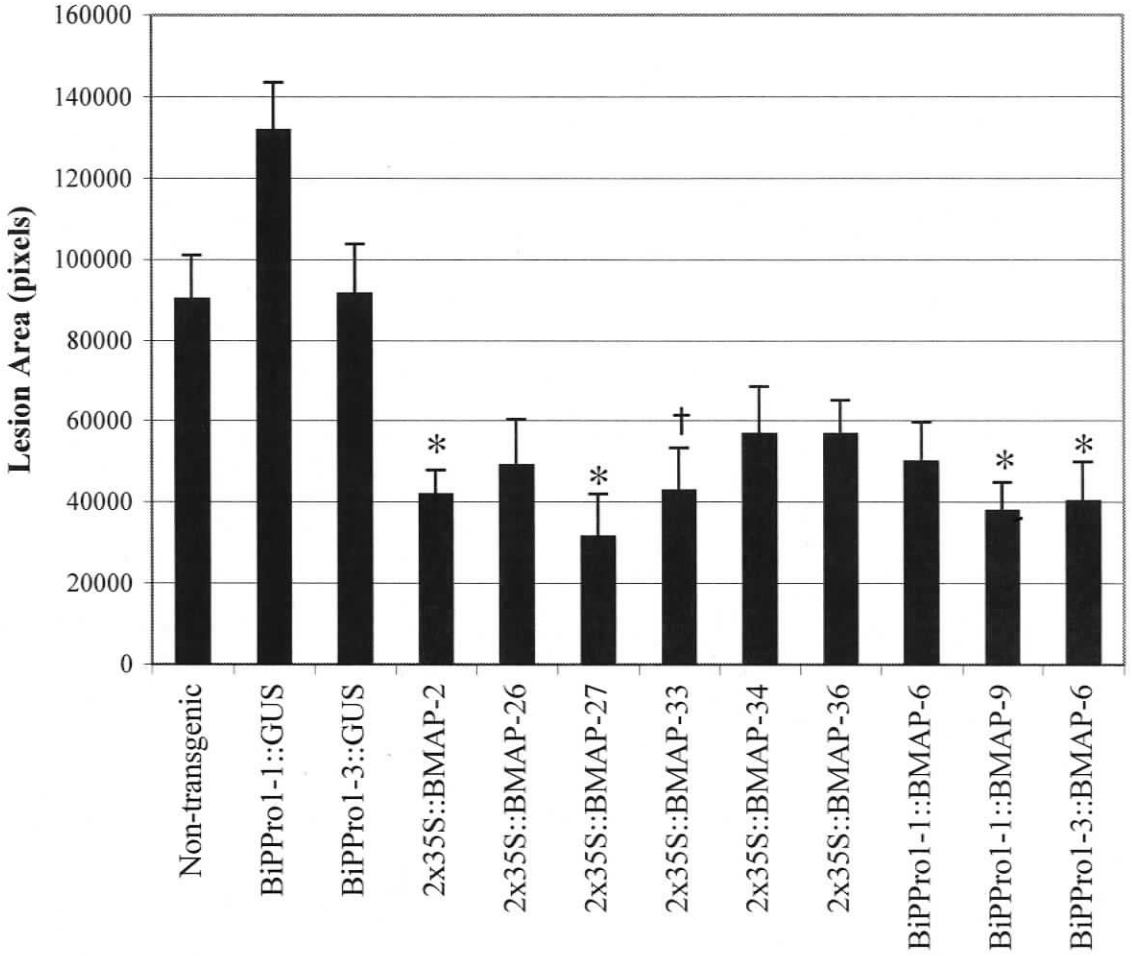


Figure 16: Resistance to *Fusarium* wilt.

Fully-expanded leaves from mature plants grown in a growth chamber were detached, transferred to a Petri plate containing moist filter paper and infected with an agar plug of *F. solani* culture. After seven days, photos were taken and the area of the lesion surrounding the agar plug was quantified. Results are the mean of 12 replicates with bars representing the standard error of means. Significant reductions in lesions sizes compared to controls are indicated by asterisks with a $p < 0.05$, or crosses with $p < 0.10$ as determined by Tukey's HSD test, n=12.

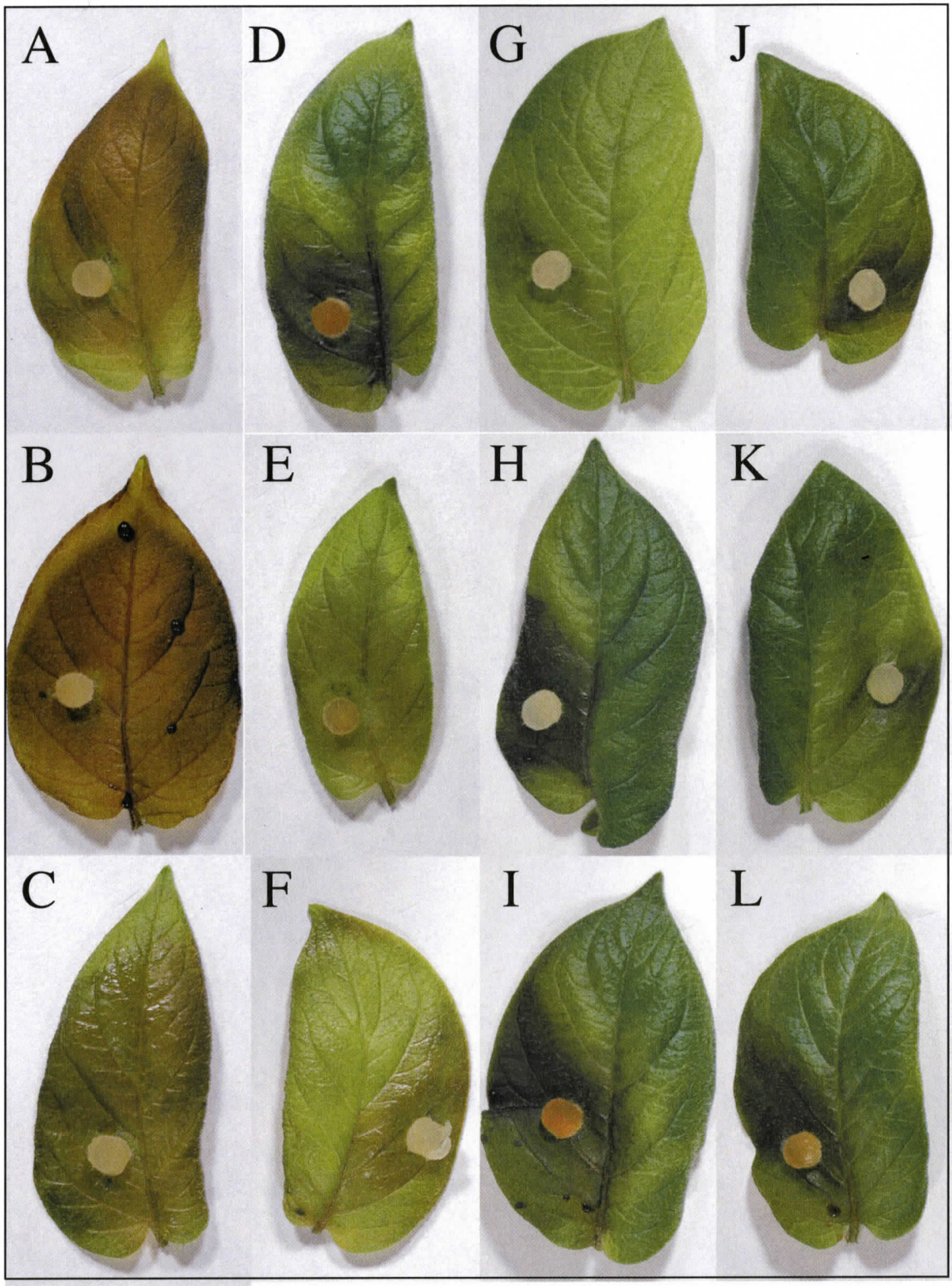


Figure 17: Detached leaf assay.

Symptoms of *Fusarium* wilt observed after 7 days of infection of detached leaves with the fungus. A) Non-transgenic, B) BiPPro1-1::GUS, C) BiPPro1-3::GUS, D-I) 2x35S::BMAP-2, 26, 27, 33, 34, and 36, respectively, J-K) BiPPro1-1::BMAP-6 and 9, respectively, L) BiPPro1-3::BMAP-6.

3.4.2 Co-cultivation of transgenics and *F. solani*

Similarly, co-cultivation of control and BMAP18-expressing plantlets with *F. solani* indicated that expression of BMAP18 inhibited chlorosis caused by the pathogen. In these experiments, an agar plug of *F. solani* culture was added to magenta jars in which 3 week old plants were growing. The fungus was allowed to grow over the surface of the medium and the date at which it contacted the stem was recorded as Day 0. While no substantial differences were observed between transgenic and control plants in regards to plant survival, the course of infection appeared to differ. Whereas the first symptom in control plants was chlorosis, transgenic plants exhibited more localized symptoms, such as stem softening and did not appear chlorotic until the final stage of the infection. In fact, the apical buds of many BMAP18-expressing plants remained green long past the time when the stem had completely softened and lower leaves wilted. The means of the results of two experiments comprising four to six replicates of each line are presented in Table 3.

Table 3: Co-cultivation of *F. solani* and transgenic potato plants.

Course of symptom development in control (non-transgenic and GUS) and BMAP18-expressing plants co-cultivated with *F. solani*. Indicated values are the average number of days until symptom development, n = 4 - 6 for each line.

	Days Post-Infection			
Line	Chlorosis	Stem Softening	Wilt	Death
Non-transgenic	3.00	4.60	5.00	9.80
BiPPro1-1::GUS	3.83	4.67	7.17	10.17
BiPPro1-3::GUS	3.00	4.00	4.25	9.00
2x35S::BMAP-2	7.00	3.67	6.67	8.33
2x35S::BMAP-26	7.00	5.20	6.80	10.00
2x35S::BMAP-27	6.00	4.00	5.60	8.80
2x35S::BMAP-33	7.60	5.40	7.80	10.60
2x35S::BMAP-34	8.00	4.00	5.50	10.00
2x35S::BMAP-36	7.40	3.60	6.00	9.20
BiPPro1-1::BMAP-6	9.20	4.00	8.00	10.80
BiPPro1-1::BMAP-9	7.67	4.50	7.00	13.33
BiPPro1-3::BMAP-6	8.25	5.00	6.50	12.50

3.4.3 Resistance of tubers to *Erwinia carotovora* soft rot

To determine whether expression of BMAP18 conferred enhanced resistance to bacterial soft rot, tubers from transgenic lines expressing BMAP18 and control plants (non-transgenic and GUS) were surface sterilized, sliced to 5 mm thick sections and infected with a suspension of *E. carotovora*. After 6 days photographs were taken and the results are summarized in Table 4. The results of Trial 2 are presented in Figure 18 as typical examples of susceptible and resistant tubers. Non-transgenic tuber slices showed susceptibility to soft rot in every trial, while BiPPro1-1::GUS control, and BMAP18-expressing lines 2x35S::BMAP-2, 2x35S::BMAP-33, 2x35S::BMAP-36, BiPPro1-1::BMAP-6, BiPPro1-1::BMAP-9 were resistant to formation of soft rot lesions.

Table 4: Resistance of tubers to bacterial soft rot.

Scoring of susceptibility of control and BMAP18-expressing tubers to bacterial soft rot 6 days post-inoculation.

Line	Trial			
	1	2	3	4
Non-transgenic	+++	+++	+++	+++
BiPPro1-1::GUS	- - -	- - -	- - -	- - -
BiPPro1-3::GUS	- - -	- - -	- - -	++ -
2x35S::BMAP-2	- - -	- - -	- - -	- - -
2x35S::BMAP-26	- - -	- - -	++ -	+++
2x35S::BMAP-27	- - -	- - -	++ -	+++
2x35S::BMAP-33	- - -	- - -	- - -	- - -
2x35S::BMAP-34	- - -	- - -	+++	+++
2x35S::BMAP-36	- - -	- - -	- - -	- - -
BiPPro1-1::BMAP-6	- - -	- - -	- - -	- - -
BiPPro1-1::BMAP-9	- - -	- - -	- - -	- - -
BiPPro1-3::BMAP-6	+++	+++	- - -	++ -

+ indicates soft rot development (susceptible)
– indicates absence of lesions (resistance)

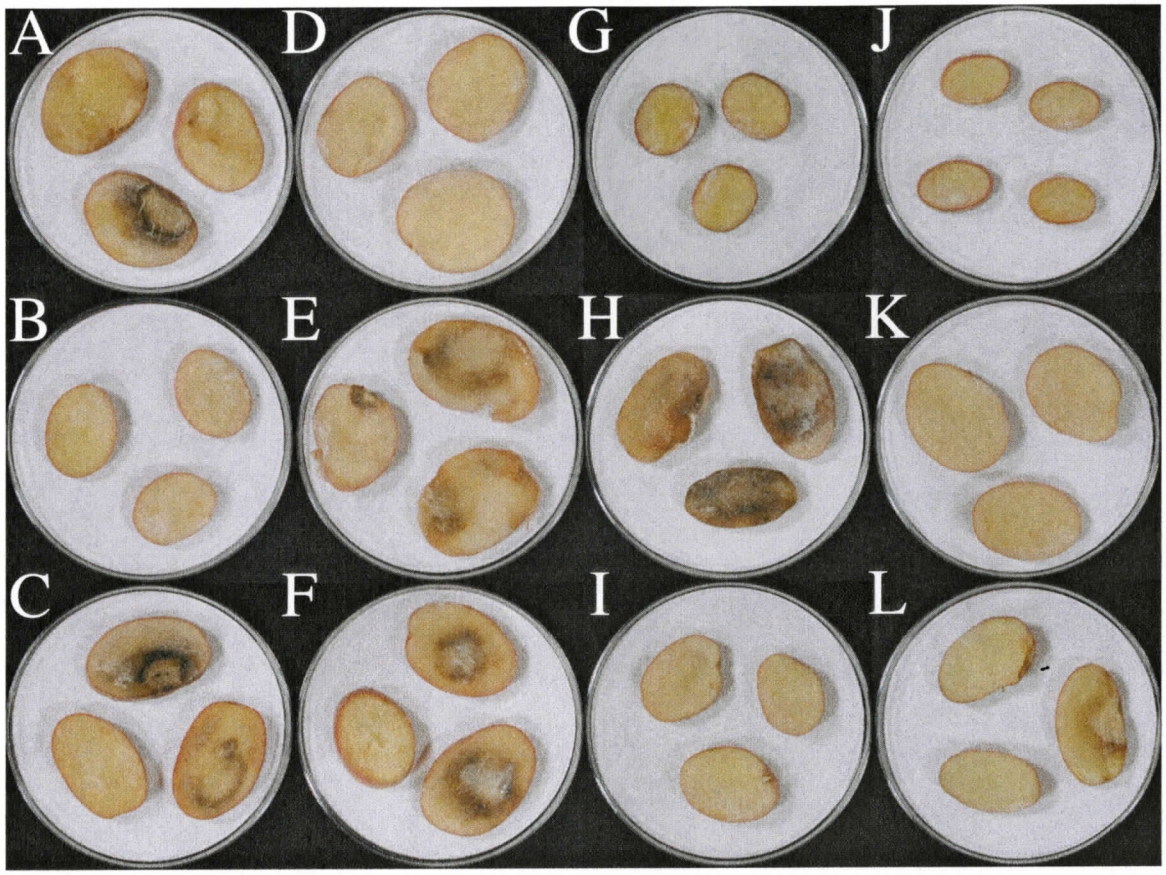


Figure 18: Bacterial soft rot assay.

Soft rot lesions were observed 6 days after inoculation with an *E. carotovora* suspension. A) Non-transgenic, B) BiPPro1-1::GUS, C) BiPPro1-3::GUS, D-I) 2x35S::BMAP-2, 26, 27, 33, 34, and 36, respectively, J-K) BiPPro1-1::BMAP-6 and 9, respectively, L) BiPPro1-3::BMAP-6. Scale: Petri dishes are 9 cm in diameter.

Chapter 4: Discussion

This study examined the feasibility of producing a highly cationic antimicrobial peptide, BMAP18, in transgenic potato for both enhancement of disease resistance and production of the peptide for human and veterinary medicinal purposes. The rapidly expanding population of the world and prevalence of disease has fostered a need for improved pharmaceuticals that are both robust, inexpensive to produce, and easy to administer. Likewise, traditional agricultural methods that rely heavily on pesticides and fungicides are not sustainable as they cause damage to both the environment and human health. The mechanism by which CAPs non-specifically target microbial cells makes them both a source of novel antibiotics for use in clinical medicine and excellent candidates for development of transgenic crops with broad-spectrum disease resistance.

Therefore, the objectives of this study were to: a) engineer the highly cationic antimicrobial peptide BMAP18 in transgenic potato as a means of large scale production of a potentially valuable pharmaceutical and, b) test transgenic potato plants for protection against potato pathogens.

4.1 Characterization of BMAP18 Activity In Vitro

4.1.1 BMAP18 is active against phytopathogenic bacteria and fungi

The *in vitro* activity of BMAP18 against a variety of clinically important bacteria, fungi and protozoa has previously been reported (Skerlavaj *et al.*, 1996; Benincasa *et al.*, 2003; Haines *et al, unpublished data*). This study investigated the activity of BMAP18 against plant pathogens. *Erwinia carotovora* and *Fusarium solani* were chosen as representatives of commercially important bacterial and fungal pathogens of potato. Both infect potato plants in the field as well as tubers during storage. *E. carotovora* ssp. *atroseptica* is the causative agent of potato blackleg, while *E. carotovora* ssp. *carotovora* causes bacterial soft rot. Dry rot caused by *Fusarium solani* has been identified as one of the most important diseases affecting post-harvest potatoes in the United States (Loria, 1993). In addition, this fungal pathogen may also cause *Fusarium* wilt in the field.

The activity of various CAPs against these agronomically important pathogens has been previously examined. The MIC of peptides tested against *E. carotovora* ranged from 1 to 30 μM (Nordeen *et al.*, 1992; Li *et al.*, 2001) depending on the peptide. In comparison, the MIC value determined for BMAP18 against *E. carotovora* ssp. *carotovora* was less than 1 μM (0.853 μM), which indicates that BMAP18 is especially active against this bacterial pathogen. MIC values for CAPs active against *F. solani* range from 2.5 to 7.5 μM (Yevtushenko,

unpublished data; Yevtushenko *et al.*, 2005), which fits the value determined for BMAP18 (3.41 μM) very well. Similarly, the MIC of BMAP18 against the fungal pathogens *Candida albicans* and *Cryptococcus neoformans* were found to be 16 and 4 μM, respectively (Skerlavaj *et al.*, 1996). Therefore, BMAP18 has potent antibacterial and antifungal activity and is an excellent candidate for engineering disease resistance in potato.

4.1.2 BMAP18 inhibits growth of clinically important pathogens

Expression of BMAP18 in transgenic potato may also provide a means for economical production of the CAP for pharmaceutical purposes. To this end the activity of BMAP18 against a variety of human pathogens was re-examined. The MICs for growth of *E. coli* and *P. aeruginosa* were found to be in the low micromolar range; whereas, Gram-positive bacteria were more resistant (see Table 2). Benincasa *et al.* (2003) studied a variety of clinical isolates of bacteria and found that the MIC of BMAP18 for Gram-negative bacteria ranged between 0.5 and 16 μM the majority being 4 μM; whereas, the range of MICs for Gram-positive bacteria was from 0.25 to >32 μM with the majority at >32 μM. Therefore, the MIC values reported in Table 2 are in agreement with previous findings.

The activity of BMAP18 against *Trypanosoma brucei* has not yet been published, however, the MIC of its parent peptide, BMAP-27, against PCF trypanosomes was shown to be 9.69 to 19.4 μM (Haines *et al.*, 2003). The lower MIC value reported here (3.41 μM) indicated that the truncated peptide is more effective

against PCF trypanosomes than the parent peptide. This contrasts the effect of truncation of the peptide on antibacterial activity. Skerlavaj *et al.* (1996) showed the MIC of BMAP18 against clinically relevant bacteria and fungi was either equal to or two- to sixteen-fold greater than the MIC of BMAP-27 against the same pathogens. However, because the membranes of protozoa are distinct from bacteria and from fungi, the mechanism by which they are permeabilized is likely to differ and may be facilitated by removal of the C-terminal hydrophobic tail. These data confirmed that BMAP18 is a potent anti-trypanosomal agent and has potential as a candidate for treatment of African Trypanosomiasis.

For treatment of African Trypanosomiasis using transgenic potato tubers accumulating BMAP18, the peptide must retain its activity after boiling to be effective. It is proposed that these potatoes may be boiled and ingested as an edible treatment. Alternatively, boiling of transgenic tubers may facilitate isolation of the peptide from endogenous tuber proteins that are heat-labile. Investigation of the heat-stability of the peptide showed no difference between the MIC of boiled and control peptide against PCF trypanosomes; therefore, BMAP18 produced in potato tubers is hypothesized to retain its activity through boiling.

4.1.3 BMAP18 exhibits low phytotoxicity

For expression of BMAP18 in transgenic potato to be practical, the CAP must not be toxic to potato at the levels it is anticipated to accumulate. Previous studies in which CAPs have been expressed in transgenic potato using the enhanced CaMV

35S promoter with the AMV translational enhancer accumulated low microgram amounts of recombinant peptide per gram of fresh tissue without affecting plant health (Osusky *et al.,* 2000; Osusky, *et al.,* 2005). In this study, exogenous BMAP18 did not affect the growth of plantlets when they were grown in media containing 100 μg peptide/mL. Also, it was found that the concentration of BMAP-18 required to kill greater than 90% of protoplasts was 75 μg/mL (32 μM), which is roughly ten-fold less than the concentration required to inhibit germination of *F. solani* and more than 30 times less than the MIC for *E. carotovora.*

In comparison to other CAPs, BMAP18 exhibited moderate phytotoxicity. It was less toxic than the cecropin variants D5-C (Qui *et al.,* 1995), cecropin SB-37 (Nordeen *et al.,* 1992) and CEMA (Yevtushenko *et al.,* 2005), which cause 100% mortality at 5 μM, 10 μM and 13 μM respectively, and less toxic than MsrA2 (Yevtushenko, *unpublished data*) which is lethal to protoplasts at 15 μM. However, it was more toxic than temporin A (Yevtushenko, *unpublished data*) and cecropin B (Mills *et al,* 1993) that require concentrations of 80 and 100 μM respectively to kill 100% of protoplasts. Nonetheless, accumulation of MsrA2 to 6-7 μg/g fresh leaf tissue in transgenic tobacco had no deleterious effects on plant health or morphology (Yevtushenko, *unpublished data*), thus, expression of BMAP18 in transgenic potato at similar levels is not expected to adversely affect plant health. Therefore, production of BMAP18 in transgenic potato is feasible.

4.2 Expression of BMAP18 in Transgenic Potato

4.2.1 Potatoes as recombinant biofactories

Potato was chosen as the production platform for this study for the following reasons. As storage organs, potato tubers are capable of accumulating large amounts of protein and are suited for long-term storage in the absence of refrigeration, making shipping of the transgenic tubers simple and inexpensive (Artsaenko *et al.*, 1998). In addition, accumulation of recombinant proteins in the aerial tissues, such as leaves and stems, would add value to these previously unused materials. Agricultural practices for planting, cultivating, and harvesting potatoes are well established and practiced worldwide as potatoes may be grown in most climatic zones (Mullins *et al.*, 2006). Also, since its advent in 1986, transformation technology for potato has been well studied and made relatively facile (Artsaenko *et al.*, 1998). The clonal propagation traditionally practiced in potato agriculture is also advantageous as it adds a level of consistency and reproducibility to the transgenic material. This is desirable from a commercial stand-point as it may help to normalize expected performance and yields.

4.2.2 Expression of BMAP18 is highest in 2x35S::BMAP lines

To produce BMAP18 in transgenic potato, expression cassettes containing the BMAP18 gene under control of three different promoters (Figure 9) were prepared and inserted into the potato genome by *Agrobacterium*-mediated transformation. For strong, constitutive expression of BMAP18, transcription was directed by the enhanced CaMV 35S promoter with the AMV translational

enhancer (2x35S). This promoter construct has been shown to direct high-level constitutive expression of transgenes in plants (Datla *et al.*, 1993). The plant-derived Douglas-fir luminal binding protein promoter (PmBiPPro) was included for tissue-specific and wound-inducible expression of BMAP18. Functional analysis of the full-length (PmBiPPro1-1) promoter in transgenic tobacco and *Arabidopsis* indicated that expression was directed to actively dividing tissues, such as the primary root elongation zone and expanding leaves and leaf primordia, and secretory tissues. Deletion of 1000 bp from the 5' end (PmBiPPro1-3) led to increased steady-state expression in all tissues and extended staining to vascular tissues, lateral roots and the root tip. In addition, both promoters exhibited wound-inducibility in *Arabidopsis* cotyledons (Forward *et al.*, 2002).

Northern blot analyses of BMAP18 mRNA accumulation in transgenic lines indicated that expression was highest in those transgenic lines harboring the pTRT1211 T-DNA construct. Accumulation of BMAP18 transcripts in BiPPro1-1::BMAP and BiPPro1-3::BMAP lines was only discernible after exposure of the film to the membrane for 14 days; whereas, one day of exposure was sufficient to detect transcripts in most 2x35S::BMAP lines. This indicated that the PmBiP promoter drives relatively low-level expression in potato leaf tissues. However, the expression in other tissues or under stress conditions was not investigated.

The observed variation in BMAP18 expression levels may have resulted from a number of phenomena. Expression levels depend not only on the activity of the promoter, but also on factors such as transgene copy number, source, and positioning within the potato genome. With increased copy number, gene expression may increase or decrease in a non-linear fashion (Bauer *et al.*, 1998). For example, a well controlled study of β-glucuronidase (GUS) expression under the control of the CaMV 35S promoter indicated that single copy transformants yielded the highest expression level and that multiple gene copies were often methylated (Hobbs *et al.*, 1990). Methylation of the CaMV 35S promoter is thought to result from recognition of viral sequences by plant surveillance machinery (Mishiba *et al.*, 2005) causing transcriptional gene silencing. However, promoter methylation is also common in transcriptionally inactive regions of DNA. Because integration of the T-DNA construct into the plant genome is virtually random, the transgene may be inserted in transcriptionally active euchromatin or transcriptionally-inactive heterochromatin resulting in positional effects that may either increase or decrease expression. For example, localization of a transgene downstream of an endogenous regulatory DNA element, such as an enhancer, may amplify transcript levels, whereas insertion into heterochromatin would result in silencing. Positional effects on transgene expression are also subject to spatio-temporal changes (van Leeuwen *et al.*, 2001). Therefore, the variation in BMAP18 expression observed between lines transformed with the same vector is to be expected.

4.3 Putative BMAP18 was detected in transgenic lines

To determine whether transgenic lines with strong expression of the BMAP18 gene stably accumulated the peptide, a series of analyses were performed on selected lines. First, polyclonal antibodies were prepared for detection of BMAP18 by immunoblot analysis; however, detection of the synthetic peptide on a polyvinylidene fluoride (PVDF) membrane was unsuccessful (Figure 14). Second, total protein extracts and cationic protein fractions were analyzed by MALDI-TOF MS, however, contaminating compounds, possibly phenolics, prevented the detection of any plant peptides by this method. Fractionation of the extracts by reverse phase HPLC to eliminate these contaminants was not done as pure BMAP18 elutes from a reverse phase column as a broad, irregular peak, making its detection in a mixture impossible (Smith, D., Proteomics Centre, UVic, *personal communication*). Thirdly, cationic protein fractions isolated from non-transgenic and BMAP18-expressing 2x35S::BMAP lines were analyzed by Tricine-SDS-PAGE for high resolution analysis of small proteins and peptides (Figure 13). A low molecular weight peptide present solely in transgenic plant protein samples with a migration distance corresponding to that of synthetic BMAP18 was observed. However, the relative level of accumulated peptide did not correlate with the amount of transcripts. Given the difficulties of detection of the peptide this is not surprising.

4.3.1 Antibody production and immunoblot analysis

Positive identification of putative BMAP18 may be obtained through either mass spectrometry followed by peptide sequencing, or immunochemistry. For this purpose, anti-BMAP18 antibodies were raised in rabbits and mice. The polyclonal and monoclonal antibodies generated were shown to specifically detect BMAP18-BSA, but not the free peptide. Binding of synthetic BMAP18 to the PVDF membrane through hydrophobic interactions may have induced a conformational change that prohibited recognition of the peptide by the antibodies. Similar difficulties were encountered by Li and colleagues (2001) during immunodetection of a magainin II variant (Myp30). The peptide was detected but results were inconsistent. They reasoned that the high positive charge (+3) and small size (24 amino acids) of the CAP prohibited its stable interaction with synthetic membranes (Li *et al.*, 2001). BMAP18 consists of 18 amino acids and has a net charge of +10, making it even smaller and more charged than Myp30. In contrast, MsrA2, which was successfully detected by Osusky *et al.* (2005), contains 31 residues and has a net charge of +2. In addition, a 46 amino acid CAP with a net charge of +6, and a 36 amino acid CAP with a net charge of +7 were successfully identified using immunoblot analysis (Ponti *et al.*, 2003; Florack *et al.*, 1995). Also, the hydrophilicities of these detected peptides range from –0.2 to +0.3, while the hydrophilicity value of BMAP18 is +0.9 (Hopp and Woods, 1981). It is likely that these larger, more hydrophobic CAPs are able to bind synthetic membranes more stably than smaller, more charged peptides and have epitopes that are not as easily obscured.

4.4 Expression of BMAP18 in Transgenic Potato Confers Enhnaced Disease Resistance

In vivo assays showed that BMAP18-expressing transgenic potato lines exhibited increased resistance to phytopathogens, supporting the stable accumulation of the peptide. Assays for antimicrobial activity of recombinant peptides have been previously used for indirect assessment of stable accumulation. For example, DeGray *et al.* (2001) were unable to identify recombinant MSI-99 in transgenic protein extracts on a 16% polyacrylamide gel, but the demonstrated high level of disease resistance achieved through expression of the CAP served as indirect evidence of stable accumulation of the peptide.

4.4.1 BMAP18-expressing plants exhibit enhanced resistance to *Fusarium* wilt

Resistance to *Fusarium* wilt was assayed in both detached, mature leaves and *in vitro* grown plantlets. In the detached leaf assay, the inoculum was applied as a plug of fungal culture with the mycelium placed in direct contact with the surface of the leaf. All BMAP-18 expressing lines developed smaller lesions than control (non-transgenic and GUS) plants, with the greatest enhanced resistance occurring in lines in which stably accumulated peptide was detected. No previous studies have examined resistance of transgenic potato to *F. solani* by this method, however, this approach has been applied to assay for resistance to other potato-specific fungal pathogens (Osusky *et al.*, 2005). Lesion development was shown to be similarly reduced in CAP-expressing lines when compared to controls. The

observed enhanced resistance to lesion development in response to infection with *F. solani* supports the production and identification of BMAP18 and its ability to enhance resistance to *F. solani*.

The *F. solani* co-cultivation assay was performed using *in vitro* grown plants according to the method of Osusky *et al.* (2000). The time required for the first symptoms to develop did not differ between control and BMAP18-expressing lines, however, the course of symptom development varied, indicating that expression of BMAP18 attenuated the pathogeneity of the fungus. This may be through direct activity of BMAP18 against the developing hyphae, or modulation of the innate defense mechanisms of the plant (Kumar, R., *personal communication*). These results indicate that expression of BMAP18 moderately decreased susceptibility of transgenic potato plants to *Fusarium* wilt. In contrast, expression of a cecropin-melittin (ECEMA) hybrid considerably increased survival of transgenic potato plants grown in the presence of *F. solani.* Control plants exhibited symptoms of wilt within six days post-infection and died within eleven days, whereas transgenic potato lines expressing ECEMA remained healthy and symptom free 19 days after infection (Osusky *et al.*, 2000).

While *in vitro* studies are a good way to identify potential drug candidates or other proteins of interest, they do not always mimic *in* vivo results. A comparison of the *in vitro* and *in vivo* activity of BMAP18 to that of its parent peptide BMAP-27 indicated that though the truncated peptide retains the potent antimicrobial

activity of its parent, it is much less effective at preventing disease in an infected mouse model (Benincasa *et al.*, 2003). The activity of the peptide may have been impeded by physiological conditions such as pH, salt content, or proteases. The inability of BMAP18-expressing transgenic potato plants to resist *Fusarium* wilt when co-cultivated with *F. solani* may stem from similar complications. Alternatively, the intracellular accumulation of the CAP may prevent its contact with the intercellular pathogen, thereby reducing its protective capacity (Alan *et al.*, 2004).

4.4.2 Resistance to soft rot in BMAP18 transgenic lines

As opposed to *F. solani* which initiates infection intercellularly, *E. carotovora* is a necrotic pathogen that secretes enzymes to lyse plant cells allowing the release of BMAP18 accumulated in the cytosol to act against the bacteria. Resistance to bacterial infection was determined by assaying tubers of control and BMAP18-expressing plants for development of bacterial soft rot caused by *E. carotovora*. All transgenic tubers tested exhibited enhanced resistance to soft rot when compared to non-transgenic tubers. However, GUS control tubers were similarly resistant. GUS controls were included in these experiments to test whether the act of transformation alone, or the kanamycin resistance selection marker may have an independent effect on disease susceptibility. The enhanced resistance of GUS controls to soft rot is an unexpected finding and needs further investigation.

Other studies in which CAP-expressing transgenic tubers were assayed for resistance to soft rot have shown large reductions in the amount of rotten tissue or

lesion diameter compared to controls. For example, Osusky *et al.* demonstrated reduced weight loss due to soft rot in transgenic potato tubers expressing a temporin A variant ,MsrA3 (2004), a cecropin-melittin hybrid, ECEMA (2000), and a modified dermaseptin, MsrA2 (2005). Similarly, no symptoms were observed in three of the 2x35S::BMAP lines or the two BiPPro1-1::BMAP lines assayed. These results indicate that expression of BMAP18 prevents the onset of bacterial soft rot. In addition, despite the low level of transcripts accumulated by BiPPro1-1::BMAP lines, the full-length PmBiP promoter was equally effective at conferring resistance to soft rot as the enhanced CaMV 35S promoter. In contrast, the truncated PmBiP promoter did not drive sufficient expression of BMAP18 to prevent bacterial soft rot. These results are similar to those of Li *et al.* (2004) who compared the efficacy of expression of a cecropin B analog (shiva-1) in transgenic potato under control of the CaMV 35S promoter or the pathogen-inducible tomato phenylalanine lyase (LePAL) promoter. The LePAL promoter is similar to the PmBiP promoter in that both contain elements for basal level constitutive expression as well as wound-inducible expression. Enhanced resistance to soft rot was achieved in all lines transformed with the LePAL promoter; whereas, lines transformed with the CaMV 35S promoter ranged from highly susceptible to highly resistant (Yi *et al.*, 2004). Therefore, an inducible plant-derived promoter may provide more reproducible, reliable disease resistance than the viral 35S promoter. Also, use of homologous plant promoters is preferable as it may alleviate some of the public concern regarding insertion of viral DNA into crops.

Chapter 5: Conclusions and Future Directions

5.1 Conclusions

The purpose of this study was to evaluate whether BMAP18 could be stably produced in transgenic potato and whether expression of the peptide would enhance resistance to phytopathogens. Demonstration of its potent broad-spectrum antimicrobial activity and low phytotoxicity identified this highly cationic peptide as a potential candidate for expression in transgenic potato for production and conferral of disease resistance. Transformation of potato with a synthetic BMAP18 gene under control of the enhanced CaMV 35S promoter and the Douglas-fir BiP promoter led to stable integration of the transgene and various levels of mRNA accumulation. While both promoters were effective at driving enhanced resistance to *Fusarium* wilt and bacterial soft rot, the high levels of transcripts accumulated in 2x35S::BMAP transgenic lines may be more suitable for economical peptide production. Therefore, for molecular farming of BMAP18 a strong constitutive promoter is preferred, while a stress-inducible plant-derived promoter is sufficient to confer disease resistance. In summary, production of BMAP18 in transgenic potato can be achieved and leads to enhanced resistance to bacterial and fungal phytopathogens.

5.2 Future Directions

Further work is needed to improve the level of peptide accumulation, quantify and purify the recombinant BMAP18, and to assay for activity against a much broader

range of plant pathogens as well as trypanosomes. Work is in progress to detect and quantify cationic peptides from a mixture by mass spectroscopy directly following affinity purification (Pearson, T., *personal communication*). To this purpose, anti-BMAP18 antibodies have been coupled to a solid support and shown to bind synthetic BMAP18 in a reversible fashion, such that it may be identified by a MALDI-TOF MS placed in-line with the affinity column. Also, efforts should be directed towards purification of BMAP18 from transgenic potato material. This would allow evaluation of the anti-trypanosomal of the recombinant CAP. Attempts to assay for anti-trypanosomal activity in transgenic leaf extracts were unsuccessful as even non-transgenic control extracts were toxic to the cells.

To improve BMAP18 accumulation and/or further enhance the antimicrobial potential of the peptide for defense against phytopathogens, alternative constructs may be used. To improve the yield of the peptide beyond that achieved through strong constitutive expression from the enhanced CaMV 35S promoter, efforts must be focused on increasing peptide stability. This may be achieved through retention of the recombinant product in the endoplasmic reticulum or by expressing it as a fusion protein (Coca, *et al.*, 2006; Okomoto *et al.*, 1998; Jones *et al., 2004*). Donini *et al.* (2005) achieved a high yield of Killer Peptide by fusion to the Potato Virus X coat protein. Proteolytic degradation of recombinant proteins produced in plants may also be prevented by co-transformation with genes that encode protease inhibitors (Rivard *et al.*, 2006). Alternatively, the

technology exists to transform the chloroplast of potato (Sidorov *et al.*, 1999; Nguyen *et al.*, 2005). Integration of transgenes into the chloroplast genome has many advantages: 1) each plant cell contains multiple chloroplasts thus the potential copy number of a given transgene is multiplied by the number of chloroplasts per cell, 2) because integration occurs via homologous recombination, gene expression is not subject to positional effects, 3) chloroplasts are maternally inherited thus there is no risk of transmission of the transgene to wild relatives through pollen, 4) there is the possibility of gene stacking through polycistronic operon expression, and 5) transgene silencing does not occur in plastids (Sidorov *et al.*, 1999). However, reports of chloroplast transformation are infrequent as the procedure is challenging and produces a low transformation frequency compared to that of *Agrobacterium*-mediated transformation. Nonetheless, there is one report of transgenic expression of a CAP in tobacco chloroplasts. DeGray *et al.* (2001) transformed tobacco chloroplasts with a magainin 2 analog, MSI-99. In contrast to other reports which show a reduction in symptoms caused by phytopathogens in transgenic lines compared to controls, DeGray and colleagues reported 100% protection against *Pseudomonas syringae* pv. *tabaci* and *Colletotrichum destructivum*, as well as very high *in vitro* antimicrobial activity (86-96% growth inhibition) of the transgenic plant extracts. These results confirm that transformation of the plastid genome with a CAP gene results in high level disease resistance with no adverse effect on plant health (DeGray *et al.*, 2001). Any of the above described methods may be used to improve BMAP18 accumulation and make commercial production of the peptide

for treatment of African Trypanosomiasis and possibly other diseases commercially viable.

Literature Cited

Alan AR, Blowers A, Earle ED (2004) Expression of a magainin-type antimicrobial peptide gene (MSI-99) in tomato enhances resistance to bacterial speck disease. Plant Cell Reports **22**: 388-396.

Allefs SJHM, DeJong ER, Florack DEA, Hoogendoorn C, Stiekema WJ (1996) Erwinia soft rot resistance of potato cultivars expressing antimicrobial peptide tachyplesin I. Molecular Breeding **2**(2): 97-105.

Andersen JH, Jenssen H, Sandvik K, Gutteberg TJ (2004) Anti-HSV activity of lactoferrin and lactoferricin is dependent on the presence of heparan sulphate at the cell surface. Journal of Medical Virology **74**(2): 262-271.

Anzai H, Yoneyama K, Yamaguchi I (1989) Transgenic tobacco resistant to a bacterial disease by the detoxification of a pathogenic toxin. Molecular and General Genetics: MGG **219**: 492-494.

Arrighi RBG, Nakamura C, Miyake J, Hurd H, Burgess JG (2002) Design and activity of antimicrobial peptides against sporogonic-stage parasites causing murine malarias. Antimicrobial Agents and Chemotherapy **46**(7): 2104-2110.

Artsaenko O, Kettig B, Fiedler U, Conrad U, Düring K (1998) Potato tubers as a biofactory for recombinant antibodies. Molecular Breeding **4**: 313-319.

Bauer M, Libantova J, Moravcikova J, Bekesiova I (1998) Transgenic tobacco plants constitutively expressing acidic chitinase from cucumber. Biologia **53**(6): 749-758.

Benincasa M, Skerlavaj B, Gennaro R, Pellegrini A, Zanetti M (2003) In vitro and in vivo antimicrobial activity of two α-helical cathelicidin peptides and of their synthetic analogs. Peptides **24**: 1723-1731.

Berrocal-Lobo M, Segura A, Moreno M, López G, García-Olmedo F, Molina A (2002) Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. Plant Physiology **128**: 951-961.

Bowman HG (1995) Peptide antibiotics and their role in innate immunity. Annual Review of Immunology **13**: 61-92.

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. Nature Reviews Microbiology **3**: 238-249.

Brown KL, Hancock REW. (2006) Cationic host defense (antimicrobial) peptides. Current Opinion in Immunology **18**: 234-30.

Bucki R, Jamney PA (2006) Interaction of the gelsolin-derived PBP 10 peptide with lipid bilayers and cell membranes. Antimicrobial Agents and Chemotherapy **50**(9): 2932-2940.

Buzeli RAA, Cascardo JCM, Rodrigues LAZ, Andrade MO, Almeida RS, Loureiro ME, Otoni WC, Fontes EPB (2002) Tissue-specific regulation of *BiP* genes: a cis-acting regulatory domain is required for *BiP* promoter activity in plant meristems. Plant Molecular Biology **50**: 757-771.

Chakrabarti A, Ganapathi TR, Mukherjee PK, Bapat VA (2003) MSI-99, a magainin analogue, imparts enhanced disease resistance in transgenic tobacco and banana. Planta **216**: 587-596.

Cheon BY, Kim HJ, Oh KH, Bahn SC, Ahn JH, Choi JW, Ok SH, Bae JM, Shin JS (2004) Overexpression of human erythropoeitin (EPO) affects plant morphologies: retarded vegetative growth in tobacco and male sterility in tobacco and *Arabidopsis*. Transgenic Research **13**: 541-549.

Chernysh S, Kim SI, Bekker G, Pleskach VA, Filatova NA, Anikin VB, Platonov VG, Bulet P (2002) Antiviral and antitumor peptides from insects. Proceedings of the National Academy of Sciences **99**(20): 12628-12632.

Cionei CD, Sigurdardottir T, Schmidtchen A, and Bodelsson M (2005) Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrobial Agents and Chemotherapy **49**(7): 2845-2850.

Coca M, Peñas G, Gómez J, Campo S, Bortolotti C, Messeguer J, San Segundo B (2006) Enhanced resistance to the rice blast fungus *Magnaporthe grisea* conferred by expression of a *cecropin A* gene in transgenic rice. Planta **223**: 392-406.

Datla RSS, Bekkaoui F, Hammerlinkl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Science **94**: 139-149.

De la Fuente-Martinez JM, Mosqueda-Cano G, Alvarez-Morales A, Herrera-Estrella L (1992) Expression of a bacterial phasolotoxin-resistant ornithyl transcarbamylase in transgenic tobacco confers resistance to *Pseudomonas syringae* pv. *phaseolicola.* Nature Biotechnology **10**: 905-908.

De Lucca AJ, Bland JM, Grimm C, Jacks TJ, Cary JW Jaynes JM, Cleveland TE, Walsh TJ (1998) Fungicidal properties, sterol binding, and proteolytic resistance of the synthetic peptide D4E1. Canadian Journal of Microbiology **44** (6): 514-520.

DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H (2001) Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiology **127**(3): 852-862.

Dieryck W, Pagnier J, Poyart C, Marden MC, Gruber V, Bournat P, Baudino S, Mérot B (1997) Human haemoglobin from transgenic tobacco. Nature 386: 29-30.

Donini M, Lico C, Bascheri S, Conti S, Magliani W, Polonelli L, Benvenuto E (2005) Production of n engineered killer peptide in *Nicotiana benthamiana* by using a Potato virus X expression system. Applied and Environmental Microbiology **71**(10): 6360-6367.

Fish WR, Muriuki CS, Grab DJ, Lonsdale-Eccles JD (1989) Disulphide bond involvement in the maintenance of the cryptic nature of the cross-reacting determinant of metacyclic forms of *Trypanosoma congolense*. Biochemistry **28**(13): 5415-5421.

Florack D, Allefs S, Bollen R, Bosch D, Visser B, Stiekema W (1995) Expression of giant silkmoth cecropin-B genes in tobacco. Transgenic Research **4**(2): 132-141.

Forward BS, Osusky M, and Misra S (2002) The Douglas-fir BiP promoter is functional in *Arabidopsis* and responds to wounding. Planta **215**: 569-576.

Friedrich CL, Moyles D, Beveridge TJ, Hancock REW (2000) Antibacterial action of structurally diverse cationic peptides on gram-positive bacteria. Antimicrobial Agents and Chemotherapy **44**(8): 2086-2092.

Gallo RL, Ono M, Povsic T, Page C, Eriksson E, Klagsbrun M, Bernfield M (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. Proceedings of the National Academy of Sciences **91**: 11035-11039.

Gennaro R, Zanetti M (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers **55**: 31-49.

Giacometti A, Cirioni O, Ghiselli R, Bergnach C, Orlando F, D'Amato G, Mocchegiani F, Silvestri C, Del Prete MS, Skerlavaj B, Saba V, Zanetti M, Scalise G (2004) The antimicrobial peptide BMAP-28 reduces lethality in mouse models of staphylococcal sepsis. Critical Care Medicine **32**(12): 2485-2490.

Gil F, Brun A, Wigdorovitz A, Catala R, Martinez-Torrecuadrada JL, Casal I, Salinas J, Borca MV, Escribano JM (2001) High-yield expression of a viral peptide vaccine in transgenic plants. FEBS Letters **488**(1-2): 13-17.

Gurr SJ, Rushton PJ (2005) Engineering plants with increased disease resistance: what are we going to express? Trends in Biotechnology **23**(6): 275-282.

Haines LR, Hancock REW, Pearson TW (2003) Cationic antimicrobial peptide killing of African trypanosomes and *Sodalis glossinidius*, a bacterial symbiont of the insect vector of sleeping sickness. Vector-Borne and Zoonotic Diseases **3**(4): 175-186.

Hancock REW. Diamond G (2000) The role of cationic antimicrobial peptides in innate host defenses. Trends in Microbiology **8**(9): 402-410.

Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weerings E, Oomen LCJM, Veerman ECI, Amerongen AVN, Abee T (1999) The Journal of Biological Chemistry **274**(11): 7286-7291.

Hightower R, Baden C, Penzes E, Dunsmuir P (1994) The expression of cecropin peptide in transgenic tobacco does not confer resistance to *Pseudomonas syringae* pv *tabaci.* Plant Cell Reports **13**(5): 295-299.

Hilpert K, Volkmer-Engert R, Walter T, Hancock REW (2005). High-throughput generation of small antibacterial peptides with improved activity. Nature Biotechnology **23**: 1008-1012.

Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Molecular Biology **15**: 851-864.

Holsters M, de Waile D, Depicker A, Messens E, van Montagu M, Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Molecular and General Genetics: MGG. **163**: 181-187.

Hopp T, Woods KR (1981) Prediction of protein antigenic determinants from amino acid sequences. Proceedings of the National Academy of Sciences **78**: 3824-3828.

Horn ME, Pappu KM, Bailey MR, Clough RC, Barker M, Jilka JM, Howard JA Streatfield SJ (2004) Advantageous features of plant-based systems for the development of HIV vaccines. Journal of Drug Targeting **11**(8-10): 539-545.

Hu Y, Aksoy S (2005) An antimicrobial peptide with trypanocidal activity characterized from *Glossina morsitans morsitans*. Insect Biochemistry and Molecular Biology **35**: 105-115.

Jin F, Xu W, Wang L, Zhang W, Gu D (2006) Expression of recombinant hybrid peptide cecropinA(1-18)-magainin2(1-12) in *Pichia pastoris*: purification and characterization. Protein Expression and Purification **50**(2): 147-156.

Jones RW, Ospina-Giraldo M, Clemente T (2004) Prosystemin-antimicrobial-peptide fusion reduces tomato late blight lesion expansion. Molecular Breeding **14**: 83-89.

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236**(4806): 1299-1302.

Keller H, Pamboukdjian N, Ponchet M, Poupet A, Delon R, Verrier JL, Roby D, Ricci P (1999) pathogen-induced elicitin production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. The Plant Cell **11**: 223-236.

Kim HK, Chun DS, Kim JS, Yun CH, Lee JH, Hong SK, Kang DK (2006) Expressing of the cationic antimicrobial peptide lactoferricin fused with the anionic peptide in *Escherichia coli*. Applied Microbiology and Biotechnology **72**: 330-338.

Kobayashi S, Chikushi A, Tougu S, Imura Y, Nishida M, Yano Y, Matsuzaki K (2004) Biochemistry **43**: 15610-15616.

Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS Thanavala Y (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proceedings of the National Academy of Sciences **98**(20): 11539-11544.

Kooter JM, Marjori AM, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends in Plant Science **4**(9): 340-347.

Kurata S, Ariki S, Kawabata S (2006) Recognition of pathogens and activation of immune responses in *Drosophila* and horseshoe crab innate immunity. Immunobiology **211**: 237-249.

Lee DG, Kim PI, Park Y, Park SC, Woo ER, Hahm, KS (2002) Antifungal mechanism of SMAP29(1-18) isolated from sheep[myeloid mRNA against *Trichosporon beigelii*. Biochemical and Biophysical Research Communications **295:** 591-596.

Lee DG, Park Y, Kim PI, Jeong HG, Woo ER, Hahm KS (2002) Influence on the plasma membrane of *Candida albicans* by HP(2-9)-magainin 2(1-12) hybrid peptide. Biochemical and Biophysical Research Communications **274**(4): 885-889.

Lehmann J, Retz M, Sidhu SS, Suttmann H, Sell M, Paulsen F, Harder J, Unteregger G, Stockle M (2006) Antitumor activity of the antimicrobial peptide Magainin II against bladder cancer cell lines. European Urology **50**(1): 141-147.

Li Q, Lawrence CB, Xing HY, Babbitt RA, Bass WT, Maiti IB, Everett NP (2001) Enhanced disease resistance conferred by expression of an antimicrobial magainin analog in transgenic tobacco. Planta **212**: 635-639.

Loria R (1993) Fusarium Dry Rot of Potato. Vegetable MD Online. Department of Plant Pathology, Cornell University, Ithaca, New York. http://vegetablemdonline.ppath.cornell.edu/factsheets/Potato_Fusarium.htm, November 17, 2006.

Ma J, Kennedy-Stoskopf S, Jaynes JM, Thurmond T, Tompkins WA (2002) Inhibitory activity of synthetic peptide antibiotics on feline immunodeficiency virus infectivity in vitro. Journal of Virology **76**(19): 9952-9961.

Ma JKC, Hiatt A, Hein M, Vine ND, Wang F, Stabilia P, van Dolleweerd C, Mostov K, Lehner T (1995) Generation and assembly of secretory antibodies in plants. Science **268**: 716-719.

Matsuzaki K, Murase O, Fujii N, Miyajima K (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry **35:** 11361-11368.

McPhee JB, Hancock REW (2005) Function and therapeutic potential of host defense peptides. Journal of Peptide Science **11**: 677-687.

Mills D, Hammerschlag FA (1993) Effect of cecropin B on peach pathogens, protoplasts, and cells. Plant Science **93**(1-2): 143-150.

Mishiba K, Nishihara M, Nakatsuka T, Abe Y, Hirano H, Yokoi T, Kikuchi A, Yamamura S (2005) Consistent transcriptional silencing of 35S-driven transgenes in gentian. Planta **44**(4): 541-556.

Morassutti C, De Amicis F, Bandiera A, Marchetti S (2005) Expression of SMAP-29 cathelicidin-like peptide in bacterial cells by intein-mediated system. Protein Expression and Purification **39**: 160-168.

Morassutti C, De Amicis F, Skerlavaj B, Zanetti M, Marchetti S (2002) Production of a recombinant antimicrobial peptide in transgenic plants using a modified VMA intein expression system. FEBS Letters **519**: 141-146.

Morin KM, Arcidiancono S, Beckwitt R, Mello CM (2006) Recombinant expression of indolicidin concatamers in *Escherichia coli.* Applied Microbiology and Biotechnology **70**(6): 698-704.

Motzkus D, Schulz-Maronde S, Heitland A, Schulz A, Forssmann WG, Jubner M, Maronde E (2006) The novel beta-defensin DEFB123 prevents lipopolysaccharide-mediated effects in vitro and in vivo. Federation of American Societies for Experimental Biology (FASEB) Journal **20**(10): 1701-1702.

Mullins E, Milbourne D, Petti C, Doyle-Prestwich B, Meade C (2006) Potato in the age of biotechnology. Trends in Plant Science **11**(5): 254-260.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays for tobacco tissue culture. Physiologia Plantarum **15**: 473-497.

Nagaoka I, Tamura H, Hirata M (2006) An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via activation of formyl-peptide receptor-like 1 and P2X(7). Journal of Immunology **176**(5): 3044-3052.

Nguyen TT, Nugent G, Cardi T, Dix PJ (2005) Generation of homoplasmic plastid transformants of a commercial cultivar of potato (*Solanum tuberosum* L.). Plant Science **168**(6): 1495-1500.

Nordeen RO, Sinden SL, Jaynes JM, Owens LD (2001)Activity of cecropin SB37 against protoplasts from several plant species and their bacterial pathogens. Plant Science **82**: 101-107.

Oh DH, Kwon CS, Sano H, Chung WI, Koizumi N (2003) Biochemical and Biophysical Research Communications **301**: 225-230.

Okamoto M, Mitsuhara I, Ohshima M, Natori S, Ohashi Y (1998) Enhanced expression of an antimicrobial peptide sarcotoxin IA by GUS fusion in transgenic tobacco plants. Plant and Cell Physiology **39**(1): 57-63.

Oren Z, Shai Y (1998) Mode of action of linear amphipathic α-helical antimicrobial peptides. Biopolymers **47**: 451-463.

Osusky M, Osuska L, Kay W, Misra S (2005) Genetic modification of potato against microbial diseases: in vitro and in planta activity of a dermaseptin B1 derivative, MsrA2. Theoretical and Applied Genetics **111**: 711-722.

Osusky M, Zhou G, Osuska L, Hancock REW, Kay WW, Misra S (2000) Transgenic plants expressing cationic peptide chimers exhibit broad-spectrum resistance to phytopathogens. Nature Biotechnology **18**: 1162-1166.

Osusky M, Osuska L, Hancock REW, Kay WW, Misra S (2004) Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot. Transgenic Research **13**(2): 181-190.

Pazgier M, Lubkowski J (2006) Expression and purification of recombinant human α-defensins in *Escherichia coli*. Protein Expression and Purification **49**:1-8.

Ponti D, Mangoni ML, Mignogna G, Simmaco M, Barra D (2003) An amphibian antimicrobial peptide variant expressed in *Nicotiana tabacum* confers resistance to phytopathogens. The Biochemical Journal **370**: 121-127.

Ramanathan B, Davis EG., Ross CR, Blecha F (2002) Cathelicidins: microbicidal activity, mechanisms of action, and roles in innate immunity. Microbes and Infection / Institut Pasteur **4**: 361-372.

Risso A, Braidot E, Sordano MC, Vianello A, Macri F, Skerlavaj B, Zanetti M, Gennaro R, Bernardi P (2002) BMAP-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore. Molecular and Cellular Biology **22**(6): 1926-1935.

Risso A, Zanetti M, Gennaro R (1998) Cytotoxicity and apoptosis mediated by two peptides of innate immunity. Cellular Immunology **189**: 107-115.

Rivard D, Anguenot R, Brunelle F, Le VQ, Vezina LP, Trepanier S, Michaud D (2006) An in-built proteinase inhibitor system for the protection of recombinant proteins recovered from transgenic plants. Plant Biotechnology Journal **4**(3): 359-368.

Schaefer SC, Gasic K, Cammue B, Broekart W, van Damme EJM, Peumans WJ and Korban, S.S. (2005) Enhanced resistance to early blight in transgenic tomato lines expression heterologous plant defense genes. Planta **222**: 858-866.

Schägger H (2006) Tricine-SDS-PAGE. Nature Protocols **1**: 16-22.

Shai Y (2002) Mode of action of membrane active antimicrobial peptides. Biopolymers **66**: 236-248.

Shi Y, Cromie MJ, Hsu FF, Turk J, Groisman EA (2004) PhoP-regulated *Salmonella* resistance to the antimicrobial peptides magainin 2 and polymyxin B. Molecular Microbiology **53**(1): 229-236.

Shinnar A E, Butler KL, Park HJ (2003) Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. Bioorganic Chemistry **31**(6): 425-436.

Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PTJ, Staub JM, Nehra NS (1999) Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. The Plant Journal: for cell and molecular biology **19**(2): 209-216.

Sidorov VA, Yevtushenko DP, Shakovsky AM, Gleba YY (1994) Cybrid production based on mutagenic inactivation of protoplasts and rescuing of mutant plastids in fusion products: potato with a plastome from *S. ulbocastanum* and *S. pinnatisectum.* Theoretical and Applied Genetics **88**: 525-529.

Sidorov VA, Zubko MK, Kuchko AA, Komarnitsky IK, Gleba YY (1987) Somatic hybridization in potato: use of γ-irradiated protoplasts of *Solanum pinnatisectum* in genetic reconstruction. Theoretical and Applied Genetics **74**: 364-368.

Skerlavaj B, Gennaro R, Bagella L, Merluzzi L, Risso A, Zanetti M (1996) Biological Characterization of Two Novel Cathelicidin-derived Peptides and Identification of Structural Requirements for Their Antimicrobial and Cell Lytic Activities. The Journal of Biological Chemistry **271**(45): 28375-28381.

Tai TH, Dahlbeck D, Clark ET, Gajiwala P, Pasion R, Whalen MC, Stall RE, Staskawicz BJ (1999) Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. Proceedings of the National Academy of Sciences **96**: 14153-14158.

Tanida T, Rao F, Hamada T, Euta E, Osaki T (2001) lactoferrin peptide increases the survival of *Candida albicans*-inoculated mice by upregulating neutrophil and macrophage functions, especially in combination with amphotericin B and granulocyte macrophage colony-stimulating factor. Infection and Immunity **69**(6): 3883-3890.

Thanvala Y, Mahoney M, Pal S, Scott S, Richter L, Natarajan N, Goodwin P, Arntzen CJ, Mason HS (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. Proceedings of the National Academy of Sciences 102**:** 3378-3382.

Thievissen K, Osborn RW, Acland DP, Broekart WF (2000) Specific binding sites for an antifungal plant defensin from dahlia (*Dahlia merckii*) on fungal cells are required for antifungal activity. Molecular Plant-Microbe Interactions: MPMI **13**(1): 54-61.

Thievissen K, Terras FRG, Broekart W F (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Applied and Environmental Microbiology **64**(12): 5451-5458.

Tossi A, Sandri L, Giangaspero A (2000) Amphipathic, α-helical antimicrobial peptides. Biopolymers **55**(1): 4-30.

Tran AX, Lester ME, Stead CM, Raetz CRH, Maskell DJ, McGrath SC, Cotter RJ, Trent MS (2005) Resistance to the antimicrobial peptide polymyxin requires myristoylation of *Escherichia coli* and *Salmonella typhimurium* lipid A. The Journal of Biological Chemistry **280**(31): 28186-28194.

Tsai CM, Frasch CE (1982) A sensitive stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry **119**: 115-119.

Turrini A, Sbrana C, Pitto L, Ruffini-Castiglione M, Girogetti L, Briganti R, Bracci T, Evangelista M, Nuti MP Giovanetti M (2004) The antifungal DM-AMP1 protein fro *Dahlia merckii* expressed in *Solanum melongena* is released in root exudates and differentially affects pathogenic fungi and mycorrhizal symbiosis. The New Phytologist **163**: 393-403.

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends in Biotechnology **21**(12): 570-578.

van Leeuwen W, Ruttink T, Borst-Vrenssen AW, van der Plas LH, van der Krol AR (2001) Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. Journal of Experimental Botany **52**(358): 949-959.

Vidal JR, Kikkert JR, Wallace PG, Reisch BI (2003) High-efficiency biolistic co-transformation and regeneration of 'Chardonnay' (*Vitis vinifera* L.) containing *npt-II* and antimicrobial peptide genes. Plant Cell Reports 22: 252-260.

Wirth S, Calamante G, Mentaberry A, Bussmann L, Lattanzi M, Baranao L, Bravo-Almonacid F (2004) Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems. Molecular Breeding **13**(1): 23-35.

Wu H, Zhang G, Minton JE, Ross CR, Blecha F (2000) Regulation of cathelicidin gene expression: induction by lipopolysaccharide, interleukin-6, retinoic acid, and *Salmonella enterica* serovar typhimurium infection. Infection and Immunity **68**(10): 5552-5558.

Xu A, Zhong ZX, Huang L, Peng L, Wang F, Cen P (2006) High-level production of bioactive human beta-defensin-4 in *Escherichia coli* by soluble fusion expression. Applied Microbiology and Biotechnology **72**(3): 471-479.

Yevtushenko DP, Romero R, Forward BS, Hancock REW, Kay WW, Misra S (2005) Pathogen-induced expression of a cecropin A-melittin antimicrobial peptide gene confers antifungal resistance in transgenic tobacco. Journal of Experimental Botany **56**(416): 1685-1695.

Yi JY, Seo HW, Yang MS, Robb EJ, Nazar RN, Lee SW (2004) Plant defense gene promoter enhances the reliability of *shiva-1* gene-induced resistance to soft rot disease in potato. Planta **220**: 165-171.

Zaiou M, Nizet V, Gallo RL (2003) Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL37) prosequence. The Journal of Investigative Dermatology **120**(5): 810-816.

Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. Journal of Leukocyte Biology **75**: 39-48.

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature **415**(6870): 389-395.

Zhang J, Zhang SQ, Wu X, Chen YQ, Diao ZY (2006) Expression and characterization of antimicrobial peptide ABP-CM4 in methylotrophic yeast *Pichia pastoris*. Process Biochemistry **41**: 251-256.