Transgenic Plant and Fungal Expression to Assay *in vitro* and *in planta* Activity of *Sus scrofa* beta-Defensin 1 and *Nicotiana tabacum* Defensin 1

by

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ABSTRACT

TRANSGENIC PLANT AND FUNGAL EXPRESSION TO ASSAY IN VITRO AND IN PLANTA ACTIVITY OF SUS SCROFA BETA-DEFENSIN 1 AND NICOTIANA TABACUM DEFENSIN 1

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To explore the use of defensins for transgenic plant disease resistance, expression by agroinfiltration of plants, stable transformation of plants and stable transformation of yeast were tested for porcine β-defensin 1 (pbd-1) and Nicotiana tabacum defensin 1 (Ntdef1). Attempts to screen constructs by agroinfiltration of Nicotiana benthamiana leaves revealed that agroinfiltration alone induced localized resistance against Colletotrichum destructivum. A comparison of Agrobacterium tumefaciens strains showed that the induced resistance required the transfer of type IV effectors into plant cells and was independent of salicylic acid or ethylene signaling. Stable expression of pbd-1 in N. tabacum and Pichia pastoris showed that PBD-1 purified from P. pastoris had varying degrees of antimicrobial activity against a broad range of microbes, including P. syringae pv. tabaci, C. destructivum and C. orbiculare, but in transgenic N. tabacum, the protein could not be detected and resistance increased only slightly to P. syringae pv. tabaci but not to C. destructivum or C. orbiculare. Stable expression of Ntdef1 in P. pastoris yielded a protein with no or little antimicrobial activity, and stable expression in N. tabacum did not result in detectable Ntdef1 or increased resistance to those pathogens. Although PBD-1 had strong antimicrobial activity against plant
pathogens, plant disease resistance likely did not increase because of the low level of the protein in the plants, whereas resistance did not increase with Ntdef1 likely because of low antimicrobial activity and low levels of the protein in the plant. This research demonstrates that agroinfiltration is not appropriate for testing genes for antimicrobial activity in planta, while the P. pastoris expression system is useful for producing protein for in vitro tests of a gene prior to its transfer to plants.
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List of Abbreviations

AA      amino acids
ABA     abscisic acid
AMPs    antimicrobial peptides
bp      base pairs
CaMV    cauliflower mosaic virus
cDNA    complementary DNA
CFU     colony forming units
DNA     deoxyribonucleic acid
DMPC    dimethyl pyrocarbonate
dNTP    deoxy nucleotide triphosphate (includes dATP, dTTP, dGTP and dCTP)
EDTA    disodium ethylenediamine tetraacetate
EGF     epidermal growth factor
ER      endoplasmic reticulum
ET      ethylene
ETI     effector-triggered immunity
FST     flower-specific thionin
GUS     glucuronidase
h       hour
hpi     hours post inoculation/infiltration
His     histidine
ISR     induced systemic resistance
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani Broth</td>
</tr>
<tr>
<td>LC MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>MAR</td>
<td>matrix attachment region</td>
</tr>
<tr>
<td>MOPs</td>
<td>3-(N-morpholino) propanesulfanic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog (medium)</td>
</tr>
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<td>NAA</td>
<td>1-naphthylacetic acid (synthetic auxin)</td>
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<td>Ni-NTA</td>
<td>nickel-nitritriacetic acid (chromatography)</td>
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<td>polymerase chain reaction</td>
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<tr>
<td>PR</td>
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</tr>
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<td>post transcriptional gene silencing</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP-triggered immunity</td>
</tr>
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<td>polyvinylidene difluoride</td>
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<td>ribonucleic acid</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
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<td>-----------</td>
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</tr>
<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
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<tr>
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<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPAD</td>
<td>soil plant analysis development</td>
</tr>
<tr>
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<td>sodium chloride-yeast extract agar sucrose medium</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>T4SS</td>
<td>type IV secretion system</td>
</tr>
<tr>
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<tr>
<td>TBS</td>
<td>Tris-buffer saline</td>
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<tr>
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<td>tobacco mosaic virus</td>
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<tr>
<td>TTBS</td>
<td>TBS with 0.05% Tween 20</td>
</tr>
<tr>
<td>TSA</td>
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<tr>
<td>TSB</td>
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Chapter 1. Literature Review

1. Host defence mechanisms

Innate immunity is defined as a universal and ancient form of host defense against infection (Janeway and Medzhitov, 2002). Innate immunity was previously called nonspecific immunity because of the nature of its microbial recognition, which relies on limited germ line-encoded receptors that evolved to recognize conserved products of microbial metabolism present only in invading microorganisms but not in the host. These conserved products are known as pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Innate immunity is found in all multicellular eukaryotic organisms including fungi, plants, invertebrates and vertebrates suggesting its long evolutionary history as part of host defenses (Pancer and Cooper, 2006). In contrast, adaptive immunity involves defense mechanisms mediated primarily by lymphocytes evolving to recognize detailed molecular structures of a pathogen and is found only in vertebrates (Janeway and Medzhitov, 2002; Pancer and Cooper, 2006). Although adaptive immunity provides more effective protection against some recurring pathogens for vertebrates, it takes several days to develop, while innate immunity provides a faster response as a first line of host defense (Pancer and Cooper, 2006).

Plants have innate immunity but lack adaptive immunity. Their innate immunity can take two forms: PAMP-triggered immunity (PTI) based on recognition of PAMPs, and effector-triggered immunity (ETI) based on recognition of microbial virulence effector molecules (Boller and Felix, 2009). One important element of PTI and ETI is the production of various antimicrobial compounds, such as lytic enzymes, secondary metabolites, and antimicrobial peptides (AMPs) (Nürnberger et al., 2004; Dodds and
Expression of AMPs can either be constitutive or inducible suggesting that they are not only a part of PTI and ETI but are also a part of a preformed/constitutive defense system of plants.

Innate immunity in vertebrates serves as an early response to infection and is also essential for activation and guidance of the adaptive immune response (Medzhitov and Janeway, 1997). Like plant innate immunity, vertebrate innate immunity relies on the recognition of PAMPs to distinguish self from non-self. The recognition of these molecules leads to host responses, such as opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and activation of apoptosis (Janeway and Medzhitov, 2002). AMPs contribute to various aspects of these responses, as they are present in epithelial tissues and on their surfaces, in glandular structures and in phagocytic cells (Ganz, 2003a). AMP can also function as a regulatory molecule that modulates activities of innate and adaptive immunity (Ganz, 2003a; Bowdish et al., 2005). Expression of AMPs can be either constitutive or induced by pathogens.

2. Antimicrobial peptides (AMPs)

AMPs are small polycationic, amphipathic molecules that range in size from 10 to 100 amino acids, but their amino acid sequences are highly diverse, a feature believed to be due to adaptations of host organisms to the unique collection of potential microbial pathogens in their environment (Broekaert et al., 1997; Zasloff, 2002; Hancock and Sahl, 2006). AMPs found in prokaryotes are generally known as bacteriocins, and are small, heat-stable peptides active against other bacteria but not against the producer which has developed specific immunity against its own bacteriocins (Cotter et al., 2005).
production of bacteriocins allows a bacterium to gain a competitive advantage over other bacteria occupying the same environment (Riley et al., 1998). Bacteriocins are extremely diverse as they are produced by all classes of bacteria and may be active against closely related bacteria or unrelated bacteria (Cotter et al., 2005).

In animals, AMPs can be broadly classified into four different groups based on their three-dimensional structures, amino acid composition, and number of disulphide bonds. These groups are the α-helical peptides, β-sheet peptides, extended peptides and loop peptides (Boman, 1995; Hancock and Lehrer, 1998; Hancock and Sahl, 2006). The first two groups are the most common AMPs found in animals. Animals usually produce multiple peptides from these different groups to perform overlapping or supporting functions to defend against a range of possible pathogens in different tissues (Hancock and Diamond, 2000).

Members of the α-helical peptide group have an α-helical structure with a slight bend in the centre of the molecule as a distinguishing structural feature (Powers and Hancock, 2003). They include cecropins from insects as well as their analogs in the porcine intestine known as porcine cecropin P1, magainins from frog skin, and cathelin-associated peptides from the blood cells of humans, cattle, pigs, mice, rabbits and sheep (Hancock and Lehrer, 1998). The β-sheet peptide group is distinguished by a β-sheet structure and sometimes a minor helical structure held together by two to four disulphide bonds (Powers and Hancock, 2003). They include defensins from various vertebrates, and invertebrates, protegrins from pigs and tachyplesins from horseshoe crabs (Hancock and Lehrer, 1998; Hancock and Sahl, 2006). Members of the extended peptide group have no distinct secondary structure but can be distinguished by their amino acid
sequence and being rich in certain amino acids, such as glycine, proline, tryptophan, arginine and/or histidine (Powers and Hancock, 2003). This group includes indolicidin from bovine neutrophils and PR39 from pig blood cells (Hancock and Lehrer, 1998; Zasloff, 2002). The loop peptide group is distinguished by having only one disulfide bond which acts to form the loop structure (Powers and Hancock, 2003). This group includes bovine bactenecin isolated from cow neutrophils, and brevinins, esculentins and ranalexin from the skin of frogs in the family Ranidae (Boman, 1995; Hancock and Sahl, 2006).

Plant AMPs all share the common features of an overall positive charge and the presence of disulfide bonds (Broekaert et al., 1997; Hammami et al., 2009). They can be classified into seven families based on their primary structure, which are the thionins, defensins, lipid transfer proteins, hevein-like proteins, knottins, snakins and cyclotides (Broekaert et al., 1997; Hammami et al., 2009). Unlike animals, plants do not have linear \( \alpha \)-helix AMPs. Thionins are characterized by either eight or six cysteines, and they have been found in most plant families (Broekaert et al., 1997). Plant defensins are characterized by eight or more cysteines with a distinct spacing and predicted disulphide bonds that are different from thionins but more similar to insect defensins (Terras et al., 1995). Plant defensins differ from thionins in that thionins have two \( \alpha \)-helices and two \( \beta \)-strands, while plant defensins contain one \( \alpha \)-helix and three \( \beta \)-sheets (Broekaert et al., 1997). Lipid transfer proteins (LTPs) are larger molecules of 90 to 93 amino acids with eight cysteine residues (Broekaert et al., 1997). The thionins, defensins and lipid transfer proteins groups of plant AMPs have been relatively well studied compared to the hevein-like proteins, knottins, snakins and cyclotides. Hevein-like AMPs and knottins are
distantly related peptides with six or eight cysteine residues that have a chitin-binding property (Broekaert et al., 1997). Snakins are characterized by 12 conserved cysteines with similarity to a protein encoded by a gibberellic-acid-stimulated transcript from *A. thaliana* (Padovan et al., 2010). Cyclotides are smaller cyclic peptides of 27 to 37 amino acids characterized by a combination of a cyclic peptide internally linked by three disulphide bonds to form a cysteine-knot (Padovan et al., 2010). In addition to these, there are plant AMPs that do not fit into these groups. The four cysteine-type AMPs, which are some of the smallest AMPs ever found, include MBP-1 from maize and Ib-AMP from *Impatiens balsamina* (Broekaert et al., 1997). Like animal AMPs, the variety of AMPs in a plant allow them to work synergistically in the same tissues and complimentarily in different tissues or at different times, which can extend plant defenses to cover a broader range of microbes (Padovan et al., 2010).

Despite their diversity, all AMPs appear to function by targeting microbial membranes by exploiting the AMP’s cationic amphipathic structure. Generally, positively charged eukaryotic AMPs are attracted to negatively charged prokaryotic membranes, which lack cholesterol and are rich in anionic phospholipids, unlike eukaryotic membranes, which contain neutral phospholipids (Bevin et al., 1999). Their amphipathic nature allows them to be inserted into the microbial membranes, and this may directly cause membrane disruption due to pore formation, membrane thinning and/or changes in membrane permeability. Changes in membrane permeability may act indirectly by allowing AMPs to enter the microbial cell and to interact with intracellular targets (Zasloff, 2002; Hancock and Sahl, 2006). Some of these intracellular targets are
DNA, RNA and cellular proteins, and their binding with AMPs is believed to inhibit their synthesis (Powers and Hancock, 2003).

3. Defensins

Defensins are members of the β-sheet peptide group and are found in many kinds of eukaryotes. They are not products of a metabolic pathway like conventional antibiotics but are directly produced through transcription and translation of a gene (Bevins et al., 1999). Multiple defensin genes are often found in a single species. For example, the *Sus scrofa* genome has 13 putative defensin genes (all β defensins), the human genome has 45 defensin genes (6 α defensins and 39 β defensins) (Sang and Blecha, 2009), the *Arabidopsis thaliana* genome has 15 defensin genes (Thomma et al., 2002) and 317 defensin-like genes (Silverstein et al., 2005), and rice has 93 defensin-like genes (Silverstein et al., 2007).

Nucleic acid and amino acid sequences of different defensins are quite different but share a β-sheet with three to four disulphide bonds as a common feature in their three-dimensional structures. Different sizes and number of disulphide bonds are often characteristic for an organism (Figure 1.1). For example, all vertebrate defensins have three disulphide bonds, whereas invertebrate defensins can have 3 or 4 disulphide bonds and all plant defensins have four or more disulphide bonds. These differences in structure and number of disulphide bonds are also related to function. For example, most vertebrate defensins are active against bacteria, whereas most plant defensins are active against fungi.
Figure 1.1 Conserved disulphide bonds present in different families of defensins. Examples are human neutrophil defensin (HNP-1) in the vertebrate α-defensin class (A), porcine β-defensin 1 (PBD-1) in the vertebrate β-defensin class (B), rhesus θ-defensin 1 (RTD-1) in the vertebrate θ-defensin class (C), drosomycin from *Drosophila melanogaster* in the 4-disulphide bond invertebrate defensin class (D), and heliomicin from *Heliothis virescens* in the 3-disulphide bond invertebrate defensin class (E), tobacco defensin 1 (Ntdef1) in the plant defensin class (F) and plectasin from *Pseudoplectania nigrella* in the fungal defensin class (G). Solid black line connecting cysteines (C in red) represents disulphide bond and blue curve indicates continuation in the amino acid sequence forming a cyclic molecule. (modified from Wilmes et al., 2011 and Bulet et al., 2004)
3.1 Animal defensins

Animal defensins fall into four classes based on their structure and antimicrobial activity. These are the invertebrate defensins and the α, β and θ vertebrate defensins. Invertebrate defensins are the most diverse group of defensins and contain 33 to 46 amino acids characterized by a cysteine-stabilized α-helix β-sheet motif (CSαβ) that are also found in many modulators of membrane potential, such as scorpion toxins, plant defensins and some human β defensins (Cornet et al., 1995; Froy and Gurevitz, 1998; Bulet and Stocklin, 2005). They have been isolated mostly from the haemolymph of arthropods and granular hemocytes of mollusks (Wong et al., 2007). Most insect defensins contain three disulphide bonds, which stabilize the one α-helix and two antiparallel β-sheet conformations (Figure 1.1) (Broekaert et al., 1995). An exception is drosomycin from Drosophila melanogaster, which has four disulphide bridges and an additional N-terminus β-sheet structure (Wong et al., 2007). Most mollusk defensins contain the same secondary structures as insect defensins but with four disulphide bonds that are believed to increase protein stability in seawater (Wong et al., 2007; Wilmes et al., 2011). However, the mussel (Mytilus edulis) defensin only contains three disulphide bonds (Wong et al., 2007).

Both α and β vertebrate defensins are linear molecules of 30 to 40 amino acids with six cysteines that form three intramolecular disulphide bonds, but the α defensins have disulphide bonds between C1 and C6, C2 and C4 and C3 and C5, whereas the β defensins have disulphide bonds between C1 and C5, C2 and C4 and C3 and C6 (Figure 1.1) (Bevins et al., 1999). The α defensins are most abundant in the neutrophils and
Paneth cells of the small intestine, while β defensins are found in the epidermal and epithelial tissues (Lehrer, 2004). The θ vertebrate defensins are the 18 amino acid cyclic molecules with three intramolecular disulphide bonds derived from heterodimeric splicing of two α defensin-like precursors (Tang et al., 1999). Thus far, they have only been isolated from leukocytes of rhesus monkeys (Tang et al., 1999; Tran et al., 2002).

The invertebrate defensins are highly active against gram-positive bacteria but less active against gram-negative bacteria, filamentous fungi and yeasts (Bulet and Stocklin, 2005). However, α, β and θ vertebrate defensins have activity against gram-negative bacteria, gram-positive bacteria, filamentous fungi, yeast, and viruses (Wilmes et al., 2011). Other than direct antimicrobial activity, primate α and β defensins can also possess functions such as modulators of the host innate immune response by enhancing phagocytosis, promoting neutrophil recruitment, enhancing the production of proinflammatory cytokines, suppressing anti-inflammatory mediators and up-regulating expression of genes involved in regeneration of epithelial cells (Yang et al, 2002; Bowdish et al., 2005). Furthermore, they may have roles in host adaptive immunity as they have chemotactic activity for monocytes, T cells and dendritic cells (Yang et al, 2002). As most mammalian AMPs, including defensins, have weak antimicrobial activity under physiological conditions, their more prominent roles are probably to modulate host immune responses (Hancock and Sahl, 2006).

3.2 Pig defensins

The only type of defensins found in pigs (Sus scrofa) thus far are β-defensins, which are encoded by 13 different genes based on sequence homology to existing β-defensins (Sang et al., 2006; Sang and Blecha, 2009). These porcine β-defensins show
different tissues-specific expression patterns with most being expressed in multiple
tissues in different quantities. For example, porcine β-defensin 1 (pbd-1) is expressed
most abundantly in tongue epithelia with lower levels in the respiratory and digestive
tracts as well as many other organs (Zhang et al. 1998; Sang et al., 2006). Study of
expression of ten other defensins by Sang et al. (2006) showed that pbd-2 and pbd-3 are
expressed in bone marrow among other tissues, and pbd-3 is expressed most abundantly
in lymphoid tissues. All porcine β-defensin genes are expressed to some degree in male
reproductive tissues, but the highest levels of expression of most porcine β-defensin
genes are either in the lung or skin.

Inducible expression of porcine β-defensins has not been well characterized, but
expression of pbd-1 and pbd-2 in a porcine epithelial cell lines was induced by
Salmonella typhimurium infection but not by S. choleraesuis (Veldhuizen et al., 2009).
However, in vivo expression of pbd-1 and pbd-2 in porcine intestine did not change due
to infection with S. typhimurium (Veldhuizen et al., 2007). Thus far only PBD-1 and
PBD-2 have shown evidence of antibacterial activity. PBD-1 is active against
Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Candida albicans
and Bordetella pertussis (Shi et al., 1999; Elahi et al., 2006). PBD-2 is active against S.
typhimurium, L. monocytogenes, and Erysipelothrix rhusiopathiae (Veldhuizen et al.,
2008).

3.3 Plant defensins

Plant defensins were originally considered to be a class of thionins, referred to as
γ-thionins, but later were named defensins based on the arrangement of their cysteine
residues and disulphide bond pairings resulting in a structure similar to insect defensins
Plant defensins are small, cationic peptides of about 45 to 54 amino acids in length with an approximate size of 5 kDa (Broekaert et al., 1995). Nucleotide and amino acid sequences of different plant defensins show very limited homology to each other, whereas the three dimensional structure is conserved among plant defensins because of the three antiparallel $\beta$-sheets and one $\alpha$-helix stabilized by the four intramolecular disulphide bonds (Figure 1.1) (Garcia-Olmedo et al., 1998; Thomma et al., 2002). Like invertebrate defensins, plant defensins have a $\text{CS}\alpha\beta$ motif (Thomma et al., 2002).

Plant defensins can be classified into two groups. The first group has an N-terminus signal peptide domain for extracellular secretion followed by a mature peptide domain (Carvalho and Gomes, 2009). The second group, which is transcribed only in flower or fruit tissue of solanaceous plants, has an N-terminus signal peptide domain for secretion followed by the mature peptide domain and an additional C-terminal acidic domain, which is later removed from the defensin mature peptide (Lay et al., 2003). Both groups of defensins contain the four conserved disulphide bonds in the mature peptide domain but some members of the second group, namely petunia defensins PhD1 and PhD2, also contain an additional disulphide bond due to two extra cysteine residues, one is located after the conserved C1 and the other after the conserved C3. The presence of an extra disulphide bond does not change the structure of PhD1 and PhD2 and has no effect on their activity (Janssen et al., 2003; and Lay et al., 2003).

Defensins are found ubiquitously in plants. Examples are radish (Terras et al., 1995), petunia (Karunanandaa et al., 1994; Lay et al., 2003), alfalfa (Gao et al, 2000), arabidopsis (Epplle et al., 1997a), tomato (Milligan and Gasser, 1995; van den Heuvel et
al., 2001), pepper (Meyer et al., 1996), potato (Stiekema et al., 1988) and tobacco (Gu et al., 1992; Li and Gray, 1999; Takemoto and Kawakita, 1999; Atnaseo 2003). Within a plant species, defensins are encoded by multigene families, the members of which differ in expression pattern, such as induction by abiotic and biotic stresses, organ specific, developmentally regulated or constitutive (Epple et al., 1997a; Broekaert et al., 1997; Thomma et al., 2002; Bahramnejad et al. 2009).

Plant defensins have been isolated from all parts of plants including seeds, pods, fruits, flower parts, leaves, tubers, roots and stems (Lay and Anderson, 2005), and some are found only in certain portions of plant tissues, often the peripheral cell layers, such as guard cells, the inner surface of vascular tissue and the epidermis of cotyledons, leaf primordia and petals (Moreno et al. 1994; Broekaert et al., 1997; Epple et al., 1997a). Most plant defensins have antifungal activity and/or occasionally antibacterial activity, but some defensins have not yet been found to have any antimicrobial activity (Thomma et al., 2002). Bacterial pathogens are less affected by plant defensins than fungal pathogens (Broekaert et al., 1997). However some plant defensins have antibacterial activity, such as CtAMP1 from Clitoria ternatea, which is active against Bacillus subtilis (Osborn et al., 1995), and the So-D2, So-D6 and So-D7 defensins from Spinacia oleracea, which are active against Clavibacter michiganensis and Pseudomonas solanacearum (Segura et al., 1998). The antimicrobial activity of plant defensins suggests a role in plant innate immunity (Thomma et al., 2002; Nürnberger et al., 2004).

Apart from antimicrobial activity, plant defensins have also been shown to have other activities, such as an α-amylase inhibitor (Pelegrini et al., 2008; Zhang et al., 1997; Bloch and Richardson, 1991), protease inhibitor (Wijaya et al., 2000; Melo et al., 2002),
protein translation inhibitor (Chen et al., 2004; Chen et al., 2005; Mendez et al, 1990; Mendez et al, 1996), growth inhibitor of roots of parasitic plants (de Zélicourt et al, 2007) and ion channel blockers (Kushmanick et al., 1998). These activities are believed to contribute to plant defenses (Lay and Anderson, 2005). Furthermore some plant defensins may also have a role in plant growth and development as some defensins, namely *Medicago sativa* defensin (MsDef1, MsDef2) and *Raphanus sativus* defensins (Rs-AFP2) have been shown to inhibit *A. thaliana* root growth *in vitro* (Allen et al., 2007). In addition, transgenic tomato plants with overexpression or suppression of endogenous defensin gene (DEF2) showed altered morphology, reduced pollen viability and reduced seed production (Stotz et al., 2009a).

3.4 Tobacco defensins

In the Plant Gene Index (http://compbio.dfci.harvard.edu/tgi/) for *N. tabacum*, 12 genes are annotated as defensins, while in the Plant Gene Index for *N. benthamiana*, 11 genes are annotated as γ thionins and four genes are annotated as defensins. Defensins described from *N. tabacum* are a flower-specific thionin (FST) (Gu et al., 1992; GenBank accession number Z11748), a flower style defensin-like protein gene (NTS13) (Li and Gray, 1999; GenBank accession number X99403), a thionin-like protein (TLP) (Takemoto and Kawakita, 1999; GenBank accession number AB034956), and *N. tabacum* defensins 1, 2, 3 (Ntdef1, Ntdef2, and Ntdef3) (Atmaseo, 2003). NTDef1 is identical to TLP, while NTDef2 and NTDef3 share 93% and 85% amino acid identity with FST, respectively. NTS13 and TLP (Ntdef1) are members of the first group of defensins, and FST, Ntdef2, and Ntdef3 are members of the second group with the unique C-terminal acidic domain. However, NTDef3 deviates from the signature pattern of
cysteins of plant defensins because one of its eight cysteines is not located in a conserved position. In the *N. tabacum* Gene Index, NTdef3 was found to have the same cysteine pattern as TC156528, but despite the unusual cysteine pattern, TC156528 is tentatively annotated as a flower-specific defensin.

TLP (*Ntdef1*) was found to be expressed in both leaf and flower tissues, whereas *Ntdef2* was only expressed in flowers (Atnaseo, unpublished). Flower-specific expression was also shown for *FST*, which is typical of the expression patterns of defensins with the C-terminal acidic domain (Gu et al., 1992). Expression of *FST* was higher specifically during flower development and declined as the flower matured (Gu et al., 1992). Inducible expression of tobacco defensins has not been reported but according to information given in the gene entry, TLP (*Ntdef1*) was inducible by a fungal elicitor (Takemoto and Kawakita, 1999).

### 3.5 Mode of action of defensins

A number of models have been proposed for the mode of action of AMPs, including defensins, which generally involve disruption of microbial membranes facilitated by electrostatic interactions between the positively charged AMP molecule with the negatively charged microbial phospholipid membrane (Kagan et al., 1990; Ganz, 2003b). This is supported by the finding that the antimicrobial activity of most defensins is reduced by increasing ionic strength/salt concentration suggesting that electrostatic interactions are important (Thomma et al., 2002; Ganz et al., 2003b; Bulet and Stocklin, 2005). The carpet-wormhole model was proposed for activity of animal defensins, which involves an initial interaction with a target cell membrane through their cationic properties followed by the formation of a carpet resulting from an electrostatic adsorption
of defensins into the membrane, which is facilitated by the amphipathic property of defensins. Once defensin accumulation reaches a threshold, defensins change their arrangement in the membrane to form membrane wormholes leading to disruption of membrane integrity and entry of the defensin and/or other normally excluded molecules into the cell, subsequently causing cell death (Lehrer et al., 1993; Ganz, 2003b). With this model, the level of specificity of defensins to target membranes is determined by the charge and concentration of defensin, as well as membrane potential and the lipid composition of the target membrane (Huang, 2000). This model explains, in part, the ability of defensins to distinguish microbes from eukaryotic host cells because eukaryotic membranes are relatively neutral compared to prokaryotic membranes (Bevins et al., 1999). It also suggests a direct physical disruption of microbial membranes by defensins as well as a non-specific binding target.

However, many defensins have been found to target specific receptors on microbial membranes. Receptor binding has been shown for fungal defensins (Schneider et al., 2010), oyster defensins (Schmitt et al, 2010) and human defensins (De Leeuw et al., 2010). The receptor model suggests that while antimicrobial activity may start with charge attraction between defensins and the negatively charged lipid patch on a microbial membrane, physical membrane disruption might not occur at low concentrations of defensin; instead the microbe is killed by interaction of a defensin with a specific receptor (Wilmes et al., 2011). For example, defensins can target lipid II in gram-positive bacteria having a direct antimicrobial effect by blocking lipid II availability for bacterial cell wall synthesis (Wilmes et al., 2011).
The receptor of antifungal defensins from insects and plants may be fungal sphingolipids or a structurally related glucosylceramide (Wilmes et al., 2011). A number of antifungal plant- and insect- defensins target fungal sphingolipids or glucosylceramide, such as the plant defensins DmAMP1 from *Dahlia merckii* (Thevissen et al., 2003) and RsAFP2 from *Raphanus sativus* (Thevissen et al., 2004) and the insect defensin heliomicin from *Heliothis virescens* (Thevissen et al., 2004). Binding of defensins with fungal sphingolipids could either activate endogenous signal transduction components leading to activation of existing ion channels or transporters, or it could serve as an anchor for defensins to insert into the membrane and form pores themselves. Either case would result in K⁺ efflux and Ca²⁺ influx of fungal hyphae (Thevissen et al., 1997). Changes in ion flux are believed to cause fungal growth inhibition as the level of growth inhibition is correlated with the ability to cause Ca²⁺ influx (De Samblanx et al., 1997). Receptor binding of defensins to fungal surfaces also induces accumulation of reactive oxygen species (ROS) and apoptosis (Aerts et al., 2008).

4. Genetic engineering of AMPs and defensins

Genetic engineering of plants expressing AMPs has been developed as a means of creating disease resistant plants with an ability to kill a broad spectrum of microbes (Hancock and Lehrer, 1998). Furthermore, it is believed that AMPs are less vulnerable to the development of microbial resistance as they do not interfere with microbial biochemical pathways like conventional antibiotics, but instead physically disrupt microbial membranes (Tan et al., 2000).

AMPs from both plants and animals have been used to generate many disease resistant plant species (Tables 1.1 and 1.2). Invertebrate defensins used for this purpose
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<td>cecropin B</td>
<td>giant silk moth</td>
<td>Rice</td>
<td>rice chitinase</td>
<td>E7ΩIn</td>
<td>ND</td>
<td>Xanthomonas oryzae pv. oryzae</td>
<td>Sharma et al., 2000</td>
</tr>
<tr>
<td>sarcotoxin</td>
<td>flesh fly</td>
<td>tobacco</td>
<td>tobacco PR</td>
<td>E12Ω</td>
<td>0.002% of total leaf weight</td>
<td>Pseudomonas syringae pv. tabaci and Erwinia carotovora</td>
<td>Ohshima et al., 1999</td>
</tr>
<tr>
<td>IA</td>
<td></td>
<td></td>
<td>protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>attacin E</td>
<td>giant silk moth</td>
<td>Pear</td>
<td>attacin E</td>
<td>2XCaMV35S</td>
<td>ND</td>
<td>Erwinia amylovora</td>
<td>Reynoird et al., 1999</td>
</tr>
<tr>
<td>D4E1</td>
<td>synthetic</td>
<td>tobacco</td>
<td>not specified</td>
<td>D35S</td>
<td>ND</td>
<td>Aspergillus flavus and Verticillium dahliae</td>
<td>Cary et al., 2000</td>
</tr>
<tr>
<td>MB39</td>
<td>synthetic</td>
<td>Apple</td>
<td>barley α-</td>
<td>OSMp</td>
<td>ND</td>
<td>Erwinia amylovora</td>
<td>Liu et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>amylase</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MSI-99</td>
<td>synthetic</td>
<td>Banana</td>
<td>pea vicilin</td>
<td>Arabidopsis ubq 3</td>
<td>ND</td>
<td>Fusarium oxysporum and Mycosphaerella musicola</td>
<td>Chakrabarti et al., 2003</td>
</tr>
<tr>
<td></td>
<td>analog of frog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>magainin 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-99</td>
<td>synthetic</td>
<td>Tomato</td>
<td>pea vicilin</td>
<td>CaMV35S</td>
<td>ND</td>
<td>Pseudomonas syringae pv. tomato but not to Phytophthora infestans or Alternaria solani</td>
<td>Alan et al. 2004</td>
</tr>
<tr>
<td></td>
<td>analog of frog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>magainin 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-99</td>
<td>synthetic</td>
<td>tobacco</td>
<td>expressed in</td>
<td>16S rRNA</td>
<td>ND</td>
<td>P. syringae pv tabaci ATCC 17914 and C. destructivum</td>
<td>DeGray et al., 2001</td>
</tr>
<tr>
<td></td>
<td>analog of frog</td>
<td></td>
<td>chloroplast</td>
<td>promoter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>magainin 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gallerimycin</td>
<td>Greater wax</td>
<td>tobacco</td>
<td>gallerimycin</td>
<td>inducible</td>
<td>ND</td>
<td>Erysiphe cichoracearum and Sclerotinia minor</td>
<td>Langen et al., 2006</td>
</tr>
<tr>
<td></td>
<td>moth <em>Galleria</em></td>
<td></td>
<td></td>
<td>mannopine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>mellonella</em></td>
<td></td>
<td></td>
<td>synthase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>promoter</td>
<td></td>
<td></td>
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</table>

Table 1.1 Summary of genetic engineering of plants with genes for antimicrobial peptides (AMPs) from animals
<table>
<thead>
<tr>
<th>helomicin</th>
<th><em>Heliothis virescens</em></th>
<th>tobacco</th>
<th>tobacco PR1a</th>
<th>D35S</th>
<th>maximum of 0.1% total protein</th>
<th>slight resistance to <em>Cercospora nicotianae</em></th>
<th>Banzet et al., 2002</th>
</tr>
</thead>
<tbody>
<tr>
<td>drosomycin</td>
<td><em>Drosophila melanogaster</em></td>
<td>tobacco</td>
<td>tobacco PR1a</td>
<td>D35S</td>
<td>lower than 0.1% total protein</td>
<td>slight resistance to <em>Cercospora nicotianae</em></td>
<td>Banzet et al., 2002</td>
</tr>
<tr>
<td>human β-defensin 2</td>
<td><em>human</em></td>
<td><em>A. thaliana</em></td>
<td>plant defensin-DmAMP1</td>
<td>CaMV3S</td>
<td>0.05-0.65% TSP</td>
<td><em>Botrytis cinerea</em></td>
<td>Aerts et al., 2007</td>
</tr>
<tr>
<td>rabbit α-defensin</td>
<td>rabbit</td>
<td>tobacco</td>
<td>not reported</td>
<td>CaMV3S</td>
<td>ND</td>
<td>slight resistance to <em>Ralstonia solanacearum</em></td>
<td>Fu et al., 1998</td>
</tr>
</tbody>
</table>

**Note:** CaMV = Cauliflower Mosaic Virus, E12Ω = the 5’ enhancer sequence from CaMV35S promoter + omega sequence from TMV, E7ΩIn = synthesized high expression vector, D35S = double CaMV35S + omega sequence from TMV, FMV = Figwort Mosaic Virus, PiII = promoter from protease II inhibitor gene, OSMP = osmotin promoter, ND = Not Determined, TSP = total soluble protein, 'the transgenic plant displayed increased resistance to the tested organisms unless otherwise specified.
Table 1.2 Summary of genetic engineering of plants with genes for antimicrobial peptides (AMPs) from plants

<table>
<thead>
<tr>
<th>AMP</th>
<th>Source for defensin</th>
<th>Transgenic plant</th>
<th>Signal sequence</th>
<th>Promoter</th>
<th>Protein level</th>
<th>Pathogens/pests tested</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrD1</td>
<td><em>Brassica rapa</em></td>
<td>rice</td>
<td>BrD1 cytochrome C</td>
<td>ND</td>
<td><em>Nilaparvata lugens</em> (brown planthopper insect)</td>
<td>Choi et al., 2009</td>
<td></td>
</tr>
<tr>
<td>RsAFP2</td>
<td>radish</td>
<td>wheat</td>
<td>RsAFP2 ubiquitin</td>
<td>ND</td>
<td><em>Fusarium graminearum</em>, <em>Rhizoctonia cerealis</em></td>
<td>Li et al., 2011</td>
<td></td>
</tr>
<tr>
<td>RsAFP2</td>
<td>radish</td>
<td>rice</td>
<td>RsAFP2 ubiquitin</td>
<td>0.45 - 0.53% TSP</td>
<td><em>Magnaporthe oryzae</em>, <em>Rhizoctonia solani</em></td>
<td>Jha and Chattoo, 2010</td>
<td></td>
</tr>
<tr>
<td>Dm-AMP1</td>
<td><em>Dahlia merckii</em></td>
<td>rice</td>
<td>Dm-AMP1 ubiquitin</td>
<td>0.43% - 0.57% TSP</td>
<td><em>Magnaporthe oryzae</em>, <em>Rhizoctonia solani</em></td>
<td>Jha et al., 2009</td>
<td></td>
</tr>
<tr>
<td>MsDef1</td>
<td>alfalfa</td>
<td>tomato</td>
<td>MsDef1 CaMV 35S</td>
<td>ND</td>
<td><em>Fusarium oxysporum</em></td>
<td>Abdallah et al., 2010</td>
<td></td>
</tr>
<tr>
<td>NmDef02</td>
<td><em>Nicotiana megalosiphon</em></td>
<td>Tobacco</td>
<td>NmDef02 CaMV 35S</td>
<td>ND</td>
<td><em>Phytophthora parasitica</em>, <em>Peronospora hyoscyami</em></td>
<td>Portieles et al., 2010</td>
<td></td>
</tr>
<tr>
<td>NmDef02</td>
<td><em>Nicotiana megalosiphon</em></td>
<td>Potato</td>
<td>NmDef02 CaMV 35S</td>
<td>ND</td>
<td><em>Phytophthora infestans</em>, <em>Alternaria solani</em></td>
<td>Portieles et al., 2010</td>
<td></td>
</tr>
<tr>
<td>WjAMP-1</td>
<td>wasabi</td>
<td>Melon</td>
<td>WjAMP-1 CaMV 35S</td>
<td>ND</td>
<td><em>Fusarium oxysporum</em>, <em>Alternaria solani</em></td>
<td>Ntui et al., 2010</td>
<td></td>
</tr>
<tr>
<td>cdef1</td>
<td>chili</td>
<td>Tomato</td>
<td>cdef1 CaMV 35S</td>
<td>ND</td>
<td><em>Fusarium sp.</em>, <em>Phytophthora infestans</em></td>
<td>Zainal et al., 2009</td>
<td></td>
</tr>
<tr>
<td>BjD</td>
<td>mustard</td>
<td>Peanut</td>
<td>BjD CaMV35S</td>
<td>ND</td>
<td><em>Phaeoisariopsis personata</em>, <em>Cercospora arachidicola</em></td>
<td>Swathi Anuradha et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Dm-AMP1</td>
<td><em>Dahlia merckii</em></td>
<td>Papaya</td>
<td>Dm-AMP1 D35S</td>
<td>0.05 to 0.08% TSP</td>
<td><em>Phytophthora palmivora</em></td>
<td>Zhu et al., 2007</td>
<td></td>
</tr>
<tr>
<td>alfAFP</td>
<td>alfalfa</td>
<td>Potato</td>
<td>alfAFP FMV35S</td>
<td>maximum 2.3 ppm</td>
<td><em>Verticillium dahliae</em></td>
<td>Gao et al., 2000</td>
<td></td>
</tr>
<tr>
<td>DRR206</td>
<td>pea</td>
<td>Canola</td>
<td>DRR206 CaMV 35S</td>
<td>ND</td>
<td>slight increase in resistance to <em>Leptosphaeria maculans</em></td>
<td>Wang et al., 1999</td>
<td></td>
</tr>
<tr>
<td>RsAFP2</td>
<td>radish</td>
<td>Tobacco</td>
<td>RsAFP2 CaMV 35S</td>
<td>maximum 2.4 μg/mL total leaf protein</td>
<td><em>Alternaria longipes</em></td>
<td>Terras et al., 1995</td>
<td></td>
</tr>
<tr>
<td>wasabi</td>
<td>wasabi defensin</td>
<td>rice</td>
<td>Ubiquitin-1</td>
<td>ND</td>
<td><em>Magnaporthe grisea</em></td>
<td>Kanzaki et al., 2002</td>
<td></td>
</tr>
<tr>
<td>MiAMP1</td>
<td><em>Macadamia integrifolia</em></td>
<td>Canola</td>
<td>MiAMP1 E12Ω</td>
<td>ND</td>
<td><em>Leptosphaeria maculans</em></td>
<td>Kazan et al., 2002</td>
<td></td>
</tr>
</tbody>
</table>

Note: CaMV = Cauliflower Mosaic Virus, E12Ω = the 5' enhancer sequence from CaMV35S promoter + omega sequence from TMV, D35S = double CaMV35S + omega sequence from TMV, FMV = Figwort Mosaic Virus, ND = Not Determined, TSP=total soluble protein, ¹ the transgenic plant displayed increased resistance to the tested organisms unless otherwise specified.
are cecropin from giant silk moth (Jaynes et al., 1993; Hightower et al., 1994; Florack et al., 1995; Huang et al., 1997; Osusky et al., 2000; Sharma et al., 2000), tachyplesin from horseshoe crabs (Allefs et al., 1996), attacin E from giant silk moth (Reynoird et al., 1999) and sarcotoxin IA from flesh fly (Ohshima et al., 1999). Vertebrate AMPs used for this purpose are human \( \beta \)-defensin 2 (hBD-2) expressed in \( A. \ thaliana \) to confer increased resistance to \( B. \ cinerea \) (Aerts et al., 2007) and rabbit defensin (NP-1) expressed in tobacco for slightly increased resistance to \( Ralstonia \ solanacearum \) (Fu et al., 1998), poplar with \textit{in vitro} activity against \( E. \ coli \) and \( Fusarium \ oxysporum \) (Zhao et al., 1999) and tomato with \textit{in vitro} activity against \( B. \ subtilis \) (Zhang et al., 2000).

Plant defensins have been transferred generally to heterologous hosts (Table 1.2), for example radish defensin (RsAFP2) in tobacco giving increased resistance to \( Alternaria \ longipes \) (Terras et al., 1995), \textit{Dahlia merckii} defensin (Dm-AMP1) in papaya giving increased resistance to \textit{Phytophthora palmivora} (Zhu et al., 2007), alfalfa defensin (alfAFP) in potato providing greater resistance to \textit{Verticillium dahliae} (Gao et al., 2000), and wasabi defensin in rice giving increased protection against \textit{Magnaporthe grisea} (Kanzaki et al., 2002). In addition to defensins, other AMPs have been used such as an \( A. \ thaliana \) thionin in \( A. \ thaliana \) against \( Fusarium \ oxysporum \) (Epple et al., 1997b), an AMP from \textit{Macadamia integrifolia} (MiAMP1) in canola against \textit{Leptosphaeria maculans} (Kazan et al., 2002) and an alfalfa defensin in potato against \textit{V. dahliae} (Gao et al., 2000).

Although small AMPs have been used successfully to generate disease-resistant plants, the synthesis of AMPs in plants for therapeutic use has not been achieved for small cationic AMPs despite their potential activity against human fungal pathogens,
such as *Candida albicans* (Thomma et al., 2003; Thevissen et al., 2007). Only the larger AMP, human lactoferrin, a multifunctional glycoprotein from milk with both nutritional and therapeutic properties, has been expressed in plants, specifically in potatoes (Chong and Langridge, 2000) and tobacco (Salmon et al., 1998) for animal use. In both cases, transgenic plants contained a relatively high level of lactoferrin (0.1 to 0.3% of total extracted protein), and at least in potato, the peptide appeared to be biologically active against *E. coli* and *Salmonella paratyphi* (Chong and Langridge, 2000).

5. Obstacles and possible solutions for expression of AMPs in plants

Attempts to produce high levels of foreign proteins in plants can be limited by low levels of expression, degradation, or a combination of these two factors. Some researchers have concluded that the most important obstacle to expressing a foreign gene, particularly an animal gene in a plant, is the lack of protein stability and resulting degradation in host tissue (Sharma et al., 2000; Obembe et al., 2010; Doran, 2006). Similarly protein degradation is believed to be a reason why many AMP-transformed plants have not shown any significant increase in disease resistance. Examples are cecropin A in tobacco (Hightower et al., 1994), cecropin B in tobacco (Florack et al., 1995) and cecropin B in potato (Allefs et al., 1995). To enhance expression of foreign genes in plants, various strategies have been employed, such as developing more efficient promoters, changing codon usage to optimize for plant expression and modifying the 5’untranslated sequence to increase the level of transcription (Doran, 2006). These strategies could increase mRNA levels but to date have provided limited improvements in the amounts of foreign protein, suggesting that transcription of the transgene is not the main obstacle (Doran, 2006).
Fusion of AMPs with other proteins is a possible solution to enhance AMP accumulation by protecting them from post-translational degradation as well as limiting their toxicity to the host plant (Piers et al., 1993). An example of this approach is the fusion of an anionic coding sequence with a cationic AMP thereby allowing the two components to interact to form a stable intramolecular unit (Piers et al., 1993). This process resembles many biologically active peptides, which are synthesized as an inactive propeptide before being processed into an active form when needed by the organism; however, this approach is potentially problematic for the biological activity of the hybrid molecule.

In addition, AMPs can be modified to improve their efficacy as well as their resistance to plant proteases. Most of this work has been done with small insect AMPs. For example, Shiva-1 is a modified form of cecropin B, but exhibited higher toxicity to *Pseudomonas solanacearum* than the native form in extracts from transgenic tobacco (Jaynes et al., 1993). Another cecropin analog, MB39, exhibited higher stability in intercellular fluid from various plants, such as potato, maize, tobacco peach, but not rice, than naturally occurring cecropin (Owen and Heutte, 1997). A cecropin-melilittin chimera, CEMA, exhibited strong antimicrobial activity against various fungal pathogens of potatoes (Osusky et al., 2000). A synthetic 17-amino-acid antimicrobial peptide, D4E1, was resistant to degradation by proteases of tobacco and cotton leaf (DeLucca et al., 1998; Cary et al., 2000).

Fusion of AMP genes with appropriate signal peptide sequences to be secreted into the intercellular space may also reduce AMP proteolysis in the cytosol (Sharma et al., 2000). Plant signal peptides have been used to enhance levels of sarcotoxin IA in
tobacco (Ohshima et al., 1999) and cecropin B in rice (Sharma et al., 2000). Although fusion of cecropin B with a plant signal sequence resulted in more mRNA than fusion with an animal signal sequence or without a signal sequence, it did not result in detectable protein (Florack et al., 1995) suggesting that extracellular proteolytic degradation also occurred (Gouka et al., 1997; Owen and Heutte 1997).

The levels of a transgenic protein can be enhanced in plants by targeting them to subcellular compartments, such as the endoplasmic reticulum (ER), plastid and vacuole (Conrad and Fiedler, 1998). The ER is attractive due to the presence of molecular chaperones, and protein disulfide isomerase in the ER is thought to facilitate correct protein folding leading to increased protein stability and accumulation (Conrad and Fiedler, 1998). Production of an anti-Hepatitis B virus surface antigen (HBsAg) in tobacco leaves was about 7-fold higher when localized to the ER than to the apoplast or vacuole, and no HBsAg was detected when localized to the cytosol (Ramirez et al., 2002). Similarly, production of Lt-B in maize seed vacuoles gave 20,000-fold more protein than in the cytosol, 6-fold more than in the apoplast and 200-fold more than in the ER (Streatfield et al., 2003).

Enhanced expression of an AMP may be achieved by expression in the chloroplast following integration of the transgene into the genome of that organelle (Daniell et al., 2001). For example, somatotropin accumulation in transformed chloroplasts of tobacco was over 300-fold higher than with a similar somatotropin construct integrated into the nuclear genome (Staub et al., 2000). Expression of an AMP gene coding for a magainin 2 analog integrated into the chloroplast genome resulted in
increased resistance of tobacco to *P. syringae, Aspergillus flavus, Fusarium moniliforme* and *Verticillium dahlia* (DeGray et al., 2001).

6. Expression of AMPs in plants using *Agrobacterium tumefaciens*

6.1 *A. tumefaciens* infection process

*A. tumefaciens* is a gram-negative bacterium found to cause crown gall disease in dicotyledonous plants (Smith and Townsend, 1907). Crown gall disease can lead to reduction of yield and vigor in an infected plant, but it has a relatively small impact in agriculture as monocots are not hosts for *A. tumefaciens* (Escobar and Dandekar, 2003). Crown gall disease is a result of the integration of a set of genes (T-DNA) located on the tumour-inducing (Ti) plasmid of *A. tumefaciens* into the plant nuclear genome (Chilton et al., 1977; Thomashow et al., 1980). An outline of the infection process is shown in Figure 1.2. T-DNA also contains oncogenes, which are responsible for uncontrolled proliferation of infected cells, and opine-related genes, which provide specific nutrients for *A. tumefaciens* inside the gall (Escobar and Dandekar, 2003). Expression of these integrated foreign genes is mediated by the host cell systems of transcription and translation in the same manner as that of plant genes in the plant genome.

In addition to T-DNA, the Ti-plasmid also contains essential genes for virulence of *A. tumefaciens*, collectively called *vir* genes (Escobar and Dandekar, 2003). Sensing the presence of phenolic compounds and sugars released by wounded plant cells induces autophosphorylation of VirA transmembrane receptor kinase which in turn donates its phosphate to the cytoplasmic VirG. Phosphorylated VirG functions as the transcription factor that activates expression of other *vir* genes (Escobar and Dandekar, 2003; Pitzschke and Hirt, 2010). The production of *vir* gene products leads to transfer of
Figure 1.2 Steps in T-DNA transfer from *A. tumefaciens* to a plant cell

1) Perception of phenolic compound from plant wound sites activates VirA to phosphorylate VirG.

2) Phosphorylated virG molecule induces the expression of *vir* operon genes.

3) VirD1 and VirD2 cleave the single-stranded T-DNA/T-strand from the T-plasmid at the border region and VirD2 remains bound to the 5’ of the T-strand.

4) T-DNA/VirD2 and VirE2, VirE3, VirD5, and VirF transport across a T-pilus made of different VirB subunits and VirD4.

5) T-complex formed by coating of T-strand with VirE2.

6) T-complex interacts with plant proteins, i.e. VIP1 and importin α, to facilitate its transport into plant nucleus.

7) VirF removes VirE2 and VirD2 from T-DNA allowing it to be integrated into plant genome.

8) T-DNA integrates into plant genome with the aid of a plant DNA-repairing machinery. NPC = the nuclear pore complex (figure adapted from Citovsky et al., 2007)
T-DNA into host cells. This process involves excision of the T-DNA as a single-stranded DNA (T-strand) from the Ti-plasmid by the activity of VirD1 and VirD2 proteins (Gelvin, 2003). VirD2 remains bound to the 5’ end of the T-strand while being transported to plant cells through a type IV secretion system (T4SS).

The T4SS is a complex of 11 VirB proteins and VirD4, which form a channel across the bacterial membrane (Fronzes et al., 2009). Assembly of the T4SS complex, as well as transfer of substrates through this channel are driven by VirB4, VirB11 and VirD4, which are ATPases (Fronzes et al., 2009). The T4SS involves a pilus, which contains VirB2 subunits as a major component and VirB5 and VirB7 as minor components (Fronzes et al., 2009). In addition to VirD2/T-strand transportation, the T4SS is also responsible for transporting other Vir proteins involved in \textit{A. tumefaciens} virulence, including VirE2, VirE3, VirD5 and VirF (Vergunst et al., 2005).

Once inside the plant cell, the VirD2/T-strand is coated by VirE2 proteins forming a T-complex, which then interacts with plant proteins for movement into the plant nucleus (Lacroix et al., 2006). Both VirD2 and VirE2 contain nuclear localization signals. VirD2 has been shown to interact with members of the \textit{Arabidopsis} importin \( \alpha \) family (Deng et al., 1998; Bako et al., 2003; Bhattacharjee et al., 2008). Because importin \( \alpha \) is a part of a host nuclear transport system that binds to the nuclear localization signal of VirD2 (Bhattacharjee et al., 2008), interaction of VirD2 with importin \( \alpha \) may result in T-complex nuclear import (Ballas and Citovsky, 1997). VirE2 interacts with tobacco VirE2 interacting protein (VIP1), which then facilitates VirE2 binding to importin \( \alpha \)-1 (Tzfira et al., 2002). VIP1 function in nuclear transport can be
partially complemented by VirE3 as VirE3 can also bind VirE2 and importin α-1 (Lacroix et al., 2005).

Within the nucleus, the T-complex is targeted to host chromatin by interacting with plant proteins involved in DNA repair (Bako et al., 2003) and by VIP1 and another VirE2 interacting protein, VIP2 (Tzfira et al., 2000). To be integrated into the host genome, the T-complex is uncoated by the activity of an F-box protein, VirF, which targets proteolysis of VIP1 possibly destabilizing VirE2 by the host enzyme complex (Schrammeijer et al., 2001; Tzfira et al., 2004). Integration of T-DNA into the host genome is facilitated by the host DNA repair machinery (Citovsky et al., 2007). Another vir gene product delivered into the host cell by the T4SS is VirD5, which is transported into the host nucleus due to the presence of a nuclear localization signal but its function is not known, although it has homology to plant transcription factors (Schrammeijer et al., 2000).

*Agrobacterium tumefaciens* is unique in its use of the T4SS to deliver T-DNA and effector proteins instead of the type III secretion system (T3SS) used by most bacterial pathogens to deliver virulence effector proteins. T3SS effectors generally act to suppress PTI by interfering with PAMP perception or PAMP responses via suppression of PAMP-induced signal transduction or transcription, which leads to suppression of defense-related gene expression in plants during biotrophic growth of hemibiotrophic bacteria, such as *P. syringae* and *Xanthomonas campestris* (Espinosa and Alfano, 2004; Hann et al., 2010). Without a T3SS, *A. tumefaciens*, which is a biotrophic plant pathogen, would presumably not be able to suppress PAMP-induced signal transduction or transcription, unless some T4SS effectors were also capable of doing this.
Although effector proteins that are able to suppress PTI have not been identified in *A. tumefaciens*, studies in various plant systems including *Ageratum conyzoides* cell cultures (Ditt et al., 2001; Ditt et al. 2005), *N. tabacum* cell cultures (Veena et al., 2003), *A. thaliana* cell cultures (Ditt et al. 2006) and agroinfiltrated *N. benthamiana* leaves (Goulet et al., 2010) have shown that *A. tumefaciens* can alter expression of defense-related and general stress response genes during the infection process in the host. Although induction of defense-related genes, such as in *A. conyzoides* cell cultures, appeared to be a general responses to bacteria as this was also observed in the presence of *Escherichia coli* (Ditt et al., 2001), other responses appear to be more specific to the *A. tumefaciens* infection. For example, suppression of plant defenses by *A. tumefaciens* occurred in an attachment-dependent manner in *A. conyzoides* cell cultures by 48 h (Ditt et al., 2005), and attachment of *A. tumefaciens* to the plant cell wall is the first step in the infection process (McCullen and Binns, 2006). In *N. tabacum* cell cultures, increased defense gene expression was triggered at 3 to 12 h after inoculation with *A. tumefaciens*, but expression of these genes was suppressed by 24 to 36 h, which coincided with integration of T-DNA and Vir proteins (Veena et al., 2003). In contrast, exposure of *A. thaliana* cell cultures to *A. tumefaciens* showed that gene expression did not change significantly at 24 h, but at 48 h the majority of defense-related genes were activated while a smaller set of genes were repressed (Ditt et al. 2006). The defense-related proteins increased in the *N. benthamiana* leaf apoplast at 6 days following agroinfiltration, indicating that T-strand transfer had resulted in accumulation of these proteins (Goulet et al., 2010). These studies show that *A. tumefaciens* is able to induce
and suppress host defenses possibly by exposure to PAMPs and secreted effectors during infection, but this may not be the same for all plants.

Induction of defense-related gene expression by *A. tumefaciens* is due to perception of PAMPs, such as elongation factor Tu (EF-Tu) (Zipfel et al., 2006), cold-shock protein (Felix and Boller, 2003) and peptidoglycan (Erbs et al., 2008). Perception of bacterial PAMPs by host plants activates PTI, which constitutes plant innate immunity and can also trigger other defensive mechanisms, such as systemic acquired resistance (SAR) and RNA silencing (Dafny-Yelin et al. 2008; Citovsky et al., 2007). In *A. thaliana* lacking the EF-Tu receptor, there is enhanced sensitivity to *A. tumefaciens*, which suggests that PAMP from *A. tumefaciens* can induce PTI (Zipfel et al, 2006).

Salicylic acid (SA), a signaling molecule of SAR, has a direct growth inhibition effect on *A. tumefaciens*, and can reduce susceptibility of *N. benthamiana* to *A. tumefaciens* upon SA treatment, which is believed to be due to inhibition of vir gene expression by SA (Anand et al., 2008). Furthermore, silencing of *N. benthamiana* genes involved in SA synthesis and signaling enhanced susceptibility to *A. tumefaciens*. Together with the finding that benzo-(1,2,3)-thiadiazole-7-carbothioic acid (BTH), a SAR inducer, can reduce gall formation, this suggests that both SA and SAR play roles in suppressing infection by *A. tumefaciens* (Anand et al., 2008). Although SAR may be able to protect plants from damage caused by *A. tumefaciens*, it might not be able to inhibit *A. tumefaciens* once plant transformation has been initiated since *A. tumefaciens* has been shown to suppress SA accumulation and expression of SA-dependent genes in *A. thaliana* after T-strand integration (Gaspar et al., 2004).
Other plant signaling molecules, ethylene (ET) and indole-3-acetic acid (IAA), also interfere with plant infection by *A. tumefaciens*. IAA inhibits growth of *A. tumefaciens* and expression of its *vir* genes by competing with VirA/VirG signaling for the plant phenolic compounds (Liu and Nester 2006). ET does not inhibit *A. tumefaciens* but it indirectly inhibits *vir* gene expression as shown by the treatment of melon plants with the ET precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), which results in lowered transformation efficiency, whereas ET-insensitive *A. thaliana* shows enhanced transformation efficiency (Nonaka et al., 2008). This antagonistic effect of these signaling molecules is intercepted by *A. tumefaciens* as *A. tumefaciens* infection leads to IAA production which in turns stimulates ET synthesis and accumulation in the galls (Wachter et al., 1999) to turn off *vir* gene expression as part of a feedback inhibition (Yuan et al., 2008).

*A. tumefaciens* can inhibit RNA silencing mediated by short interfering RNAs (siRNA) to aid in successfully infecting the host (Dunoyer et al., 2006). MicroRNA (miRNA)-mediated RNA silencing is required for gall formation as miRNA-deficient plants were resistant to gall formation (Dunoyer et al., 2006). As RNA silencing was only detected in the galls, this process may require phytohormones encoded by the oncogenes (Dunoyer et al., 2006). Two miRNAs (miR393 and miR167) involved in regulating auxin signaling suppression are repressed in the galls suggesting that their suppression promotes auxin activity, which in turn promotes RNA silencing (Dunoyer et al., 2006). In contrast, agroinfiltrated leaves of *N. tabacum* have miR393 but not miR167 induced by oncogenic strains of *A. tumefaciens* (Pruss et al., 2008).
6.2 *Agrobacterium*-mediated stable transformation

The ability of *A. tumefaciens* to transfer DNA into plant cells has been extensively exploited for plant transformation (Twyman et al., 2003). Efficient transfer of foreign genes into plants was made possible by the development of a binary vector system which separated the Ti plasmid into two plasmids, a *vir* helper plasmid and a binary plasmid (Hoekema et al., 1983; de Frammond et al., 1983; Lee and Gelvin, 2008). The *vir* helper plasmid retains the *vir* region but without the T-DNA region. Laboratory strains of *A. tumefaciens* used for transformation that carry the *vir* helper plasmid are called “disarmed” strains of *A. tumefaciens* as they cannot induce tumour formation in plants but are capable of gene transfer provided that a binary vector is present. The binary vector is capable of replicating in both *E. coli* and *Agrobacterium* allowing for engineering of the gene (Hoekema et al., 1983; de Frammond et al., 1983). In the binary vector, the tumour generating genes have been removed from the T-DNA, leaving only the left and right border direct repeats, required for recognition and excision of the T-strand. Genes for resistance to antibiotics or other agents are also incorporated into the binary vector to facilitate the selection of transformed tissues together with the incorporation of various restriction sites for insertion of the foreign DNA (Lee and Gelvin, 2008).

The *Agrobacterium*-mediated plant transformation process starts with cocultivation of *A. tumefaciens* with plant tissues, usually leaf discs, followed by selection of the transformed tissues and regeneration of transformed plants from callus. *Agrobacterium*-mediated plant transformation has been used widely as a means for transferring foreign genes into plants for both basic and applied research. Most research
involving AMP transgenes have utilized this technique to create stably-transformed plants (e.g., Ohshima et al., 1999; Kanzaki et al., 2002; Aerts et al., 2007). Although stable transformants can give stable expression levels especially for long-term or field studies, they are time consuming to make due to a lengthy period of selection and regeneration.

6.3 Agroinfiltration

Another option for synthesizing foreign proteins in plants is agroinfiltration, a transient expression system wherein the foreign protein can be detected 24 to 48 h after the introduction of *A. tumefaciens* into plant tissue. Regeneration of transgenic plants from callus is not required (Kapila et al., 1997). This technique also enables gene expression in plant species where stable transformation or plant regeneration protocols have not been developed (Kapila et al., 1997; Wydro et al., 2006). With this method, a binary vector with an expression cassette, identical to those used for stable transformation, is used. The method for agroinfiltration generally includes preparation of an *A. tumefaciens* suspension culture carrying a binary vector with the gene of interest and delivering the culture to the intercellular space of detached or intact plant tissues by direct injection with a syringe or vacuum infiltration (Kapila et al., 1997; Voinnet et al., 2003; Wydro et al., 2006). The *A. tumefaciens* suspension culture is prepared in a buffer containing acetosyringone for induction of vir gene expression to allow for the delivery of the T-strands into plant cells (Voinnet et al., 2003; Wydro et al., 2006).

Agroinfiltration is an ideal method for testing constructs and producing protein for functional analysis prior to making stable transgenic plants because transient expression has a much shorter turnover time than stable transformation (Twyman et al.,
2003). Also, as genome integration is not required, expression is not affected by the location of T-DNA integration in the plant genome (Kapila et al., 1997). Agroinfiltration has been used in many applications, such as the expression of a functional tumor-specific antibody, T84.66, in tobacco leaves, which gave a yield of protein comparable to stable transformation (Vaquero et al., 1999) or even slightly higher for the T84.66/GS8 diabody, which is a bispecific antibody (Vaquero et al., 2002). Other applications of agroinfiltration include screening a bacterial toxin gene and a plant proteinase inhibitor gene for their anti-insect activities (Leckie and Stewart Jr., 2011), and delivery of virus-induced gene silencing (VIGS) vectors (Velasquez et al., 2009).

The usefulness of agroinfiltration, however, is limited by the rapid decline of transgene expression after a short peak period at 60 to 72 h post-infiltration (Voinnet et al., 2003). Post-translational gene silencing (PTGS) is believed to be one of the factors responsible for this decline (Johansen and Carrington, 2001). Co-infiltration with a construct expressing a viral protein suppressor of PTGS, such as p19 (19 kDa protein), effectively enhances transient protein expression by agroinfiltration. For example, the level of green fluorescent protein (GFP) in *N. benthamiana* was 50 times higher in the presence of P19 (Voinnet et al., 2003), and production of a bacterial antigen, GS60 from *Mannheimia haemolytica*, in *N. benthamiana* was higher with p19 compared to agroinfiltration without p19, but the level of protein increase was not stated (Lee et al., 2008a). P19 is a pathogenicity factor of the tomato bushy stunt virus (TBSV) that helps it to systemically invade the host (Scholthof et al., 1995).
7. Pichia pastoris expression system

*P. pastoris* is a methylotrophic yeast widely used as an expression system for recombinant proteins (Cereghino et al., 2002). Because of *P. pastoris*’s ability to metabolize methanol, it was originally exploited as a means to convert methanol to a protein source for animal feed, but as the cost of methanol increased and soybean prices decreased, such use became uneconomical (Cregg et al., 2000). *P. pastoris* was then developed into a protein expression system. Methanol metabolism of *P. pastoris* is initiated by the activity of alcohol oxidase (AOX), which is coded by the *AOX1* and *AOX2* genes (Cregg et al., 2000). *AOX1* expression is tightly regulated as it is repressed in cells grown on glucose and most other carbon sources but induced by methanol (Cereghino et al., 2002). The *AOX1* promoter was incorporated into the *P. pastoris* expression vector for inducible expression of foreign genes (Cregg et al., 2000). Later a constitutive expression vector was developed utilizing the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (GAP) (Cereghino et al., 2002). Foreign proteins produced with *P. pastoris* can accumulate inside the cell or be secreted into the growth medium. Extracellular production elimination the need for cell lysis, and because *P. pastoris* secretes low levels of native proteins, recovery of foreign proteins is simplified (Macauley-Patrick et al., 2005). The most commonly used secretion signal sequence is the *Saccharomyces cerevisiae* α-factor secretion peptide although native secretion signals have also been used (Cereghino et al., 2002). However, secretion of foreign proteins has not always been achieved or proteins that were secreted were not always correctly processed. Such outcomes are not necessarily determined by the choice of secretion signal alone, but likely by the combination of the signal peptide and the
foreign protein being secreted (Cereghino et al., 2002). The best combination of secretion signal for a particular protein cannot be predicted and therefore relies on trial and error so that many expression studies report the use of multiple constructs.

Stable transformants of \textit{P. pastoris} are generated through homologous recombination between the linearized expression vector and the \textit{P. pastoris} genome (Cregg et al., 2000). Higher levels of expression are generally achieved by inserting multiple copies of genes (Clare et al., 1991). The \textit{P. pastoris} expression system has been used for expression of proteins from all origins and functional groups including plant and animal defensins, for example corn defensin (Kant et al., 2009), peach defensin (Wisniewski et al., 2003), human \(\alpha\)-defensin 5 (HD5) (Hsu et al., 2009), sheep \(\beta\)-defensin 1 (sBD-1) (Zhao and Cao, 2011) and PBD-1 (Jiang et al., 2006). The frequent use of \textit{P. pastoris} is due to its advantages of being easily manipulated genetically, having inducible and constitutive expression, choice of intracellular or extracellular protein production, and ability to perform eukaryotic protein modifications, such as glycosylation and disulfide-bond formation (Cregg et al., 2000).

8 Plants and pathogens

8.1 \textit{Nicotiana tabacum}

The genus \textit{Nicotiana} is a member of the nightshade or Solanaceae family and contains 76 species originating from North America, South America, Australia and Africa (Chase et al., 2003; Knapp et al., 2004). \textit{N. tabacum} is an amphidiploid/allotetraploid species in the genus with 48 chromosomes originally generated from hybridization between \textit{N. sylvestris} and either \textit{N. tomentosiformis} Goodspeed or \textit{N. otophora} Grisebach whose origins are all in South America (Lewis and Nicholson, 2007;
Kitamura et al., 2001). In plant research, *N. tabacum* has been used extensively as a model plant due to its high biomass, low maintenance and high seed production. In plant pathology, studies with *N. tabacum* have contributed to our understanding of the interaction between the tobacco mosaic virus (TMV) and the plant hosts, particularly in relation to the development of SAR (Ross 1961; Vallad and Goodman, 2004). Transformation protocols for *N. tabacum* are well established allowing for the development of transgenic plants with traits designed specifically for the study of plant-microbe interactions. Examples are *N. tabacum* cv. Xanthi containing bacterial gene coded for salicylate hydroxylase (*nahG*), which converts SA to catechol resulting in the inability of the plant to accumulate salicylic acid (Gaffney et al., 1993) and *N. tabacum* cv. Samsun containing a mutated *Arabidopsis etr-1* gene encoding a defective ethylene receptor resulting in a plant with ethylene insensitivity (Knoester et al., 1998). Transgenic *N. tabacum* has also been used to produce foreign proteins, such as anti-Hepatitis B virus surface antigen (HBsAg) (Ramirez et al., 2002), human interleukin-10 (Menassa et al., 2001), somatotropin (Staub et al., 2000) and the AMP, magainin 2 analog (DeGray et al., 2001). In this respect, an advantage of using *N. tabacum* includes existing large-scale processing technology and the fact that it is not a food/feed crop (Twyman et al., 2003).

**8.2 Nicotiana benthamiana**

*N. benthamiana* originated from Australia and is an amphidiploid species with 38 chromosomes (Goodspeed, 1954). *N. benthamiana* may have resulted from hybridization between *N. suaveolens* and *N. debneyi* (Goodspeed, 1954). *N. benthamiana* has been used extensively in the area of plant virology because of its susceptibility to a wide
variety of viral pathogens as a result of a mutation in its RNA-dependent RNA polymerase gene (Yang et al., 2004). As a result, it has become a model system for plant genetics as one can silence plant genes through the virus-induced gene silencing (VIGS) technique, and is especially useful for transiently expressing genes via agroinfiltration (Goodin et al., 2008). Furthermore, N. benthamiana can be transformed with high efficiency and easily maintained due to its short stature, short regeneration time and high seed production (Goodin et al., 2008).

8.3 Pseudomonas syringae pv. tabaci

P. syringae pv. tabaci is a gram-negative hemibiotrophic bacterium that is the causal agent of wildfire in N. tabacum but it can also infect other Nicotiana species (Shew and Lucas, 1991; Hann and Rathjen, 2007), soybeans and field beans (Nyvall, 1989). Wildfire disease of tobacco is likely to occur in seedbeds under wet conditions and can cause death in young seedlings. Field tobacco infected by P. syringae pv. tabaci through stomates or wounds start to develop water-soaked spots, which eventually become necrotic with characteristic chlorotic halos around the spots (Nyvall, 1989; Shew and Lucas, 1991). The presence of the chlorotic halo is a result of ammonia accumulation due to inhibition of glutamine synthetase by tabtoxin produced by P. syringae pv. tabaci (Thomas et al., 1983). A related strain of P. syringae pv. tabaci is P. syringae pv. angulata, which does not produce tabtoxin, but causes angular leaf spot of tobacco, which is less severe than wildfire (Nyvall, 1989; Shew and Lucas, 1991).

P. syringae pv. tabaci has been used to study plant-microbe interactions. Effectors from the type III secretion pathway, such as AvrPto and HrpW in P. syringae pv. tomato DC3000 (Ronald et al., 1992; Charkowski et al., 1998), AvrPphE/HopX from P.
syringae pv. phaseolicola (Mansfield et al., 1994; Lindeberg et al., 2005) have been examined. Furthermore, the genomic sequence of *P. syringae* pv. *tabaci* 11528 is available (Studholme et al., 2009). Sequence analysis showed that this strain contains for the most part genes similar to those found in previously sequenced *P. syringae* genomes of pv. *tomato*, *phaseolicola* and *syringae* but also contains unique sequences making up 5.72% of the predicted genes. *P. syringae* pv. *tabaci* 11528 is missing two of the core set of the Hrp (Hypersensitive response and pathogenicity) Outer Protein genes that are conserved in other sequenced *P. syringae* genomes, but it possesses three unique Hop effectors not found in any of the other sequenced *P. syringae* genomes, which could contribute to its difference from other pathovars. Availability of the genome sequences of *P. syringae* pv. *tabaci* allows for the identification of genes of probable impact on its host, such as type III secretion system effectors, making it a useful model for studying plant-microbe interactions.

8.4 Colletotrichum species

*Colletotrichum* is an ascomycete fungus that is the causal agent of anthracnose or blight in various plants including soybean, cowpea, alfalfa and tobacco (Nyvall, 1989; Perfect et al., 1999). Infection by *Colletotrichum* species is commonly initiated by direct penetration of the plant surface by means of appressoria (Bailey et al., 1992). Following penetration, infection by different *Colletotrichum* species assumes different modes (Bailey et al., 1992). Many *Colletotrichum* species are classified as intracellular hemibiotrophic pathogens characterized by a two-stage infection. Infections start with a symptomless biotrophic phase during the initial establishment of the pathogens in the plant, followed by a necrotrophic phase, when host cells are damaged and lesions are
visible. Other *Colletotrichum* species are classified as subcuticular intramural species. These are characterized by initial hyphal growth under the cuticle within the cell walls, followed by dissolution of the wall structure resulting in visible disease symptoms. Some *Colletotrichum* species exhibit a combination of both infection types. Their hemibiotrophic life style and ability to grow easily in culture makes several *Colletotrichum* species preferred model pathogens for studies of fungal-plant interactions (Perfect et al., 1999; Bailey et al., 1992).

*C. destructivum* and *C. orbiculare* are both intracellular hemibiotrophic species which cause anthracnose in *N. tabacum* and *N. benthamiana* (Shen et al., 2001a, Shen et al., 2001b). The main difference between *C. destructivum* and *C. orbiculare* is that during the biotrophic phase, *C. destructivum* hyphae are restricted to the initial infected epidermal cell, while *C. orbiculare* hyphae grow into the adjacent epidermal cells (Shen et al., 2001a, Shen et al., 2001b). As a result, *C. destructivum* has a biotrophic phase that lasts for only 60 h compared to *C. orbiculare* that has a biotrophic phase that lasts for 72-96 h. Hyphae of the necrotropic phase appear as thin secondary hyphae and develop from side branches and the tips of the biotrophic large primary hyphae for *C. orbiculare* and the tips of the biotrophic multi-lobed infection vesicles for *C. destructivum*. Eventually, masses of conidia borne in acervuli are released from the plant surface by splashing water.

In tobacco, anthracnose causes lesions, which appear as water-soaked spots on leaves (Shen et al., 2001a). These spots expand in wet weather, and the infected leaves later dry out and become wrinkled, distorted and may eventually die (Nyvall, 1989; Shew and Lucas, 1991). Anthracnose is primarily a seedling disease, and it can cause death in...
small seedlings under favorable conditions (Nyvall, 1989; Shew and Lucas, 1991). *C. destructivum* tolerates a wide temperature range (18 to 32°C) and prefers high humidity for disease symptoms to develop (Nyvall, 1989). Humidity is considered the major factor determining the appearance and development of tobacco anthracnose (Shew and Lucas, 1991).

Both SA and ET appear to have important roles during infection of *C. destructivum* and *C. orbiculare*. Infection with *C. destructivum* results in ET accumulation in *N. tabacum*, and *etr-1* tobacco that is ET-insensitive was more susceptible to *C. destructivum* than wild type plants (Chen et al., 2003). Infection by either *C. destructivum* or *C. orbiculare* induces the expression of ET-dependent plant defense genes in *N. benthamiana* (Dean et al., 2005). Application of (2R,3R) butanediol activated ET/jasmonic acid (JA)-dependent induced systemic resistance (ISR), induced ET-dependent gene expression and increased resistance to *C. orbiculare* (Cortes-Barco et al., 2010). Infection by either *C. destructivum* or *C. orbiculare* can induce the expression of SA-dependent plant defense genes in *N. benthamiana* (Dean et al., 2005), and application of acibenzolar-s-methyl (1,2,3-benzothiadiazole-7-thiocarboxylic acid-s-methyl-ester) activates SAR, induces SA-dependent gene expression and increases resistance to *C. orbiculare* (Cortes-Barco et al., 2010). Thus, infections by *C. destructivum* or *C. orbiculare* are a good model system for studying different types of induced resistance in plant-microbe interactions.
Chapter 2. Agroinfiltration induced resistance against *Collectotrichum destructivum*

1. Introduction

*Agrobacterium tumefaciens* is a gram-negative bacterium which causes crown gall disease in various plant species with symptoms of tumour-like growth of the infected tissues (Smith and Townsend, 1907). Virulent strains of *A. tumefaciens* contain a Ti-plasmid, which encodes virulence (Vir) proteins for processing, transfer and integration of the transferred DNA (T-DNA) into the plant genome (Stachel et al., 1985). The *vir* genes are normally not expressed except for a two component-regulatory system encoded by *virA* and *virG* that activates expression of the other *vir* genes after sensing phenolic compounds and sugars released by wounded plant cells (Stachel and Nester, 1986).

Processing of the T-DNA results in excision of single-stranded T-DNA (T strands) with the VirD2 protein attached to the 5’ end (Stachel et al., 1986; Herrera-Estrella et al., 1988). Together with VirD2 proteins, the T strands are delivered into a plant cell by a T4SS, which is regulated and encoded by 11 genes in the *virB* operon and the *virD4* gene (Christie, 2004). After the T-DNA is incorporated into the plant genome, oncogenes and opine genes on the T-DNA result in the production of the phytohormones auxin and cytokinin resulting in gall formation, and synthesis and secretion of opine into the apoplast (Klee et al., 1984; Lichtenstein et al., 1984; Guyon et al., 1980). The Ti-plasmid can be 'disarmed' by deletion of the tumour-inducing genes turning *A. tumefaciens* into a delivery system for transforming plants.

*A. tumefaciens* can be used to introduce foreign genes into plants through both stable and transient expression (Twyman et al., 2003). Stable transformation allows for expression of the gene of interest throughout the life of the transgenic plant, but the
process of creating a stably transformed plant is time consuming as the plant tissue has to
go through selection for the transgene, regeneration from callus and usually selection for
homozygous lines. Agroinfiltration is an alternative where transient transformation
occurs when a culture of *A. tumefaciens* containing an expression cassette with the gene
of interest is introduced directly into the plant leaves (Kapila et al., 1997). For
agroinfiltration, *A. tumefaciens* is grown in liquid culture, resuspended and incubated in a
buffer containing acetosyringone and then infiltrated into intact plant tissue by direct
injection with a syringe or vacuum infiltration (Kapila et al., 1997; Voinnet et al., 2003;
Wydro et al., 2006). The plant cells surrounding the infiltrated *A. tumefaciens* receive the
T-DNA, and multiple copies are integrated into the plant genome, as in stable
transformation, and/or remain as single T-DNA strands in the plant nucleus (Kapila et al.,
1997; Wydro et al., 2006; Pitzschke and Hirt, 2010). The foreign protein is then
expressed from the integrated or non-integrated T-DNA.

Since the transformed cells do not need to be regenerated into entire transgenic
plants, foreign gene expression can be detected in the infiltrated area within 2 days of
agroinfiltration (Kapila et al., 1997). This method has been utilized for many
applications, such as production of antibodies (Vaquero et al., 1999), infection of cloned
plant viruses (Annamalai et al., 2008), introduction of tagged proteins to detect their sub-
cellular localization and interaction (Goodin et al., 2002), expression of fungal avirulence
genes (such as *Avr9* and *Avr4*) and expression of plant resistance genes (such as *Cf-9* and
*Cf-4*) (Van der Hoorn et al., 2000).

Agroinfiltration can also affect plant-pathogen interactions. Robinette and
Matthysse (1990) reported that infiltration of tobacco leaves with *A. tumefaciens*
inhibited the hypersensitive response (HR) to *P. syringae pv. phaseolicola* and suggested that this was due to the presence of additional auxin produced from genes on the T-DNA. Agroinfiltrated tobacco leaves also had a reduced HR-associated necrosis due to avirulent *P. syringae pv. tomato* but were more resistant to infection by virulent *P. syringae pv. tabaci*, and these effects were purportedly due to interference by agroinfiltration with the typical *P. syringae*-elicited SA and abscisic acid (ABA) in the plant (Rico et al., 2010). Similarly, agroinfiltrated tobacco leaves were more resistant to subsequent infection by TMV, perhaps due to induction of SA-dependent defense responses (Pruss et al., 2008). Such changes may be related to alterations by *A. tumefaciens* of plant defense gene expression. Arabidopsis cell cultures inoculated with *A. tumefaciens* showed up-regulation and down-regulation of different sets of defense-related genes (Ditt et al. 2006). Similarly, defense-related proteins increased, while cell wall modifying proteins decreased in the *N. benthamiana* leaf apoplast after agroinfiltration (Goulet et al., 2010).

Activation of plant defenses by plant pathogenic bacteria can be due to recognition of PAMPs, and this form of resistance is known as PAMP-triggered immunity (PTI). Thus far, PAMPs identified in *A. tumefaciens* are elongation factor Tu (EF-Tu) (Zipfel et al., 2006), cold-shock protein (Felix and Boller, 2003) and peptidoglycan (Erbs et al., 2008). However, a typical PAMP in other plant pathogenic bacteria, flagellin, did not induce defense responses for *A. tumefaciens* when added to tomato tissue culture cells (Felix et al., 1999). PAMPs may be important in inducing plant gene expression by *A. tumefaciens* since defense gene up-regulation in *Ageratum conyzoides* cell cultures by *A. tumefaciens* was non-specific because it also occurred with *Escherichia coli* (Ditt et al., 2001).
Another form of resistance is effector-triggered immunity (ETI) that is activated by recognition of effectors that are normally secreted by pathogens to manipulate the host to promote development of disease. Effectors of *A. tumefaciens* secreted by the T4SS are VirD2 attached to the T-strands, VirD5, VirE2, VirE3 and VirF (Gelvin, 2010). Inside plant cells, VirD2 acts in nuclear localization of the T-complex (Howard et al., 1992), VirD5 contains a nuclear localization signal and eukaryotic DNA binding motif acting as a plant transcriptional activator to optimize T-DNA transport or integration (Schrammeijer et al., 2000), VirE2 forms a complex with the single T-strand for interaction with plant proteins and integration into the plant genome (Ward and Zambryski, 2001), VirE3 may interact with VirE2 to mediate its transfer to the nucleus as well as act as a plant transcriptional activator to induce gene expression for tumour formation (Lacroix et al., 2005; Garcia-Rodriguez et al., 2006), and VirF is an F-box protein tagging protein for proteolysis to remove proteins from T-complexes (Schrammeijer et al., 2001; Tzfira et al., 2004).

Although the roles of these T4SS effectors in plant cells are not well understood, all are believed to interact directly or indirectly with the T-DNA/VirD2 complex to facilitate its transport to the nucleus and integration into the genome (Gelvin, 2010). However, the T4SS effectors may also be important in affecting plant gene expression. Although defense genes in tobacco cell cultures were first activated upon exposure to *A. tumefaciens*, they were later suppressed during the transfer of T-DNA (Veena et al., 2003).

PTI and ETI can cause a localized response but can also induce a systemic response that is mediated by the signaling molecules of SA for SAR, and ET with JA for
ISR (Vallad and Goodman, 2004). Activation of these defense mechanisms could provide protection against subsequent infections by a broad range of microbial pathogens. Furthermore, such defense responses could also include priming, a process which potentiates the infected plant to be ready to ward off subsequent infection by other pathogens (Conrath et al., 2006)

To examine the effects of *A. tumefaciens* on induction of defense responses, agroinfiltration of *N. benthamiana* leaves was performed followed by inoculation with *C. destructivum*, the causal agent of anthracnose (Nyvall, 1989), to determine the timing of resistance, changes in plant gene expression, the localized versus systemic nature of the resistance, and the potential role of plant-signaling molecules. Factors from *A. tumefaciens*, such as PAMPs, T-DNA, T4SS and effectors, were examined to assess their contribution to inducing resistance, as well as the significance of this induced resistance to the use of agroinfiltration as a technique to test foreign genes, such as animal defensins, for their ability to increase plant disease resistance.

2. Materials and methods

2.1 Plant materials

Seedlings of *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Xanthi and cv. Samsun were transplanted 2 and 3 weeks respectively after germination to individual 6 x 8-cm pots, and grown at a constant temperature of 21 °C under artificial light with a 12-h photoperiod and photon flux density of 150 µmol/m²/s. Liquid fertilizer (20-8-20 (N-P-K)) was provided weekly thereafter until the plants were 5 to 7 weeks old. A mutant line, *N. tabacum* cv. Xanthi with *nahG*, that cannot accumulate salicylic acid (Gaffney et al.,
1993) and another mutant line, *N. tabacum* cv. Samsun, *etr-1*, that is insensitive to ethylene (Knoester et al., 1998) were grown under the same conditions.

2.2 Bacterial and fungal inoculations

The *A. tumefaciens* strains used in this study are listed in Table 2.1. To isolate the E2 strain from Euonymus, galls were collected from stems on a bush growing in Guelph, Ontario and surface sterilized with 20% bleach, then rinsed three times with sterile distilled water before being peeled and cut into approximately 2-mm pieces. The pieces were incubated in sterile distilled water for 1 h, and then the liquid was collected, diluted and plated onto LB, PDA (Bioshop, Burlington, ON), and Nutrient agar (Difco, Mississauga, ON) supplemented with 0.01% yeast extract (Bioshop). Plates were incubated at 28 °C for 48 h. Single colonies were streaked onto LB agar, and then again on LB until the colonies appeared uniform.

To test for virulence, selected bacterial colonies were grown at 28 °C for 2 days in liquid LB media, centrifuged at 4900 xg and resuspended at 2 x 10⁹ CFU/mL in 10 mM MgCl₂ with 150 µg/mL acetosyringone. The cell suspension was incubated at room temperature for 3 h, and 25 µL was pipetted onto a wounded leaf of *Kalanchoe daigremontiana*. Gall formation was monitored for 1 month after inoculation, and strain E2 was selected because it consistently formed galls on *K. daigremontiana*. To evaluate the level of virulence of *A. tumefaciens* strains A348, E2, UGAt-biotype I and UGAt-biotype II, *N. benthamiana* stems were wounded by needle and bacterial inoculum prepared as above was injected into the wound. Gall formation was monitored weekly, and gall fresh weight was determined 6 weeks after inoculation after excision from stems of three *N. benthamiana* plants.
Table 2.1 *Agrobacterium tumefaciens* strains used in this research. Wild-type/virulent strains are able to cause tumors. Disarmed strains are common laboratory strains used for transformation, and cannot cause tumors, but can transfer T-DNA. There is no binary vector in these described strains. Mutant strains were derived from either strain A348 or C58 and contain mutation in *vir* genes or genes involved in formation of the pilus or flagella.

<table>
<thead>
<tr>
<th>Category</th>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type/virulent</td>
<td>E2</td>
<td>Virulent on <em>Kalanchoe</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UGAt</td>
<td>Virulent on <em>Kalanchoe</em></td>
<td></td>
</tr>
<tr>
<td>Biotype I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UGAt</td>
<td>Virulent on <em>Kalanchoe</em></td>
<td></td>
</tr>
<tr>
<td>Biotype II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A348</td>
<td>C58 background, pTiA6NC Ti plasmid, virulent</td>
<td>Garfinkel et al., 1981</td>
</tr>
<tr>
<td>Dis-armed</td>
<td>C58C1</td>
<td>C58 background, pMP90 Ti plasmid, gentamycin resistant</td>
<td>Koncz and Schell., 1986</td>
</tr>
<tr>
<td></td>
<td>GV3101</td>
<td>C58 background, pMP90 Ti plasmid, gentamycin resistant</td>
<td>Holsters et al., 1980</td>
</tr>
<tr>
<td></td>
<td>LBA4404</td>
<td>Ach5 background, pAL4404 Ti plasmid, streptomycin resistant</td>
<td>Hoekema et al., 1983</td>
</tr>
<tr>
<td></td>
<td>EHA101</td>
<td>C58 background, Ti plasmids are derivative of pTiB6, pTiCl58, and pTiBo542, kanamycin resistant</td>
<td>Hood et al., 1993</td>
</tr>
<tr>
<td></td>
<td>EHA105</td>
<td>C58 background, Ti plasmids are derivative of pTiB6, pTiCl58, and pTiBo542, kanamycin resistant</td>
<td>Hood et al., 1993</td>
</tr>
<tr>
<td>Mutant</td>
<td>PC1000</td>
<td>A348 with deletion of the <em>virB</em> operon on pTiA6NC</td>
<td>Fernandez et al., 1996a</td>
</tr>
<tr>
<td></td>
<td>CB1001</td>
<td>C58 with deletion of the <em>virB1</em> on pTiC58; involved in pilus formation and T-complex transport but not absolutely required.</td>
<td>Schmidt-Eisenlohr et al., 1999b</td>
</tr>
<tr>
<td></td>
<td>CB1002</td>
<td>C58 with deletion of the <em>virB2</em> on the pTiC58 VirB2 is a major pilin subunit.</td>
<td>Aly et al., 2008</td>
</tr>
<tr>
<td></td>
<td>PC1004</td>
<td>A348 with deletion of <em>virB4</em> from pTiA6NC virB4 provides energy for T-complex transport.</td>
<td>Berger and Christie, 1993</td>
</tr>
<tr>
<td></td>
<td>CB1005</td>
<td>C58 with deletion of <em>virB5</em> on the pTiC58 virB5 is an extracellular component of the T-pilus</td>
<td>Schmidt-Eisenlohr et al., 1999a</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------</td>
<td></td>
</tr>
</tbody>
</table>
| PC1007   | A348 with deletions of *virB7*  
*virB7* is important for T-pilus assembly. | Berger and Christie, 1994 |
| WR5000   | A348 derivative with replacement of *virE2* with nptII gene from Tn5.  
*virE2* protein (together with *virD2*) forms complex with T-DNA and contains nuclear localization signal. Kanamycin resistant | Dombek and Ream, 1997 |
| CB2004   | C58 with deletion of the *virD4* on the pTiC58  
*virD4* protein is required for intercellular translocation but not involved in T-pilus biogenesis. | Aly and Baron, 2007 |
| PC2000   | A348 with a *tatC* null mutation; attenuated virulence, flagellation and mobility, but no affect on T-DNA transfer | Ding and Christie, 2003 |
| NT1REB   | C58 derivative with deletion of *flaABC*, contain pJK270 (pTiC58*traC*:Tn5) Ti plasmid  
Non-motile, no flagella (bald), erythromycin resistant | Chesnokova et al., 1997 |
For infiltration of *N. benthamiana* and *N. tabacum* leaves, *A. tumefaciens* strains were grown in liquid LB media overnight for 18 to 20 h at 28°C starting from stocks on LB agar. Subcultures were then transferred to fresh liquid LB media, grown overnight, and cells collected by centrifugation at 4900 xg for 10 minutes at room temperature. The bacteria were then washed twice with 10 mM MgCl₂ containing 150 µg/mL acetosyringone, and resuspended in the same solution used for the washing. Wild-type/virulent and disarmed strains of *A. tumefaciens* (Table 2.1) were adjusted to OD₆₀₀ = 1 (2 x 10⁹ CFU/mL), and mutant strains (Table 2.1) were diluted to 2 x 10⁶ cells/mL. The suspensions were incubated for 3 h at room temperature and then infiltrated with a needle-less syringe into the underside of the first fully matured leaf of a *N. benthamiana* plant until the entire leaf had a dark, water-soaked appearance.

*Colletotrichum destructivum*, strain N150, was grown on sodium chloride-yeast extract agar sucrose (SYAS) medium, consisting of 0.5% NaCl, 0.3% yeast extract, 2% agar, and 1% sucrose (Manandhar et al., 1986) under constant fluorescent light for 9 to 12 days to induce spore formation. Spores were scraped from the media surface with a spatula, suspended in sterile water and incubated for 20 min to allow media and hyphae to settle out. Spore concentrations were determined with a haemocytometer, adjusted to 1 x 10⁶ spores/mL for *N. benthamiana* and 5 x 10⁴ spores/mL for *N. tabacum*. The spore suspension was applied to leaves until run-off by an air pressure sprayer, and then plants were incubated under high humidity in the dark for 72 h. The numbers of lesions on each leaf were counted, and the leaf area was measured by a LI-3100 area meter (Li-Cor Inc., Lincoln, NE) to calculate the number of lesions per cm².
2.3 Induction of resistance to *C. destructivum* N150 by infiltration with *A. tumefaciens*

Three plants with one leaf per plant were infiltrated with each *A. tumefaciens* strain. The controls were non-infiltrated leaves and leaves infiltrated with 10 mM MgCl₂ and 150 µg/mL acetosyringone. After a period of 96 h after infiltration, leaves were inoculated with *C. destructivum*. The experiment was repeated 3 to 5 times. To test for systemic resistance, the first fully expanded *N. benthamiana* leaf was infiltrated with *A. tumefaciens* strains A348 and PC1000. At 4 or 7 days after infiltration, the leaf above the infiltrated one was inoculated with *C. destructivum* N150, and the number of lesions per cm² was determined. To test for localized resistance, half of the leaf lateral to the midvein was infiltrated with *A. tumefaciens* strains A348 or PC1000, and then 96 h later, the whole leaf was sprayed with *C. destructivum* N150. The number of lesion/cm² was determined for each half of the leaf by cutting the leaf along the midvein before using the LI-3100 area meter.

To evaluate the role of SA and ET in induced resistance by *A. tumefaciens* infiltration, the entire first fully expanded leaves of the *N. tabacum nahG* and *etr-1* mutant plants were each infiltrated with 2 x 10⁶ cells/mL of either *A. tumefaciens* strain A348 or PC1000. After 96 h, the infiltrated leaves were inoculated with *C. destructivum* N150, and 72 h later the number of lesions per cm² was determined.

2.4 Effect of infiltration with *A. tumefaciens* on leaf area and chlorophyll content

A suspension of 2 x 10⁶ CFU/mL of *A. tumefaciens* A348 or PC1000 was prepared as mentioned above and infiltrated into the lateral half of the first fully expanded leaf of *N. benthamiana*. The non-infiltrated half of the same leaf was used as
the control. The areas of both halves of the leaves were measured daily for 7 days by tracing the margin of the infiltrated and non-infiltrated halves of the leaf onto clear plastic sheets, which were later photocopied onto paper. The image of the leaf on the paper was excised and used for leaf area measurement with the LI-3100 area meter. Leaf colour was assessed each day by a chlorophyll meter (Soil Plant Analysis Development (SPAD)-502, Minolta, Osaka, Japan). An average of three readings from different areas on each side of the leaf was recorded. For each *A. tumefaciens* strain and control, one leaf per plant and three plants were used.

2.5 Gene expression

Infiltration with *A. tumefaciens* strains A348 or PC1000 was performed on *N. benthamiana* as previously described, but the entire leaf was infiltrated. One leaf each from two plants was collected at 0, 12, 24, 48, 72, 96 hpi. At 96 hpi, plants were inoculated with *C. destructivum* N150, and leaf samples were collected at 6, 24, 48 h after infection (102, 120, 144 hpi). Leaves from non-infiltrated plants and leaves infiltrated with 10 mM MgCl$_2$ and 150 µg/mL acetosyringone were also collected at the same time to serve as controls. All samples were stored at -80°C prior to RNA extraction.

For RNA extraction, approximately 100 mg of the leaf tissue was homogenized in 1 mL of TRIzol Reagent® (Invitrogen), and RNA extraction was performed according to the manufacturer’s instructions. The final RNA pellet was dissolved in 25 µL sterile DMPC (dimethyl pyrocarbonate)-treated water (1 mL DMPC dissolved in 50 mL of 95% ethanol plus deionized water to 1000 mL).
RNA quality was evaluated on a 1.2% formaldehyde-agarose gel and was quantified with a Spectramax a 384 Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). The RNA concentration was adjusted to approximately 1 μg/μL. RNA samples were then treated with RQ1 RNase-Free DNase A (Promega, Madison, WI) following the manufacturer’s instructions.

2.6 Relative RT-PCR

First strand cDNA synthesis was performed with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase according to the instructions provided by the manufacturer (Invitrogen, Burlington, ON). Approximately 3 μg of RNA was used for cDNA synthesis with 0.1 nmoles oligo (dT)$_{18}$ (Fermentas, Burlington, ON). The primers used for this study are listed in Table 2.2. Multiplex PCRs with two sets of primers were done with primers for the gene under study and primers for the $NbEF-1α$ as a constitutive control (Dean et al., 2002); 250 ng of cDNA and 1.5 units of Taq DNA Polymerase (New England BioLabs, Pickering, ON) were added to each 15-μL PCR reaction: 1x PCR buffer (Invitrogen), 2.33 mM MgCl$_2$, 0.33 mM dNTPs (Promega). Primer concentration was 0.67 μM of each primer, except for 0.33 μM TobefA and TobefS357 for $NbEF-1α$. A Tpersonal thermocycler (Biometra, Goettingen, Germany) was used for PCR with the lid temperature set at 105°C. The reaction was started with a denaturing step at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, an annealing temperature specific for each set of primers (Table 2.2) for 1 min, 72°C for 1 min and 5 s, and a final elongation step at 72°C for 5 min. As a control, PCR was also performed using TobefA and TobefS375 primers with RNA instead of cDNA as a template. PCR products were separated in a 1.2% agarose TAE gel, and after staining, the images were
Table 2.2 Primers used for relative RT-PCR of *N. benthamiana* genes in leaves infiltrated with *A. tumefaciens* strains. Primers sequences are obtained from Cortes-Barco et al., 2010. S= G and C, R= A and G

<table>
<thead>
<tr>
<th>Primer pairs and sequences</th>
<th>Co-amplification partner/annealing temperature</th>
<th>Target gene and product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>internal control-375</td>
<td>NbPR5/60°C</td>
<td><em>NbEF-1 α</em> 375</td>
</tr>
<tr>
<td>TobefA: 5'-CTTCGTGGTGCATCTCAAC-3’  TobefS375: 5'-CTCCAAGGCTAGGTATGATG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>internal control-700</td>
<td>NbPR1a, NbPR2, NbPR1b-1b /53°C or 60°C</td>
<td><em>NbEF-1 α</em> 700</td>
</tr>
<tr>
<td>TobefA: 5'-CTTCGTGGTGCATCTCAAC-3’  TobefS700: 5'-CAAGTATGCGCTGGTGCT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>internal control-700</td>
<td>NbPR1a, NbPR2, NbPR1b-1b /53°C or 60°C</td>
<td><em>NbEF-1 α</em> 700</td>
</tr>
<tr>
<td>NbPR1aF: 5'-TGGSATTTTCTCTTTTTCAC-3’  NbPR1aR: 5'-CCTGGAGGATCATAGTTGC-3’</td>
<td>internal control-700/53°C</td>
<td><em>NbPR1a</em> 478 bp</td>
</tr>
<tr>
<td>NbPR5F164: 5'-GGCAGGCAGCTCAACTCG-3’  NbPR5R817: 5'-CGAACAAGAGAATCTGACCAC-3’</td>
<td>internal control-375/60°C</td>
<td>Acidic <em>NbPR5</em> 653 bp</td>
</tr>
<tr>
<td>NbPR2F: 5'-CATCACAGGGTTCGTTTAGGA-3’  NbPR2R: 5'-GGGTTCCTTGTGTCTCATCA-3’</td>
<td>internal control-700/53°C</td>
<td><em>basic</em> <em>NbPR2</em> 442 bp</td>
</tr>
<tr>
<td>NbPR1b-1b-F20: 5'GTTCGTGTTTTTATTACCTTTGC-3’  NbPR1b-1b-R431: 5'-GGTGGATCATAATTGATGT-3’</td>
<td>internal control-700/60°C</td>
<td><em>NbPRb-1b</em> (TC7078) 411 bp</td>
</tr>
</tbody>
</table>
captured as tiff files for analysis by Scion Image software version 4.0.3.2 (Scion Corporation, MD). The expression levels of the gene of interest were determined by the band intensity of the gene of interest divided by the band intensity of the PCR product of NbEF-1α in the same lane.

3. Results

3.1 Induced resistance to *C. destructivum* N150 infection following infiltration with *A. tumefaciens* C58C1

Infiltration of the first fully mature leaf of *N. benthamiana* with *A. tumefaciens* C58C1 that is disarmed (i.e. lacking T-DNA but containing the Ti plasmid) caused the infiltrated leaf to curl downward and yellow slightly in 2 to 3 days following infiltration. Lesion numbers due to *C. destructivum* N150 infection relative to the non-infiltrated control gradually declined after infiltration, which became significant by 48 h after infiltration (Figure 2.1A and B). This reduction continued to increase up to 96 h post-infiltration, at which point the number of lesions was only 10% of the number on the non-infiltrated control. In comparison, the lesion number on leaves infiltrated with MgCl₂ was always approx. 80% of that of the non-infiltrated leaves. Based on these results, 96 h post-infiltration was the time point chosen to infect the leaves with *C. destructivum* N150 for subsequent experiments.

The lesion number following infection with *C. destructivum* was evaluated at 66 HPI (Figure 2.1A) or 72 HPI (Figure 2.1B). As lesions were easier to count at 72 HPI than at 66 HPI, lesions were counted at 72 HPI in all subsequent experiments.
Figure 2.1 Severity of *C. destructivum* N150 infection on *N. benthamiana* leaves which were previously infiltrated with *A. tumefaciens* C58C1 or MgCl₂ for 2, 24, 48, 72 and 96 h. Lesion number on infiltrated plants is presented as a percentage of lesion number on non-infiltrated control leaves assessed at 66 h (A) or 72 h (B) after N150 infection. The concentration of C58C1 used for infiltration was $2 \times 10^9$ CFU/mL, and N150 concentration was $1 \times 10^5$ spores/mL. Hour post infiltration is the time between when *N. benthamiana* leaves had been infiltrated with *A. tumefaciens* C58C1 or MgCl₂ and then inoculated with *C. destructivum* N150. Standard error bars were calculated from six replications. (— = C58C1 and — = MgCl₂)
3.2 Induced resistance to *C. destructivum* N150 by infiltration with wild type and disarmed strains of *A. tumefaciens*

Leaves infiltrated with wild-type strains (i.e. containing Ti-plasmid with oncogenic T-DNA) showed lesion numbers ranging from 28% to 77% of those found on the MgCl$_2$ control (Table 2.3). Leaves infiltrated with wild-type *A. tumefaciens* strain A348 became so necrotic that the level of infection with *C. destructivum* could not be evaluated. Leaves infiltrated with strain UGAt-biotype II had the highest number of lesions, and which was not significantly different from that of the MgCl$_2$ control. Infiltration with strains UGAt-biotype I and E2 significantly increased resistance to *C. destructivum* N150 compared to infiltration with MgCl$_2$.

To test for virulence, inoculation of *K. daigremontiana* leaves with strains A348 and E2 at 2 x 10$^9$ CFU/mL were performed, which resulted in visible galls by 14 days, but inoculation of UGAt-biotype I and II at the same concentration did not result in any visible galls even after 30 days. Stem inoculation of *N. benthamiana* also gave similar results with A348 producing visible galls in 1 week after inoculation, while strain E2 produced much smaller galls, which enlarged but remained smaller than those of A348 by 2 weeks after inoculation. UGAt-biotypes I and II produced small galls by 2 to 4 weeks after inoculation, similar in size to that of E2 at 1 week after inoculation. The galls produced by the UGAt-biotype II developed earlier and were larger than those produced by the UGAt-biotype I. The total fresh weight of the galls from three stems taken 6 weeks after inoculation was 2.72 g for strain A348, 0.59 g for strain E2, 0.054 g for UGAt-biotype I, and 0.302 g for UGAt-biotype II. According to the gall fresh weight,
Table 2.3 Severity of *C. destructivum* N150 symptoms on *N. benthamiana* leaves pre-infiltrated with wild-type strains of *A. tumefaciens* or pre-infiltrated with *A. tumefaciens* A348 or MgCl₂. *N. benthamiana* leaves were infiltrated with 2 × 10⁹ CFU/mL of each *A. tumefaciens* strain for 96 h before inoculation with about 1 × 10⁵ spores/mL of *C. destructivum* N150. The number of lesions per cm² leaf area was assessed 72 h after the infection. Non-infiltrated *N. benthamiana* leaves and *N. benthamiana* leaves infiltrated with MgCl₂ served as control treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm²</th>
<th>% of MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrated</td>
<td>11.3 a</td>
<td>135 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>8.7 b</td>
<td>100 b</td>
</tr>
<tr>
<td>UGAt-Biotype II</td>
<td>6.9 bc</td>
<td>77 bc</td>
</tr>
<tr>
<td>UGAt-Biotype I</td>
<td>5.2 c</td>
<td>61 c</td>
</tr>
<tr>
<td>E2</td>
<td>2.7 d</td>
<td>28 d</td>
</tr>
<tr>
<td>A348</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

1 Disease severity was measured as the number of lesions/cm² caused by *C. destructivum* N150.
2 Disease severity was expressed as the percentage of lesions/cm² compared to MgCl₂ control.
3 Data represent the average of four replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS (SAS Institute Inc., version 9.1.3, Cary, NC) Proc GLM at *p* = 0.05. ND - lesion count could not be determined after infiltration with *A. tumefaciens* A348 at a concentration of 2 × 10⁹ CFU/mL as the infiltration itself led to the development of leaf necrosis.
symptoms caused by strain E2, UGAt-Biotype I and II was found to be 22%, 2%, and 10%, respectively, of the gall fresh weight on stems infected with strain A348.

As *A. tumefaciens* strain A348 caused necrosis when infiltrated at $2 \times 10^9$ CFU/mL, different concentrations of *A. tumefaciens* strain A348 ($2 \times 10^9$, $2 \times 10^8$, $2 \times 10^7$, $2 \times 10^6$, $2 \times 10^5$, $2 \times 10^4$, $2 \times 10^3$, $2 \times 10^2$ CFU/mL) were infiltrated, which showed that yellowing and curling of the leaves as well as the amount of necrosis declined as the concentration of infiltrated bacteria decreased. Infiltration of this strain at $2 \times 10^6$ CFU/mL induced leaf curling and yellowing but did not produce necrosis. However, it still induced resistance to fungal infection, demonstrating that necrosis was not necessary for the induced resistance. At lower concentrations ($2 \times 10^5$ to $2 \times 10^2$ CFU/mL), induced resistance was not observed (data not shown). This concentration was used in further experiments with *A. tumefaciens* strain A348 and all mutant *A. tumefaciens* strains.

All leaves infiltrated with disarmed strains of *A. tumefaciens* showed significant reductions in the number of *C. destructivum* N150 lesions compared to the two controls (Table 2.4). Among these disarmed strains, C58C1 induced the highest level of resistance. These results indicate that the induced resistance did not require T-DNA to be transferred.

Agroinfiltration appeared to suppressed the hypersensitive response of *N. benthamiana* leaves that were infiltrated with *P. syringae pv. tomato* strain 06T2 resulting in a hypersensitive response within 24 h. Leaves of *N. benthamiana* infiltrated with *A. tumefaciens* strain A348 at $2 \times 10^6$ CFU/mL or strain C58C1 and strain PC1000, *virB* operon mutant, at $2 \times 10^9$ CFU/mL followed 4 days later by inoculation with
Table 2.4 Severity of *C. destructivum* N150 symptoms on *N. benthamiana* leaves pre-infiltrated with different disarmed strains of *A. tumefaciens*, which do not contain T-DNA on the Ti-plasmid, or pre-infiltrated with *A. tumefaciens* A348 or MgCl₂. *N. benthamiana* leaves were infiltrated with 2 x 10⁹ CFU/mL of each *A. tumefaciens* strain for 96 h before inoculation with about 1 x 10⁵ spores/mL of *C. destructivum* N150. The number of lesions per cm² leaf area was assessed 72 h after the infection. Non-infiltrated *N. benthamiana* leaves and *N. benthamiana* leaves infiltrated with MgCl₂ served as control treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm²</th>
<th>% of MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrated</td>
<td>9.8 a</td>
<td>109 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>9.1 a</td>
<td>100 a</td>
</tr>
<tr>
<td>EHA101</td>
<td>6.5 b</td>
<td>71 b</td>
</tr>
<tr>
<td>EHA105</td>
<td>5.9 b</td>
<td>66 b</td>
</tr>
<tr>
<td>LBA4404</td>
<td>4.9 c</td>
<td>52 c</td>
</tr>
<tr>
<td>GV3101</td>
<td>2.9 d</td>
<td>31 d</td>
</tr>
<tr>
<td>C58C1</td>
<td>2.1 d</td>
<td>22 d</td>
</tr>
</tbody>
</table>

1 Disease severity was measured as the number of lesions/cm² caused by *C. destructivum* N150.
2 Disease severity was expressed as the percentage of lesions/cm² compared to MgCl₂ control.
3 Data represent the average of three replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at *p* = 0.05.
*P. syringae* pv. *tomato* strain 06T2 resulted in a visual suppression of necrosis due to hypersensitive response so that only approximately 40 to 70% of the area was necrotic compared to complete necrosis in the infiltrated area of the MgCl2 infiltrated control (data not shown).

### 3.3 Induced resistance to *C. destructivum* N150 by infiltration with mutant strains of *A. tumefaciens*

Two PAMP mutant strains of *A. tumefaciens* were examined to determine if agroinfiltration-induced resistance in the leaves of *N. benthamiana* infiltrated with *A. tumefaciens* is dependent on certain PAMPs. These strains were PAMP mutants, NT1REB (*flaABC*), which has a deletion in genes involved in flagellum synthesis, (Chesnokova et al., 1997), and the attachment mutant, PC2000 (*tattc*), which has a mutation in the twin-arginine translocation (TAT) system required for flagella formation and chemotactic responses (Ding and Christie, 2003). These mutants induced the same level of resistance as wild-type strain A348 indicating that the flagella did not play a role in the induced resistance (Table 2.5).

In addition, mutants affecting the T4SS and the effectors that enter the plant cell through the T4SS were examined to determine if agroinfiltration-induced resistance in the leaves of *N. benthamiana* infiltrated with the bacteria is dependent on the T4SS. These mutants were derived either form strain C58 or A348 (Table 2.1). The T4SS related mutants were PC1000 with a deletion of the entire *virB* operon, which forms the structural components of T4SS (Fernandez et al., 1996a), CB2004 with a deletion of *virD4*, which encodes an ATPase necessary for substrate transport through the T4SS (Aly...
Table 2.5 Severity of *C. destructivum* N150 symptoms on *N. benthamiana* leaves pre-infiltrated with mutant strains of *A. tumefaciens* or pre-infiltrated with *A. tumefaciens* A348 or MgCl2. *N. benthamiana* leaves were infiltrated with about $2 \times 10^6$ CFU/mL of each *A. tumefaciens* strains or with the control treatments for 96 h before the treated leaves were infected with about $1 \times 10^5$ spores/mL *C. destructivum* N150. N150 lesions were assessed 72 h after the infection. Mutant strains used are either unable to transfer T-DNA or lacking known bacterial elicitors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm²</th>
<th>% of MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrated</td>
<td>10.1 a</td>
<td>130 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>8.3 b</td>
<td>100 bc</td>
</tr>
<tr>
<td>WR5000 (virE2)</td>
<td>8.7 ab</td>
<td>114 ab</td>
</tr>
<tr>
<td>PC1000 (virB)</td>
<td>8.6 ab</td>
<td>107 ab</td>
</tr>
<tr>
<td>CB2004 (virD4)</td>
<td>8.1 b</td>
<td>105 b</td>
</tr>
<tr>
<td>NT1REB (flaABC)</td>
<td>5.8 c</td>
<td>78 c</td>
</tr>
<tr>
<td>PC2000 (tatC)</td>
<td>4.5 c</td>
<td>53 d</td>
</tr>
<tr>
<td>A348</td>
<td>4.3 c</td>
<td>52 d</td>
</tr>
</tbody>
</table>

1 Disease severity was measured as the number of lesions/cm² caused by *C. destructivum* N150.
2 Disease severity was expressed as the percentage of lesions/cm² compared to MgCl₂ control.
3 Data represent the average of five replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at $p = 0.05$. 
and Baron, 2007), CB1001 with a deletion of \textit{virB1}, which participates in assembly of the T-pilus but is not required for substrate transport (Schmidt-Eisenlohr et al., 1999b), CB1002 with the deletion of \textit{virB2}, which encodes a major extracellular structural component of the T-pilus (Aly et al., 2008), PC1004 with a deletion of \textit{virB4}, which encodes an ATPase required for substrate translocation across T4SS and stabilization of other VirB proteins during T-pilus assembly (Berger and Christie, 1993), CB1005 with the deletion of \textit{virB5}, which encodes a minor extracellular structural component of the T-pilus (Schmidt-Eisenlohr et al., 1999a), and PC1007 with the deletion of \textit{virB7}, which encodes a lipoprotein of the bacterial outer membrane forming part of the extracellular component of T4SS required for T-pilus assembly and function (Berger and Christie, 1994; Fernandez et al., 1996b). Another mutant, WR5000, with a deletion of \textit{virE2}, which encodes an effector protein which passes through T4SS and interacts with T-DNA and host proteins (Dombek and Ream, 1997) was also tested. Most of these strains had been created from \textit{A. tumefaciens} strain A348 except for CB2004 and NT1REB, which were created from a wild-type C58 (Table 2.1). All leaves infiltrated with mutants in the \textit{virB} operon, \textit{virB2}, \textit{virB4}, \textit{virB5} and \textit{virB7} and \textit{virD4}, and the effector protein that passes through T4SS, \textit{virE2} lost the ability to induce resistance, and thus displayed the same levels of disease (expressed as the number of lesions/cm\(^2\)) as the non-infiltrated and MgCl\(_2\)-infiltrated leaves (Tables 2.5 and 2.6). However, the \textit{virB1} mutant, which is the only mutant not required for T4SS transport, did not lose the ability to induce resistance. This shows that the T4SS and at least one effector that passes through it are critical for induced resistance. This type of resistance was named T4SS-dependent agroinfiltration induced resistance.
Table 2.6 Severity of *C. destructivum* N150 symptoms on *N. benthamiana* leaves pre-infiltrated with different *virB* mutant strains of *A. tumefaciens*, strain A348 or MgCl₂. *N. benthamiana* leaves were infiltrated with about $2 \times 10^6$ CFU/mL of each *A. tumefaciens* strain or with the control treatments for 96 h before the treated leaves were infected with about $1 \times 10^5$ spores/mL *C. destructivum* N150. N150 lesions were assessed 72 h after the infection. *virB* mutant strains lack different elements for T-pilus assembly or T-DNA transfer.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm²</th>
<th>% of MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrated</td>
<td>9.9 a</td>
<td>124 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>8.0 b</td>
<td>100 a</td>
</tr>
<tr>
<td>PC1004 (<em>virB4</em>)</td>
<td>9.3 ab</td>
<td>120 a</td>
</tr>
<tr>
<td>PC1007 (<em>virB7</em>)</td>
<td>9.2 ab</td>
<td>118 a</td>
</tr>
<tr>
<td>PC1000 (<em>virB</em>)</td>
<td>8.6 ab</td>
<td>110 a</td>
</tr>
<tr>
<td>CB1005 (<em>virB5</em>)</td>
<td>8.5 ab</td>
<td>109 a</td>
</tr>
<tr>
<td>CB1002 (<em>virB2</em>)</td>
<td>8.1 b</td>
<td>102 a</td>
</tr>
<tr>
<td>CB1001 (<em>virB1</em>)</td>
<td>5.1 c</td>
<td>65 b</td>
</tr>
<tr>
<td>A348</td>
<td>4.1 c</td>
<td>52 b</td>
</tr>
</tbody>
</table>

1 Disease severity was measured as the number of lesions/cm² caused by *C. destructivum* N150.
2 Disease severity was expressed as the percentage of lesions/cm² compared to MgCl₂ control.
3 Data represent the average of four replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at $p = 0.05$. 

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3.4 Systemic versus localized induced resistance to *C. destructivum* N150 with *A. tumefaciens* in *N. benthamiana*

To test for systemic resistance, the upper leaves of the plant were inoculated with *C. destructivum* N150 at 96 or 168 h after a leaf at one to two leaf positions lower had been infiltrated with *A. tumefaciens* strains A348 or PC1000. The upper leaves displayed the same fungal lesion numbers regardless of the different treatments on the lower leaves indicating that the induced resistance was not systemic under the conditions tested (Table 2.7).

Infiltration was also performed on a half-leaf lateral to the midvein on a *N. benthamiana* plant 96 h prior to fungal inoculation. The numbers of lesion/cm² on the non-infiltrated half of a treated leaf were not different from untreated leaves or leaves treated with buffer or strain PC1000. In contrast, lesion numbers on the half of the leaf that was infiltrated with *A. tumefaciens* A348 were reduced by more than 50% (Table 2.8). These results show that the induced resistance is highly localized, not spreading across the mid-vein of the leaf by 96 h.

3.5 Effect of infiltration with *A. tumefaciens* on the appearance of *N. benthamiana* leaves

Infiltration of leaves of *N. benthamiana* showed that the size of the infiltrated half leaf was larger and more yellow than the non-infiltrated half, and infiltrated leaves curled downward (Figure 2.2). To quantify these changes, half leaves were infiltrated with *A. tumefaciens* strains A348, PC1000 or MgCl₂, and the leaf area and chlorophyll content were measured and expressed as percentages of the non-infiltrated half of the same leaf (Figures 2.3 and 2.4). The leaf halves infiltrated with *A. tumefaciens* strain PC1000 and
Table 2.7 Assessment of systemic spread of resistance induced by infiltration with *A. tumefaciens* strains A348 and PC1000. *N. benthamiana* leaves were infiltrated with 2 x 10^6 CFU/mL of each *A. tumefaciens* strain; control leaves were non-infiltrated or infiltrated with MgCl2. After infiltration, an upper leaf (one or two leaves above the infiltrated leaf) was infected with about 1 x 10^5 spores/mL *C. destructivum* and disease symptoms were evaluated 72 h after the infection.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm^2 (4 days)</th>
<th>Number of lesions/cm^2 (7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrated</td>
<td>10.7 a</td>
<td>5.8 a</td>
</tr>
<tr>
<td>MgCl2</td>
<td>9.6 a</td>
<td>6.1 a</td>
</tr>
<tr>
<td>PC1000</td>
<td>10.4 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>A348</td>
<td>9.6 a</td>
<td>5.4 a</td>
</tr>
</tbody>
</table>

1 Disease severity was measured as the number of lesions/cm^2 caused by *C. destructivum* N150.
2 Data represent the average of four replicates of lesions/cm^2 taken from upper leaf inoculated with *C. destructivum* N150 at 4 days after the lower leaf has been treated of four replicates.
3 Data represent the average of five replicates of lesions/cm^2 taken from upper leaf inoculated with *C. destructivum* N150 at 7 days after the lower leaf has been treated of four replicates.
4 Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at p = 0.05.
Table 2.8 Assessment of localized resistance within a leaf induced by infiltration with *A. tumefaciens* strains A348 and PC1000. One lateral half of *N. benthamiana* leaves were infiltrated with 2 x 10^6 CFU/mL of each *A. tumefaciens* strain; the other control halves were non-infiltrated. Four days after infiltration the whole leaves were inoculated with 1 x 10^5 spores/mL of *C. destructivum* N150 and disease symptoms were evaluated 72 h after the inoculation for each leaf side. Disease severity (lesions/cm^2) was evaluated for each treatment between the infiltrated and non-infiltrated halves. For the non-infiltrated control, both sides of the leaf were not infiltrated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lesions/cm^2 non-infiltrated</th>
<th>Lesions/cm^2 treated</th>
<th>Ratio lesions/cm^2 on untreated : lesions/cm^2 treated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infiltrated</td>
<td>5.6 a</td>
<td>5.9 a</td>
<td>100 b</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5.7 a</td>
<td>5.4 a</td>
<td>110 b</td>
</tr>
<tr>
<td>PC1000</td>
<td>5.7 a</td>
<td>5.1 a</td>
<td>110 b</td>
</tr>
<tr>
<td>A348</td>
<td>5.8 a</td>
<td>2.5 b</td>
<td>250 a</td>
</tr>
</tbody>
</table>

1 Disease severity was measured as number of lesions/cm^2 caused by *C. destructivum* N150.
2 Disease severity was measured as lesions/cm^2 and expressed as a percentage of the ratios between symptoms on the non-infiltrated side and symptoms on the infiltrated side.
3 Data represent the average of seven replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at \( p = 0.05 \).
Figure 2.2 Appearance of an *N. benthamiana* non-infiltrated leaf and leaf infiltrated with MgCl₂, or *A. tumefaciens* strains A348 or PC1000 at 7 days after infiltration. A = non-infiltrated control, B = whole leaf infiltrated with strain A348, C = whole leaf infiltrated with strain PC1000, D = half leaf infiltrated with MgCl₂, E = half leaf infiltrated with strain A348, F = half leaf infiltrated with strain PC1000. The infiltrated side is labelled.
Figure 2.3 Area of the treated half leaf relative to the non-infiltrated half leaf of *N. benthamiana*. One lateral half of the leaf was infiltrated with $2 \times 10^6$ CFU/mL of an *A. tumefaciens* strain or a control treatment (non-infiltrated or MgCl$_2$). The leaf area of each half of the leaf was measured each day over the period of 7 days after infiltration. Relative leaf area was calculated as (leaf area on treated side/ leaf area on non-infiltrated side of the same leaf) x 100%. Data represent the average of five replicates. Lines with significantly different slopes (b value) were designated with different letters according to Fisher’s protected LSD test. LSD was calculated from a standard error of 0.3063 generated by SAS Proc MIXED at $p = 0.05$. (Treatments were $\bullet = $ A348, $\square = $ PC1000, $\triangle = $ MgCl$_2$, $\times = $ non-infiltrated).
Figure 2.4 Estimated relative chlorophyll content of the treated half leaf relative to the non-infiltrated half leaf of *N. benthamiana*. One lateral half of each leaf was infiltrated with 2 x 10^6 CFU/mL of a *A. tumefaciens* strain or a control treatment (non-infiltrated or MgCl₂). Intensity of leaf colour was measured using chlorophyll meter on each half of the leaf each day over the period of 7 days after infiltration. Chlorophyll content was estimated from SPAD readings and calculated as (SPAD units on treated side/ SPAD units on non-infiltrated side of the same leaf) x 100% (♦ = A348, □ = PC1000, ▲ = MgCl₂, × = non-infiltrated). Data represent the average of four replicates. Lines with significantly different slopes (b value) were designated with different letters according to Fisher’s protected LSD test. LSD was calculated from a standard error of 0.3056 generated by SAS Proc MIXED at $p = 0.05$. (Treatments were ♦ = A348, □ = PC1000, ▲ = MgCl₂, × = non-infiltrated).
MgCl₂ underwent a slight decrease in size compared to the non-infiltrated control, but the two treatments were not different from each other (Figures 2.3). The leaf halves infiltrated with *A. tumefaciens* strain A348 were significantly larger in size over time than those of other treatments or the non-infiltrated controls. In addition, the chlorophyll content of the half leaves infiltrated with *A. tumefaciens* strain A348 showed a significant decrease over time compared to the non-infiltrated control (Figures 2.4). These results demonstrate that induced resistance is associated with leaf growth promotion and a loss of photosynthetic pigment.

3.6 Expression of genes associated with SAR and ISR following infiltration with *A. tumefaciens* and a subsequent infection by *C. destructivum* N150 in *N. benthamiana*

Expression of the SAR-related gene, *NbPR1a* (Cortes-Barco et al., 2010), in leaves infiltrated with *A. tumefaciens* strains A348 or PC1000 was similarly induced by 48 hpi and remained high until 96 hpi just prior to inoculation with *C. destructivum*. After *C. destructivum* N150 inoculation, the expression of *NbPR1a* decreased to the same level as the controls for both strains of *A. tumefaciens* (Figure 2.5). Expression of another SAR-related gene, acidic *NbPR5* (Cortes-Barco et al., 2010), showed a very similar pattern to that of *NbPR1a* (Figure 2.6).

The expression levels of the ISR-related genes, basic *NbPR2* and *NbPRb-1b* (Cortes-Barco et al., 2010), were induced by infiltration with *A. tumefaciens* strain PC1000 (Figures 2.7 and 2.8). However, infiltration with *A. tumefaciens* strain A348 did not induced expression of basic *NbPR2* and *NbPRb-1b* above the level of the two control treatments (Figures 2.7 and 2.8). After infection with *C. destructivum* N150, expression of basic *NbPR2* and *NbPRb-1b* were elevated in all *A. tumefaciens* treatments as well as
Figure 2.5 Expression of NbPR1a in response to infiltration with A. tumefaciens. Transcription of NbPR1a was measured between 0 and 96 h post infiltration (hpi) in leaves infiltrated with wild-type (A348) or virB (PC1000) mutant strain of A. tumefaciens compared to the untreated (control) and MgCl₂ infiltrated leaves. After 96 hpi, infiltrated leaves were infected with C. destructivum (shown by arrow), and NbPR1a expression was examined for evidence of priming. Each data point represents the average of 6 replications for the 0-96 hpi time points, and 4 replications for the priming experiment from 102 hpi to 144 hpi. Standard error bars are shown. Note that data for the expression of NbPR1a before and after C. destructivum infection were from different sets of experiments. Relative NbPR1a expression levels were determined in relation to those of nbEF-1α. Hours post infiltration are the times at which RNA samples for leaves were collected after infiltration. (○ = non-infiltrated, ▲ = MgCl₂, ▼=A348, ■= PC1000)
Figure 2.6 Expression of acidic NbPR5 in response to infiltration with *A. tumefaciens*. Transcription of acidic NbPR5 was measured between 0 and 96 h post infiltration (hpi) in leaves infiltrated with wild-type (A348) or *virB* (PC1000) mutant strain of *A. tumefaciens*, compared to the untreated (control) and MgCl2 infiltrated leaves. At 96 hpi, infiltrated leaves were infected with *C. destructivum* (shown by arrow), and acidic NbPR5 expression was examined for evidence of priming. Each data point represents the average of 6 replications for the 0-96 hpi time points, and 4 replications for the priming experiment from 102 hpi to the end of the experiment. Standard error bars are shown. Note that data for the expression of acidic NbPR5 before and after *C. destructivum* infection were from different sets of experiments. Relative acidic NbPR5 expression levels were determined in relation to *NbEF-1α* expression. Hours post infiltration are the times at which RNA samples for leaves were collected after infiltration. ( –– = non-infiltrated, – = MgCl2, =A348, = PC1000)
Figure 2.7 Expression of basic NbPR2 in response to infiltration with A. tumefaciens. Transcription of basic NbPR2, was measured between 0 and 96 h post infiltration (hpi) in leaves infiltrated with wild-type (A348) or virB (PC1000) mutant strains of A. tumefaciens, compared to the untreated (control) and MgCl2 infiltrated leaves. At 96 hpi, infiltrated leaves were infected with C. destructivum (shown by arrow), and basic NbPR2 expression was measured for evidence of priming. Each data point represents the average of 6 replications for the 0-96 hpi time points, and 4 replications for the priming experiment from 102 hpi to the end of the experiment. Standard error bars are shown. Note that data for the expression of basic NbPR2 before and after C. destructivum infection were from different sets of experiments. Relative levels of basic NbPR2 expression were determined in relation to the levels of NbEF-1α expression. Hours post infiltration are the times at which RNA samples for leaves were collected after infiltration. (○ = non-infiltrated, □ = MgCl2, ▲ = A348, ■ = PC1000)
Figure 2.8 Expression of NbPRb-1b in response to infiltration with A. tumefaciens. Transcription of NbPRb-1b was measured between 0 and 96 h post infiltration (hpi) in leaves infiltrated with wild-type (A348) or virB (PC1000) mutant strain of A. tumefaciens, compared to the untreated (control) and MgCl₂ infiltrated leaves. At 96 hpi, infiltrated leaves were infected with C. destructivum (shown by arrow), and NbPRb-1b expression was examined for evidence of priming. Each data point represents the average of 6 replications for the 0-96 hpi time points, and 4 replications for the priming experiment from 102 hpi to the end of the experiment. Standard error bars are shown. Note that data for the expression of NbPRb-1b before and after C. destructivum infection were from different sets of experiments. Relative levels of NbPRb-1b expression were determined in relation to NbEF-1α expression. Hours post infiltration are the times at which RNA samples for leaves were collected after infiltration. (  = non-infiltrated, = MgCl₂, = A348, = PC1000)
in the two controls (Figures 2.7 and 2.8). These results indicate that genes associated with SAR or ISR were not specifically increased in expression by the active inducer of resistance, *A. tumefaciens* strain A348, compared to non-inducers of resistance, *A. tumefaciens* strain PC1000, buffer and no infiltration.

3.7 Involvement of SA and ET as signalling molecules in induced resistance to *C. destructivum* by *A. tumefaciens* infiltration in *N. benthamiana*

Infiltration of wild-type *N. tabacum* cv. Xanthi leaves with strain A348 or PC1000 significantly reduced lesion numbers compared to the buffer control, but A348 induced resistance significantly more than PC1000 demonstrating that it was more effective with a T4SS (Table 2.9). Infiltration of *nahG N. tabacum* cv. Xanthi leaves with A348 or PC1000 also significantly reduced lesion numbers compared to the buffer control. Unlike in the wild type plants, however, the level of induced resistance in *nahG* plants no longer differed between A348 containing a T4SS and PC1000 missing a T4SS, demonstrating a loss of the greater effectiveness provided by a T4SS with greatly reduced SA (Table 2.9). Without agroinfiltration, there was an average of 4.9 versus 3.6 lesions/cm² in *nahG* and wild type *N. tabacum* cv. Xanthi, respectively, which were significantly different.

Infiltration of wild-type *N. tabacum* cv. Samsun leaves with A348 or PC1000 resulted in lower lesion numbers than in the buffer control, although A348 induced resistance significantly more than PC1000 just as in *N. tabacum* cv. Xanthi leaves (Table 2.10). For the *etr-1* cv. Samsun leaves, however, the lesion numbers of *C. destructivum* N150 on A348- and PC1000- infiltrated leaves were not significantly different from each other and from those on the buffer control indicating both strains were no longer able to
Table 2.9 Involvement of salicylic acid, as a signaling molecule for the SAR pathway, in the resistance induced by infiltration of *N. tabacum* leaves with *A. tumefaciens*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type²</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>3.4 a</td>
</tr>
<tr>
<td>PC1000</td>
<td>2.6 b</td>
</tr>
<tr>
<td>A348</td>
<td>1.9 c</td>
</tr>
</tbody>
</table>

¹ Leaves of wild-type *N. tabacum* (Xanthi) and *N. tabacum* unable to accumulate salicylic acid due to the presence of salicylate hydroxylase (*nahG*) which convert SA to catechol were infiltrated with 2 x 10⁶ CFU/mL of each *A. tumefaciens* strain or MgCl₂. At 96 h post infiltration, the infiltrated leaves, as well as non-infiltrated control leaves were infected with about 5 x 10⁴ spores/mL *C. destructivum*, and disease symptoms were evaluated 72 h after the infection.

² Data represent the average of five replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at *p* = 0.05.
Table 2.10 Involvement of ethylene, as a signaling molecule for the ISR pathway, in the resistance induced by infiltration of *N. tabacum* leaves with *A. tumefaciens*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of lesions/cm²</th>
<th>Wild-type²</th>
<th>etr-1²</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>3.4 a</td>
<td>3.9 a</td>
<td></td>
</tr>
<tr>
<td>PC1000</td>
<td>2.1 b</td>
<td>2.9 a</td>
<td></td>
</tr>
<tr>
<td>A348</td>
<td>1.5 c</td>
<td>3.2 a</td>
<td></td>
</tr>
</tbody>
</table>

¹ Leaves of wild-type *N. tabacum* cv. Samsun and ethylene insensitive *N. tabacum*, due to the presence of a mutated ethylene receptor gene (*etr-1*) were infiltrated with 2 x 10⁶ CFU/mL of each *A. tumefaciens* strain or MgCl₂. At 96 h post infiltration, the infiltrated leaves, as well as non-infiltrated control leaves were infected with about 5 x 10⁴ spores/mL of *C. destructivum* and disease symptoms were evaluated 72 h after the infection.

² Data represent the average of six replicates. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at *p* = 0.05.
induce resistance if ET could not be detected (Table 2.10). Without agroinfiltration, there was an average of 4.1 versus 3.5 lesions/cm² in etr-1 and wild type \textit{N. tabacum cv. Samsun}, respectively, which were not significantly different.

4. Discussion

Agroinfiltration of \textit{N. benthamiana} leaves with \textit{A. tumefaciens} C58C1 with or without a pig β-defensin gene (pbd-1) in the binary vector resulted in induced resistance to subsequent infection with \textit{C. destructivum}, and this masked any possible increase in resistance due to the pig β-defensin. Strain C58C1 is typical of strains commonly used in agroinfiltration in that it is disarmed, and cannot induce tumours, but is capable of gene transfer and utilizes a binary vector system, where the \textit{vir} region and the T-DNA region of the Ti-plasmid are separated into two vectors, a \textit{vir} helper plasmid and a binary vector (Hoekema et al., 1983; de Frammond et al., 1983; Lee and Gelvin, 2008). The induced resistance occurred rapidly as inoculation with \textit{C. destructivum} at the agroinfiltration site at 2 h after infiltration with \textit{A. tumefaciens} C58C1, resulted in a reduction in the number of lesion/cm² by 40%. Inoculation with \textit{C. destructivum} 4 days after agroinfiltration resulted in a 90% reduction. This is the first report of agroinfiltration-induced resistance against a fungus, \textit{C. destructivum}, but agroinfiltration with other disarmed strains have shown even greater induced resistance. Infiltration of \textit{A. tumefaciens} GV3101 reduced \textit{P. syringae pv. tabaci} populations in the agroinfiltrated area of tobacco leaves 2 days later by approximately 100-fold at 3 to 5 dpi, and 40-fold at 11 dpi, which coincided with reduced disease symptoms compared to the MgCl₂-infiltrated control (Rico et al., 2010). Agroinfiltration with \textit{A. tumefaciens} ASE followed by TMV inoculation in the
agroinfiltrated area 3 days later resulted in no TMV symptoms by 4 dpi (Pruss et al., 2008).

Perception of bacterial PAMPs, which are essential conserved microbial compounds recognized by plants (Nürnberger et al., 2004), or bacterial effectors, which are specific secreted molecules required for virulence, usually by suppression of plant defense responses (da Cunha et al., 2007), can lead to induced resistance in plants (Thomma et al., 2011). To determine what factors of *A. tumefaciens* were triggering induced resistance, wild-type, disarmed and mutant strains of *A. tumefaciens* were infiltrated into *N. benthamiana* leaves and inoculated 4 days later with *C. destructivum*.

Wild-type oncogenic strains of *A. tumefaciens* were able to induce different levels of resistance to *C. destructivum*, and the amount of induced resistance appeared to correlate with the virulence of the strain. Strain UGAt-biotype II did not produce galls on *K. daigremontiana* leaves, and only induced small gall formation on *N. benthamiana* stems. It also did not induce resistance against *C. destructivum*. In contrast, strains E2 and A348 produced the largest galls and induced the most resistance. Strain A348 was so virulent that it could not be used for infiltration at the same concentration as other wild-type strains without causing necrosis to *N. benthamiana* leaves. However, when diluted by 1000x, A348 could induce resistance without causing leaf necrosis.

Disarmed strains also induced resistance against *C. destructivum*, with strains GV3101 and C58C1 inducing the most resistance. The Ti-plasmid type was more important in determining the intensity of induced resistance than the chromosomal background. For example, strains GV3101 and C58C1, which both contain pMP90, induced similar levels of resistance, and strains EHA101 and EHA105, which both
contain pTiBo542, also induced similar levels of resistance. By comparison, strains, like EHA101, EHA105, GV3101 and C58C1, which all have a C58 chromosomal background, produced a wide range of induced resistance. As the Ti-plasmid contains all the *vir* genes, this implies that it is the *vir* gene products that are primarily responsible for the induced resistance in *N. benthamiana*.

However, it is possible that differences in levels of agroinfiltration-induced resistance in *N. benthamiana* are due to a particular Ti-plasmid placed in a particular chromosomal background. Different disarmed strains of *A. tumefaciens* have different transformation efficiencies (Akama et al., 1992; Wroblewski et al.; 2005, Wydro et al., 2006). For example, agroinfiltration of *N. benthamiana* leaves with the GUS expression vector, pTFS40, gave stronger GUS staining with strain C58C1 compared to strain GV3101 (Wroblewski et al., 2005). GFP expression in *N. benthamiana* leaves using pCAMBIA(gfp)1302 in strain LBA4404 yielded higher GFP activity than the same plasmid in strain EHA105 (Wydro et al., 2006).

The ability of disarmed strains to produce agroinfiltration-induced resistance has previously been reported. Infiltration with the disarmed *A. tumefaciens* strain GV3101, resulted in induced resistance against *P. syringae pv. tabaci* and suppressed the HR caused by *P. syringae pv. tomato* in *N. tabacum*, indicating that oncogenes were not required for agroinfiltration-induced resistance (Rico et al. 2010). *N. tabacum* leaves infiltrated with wild-type *A. tumefaciens* strain C58 or strain C58 with a T-DNA deletion gave comparable levels of resistance to TMV (Pruss et al., 2008). Furthermore, the addition of DNA between the left and right borders, such as kanamycin resistance genes, did not interfere with the ability to induce resistance to TMV (Pruss et al., 2008). These
results indicate that agroinfiltration-induced resistance does not require oncogenes in the T-DNA, and therefore disarmed strains should be effective. However, the presence of oncogenes was important for suppression of HR caused by *P. syringae* pv. *phaseolicola* in *N. tabacum* based on reduction of HR symptoms (Robinette and Matthysse, 1990).

Further support for a role for oncogenes is that *Arabidopsis* plants infected with wild-type *A. tumefaciens* strains C58 showed as much as four times more plant gene expression than when infected with the disarmed strain, GV3101, that had the same background as C58 (Lee et al, 2009a).

Agroinfiltration with the flagella mutant, NT1REB that lacks *flaA*, *flaB* and *flaC* genes and therefore cannot synthesize flagella (Chesnokova et al., 1997) or the twin-arginine translocation (TAT) mutant PC2000 that is missing the *tatc* gene important for flagellum formation and chemotactic responses (Ding and Christie, 2003) induced the same level of resistance as strain A348, which is in the same C58 background (Table 2.5). This suggests that the PAMP, flagellin, is not an inducer of resistance in *N. benthamiana*. Purified flagellin from *P. syringae* pv. *tabaci* triggered immunity in tomato, tobacco, potato and arabidopsis cell cultures, but purified flagellin from *A. tumefaciens*, which lacks the conserved flagellin sequence shared by other bacterial pathogens including different species of *Pseudomonas*, was not able to activate the same responses in tomato cell cultures (Felix et al., 1999). Therefore, it is not unexpected that agroinfiltration-induced resistance would not be activated by flagellin.

Infiltration with mutant *A. tumefaciens* strain PC1000 lacking the entire *virB* operon and four other *virB* genes, *virB2*, *virB4*, *virB5* and *virB7*, encoding proteins important for T-pilus structure and function (Christie, 2004) did not induce resistance to
*C. destructivum* (Table 2.5 and 2.6). This shows that the T-pilus is important for the induction of the induced resistance. However, a *virB1* mutant which lacks VirB1 protein did induce resistance to *C. destructivum* (Table 2.6). VirB1 contains two functional domains, an N-terminal lytic transglycosylase, which digests the bacterial cell wall to allow for insertion of the transmembrane component of T4SS, and a C-terminal soluble protein, which binds to VirB2 and VirB5 to facilitate their transport outside the bacterial cell and assembly into the T-pilus (Zupan et al., 2007). VirB1 is the only VirB protein that is not absolutely essential for T-pilus function (Berger and Christie, 1994). *VirB1* mutants are not completely avirulent and can still produce galls, but the galls are smaller and take much longer to develop compared to those produced by wild-type strains (Berger and Christie, 1994; Schmidt-Eisenlohr et al., 1999b). Therefore, the *virB1* mutant could still transfer T4SS effector proteins into plant cells that could be triggering agroinfiltration-induced resistance.

It is possible that agroinfiltration-induced resistance in *N. benthamiana* is due to the VirB proteins themselves that make up the T-pilus structure rather than the molecules that are transported through the T-pilus. To address this possibility, infiltration with *A. tumefaciens* strain CB2004 lacking the *virD4* gene (Aly and Baron, 2007) was tested, and it did not induce resistance to *C. destructivum* (Table 2.5). The *virD4* mutant produces a non-functional T-pilus, despite the presence of all VirB proteins (Aly and Baron, 2007). This indicates that agroinfiltration-induced resistance in *N. benthamiana* requires more than just the presence of the VirB proteins; it requires that they function as a T4SS so that T-DNA-VirD2, VirE2, VirE3, VirF, and VirD5 can be transferred into the plant cell (Gelvin, 2010). The T-DNA itself is not a likely inducer because agroinfiltration-induced
resistance in *N. benthamiana* was caused by disarmed strains lacking the T-DNA. VirD2 is also an unlikely inducer because transport of VirD2 through T4SS requires that it is bound and transported with T-DNA (Pitzschke and Hirt, 2010). Low levels of VirD2 may be found in plant cells without T-DNA, but the levels are so low that covalent binding of VirD2 to T-DNA appears to be normally required (Vergunst et al. 2005; van Kregten et al., 2009). Thus, the lack of T-DNA would block a significant flow of VirD2 into the plant cell. This suggests that the possible inducers passing into the plant cell via the T4SS are VirE2, VirE3, VirF or VirD5.

Infiltration with mutant *A. tumefaciens* strain WR5000 lacking *virE2* (Dombek and Ream, 1997) did not induce resistance to *C. destructivum* (Table 2.5) indicating that the VirE2 protein is required. VirE2 is a multifunctional protein that is transported independently of the T-DNA through the T-pilus, binding to the T-DNA once inside the plant cell, where it protects the T-DNA and directs the T-DNA complex to the plant nucleus (Lee et al., 1999a; Ward and Zambryski, 2001). In plant cells, VirE2 also interacts with several plant proteins to facilitate T-DNA transfer into plant genome. One of these is VirE2 interacting protein (VIP1), which is important for nuclear translocation of T-DNA complex (Tzfira and Citovsky, 2000). The possible direct interaction of VirE2 with VIP1 and other plant proteins makes it a likely candidate as an activator of plant defences.

It is possible that VirE3, VirF or VirD5 proteins, which also require T4SS for transport into plant cells, are also involved in agroinfiltration-induced resistance in *N. benthamiana* as these proteins may have complementary functions with each other and with VirE2 inside the plant cell. However, *virE3*, *virF* and *virD5* mutants were not
tested. VirE3 mimics the function of VIP1 by binding to VirE2 to partially complement VIP1 allowing the T-complex to interact with another plant protein, karyopherinα, thus allowing it to be transported to the plant nucleus in the absence of VIP1. VirE3 is needed because VIP1 is not an abundant plant protein, and being able to complement VIP1 allows *A. tumefaciens* to have a broader host range (Lacroix et al., 2005). Since there is a direct interaction of VirE3 with VirE2, it may be difficult to distinguish the effects of VirE3 on agroinfiltration-induced resistance separately from that of VirE2. VirF is regarded as a host range determination factor (Jarchow et al., 1991), possibly because it helps target proteolysis of VIP1 to destabilize VirE2 resulting in uncoating of T-complexes (Schrammeijer et al., 2001; Tzfira et al., 2004). Since VirF interacts with VIP1, which also interacts with VirE2, it could be difficult to distinguish the effect of VirE3 on agroinfiltration-induced resistance separately from that of VirE2. VirD5 is a homolog of plant transcription factor and contains a nuclear localization signal (Schrammeijer et al., 2000), but its role in infection is not well understood. Although it is not known to interact with any plant protein or other Vir protein, it could still be a trigger of agroinfiltration-induced resistance. While VirE2 is definitely required for induced resistance, it may either act alone or in conjunction with one or more of the other Vir proteins that enter the plant cell via the T4SS.

The mutant strains used in these experiments contain different Ti-plasmids, either pTiC58 derivatives (*virD4* mutant and *flaABC* mutant) or pTiA6NC (*virE2* mutant, *virB* operon mutant, *tata* mutant, and wild-type A348). However, the retention or loss of induced resistance in the mutants was clear, and therefore this did not appear to be a confounding factor.
Agroinfiltration-induced resistance appears to be a form of localized acquired resistance (LAR), which is defined as strong activation of defense responses in a specific area on a plant, such as around the area of HR (Dorey et al., 1997). Resistance induced by A348 was localized to the infiltrated area, whether this was the entire leaf or only a half leaf (Table 2.7 and 2.8). Induced resistance in *N. tabacum* leaves infiltrated with the disarmed strain GV3101 or wild-type strain C58 was also localized to the infiltrated tissue (Pruss et al., 2008). One of the better described forms of LAR occurs in tissue surrounding necrotic areas formed during HR. Cells of *N. tabacum* undergoing the HR release a signal to nearby cells inducing a localized increase in gene expression (Ghannam et al., 2005). While the LAR signal is unknown, it does not appear to be SA (Costet et al., 1999).

Agroinfiltration of *N. benthamiana* with all wild-type or disarmed strains resulted in enlargement of the infiltrated leaf area relative to the buffer-treated area causing leaf curling, and the agroinfiltrated area also became chlorotic. However, disarmed strains generally produced less chlorosis and increased leaf expansion compared to the wild-type strains. Except for the UGAt-biotype II, which produced these symptoms but did not significantly induce resistance, the severity of the leaf curling and chlorosis was directly correlated with the degree of induced resistance in *N. benthamiana*. For example, wild-type strain A348 induced a high level of these symptoms and resistance, while wild-type strain UGAt-biotype I induced a low level of these symptoms and resistance. The *virB* operon, *virB2*, *virB4*, *virB5*, *virB7* and *virE2* mutants, which were unable to induce resistance, did not produce these symptoms in *N. benthamiana*, while the *virB1* mutant that was able to induce resistance produced some mild symptoms.
Chlorosis and leaf curling were affected by the bacterial and plant genotype. Infiltration of wild-type A348 into leaves of *N. tabacum* cvs. Xanthi or Samsun produced less chlorosis than in *N. benthamiana*, and there was no increase in leaf expansion in *N. tabacum* unlike in *N. benthamiana*. However, infiltration with avirulent PC1000 did not produce noticeable chlorosis or leaf curling in *N. tabacum* cvs. Xanthi or Samsun, even though it induced resistance in both those cultivars like A348. This shows that chlorosis and leaf curling was not as tightly linked to induced resistance in *N. tabacum* as it was in *N. benthamiana*. Pruss et al. (2008) reported that infiltration with wild-type strain C58 or disarmed strain GV3101 produced chlorosis in *N. tabacum* cv. Xanthi NN leaves, but GV3101 inhibited leaf expansion and C58 did not affect leaf growth. Rico et al. (2010) did not report that infiltration with disarmed or wild-type strains produced chlorosis or affected *N. tabacum* cv. Xanthi leaf expansion, but possibly this was due to the small area of the leaf that was agroinfiltrated and then inoculated with *P. syringae* pv. *tabaci*, thus masking the effects of agroinfiltration.

When strain A348 was infiltrated in *N. benthamiana* at different concentrations, necrosis was observed at $2 \times 10^9$, $2 \times 10^8$ and $2 \times 10^7$ CFU/mL, but the severity of necrosis decreased as the infiltrated bacterial concentration was lowered. By $2 \times 10^6$ CFU/mL, only severe leaf curling and chlorosis was observed. For disarmed strain C58C1, mild leaf curling and chlorosis but no necrosis were observed even at $2 \times 10^9$ CFU/mL and mild symptoms to no symptoms were observed at $2 \times 10^6$ CFU/mL. Thus, the degree of leaf curling and chlorosis is dependent upon the concentration of bacteria infiltrated, and necrosis occurs at the highest concentrations.
To determine if SA played a role in agroinfiltration-induced resistance, expression of \( NbPR1a \) and acidic \( NbPR5 \), which are marker genes for SAR (van Loon and van Strien, 1999; Cortes-Barco et al., 2010) was monitored. While there was increased expression of both genes following infiltration with A348, which induced resistance, there was also increased expression with PC1000, which does not induce resistance.

A second approach to examine the role of SA was to infiltrate A348 or PC1000 into leaves of wild type \( N. tabacum \) cv. Xanthi or a mutant line, \( nahG \), which lacks the ability to accumulate SA and thus cannot induce SAR (Gaffney et al., 1993). Unlike in \( N. benthamiana \), both A348 and PC1000 induced resistance in \( N. tabacum \) cv. Xanthi as well as cv. Samsun, which was used as the control for the \( etr-1 \) mutant. The effectiveness of PC1000 shows that there was a T4SS-independent agroinfiltration-induced resistance in \( N. tabacum \). However, A348 was more effective in inducing resistance in wild type \( N. tabacum \) cv. Xanthi as well as wild type \( N. tabacum \) cv. Samsun leaves compared to PC1000 suggesting that there was also a T4SS-dependent agroinfiltration-induced resistance in \( N. tabacum \). In the \( nahG \) plants, while both strains could induce resistance with greatly reduced SA, the greater effectiveness of A348 relative to PC1000 was lost compared to wild type plants. This demonstrates that the T4SS-dependent agroinfiltration-induced resistance in \( N. tabacum \) relied to some extent on SA.

Previous reports provide contradictory evidence for a role of SA in agroinfiltration-induced resistance. Expression of \( PR-1 \) was induced in \( N. tabacum \) leaves infiltrated with wild-type and disarmed \( A. tumefaciens \), and therefore expression of SA-dependent defenses was thought to be important (Pruss et al., 2008). However, the
nahG mutant *N. tabacum* retained a degree of agroinfiltration-induced resistance, and they believed this was due to incomplete inhibition of SA accumulation in the nahG plants (Pruss et al., 2008). On the other hand, expression of *PR1a* or SA accumulation was not detected in *N. tabacum* leaves infiltrated with disarmed strain GV3101 alone despite its ability to induce resistance to *P. syringae pv. tabaci* (Rico et al., 2010). In fact, compared to MgCl₂ infiltration, agroinfiltration with GV3101 suppressed the usual increase in *PR1a* expression and SA accumulation that follows infection by *P. syringae pv. tabaci*. Rico et al. (2010) postulated that *PR1a* induction and SA accumulation did not occur due to agroinfiltration because they used a lower concentration of bacteria (1 x 10⁷ CFU/mL) compared to 8 x 10⁸ CFU/mL used by Pruss et al. (2008). In the experiments conducted for this thesis, infiltration with 2 x 10⁶ CFU/mL still induced expression of *NbPR1a*.

To determine if ET played a role in agroinfiltration-induced resistance, expression of basic *NbPR2* and *NbPRb-1b*, indicators of ISR (van Loon and van Strien, 1999; Cortes-Barco, et al., 2010) were monitored. Expression was strongly induced in PC1000-infiltrated leaves, but only slightly induced to not induced in A348-infiltrated leaves, even though both strains induced resistance. The lower induction of those genes when the T4SS was present in A348 indicates that T4SS effectors may be suppressing their expression. This is consistent with reports that successful transfer of T-DNA results in host defenses being suppressed (Veena et al., 2003, Ditt et al. 2005). However, the level of expression of these PR genes was not directly related to the level of induced resistance, as A348-infiltrated leaves had more rather than less induced resistance compared to PC1000-infiltrated leaves.
A second approach to examine the role of ET was to infiltrate A348 or PC1000 into leaves of the wild type *N. tabacum* cv. Samsun or a mutant line, *etr-1*, which is insensitive to ET (Knoester et al., 1998). In the *etr-1* plants, induced resistance was lost completely with infiltration of either A348 or PC1000. This indicates that a loss of ET perception eliminated the ability of strains containing or missing the T4SS to induce resistance. Thus, ET, but not SA, appears to be a significant factor in both agroinfiltration-induced resistance that is T4SS-independent and T4SS-dependent.

A role for ET in T4SS-dependent agroinfiltration-induced resistance may explain some of the symptoms of agroinfiltration in *N. benthamiana*. One of the functions of ET is to promote leaf senescence, which involves breakdown of chlorophyll (Gan and Amasino, 1997). Exposure to ET can also affect plant cell growth, such as swelling of pea stem tissues (Apelbaum and Burg, 1971). Thus, ET may be responsible for the localized chlorosis and enlargement observed in agroinfiltated leaves.

The T4SS-independent agroinfiltration-induced resistance in *N. tabacum* caused by PC1000 was unexpected, as it did not occur in *N. benthamiana*. As PC1000 is missing the entire *virB* operon and thus not able to secrete the effectors that pass through the T4SS, it is more likely that one or more PAMPs in PC1000 are being recognized by *N. tabacum* but not by *N. benthamiana*. This agrees with agroinfiltration inducing a degree of resistance in *N. tabacum* even when the entire Ti plasmid was absent (Pruss et al., 2008). It is possible that wild-type *N. tabacum* plants have Pattern Recognition Receptors (PRRs) that can recognize *A. tumefaciens* PAMPs that are not found in wild-type *N. benthamiana* plants. A prominent *A. tumefaciens* PAMP is EF-Tu (Kunze et al., 2004). Unlike *A. thaliana*, *N. benthamiana* does not possess a PRR for EF-Tu, but when
the PRR of *A. thaliana* for EF-Tu was expressed in *N. benthamiana*, it could induce PAMP responses indicating that only the appropriate PRR was lacking (Zipfel et al., 2006). Certain PAMPs are recognized only by a limited range of plants, such as EF-Tu and cold-shock protein, which are recognized only by members of the Brassicaceae and Solanaceae, respectively (Thomma et al, 2011). The PAMP of PC1000 possibly recognized by *N. tabacum* is unknown, but testing a wider range of plant species will determine if *N. tabacum* or *N. benthamiana* is more typical of the resistance induced by PAMPs and T4SS effectors of *A. tumefaciens*.

There is previous evidence that PAMPs contribute to agroinfiltration-induced resistance in *N. tabacum*. Infiltration of *N. tabacum* leaves with a heat-killed strain or a strain lacking Ti-plasmid induced a low level of resistance in tobacco against TMV, which was much less than with the disarmed strain (Pruss et al., 2008). Similarly, infiltration of *N. tabacum* leaves with a heat-killed strain or a strain lacking the Ti-plasmid was much less effective at suppressing SA synthesis elicited by *P. syringae* pv. *tabaci* than a disarmed strain (Rico et al., 2010). In addition, infiltration of arabidopsis leaves with supernatant from a crude cell extract of *A. tumefaciens* cells lysed by lysozyme induced resistance against subsequent infection by *P. syringae* pv. *tomato* DC3000 indicating that the T4SS was not required (Zipfel et al., 2004). This study is the first report of agroinfiltration-induced resistance in *N. benthamiana*, and since the loss of the T4SS eliminated agroinfiltration-induced resistance in *N. benthamiana*, it appears that agroinfiltration-induced resistance is entirely T4SS-dependent in that species.

One mechanism of induced resistance is to prime defense genes for rapid expression upon subsequent infection (Conrath et al., 2006). To test for gene priming,
gene expression was also measured in agroinfiltrated leaves of *N. benthamiana* following infection with *C. destructivum*. Expression levels of *NbPR1a* and acidic *NbPR5* were not elevated upon fungal infection and appeared to decline in most cases, showing that these genes were not primed by agroinfiltration. Expression of basic *NbPR2* and *NbPRb-1b* also increased following infection by *C. destructivum* in leaves infiltrated with buffer or agroinfiltrated with A348 or PC1000. Based on these results, priming is not involved in induced resistance against *C. destructivum* by agroinfiltration. However, in tobacco leaves agroinfiltrated with GV3101, there was priming of callose deposition following infection by *P. syringae* pv. *tabaci* (Rico et al., 2010). They found that ABA levels were lowered after agroinfiltration allowing for priming of callose deposition during infection by *P. syringae* pv. *tabaci* or pv. *tomato* (Rico et al., 2010).

An alternative explanation of agroinfiltration-induced resistance could be the competition for plant resources between *A. tumefaciens* and *C. destructivum* as both attempt to colonize a leaf. In addition, as *A. tumefaciens* is a biotrophic pathogen, which requires a living plant cell to proliferate, co-infection with a hemibiotrophic pathogen, such as *C. destructivum* (Shen et al., 2001a), may lead to a response in *A. tumefaciens* to limit growth of fungi or other pathogens which might colonize the galls. Whether *A. tumefaciens* exerted any direct antagonistic mechanism against *C. destructivum* was not investigated in this study.

In conclusion, agroinfiltration-induced resistance to *C. destructivum* in *N. benthamiana* is T4SS-dependent but does not require oncogenes in the T-DNA region. However, agroinfiltration-induced resistance to *C. destructivum* in *N. tabacum* has both T4SS-dependent and T4SS-independent components, and the latter may be due to PAMP
recognition. SA plays a role in the T4SS-dependent agroinfiltration-induced resistance, while ET plays a role in both T4SS-dependent and T4SS-independent agroinfiltration-induced resistance. ET could also be responsible for the morphological changes that correlated with the degree of resistance to *C. destructivum*. Gene markers for agroinfiltration-induced resistance in *N. benthamiana* were not found, but large scale changes in gene expression, both prior to or following pathogen challenge, would be likely.
Chapter 3. Expression of porcine β-defensin1 (pbd-1) in Nicotiana tabacum and Pichia pastoris

1. Introduction

Defensins constitute a class of antimicrobial peptides (AMPs) found in many organisms as part of their system of innate immune system, which provides a basal defense mechanism for the host against pathogens (Ganz, 2003b). To date, defensins have been demonstrated in plants, insects and vertebrates. Vertebrate defensins can be classified into α-, β-, and θ-defensins according to their structures. All classes of mammalian defensins contain six cysteine residues, which form three disulphide bonds (Bevins et al., 1999).

The β-defensin-1 gene, pbd-1, (GenBank accession number AF031666) from Sus scrofa, was isolated from cDNA of healthy porcine intestinal tissues and encodes a 64 AA prepro-peptide, consisting of a 20 AA signal sequence, a 6 AA prosequence and a 38 AA mature peptide (Figure 3.1; Zhang et al., 1998). The PBD-1 peptide is a cationic molecule with six conserved cysteine residues, whose predicted disulfide bond formation is the same as that found in other vertebrate β-defensins. Expression of pbd-1 in porcine tissues was most abundant in tongue epithelia with lower levels found in the respiratory and digestive tracts, as well as in some organs/tissues, such as spleen, lymph node, brain, liver, bladder, testis, skin and heart (Zhang et al. 1998). Following the discovery of PBD-1, another 12 putative porcine β-defensins were identified based on sequence homology to existing β-defensins (Sang et al., 2006; Sang and Blecha, 2009). These β-defensins appear to have different expression patterns from pbd-1 both temporally and spatially (Sang et al., 2006; Veldhuizen et al., 2006).
A.  

<table>
<thead>
<tr>
<th>signal peptide (20 AA)</th>
<th>Pro (6 AA)</th>
<th>mature peptide (38 AA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCCACCAGCATGAGACTCCACCGCCTCCTCCTTGTATTCTCCTCATGGTCTCTGTTACCT</td>
<td>Pro</td>
<td>mature peptide</td>
</tr>
<tr>
<td>GCCACCAGCATGAGACTCCACCGCCTCCTCCTTGTATTCTCCTCATGGTCTCTGTTACCT</td>
<td>Pro</td>
<td>mature peptide</td>
</tr>
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</table>

B.  

1    GCCACCAGCATGAGACTCCACCGCCTCCTCCTTGTATTCTCCTCATGGTCTGTTACCT  
    M R L H R L L L V F L L M V L L P  
     
61   GTGCCAGGTCTACTAAAAAACATAGGAAATCTCTGTTAGCTGTTAAGGAATAAAGGCCTG  
     V P G L L K N I G N S V S C L R N K G V  
     
121  TGTATGCCGGGCAAGTGTGCTCCAAAGATGAAACAGATCGGCACCTGTGGCATGCCAA  
     C M P G K C A P K M K Q I G T C G M P Q  
     
181  GTCAAATGCTGCAAAAGGAAGTAAAAAGAAAGTGAAGAAACAACCCCACAGATATGGCTC  
     V K C C K R K  
     
241  AGAAGCTGCTCCCTTGGAAAAGCATATAAAATTTAAACTAGATTTAAATCTTTGTTCAAGG  
     C  

Figure 3.1 Diagramatic representation of nucleic acid and amino acid sequence of native porcine β-defensin-1 (PBD-1). GenBank accession number AF031666.  
A. Structure of PBD-1 protein with the 20-amino acid signal peptide, 6-amino acid propeptide sequence, and 38-amino acid mature peptide.  
B. DNA and amino acid sequences of the 6-amino acid propeptide sequence and 38-amino acid mature peptide of PBD-1. Underlined amino acids indicate the putative signal sequence, and bold letters indicate the putative mature peptide.
The PBD-1 peptide has \textit{in vitro} antibacterial and antifungal activity against \textit{Escherichia coli}, \textit{Salmonella typhimurium}, \textit{Listeria monocytogenes} and \textit{Candida albicans} (Shi et al., 1999). In addition, it protected newborn piglets from lung infections by \textit{Bordetella pertussis} following \textit{in vivo} treatment with synthetic PBD-1 (Elahi et al., 2006). These results suggest that PBD-1 has broad antimicrobial activity and is effective as a therapeutic agent for certain animal diseases. Thus, it is a good candidate for expression in other eukaryotic species, such as fungi or plants for molecular farming of antibiotic peptides. Thus far, there are only a few studies that have attempted to express vertebrate defensins in plants, which include expression of the rabbit \alpha-defensin (\textit{NP-1}) in tobacco (Fu et al., 1998), poplar (Zhao et al.,1999) and tomato (Zhang et al., 2000), and expression of the human \beta-defensin 2 (\textit{hBD-2}) in \textit{Arabidopsis thaliana} (Aerts et al., 2007). The last is the only example where the transgenic plants expressing vertebrate \beta-defensin gene were tested with a plant pathogen, and the plants had increased resistance to \textit{Botrytis cinerea}. Typically, attempts to increase plant disease resistance utilize defensin genes from plants or insects (Table1.1).

Transgenic \textit{Nicotiana tabacum} cv. Xanthi plants carrying one of two modified versions of \textit{pbd-1} were generated by Atnaseo (2003). Both versions were modified by changing the DNA sequence of the mature peptide region of \textit{pbd-1} from the native pig codon usage to codons most commonly used in alfalfa (Ikemura, 1993). Both versions were also modified to incorporate a DNA sequence coding for six histidine residues at the C-terminus of PBD-1 to facilitate protein detection. One version, named the \textit{po2+pbd-1} construct, was altered in secretion of PBD-1 in the plant by fusing the \textit{pbd-1} sequence to a 250 bp portion of the 5’ end of \textit{po2} from alfalfa, which includes a 5’ UTR, secretion
signal sequence and a portion of the negatively charged domain of the mature PO2 peptide (Figure 3.2). The po2 gene codes for an alfalfa pollen-specific protein found in the pollen cell wall and has no homology to known allergens (Qiu et al., 1997). The exact role of PO2 protein is not known, but it may be involved in pollen germination. The rationale for fusing PO2 to the PBD-1 mature peptide was to add both a plant signal peptide and extra amino acids at the N-terminus of PBD-1 to help protect PBD-1 from degradation by plant proteases. The second version, named the pbd-1+pbd-1 construct, had the native pbd-1 signal peptide sequence and prosequence, without codon modification, fused with the codon usage-modified pbd-1 sequence for the mature peptide (Figure 3.3). The po2 and native pbd-1 signal peptides represent plant and animal secretion signal sequences, respectively, that may differ in their ability to direct the PBD-1 peptide to the ER for secretion to the apoplast.

For the T0 transgenic N. tabacum plants, pbd-1 mRNA was detectable in plants with either the po2 or native pbd-1 constructs, but the PBD-1 protein was not detected by western blots with the 6xhistidine tag antibody (Atnaseo, 2003). The T0 plants also appeared to have enhanced disease resistance against P. syringae pv. tabaci compared to the untransformed plants based on an evaluation of disease symptoms after bacterial inoculation. However, testing disease resistance in T0 plants is problematic, because it is difficult to obtain large numbers of plants for replicated inoculations or inoculations with other pathogens. A preferred approach is to work with T2 plants which are homozygous for the transgene. Such plants might also show increased levels of PBD-1 protein than the T0 plants as the homozygous T2 plants have at least two copies of the transgene, which can result in higher expression levels (James et al., 2002).
Figure 3.2 Construction of the binary vector pBIDef containing the po2+pdb-1 fusion for expression in N. tabacum cv. Xanthi. A. Components of the po2+pdb-1 fusion construct and locations of primers are indicated by arrows. This vector was used as a template for amplification of the po2+pdb-1 fusion construct for expression in P. pastoris X-33 with PDPiFo and Nt1PiRe1 primers indicated on the diagram. The 6xH indicates the six histidine tag fused to the end of the mature peptide. B. The DNA and amino acid sequences of the PO2+PBD-1 fusion. Restriction sites are in bold letters, underlined amino acid sequence represents putative PO2 signal peptide, amino acid sequence with bold letters represents the PBD-1 mature peptide, and amino acid sequence between the PO2 signal peptide and the PBD-1 mature peptide is the N-terminus portion of PO2 mature peptide.
Figure 3.3 Construction of binary vector pBISD containing the **pbd-1**+**pbd-1** fusion for expression in *N. tabacum* cv. Xanthi. **A.** Components of the **pbd-1**+**pbd-1** fusion construct and locations of primers are indicated by arrows. This vector was used as a template for amplification of the **pbd-1**+**pbd-1** construct for expression in *P. pastoris* X-33 with SDPiFo and Nt1PiRe1 primers indicated on the diagram. The 6xH indicates the six histidine tag fused to the end of the mature peptide. **B.** The DNA and amino acid sequences of the PBD-1+PBD-1 fusion. Restriction sites are in bold letters, underlined amino acid sequence represents the native PBD-1 signal peptide, amino acid sequence with bold letters represents the PBD-1 mature peptide, and amino acid sequence in between the PBD-1 signal peptide and mature peptide is a prosequence.
Producing defensins as therapeutic agents in fungi, such as *Pichia pastoris*, has been very effective (Cregg et al., 2000; Macauley-Patrick et al., 2005). The advantages of this approach are the easier genetic manipulation of *P. pastoris*, the potential to produce high levels of foreign protein intracellularly or extracellularly and with either a constitutive or inducible expression, and the ability to have eukaryotic protein modification such as glycosylation, and disulfide-bond formation (Cregg et al., 2000).

*P. pastoris* has been used for synthesis of different mammalian defensins, such as human α-defensin 5 (HD5) (Hsu et al., 2009), sheep β-defensin 1 (sBD-1) (Zhao and Cao, 2011), and PBD-1 (Jiang et al., 2006). Jiang et al. (2006) tested expression of secreted *pbd-1* in *P. pastoris* using a methanol-inducible promoter, and they only reported antimicrobial activity of PBD-1 against *Staphylococcus aureus*.

The objectives of this study were to evaluate the expression of an animal antimicrobial peptide in a plant and a fungus. Production of the protein was tested in both expression systems and resistance against plant pathogens was also examined in the plant.

2. Materials and methods

2.1 Generation of transgenic plants homozygous for *pbd-1* transgenes

Five T₀ *N. tabacum* cv. Xanthi plants transformed with *po2+pbd-1* construct and five plants transformed with *pbd-1+pbd-1* construct were chosen. This was based on the appearance of reduced *P. syringae* pv. *tabaci* symptoms of foliar chlorosis and necrosis compared to that on untransformed plants when the pathogen was infiltrated into leaves (Atnaseo, 2003). T₁ seeds were produced by selfing these plants, and 60 seeds were
plated onto 1/2 strength MS (Murashige and Skoog) media (Murashige and Skoog, 1962) with 300 µg/mL kanamycin. Ten surviving T₁ seedlings per line were screened by PCR using primers 17G-1 (5’-CCACTGACGTAAGGGATG-3’) and 19-3 (5’-CAACACATGAGCGAAACCC-3’), which were designed to amplify the region between the CaMV35s promoter and terminator as described below. PCR positive T₁ seedlings from each line were transferred to soil and bagged for self fertilization. 80 T₂ seeds per line were tested on 1/2-strength MS media with 300 µg/mL kanamycin to distinguish homozygous parental T₁ plants, which are identified by 100% survival of their T₂ seedlings. PCR was again performed to test for the presence of the transgene in two random T₂ seedlings per line using primers 17G-1 and 19-3. From these, one T₁ plant per line from the original 10 tested T₁ seedlings were selected as homozygous parents of the T₂ plants, which were used for all subsequent tests. In addition, RT-PCR using the sense primer DeF (5’-GATATCGACGCCAGAAAGAACAGTGTTAGTTGCC-3’) and antisense primer 19-3, which should amplify the region between the pbd-1 prosequence and the CaMV35s terminator was done for two randomly selected T₂ seedlings per line as described below. Western analysis of total protein extracted according to Lay et al. (2003) of 2 to 4 T₂ seedlings per line using the anti-histidine antibody was also done as described below. All subsequent experiments were conducted on homozygous T₂ plants grown from seeds derived from the original two set of five T₀ lines. To create the control line DC1, T₁ seeds were germinated on 1/2 strength MS media with 300µg/mL kanamycin, but before the susceptible seedlings completely died, they were transferred to new media without antibiotic so that they could recover. These
control plants, like those containing the transgene, had gone through the transformation and tissue culture processes, but they did not have the transgene because they were selected for loss of the transgene in the T1 generation.

For fungal and bacterial inoculation assays, T2 seedlings were transplanted 3 to 4 weeks after germination into individual 6 x 8-cm pots, and grown at 21 °C under artificial light with a 12-h photoperiod at a photon flux density of 150 µmol/m²/s. Liquid fertilizer (20-8-20) was provided weekly thereafter.

2.2 Detection of \textit{pbd-1} DNA in homozygous T2 lines

Genomic DNA was extracted from homozygous T2 lines using a protocol adapted from Edwards et al. (1991). Leaf tissue of approximately 100 mg was cut from the leaves and macerated in a 1.5-mL microtube with a homogenizer stirrer RZR 50 (Caframo, Wiarton, ON) at room temperature without buffer. Then, 400 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) was added to the ground tissue followed by a brief vortexing and a 1 h incubation at room temperature. The sample was centrifuged at 21,000 x g for 1 min, and 300 µL of supernatant was transferred to a new microtube containing 300 µL isopropanol. The sample was mixed and incubated at room temperature for 2 min before another centrifugation at 4°C at 21000 xg for 20 min. The supernatant was discarded, 500 µL of 70% ethanol was added, and the microtube was briefly vortexed to wash the DNA pellet. The sample was centrifuged at 21,000 xg for 4 min, and the ethanol was discarded followed by additional centrifugation at the same speed for 1 min. Excess ethanol was removed with a micropipette, and the DNA pellet was left to dry at room temperature. The DNA pellet was dissolved in 100 µL of TE buffer and stored at –20°C.
PCR using genomic DNA from homozygous T2 lines was performed with a sense primer 17G-1 from the CaMV35S promoter and an antisense primer 19-3 from the CaMV35S terminator, which gave a 695 bp product for the po2+pbd-1 fusion and a 475 bp product for the pbd-1+pbd-1 fusion. Each 20 µL PCR reaction was composed of 500 ng genomic DNA, 0.8 units of Taq DNA Polymerase (New England BioLabs, Pickering, ON), 1xThermoPol Reaction Buffer that included 2 mM MgSO4 (New England BioLabs), 0.2 mM dNTPs (Promega), and 0.2 µM of each primer.

2.3 Detection of pbd-1 RNA in homozygous T2 lines

RNA extraction and cDNA synthesis were performed as previously described in Chapter 2. PCR was performed with a sense primer DeF (5’-GATATCGACGCGCCAAGAAGACAGTGTTAGTGCC-3’) from the mature peptide sequence of pbd-1 and an antisense primer 19-3 from the CaMV35S terminator, which gave a 242 bp for both constructs. Each PCR reaction of 20 µL contained 250 ng of cDNA, 0.8 units of Taq DNA Polymerase (New England BioLabs), 1 mM MgCl2, 0.2 mM dNTPs (Promega) and 0.2 µM of each primer in 1x PCR buffer (Invitrogen). PCR for both genomic DNA and cDNA was performed with a Tpersonal thermocycler (Biometra, Goettingen, Germany) with the lid temperature set at 104°C and a denaturing step of 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. PCR products were analyzed on a 1% agarose TAE gel.

2.4 Detection of PBD-1 protein in the homozygous T2 lines

Protein extraction was performed according to Lay et al. (2003); 600 µL of protein extraction buffer (50mM Tris-HCL, pH 8.0, 10 mM EDTA and 0.5 M NaCl) was
added to 300 mg of leaf tissue ground in a mortar and pestle. The samples were mixed for 1 min using a vortex and centrifuged at 21000 xg for 30 min before the supernatant was transferred to a new tube. Protein concentration was estimated using the Bio-Rad protein assay with bovine γ-globulin as a standard (Bio-Rad, Mississauga, ON).

Tricine-SDS-PAGE was performed according to Schagger (2006). Aliquots of 30 to 80 μg of total soluble protein per lane was electrophoresed in duplicate on 16% polyacrylamide gels for approximately 30 min at 30 V followed by 3 to 5 h at 70 to 90 V in the Mini-Protean II electrophoresis system (Bio-Rad). Protein extracts from non-transformed \textit{N. tabacum} cv. Xanthi and the transformed rescued line (DC1) were negative controls, and the 6xHis Protein Ladder (Qiagen, Mississauga, ON) was a positive control for western analysis. One gel was visualized by silver staining following the method by Schagger (2006). Proteins from the other gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in a transfer buffer consisting of 25 mM Tris-base, 192 mM glycine, 20% methanol, pH 8.3 (Towbin, et al., 1979) using the Mini Trans-Blot transfer cell (Bio-Rad).

For immunoblot analysis, the PVDF membrane was first incubated in a blocking solution (3% skim milk in TBS, 20 mM Tris-base, 500 mM NaCl, pH 7.5) for 1 h with gentle agitation. The membrane was then washed twice with TTBS (TBS with 0.05% Tween 20) for 5 min followed by incubation overnight at 4°C in a monoclonal anti-histidine antibody (Bioshop) (1:10000 dilution). The membrane was washed again with TTBS as mentioned above followed by incubation with anti-mouse IgG alkaline phosphatase (Bioshop) (1:5000 dilution) for 1 h at room temperature. All antibodies were diluted in 1% skim milk in TTBS. After incubation with the secondary antibody,
the TTBS wash was repeated with an additional wash with TBS. The membrane was developed in the alkaline phosphatase developing buffer (0.1 M Tris base, 0.5 mM MgCl₂, pH 9.5) with 300 μg/mL NBT (nitro blue tetrazolium chloride) and 150 μg/mL BCIP (5-promo-4-chloro-3-indolylphosphate p toluidine salt) (Roche, Indianapolis, IN) according to the manufacturer’s instructions.

2.5 Fungal inoculations

*Colletotrichum destructivum* strain N150 and *C. orbiculare* strain ATTC 20767 were grown on sodium chloride-yeast extract agar sucrose (SYAS) medium, consisting of 0.5% NaCl, 0.3% yeast extract, 2% agar and 1% sucrose (Manandhar et al., 1986) under constant fluorescent light for 10 to 14 days to induce spore formation. Spores were scraped from the media surface with a spatula, suspended in sterile water and incubated for 20 min to allow media and hypha to settle out. For inoculation of 5 to 6-week-old plants, *C. destructivum* strain N150 was adjusted to 5 x 10⁴ spores/mL and *C. orbiculare* strain ATTC 20767 was adjusted to 1 x 10⁵ spores/mL in sterile water using a haemocytometer. Spore suspensions were sprayed onto whole plants using an air pressure sprayer, and then incubated under high humidity in the dark for 72 h. The two first fully expanded leaves from each of two plants of each line were taken for lesion counts, and the leaf area was measured by a LI-3100 area meter (Li-Cor Inc. Lincoln, NE) to calculate the number of lesions/cm². The experiment was repeated 3 to 6 times.

2.6 Bacterial inoculations

*P. syringae* pv. *tabaci* isolate 11528R was grown on King’s B media (King et al., 1954) (20 g/L peptone (Bioshop, Burlington, ON), 1.5 g/L K₂HPO₄, 1.5 g/L MgSO₄.7 H₂O, 10 mL/L glycerol and 15 g/L agar) at 28°C for 48 h. Bacterial cells were scraped
from the plate and resuspended in appropriate assay buffers for dip or infiltration inoculation.

A dip inoculation technique was adapted from Wei et al. (2007). Briefly, 3 to 4 week-old homozygous T2 seedlings (about three-leaf stage) were removed from the soil, submerged in a 1.2 x 10^8 CFU/mL of *P. syringae* pv. *tabaci* 11528R suspension in water with 0.02% Silwet L-77 (Witco Corp., Greenwich, CT), and swirled for 5 minutes before being replanted into soil. Seedlings were kept at high humidity 1 day before inoculation and for the first week after inoculation by filling the tray with water and covering the tray with a transparent lid. Twelve plants were used per experiment, and the experiment was repeated three times. The number of dead seedlings was evaluated weekly for four weeks.

For infiltration inoculation of 5 to 6 week old plants, the first fully mature leaf of a T2 plant was infiltrated with 1.2 x 10^4 CFU/mL of *P. syringae* pv. *tabaci* 11528R according to DeGray et al. (2001). After 1 to 4 days, two leaf discs (total area of 1.27 cm^2) from the infiltrated area of each plant were homogenized in 300 μL 10 mM MgCl_2 in the 1.5 mL microfuge tube, transferred to a larger tube, and then 5 mL 10 mM sodium phosphate buffer pH 7 was added (DeGray et al., 2001). A series of 10-fold dilutions was done up to a dilution of 1:1000 using the same buffer, and 100 μL of each dilution was plated on King’s B media and incubated at 28°C overnight before counts were taken from the dilution plates that yielded 30 to 300 CFU. Two plants were used per time point per experiment, and the experiment was repeated five times.
2.7 Construction of Pichia pastoris expression vectors

To create the constructs in P. pastoris, the po2+pbd-1 or pbd-1+pbd-1 fusion regions were amplified from the plasmids, pBIDef and pBISD that had been used to generate the transgenic tobacco (Figure 3.2 and 3.3). Therefore, two constructs were made for P. pastoris, one from pBIDef and one from pBISD. The construct derived from pBIDef was named po2+pbd-1. It encodes theSaccharomyces cerevisiae α-factor secretion peptide, followed by the first 56 amino acids of the mature peptide of PO2 fused with PBD-1 mature peptide (Figure 3.4). The construct derived from pBISD was named pbd-1+pbd-1. It contained α-factor signal peptide sequence, followed by the pbd-1 prosequence and sequence for pbd-1 mature peptide (Figure 3.5). The PO2 signal peptide and native PBD-1 signal peptide sequences were not included in either of these P. pastoris X-33 expression constructs because the α-factor signal sequence contained in the pGAPZα A vector (Invitrogen, Burlington, ON) is used for secretion by P. pastoris.

To create the po2+pbd-1 construct, PCR was performed with a sense primer PDPiFo (5’-GAATTCATTAATTGTTTGGACATAACAA-3’) located in the 5’ end of po2 mature peptide sequence (Figure 3.1) and the antisense primer Nt1PiRe1 (5’-TCTAGATTTAGTGATGGATGATGG-3’) located in the 6x his-tag region. To create the pbd-1+pbd-1 construct, PCR was performed with sense primer SDPiFo (5’-GAATTCCTACTAAAAACATAGGAACGTTAGT-3’) located in the 5’ end of pbd-1 prosequence (Figure 3.2) and the antisense primer Nt1PiRe1. Underlined portions of the primers indicate an EcoRI site for primers PDPiFo and SDPiFo and an XbaI site for primer Nt1PiRe1 to facilitate insertion into the P. pastoris X-33 expression vector.
Figure 3.4 Plasmid construct for po2+pbd-1 expression in P. pastoris X-33. A. Components of the po2+pbd-1 fusion construct and location of primers. The po2+pbd-1 fusion fragment was amplified from pBIDef plasmid using PDPiFo and Nt1PiRe1 primers, which contained EcoRI and XbaI sites to accommodate insertion into pGAPZα expression vector in frame with the α-factor signal peptide. B. The DNA and amino acid sequences of the PO2+PBD-1 fusion. Restriction sites are in bold letters, underlined DNA sequences are primer sites. Amino acid sequence in bold letters is the putative PO2+PBD-1 fusion peptide. Arrows indicate possible signal peptide cleavage sites.
Figure 3.5 Plasmid construct for pig defensin (PBD-1) expression in *P. pastoris* X-33.

A. Components of the *pdb-1+pdb-1* and location of primers. Sequence for *pdb-1* prosequence and mature peptide were amplified from pBISD plasmid using SDPiFo and Nt1PiRe1 primers, which contained *Eco*R1 and *Xba*I sites to accommodate insertion into pGAPZα expression vector in frame with the α-factor signal peptide. B. The DNA and amino acid sequences of the *pdb-1+pdb-1* expression construct. Restriction sites are in bold letters, the underlined DNA sequences are primer sites. The amino acid sequence in bold letters is a putative PBD-1+PBD-1 peptide. Arrows indicate possible signal peptide cleavage sites.
pGAPZα A (Invitrogen). The pGAPZα A vector was generously provided by Dr. P. Pauls, Dept. Plant Agriculture, University of Guelph, Guelph, ON.

PCR was performed in a 50-µL reaction mixture of 1x High Fidelity PCR buffer, 0.2 mM dNTPs (Promega), 2 mM MgSO4, 0.2 µM of each primer, 1 unit of Platinum® Taq DNA polymerase High Fidelity (Invitrogen) and 50 ng of template DNA. A Tpersonal thermocycler (Biometra) was used for PCR with the lid temperature set at 104°C and a denaturing step of 95°C for 2 min, followed by 25 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. PCR products were evaluated on a 1% TAE gel and the fragment was purified from the gel using the QIAquick gel extraction kit (Qiagen, Mississauga, ON). The PCR product and expression vector pGAPZα A were cut with EcoRI and XbaI and checked by electrophoresis with a 1% agarose TAE gel. The expected digested pGAPZα A fragment and digested PCR product were excised from the agarose gel and purified with the QIAquick gel extraction kit (Qiagen). Ligation was achieved with T4 DNA ligase (Invitrogen) incubated overnight at room temperature. The ligation product was used to transform competent E. coli strain DH5α by means of heat-shock at 42°C for 90 s (Sambrook et al., 1989). Cells were selected on low-salt LB media (1% tryptone, 0.5% NaCl, 0.5% yeast extract (Bioshop) supplemented with 25 µg/mL Zeocin (Invitrogen).

Recovered colonies were tested by colony PCR using pGAP forward (5’-GTCCCTATTTCAATCAATTGAA-3’) and AOXI reverse (5’-GCAAATGGCATTCTGACATCC-3’) primers, both located on pGAPZα vector locations that were upstream and downstream, respectively, of the inserted fragment. Cells from each colony were removed with a pipette tip and added to a 20 µL PCR
reaction mixture composed of 1 unit of *Taq* DNA Polymerase, 1xThermoPol Reaction Buffer including 2 mM MgSO₄ (New England BioLabs), 0.2 mM dNTPs (Promega), and 0.2 μM of each primer and PCR was performed with a Tpersonal thermocycler (Biometra) with the lid temperature set at 104°C and a denaturing step of 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. The PCR products were verified by DNA sequencing (Laboratory Services, University of Guelph, Guelph, ON).

2.8 *Pichia pastoris* transformation

Plasmid DNA was purified from the *E. coli* clones containing the desired sequences and linearized by digestion with *AvrII* (Fermentas). The linearized plasmid DNA was used for *P. pastoris* X-33 transformation by electroporation following the protocol provided for pGAPZ products (Invitrogen). The *P. pastoris* X-33 culture was a generous gift from Dr. C. Hall, School of Environmental Sciences University of Guelph, Guelph, ON. Electroporation of *P. pastoris* X-33 was performed with a MicroPulser Electroporator (BioRad, Mississauga, ON) to introduce the linearized plasmid DNA into *P. pastoris* cells where it can be stably integrated into the *P. pastoris* genome via homologous recombination. Colonies were selected on Yeast Extract Peptone Dextrose Medium (YPD-1% yeast extract, 2% peptone, 2% dextrose, and 2% agar for solid medium) plates supplemented with 100 μg/mL Zeocin (Invitrogen). Plates were incubated at 30°C for 2-5 days, and the recovered colonies were transferred to a fresh plate. Selected clones were verified by PCR with the PDPiFo and *AOX1* primers for the *po2+pbd-1* construct and the SDPiFo and *AOX1* primers for the *pbd-1+pbd-1* construct. PCR was performed as mentioned above for screening *E. coli* colonies.
P. pastoris X-33 cells containing the po2+pbd-1 or pbd-1+pbd-1 construct were cultured overnight in 5 mL YPD broth supplemented with 100 µg/mL Zeocin. Subcultures were made from the overnight culture at a 1:1000 dilution in a fresh YPD broth without Zeocin. These P. pastoris X-33 cultures were grown for 3 days at 28 °C and 250 rpm. Then, the P. pastoris X-33 suspension was centrifuged at 3000 xg for 5 to 10 min. To purify the defensin proteins which contained a poly-His tag from the supernatant, the supernatant was mixed with the Ni-NTA agarose (Qiagen) at a ratio of 50 mL supernatant per 400 µL of the Ni-NTA agarose. The mixture was incubated overnight at 4 °C with gentle agitation. The resin from 100 mL supernatant was collected in a 10 mL Poly-Prep column (BioRad) and washed twice with 2 mL wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The polyhistidine-tagged proteins were eluted four times with 1 mL elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The four eluates were checked for the presence of the poly-His tagged proteins by 16% Tricine SDS-PAGE (Schagger, 2006) and western blot as described above using the histidine tag antibody. All eluted solutions containing the desired proteins were combined, the salt removed, and the protein concentrated using Amicon Ultra Centrifugal Filters with a 3000 MW cutoff (Milipore, Billerica, MA) following the manufacturer’s instructions. The elution buffer was exchanged with water by reconstituting the concentrate with the same volume of water and repeating the centrifugation. This process was repeated three times to remove NaCl and imidazole from the protein solution, and the concentrated protein was collected after the final centrifugation. Partially purified PBD-1 protein was filtered through a 0.22 µm
Ultrafree-MC Centrifugal Filter (Milipore) and stored at -20 °C. The protein concentration was estimated with Bio-Rad Protein assay using bovine γ-globulin as a standard (BioRad).

2.10 PBD-1 protein analysis by slot and western blots

For slot blots, a PVDF membrane was assembled onto the Hybrid-Slot™ Manifold (BRL, Gaithersburg, MD) after being soaked in methanol. As a vacuum was applied to the manifold, 100 µL of TSB was loaded in each slot followed by 200 µL of YPD broth that had P. pastoris X-33 growing in it for 3 days. After the YPD broth was drawn through the PVDF membrane, an additional 200 µL aliquot of TSB was added to the slot to wash the membrane. The membrane was then removed from the manifold and placed in blocking solution (3% skim milk in TBS) for 1 h with gentle agitation. Immunoblot analysis was performed as mentioned above for transgenic plants. Tricine SDS-PAGE and western analysis was performed as mentioned previously for the transgenic plants.

2.11 Peptide and PBD-1 protein identification

Desalted protein extracts partially purified by Ni-NTA agarose were separated on tricine-SDS-PAGE and stained with 0.1% Coomassie blue R250 (Bio-Rad), 40% methanol, and 10% acetic acid overnight and destained in 5% methanol, 7% acetic acid until proteins bands were visible. Protein bands were excised and sent to The Advanced Protein Technology Centre (APTC) of The Hospital for Sick Children, Toronto, ON, where in gel digestion, LC MS/MS and analysis of LC MS/MS data were conducted to identify the components of each band. See Appendix A for more detail.
2.12 Antifungal activity of PBD-1 protein produced in \textit{P. pastoris}

Conidia of \textit{C. destructivum}, strain N150 and \textit{C. orbiculare}, strain ATTC 20767 were collected as previously mentioned and adjusted to $1 \times 10^5$ spores/mL in SYAS broth (0.5\% NaCl, 0.3\% yeast extract and 1\% sucrose). Filtered-sterilized partially purified PBD-1 protein was adjusted to 25, 50, 100, 200 and 400 $\mu$g/mL in sterile water. The assay was adapted from Broekaert et al. (1990) and van der Weerden et al. (2008) and was performed in 96-well plates (Sarstedt, Montreal, QC). In each well, 50 $\mu$L of spore suspension was mixed with 50 $\mu$L of PBD-1 protein, but in control wells, only 50 $\mu$L sterile water was used. The contents were mixed by the plate shaker inside a SpectraMax Plus 348 Microplate Reader (Molecular Devices, Sunnyvale, CA), and the plate was incubated at 25 $^\circ$C until the OD$_{595}$ of the water control reached a value of 0.2 (approximately 40 to 50 h depending on strain) before the OD$_{595}$ readings were taken on the other wells.

2.13 Antibacterial activity of PBD-1 protein produced in \textit{P. pastoris}

Bacterial species used for the antibacterial activity assay of PBD-1 protein are listed in Table 3.1. All bacteria were cultured in TSB (3\% tryptic soy broth, Sigma-Aldrich, Oakville, ON), except for \textit{Clavibacter michiganensis} subsp. \textit{michiganensis} which was grown in media 802 of NBRC (NITE Biological Resource Center, Chiba, Japan), containing 1\% polypeptone (BioBasics, Markam, ON), 0.2\% yeast extract (Bioshop) and 0.1\% MgSO$_4 \cdot 7$H$_2$O, pH 7. With the exception of \textit{P. aeruginosa} K799 and \textit{Escherichia coli} UB1005, which were cultured at 37$^\circ$C, all other bacteria were cultured at 28$^\circ$C. After overnight growth at 250 rpm, 50 $\mu$L of the \textit{E. coli} UB1005 suspension or
Table 3.1 List of bacterial strains used to test antimicrobial activity of PBD-1 (Chapter 3) or Ntdef1 (Chapter 4) produced by *P. pastoris* X-33.

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em> strain UB1005, and <em>Pseudomonas aeruginosa</em> strain K799</td>
<td>Dr. R. Hancock, Dept. Microbiology and Immunology, University of British Columbia, Vancouver, BC</td>
</tr>
<tr>
<td><em>Erwinia carotovora</em> subsp. <em>carotovora</em> Ecc</td>
<td>Linda Veldhuis, School of Environmental Science, University of Guelph, Guelph, ON</td>
</tr>
<tr>
<td><em>Xanthomonas axonopodis</em> pv. <em>phaseoli</em> strain UG18</td>
<td>Dr. G. Perry, Dept. Plant Agriculture, University of Guelph, Guelph, ON</td>
</tr>
<tr>
<td><em>Agrobacterium tumefaciens</em> strain A348</td>
<td>Dr. P. J. Christie, Dept. of Microbiology and Molecular Genetics, University of Texas, Houston, TX</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> strain 342</td>
<td>Dr. D. Monje Johnston, Dept. Plant Agriculture, University of Guelph, Guelph, ON</td>
</tr>
<tr>
<td><em>P. chlororaphis</em> strain B21, <em>Bacillus subtilis</em> strain GB03, and <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> Cmm</td>
<td>Coralie Sopher, School of Environmental Science, University of Guelph, Guelph, ON</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tomato</em> strain 06T2, <em>P. syringae</em> pv. <em>tabaci</em> strain 11528R, <em>P. syringae</em> pv. <em>tabaci</em> strain ZA Patrick <em>P. aeruginosa</em> strain PA01,</td>
<td>Dr. P.H. Goodwin, School of Environmental Science, University of Guelph, Guelph, ON</td>
</tr>
</tbody>
</table>
100 µL of the other bacterial species were transferred to 50 mL of the appropriate broth and temperature. For *E. coli* UB1005, *E. carotovora* subsp. *carotovora*, *P. aeruginosa* K799, *P. aeruginosa* PA01, *P. syringae* pv. *tabaci* ZA Patrick, *K. pneumonia* 342, *X. axonopodis* pv. *phaseoli* UG18, *P. chlororaphis* B21, and *B. subtilis* GB03, subcultures were grown for 3 to 5 h to an OD$_{600}$ of 0.3 to 0.4 at 250 rpm. For *C. michiganensis* subsp. *michiganensis*, *A. tumefaciens* A348, *P. syringae* pv. *tomato* 06T2, and *P. syringae* pv. *tabaci* 11528R, subcultures were grown for 15 to 18 h to an OD$_{600}$ of approximately 1.0 as it took over 7 h to reach an OD$_{600}$ of 0.3 to 0.4. After reaching the expected density, bacterial cells were centrifuged at 2300 x g for 5 min and washed with 10 mM sodium phosphate buffer pH 7.4. Cell concentrations were adjusted to approximately $10^6$ CFU/mL in 1% TSB in 10 mM sodium phosphate buffer pH 7.4.

PBD-1 protein was diluted in 10 mM sodium phosphate buffer pH 7.4, and 3 µL of each bacterial culture was added to 27 µL of PBD-1 protein to give final concentrations of 500, 300, 100, 25 µg/mL PBD-1 in 500 µL microtubes (Diamed, Mississauga, ON). The tubes were incubated in a water bath for 3 h at 28 or 37°C, depending on the strain. The assay mixture was then diluted in 10 mM sodium phosphate buffer pH 7.4 before being plated on TSA (Tryptic Soy Agar, TSB with 1.2% agar) and grown for 18 to 48 h at the appropriate temperature for that organism. Bacterial growth was evaluated by dilution plating and counting CFUs. The experiment was repeated at least 3 times for each bacterial strain. Within each replicate, three plates were counted for each protein concentration inoculated with the bacterial suspension.
3. Results

3.1 Developing T2 plants homozygous for pbd-1 transgenes

Five po2+pbd-1 construct T0 lines, 7, 9, 14, 22 and 24, and five pbd-1+pbd-1 construct T0 lines, 9, 11, 12, 13, and 15, were selected for generating seed based on the preliminary resistance test results of the decrease in disease symptoms caused by P. syringae pv. tabaci PBIC4. From the screening of kanamycin resistant T1 seedlings, seven to ten seedlings testing positive for DNA of pbd-1 by PCR from each of the five po2+pbd-1 lines or five pbd-1+pbd-1 lines were chosen as parents. Eighty T2 seeds collected from each of these plants were again screened on kanamycin to select for 100% survival in order to identify their parents as homozygous. One T1 homozygous plant from each of the original T0 transformed lines was selected based on consistent positive PCR results for DNA of pbd-1 and 100% survival on kanamycin of its T2 progenies. For the po2+pbd-1 construct, the chosen homozygous T1 lines were 7-5, 9-8, 14-5, 22-6 and 24-6, and for pbd-1+pbd-1 construct, the chosen homozygous T1 lines were 9-4, 11-3, 12-7, 13-4 and 15-1. The T2 seed from these T1 parental lines were used in all experiments hereafter and are referred to by the name of their T1 parent line.

All plants, except for line 14-5 of the po2+pbd-1 construct, appeared morphologically normal like non-transgenic plants. Line 14-5 displayed a stunted stem, thickened small rugose leaves with altered vein patterns, fewer flowers and fewer seeds. Some flowers appeared stunted with misshaped ovules resulting in reduced or no seed production from some flowers. PCR and RT-PCR test confirmed the presence of the pbd-1 gene constructs and their expression in all 10 selected lines (Table 3.2, Figure 3.6 and 3.7).
Table 3.2 Screening of the five selected homozygous lines of *N. tabaccum* Xanthi expressing *po2+pbd-1* or *pbd-1+pbd-1* for the presence of the transgene by PCR and its expression by RT-PCR. PCR was performed on genomic DNA using 17G-1 and 19-3 primers. For RT-PCR, cDNA was synthesized from oligo (dT)\textsubscript{18} and PCR was performed using primers 19-3 and a defensin specific primer DeF.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Line</th>
<th>Presence of <em>pbd-1</em> tested by PCR</th>
<th>Expression of <em>pbd-1</em> tested by RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO2+<em>pbd-1</em></td>
<td>7-5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>9-8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>14-5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>22-6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>24-6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBD-1+<em>pbd-1</em></td>
<td>9-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>11-3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12-7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>13-4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>15-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>controls</td>
<td>Xanthi</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DC1</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\) The specific tobacco lines were selected on the basis of reduced visual disease symptoms in the T\(_0\) plants inoculated with *P. syringae pv. tabaci* strain PBIC4. The five T\(_0\) lines showing the least symptoms were selected. The T\(_2\) homozygous tobacco plants were selected on the basis of kanamycin resistance. DC1 is a line which has gone through the transformation and tissue culture selection process but does not contain the transgenic construct.
Figure 3.6 The presence and expression of pbd-1 transgenes in selected T₂ homozygous plants of N. tabacum cv. Xanthi as determined by PCR and RT-PCR. A. PCR analysis of tobacco plants transformed with the fusion po2+pbd-1. The PCR reaction was performed with primers 17G-1 and 19-3, which produced an expected product of 695 bp indicated by an arrow. B. The RT-PCR reaction with primers DeF and 19-3, which gave the expected product of 242 bp indicated by an arrow. Wild type cv. Xanthi (Xan) and a transformed rescued line (DC1) were negative controls, and the pBIDef plasmid was a positive control (+). M represents FastRuler Middle Range DNA ladder, and the selected plants were 7-5, 9-8, 14-5, 22-6, and 24-6 of the T₂ homozygous lines of N. tabacum cv. Xanthi containing the po2+pbd-1 fusion construct.
**Figure 3.7** The presence and expression of *pbd-1* transgenes in selected T2 homozygous lines of *N. tabacum* cv. Xanthi as determined by PCR and RT-PCR. **A.** PCR analysis of tobacco plants transformed with the *pbd-1+pbd-1*. The PCR reaction was performed with primers 17G-1 and 19-3, which produced an expected product of 475 bp indicated by the arrow. **B.** Results from the RT-PCR reaction with primers DeF and 19-3, which gave the expected product of 242 bp indicated by arrow. Wild type cv. Xanthi (Xan) and a transformed rescued line (DC1) were negative controls, and the pBISD plasmid was a positive control (+). M represents FastRuler Middle Range DNA ladder, and the selected plants were 9-4, 11-3, 12-7, 13-4, and 15-1 of the T2 homozygous lines of *N. tabacum* cv. Xanthi containing the *pbd-1+pbd-1* construct.
Western blots of leaves from the T2 plants for the PBD-1 protein using antibodies against the histidine tag never resulted in 6xhis tag detection even when 30 to 80 μg of total soluble protein was added per well. The lower limit of detection by western blot analysis used in this work was approximately 10 ng based on the detection of the 6xhis protein ladder. This suggested that PBD-1 is either not present in these plants or at a level lower than the detection limit of 0.03 to 0.01% of the total soluble leaf protein.

3.2 Evaluation of resistance of the homozygous lines to *P. syringae* pv. *tabaci* 11528R

All the tobacco lines, except for line 9-8 of the *po2+pbd-1* construct which had a low germination rate, were tested for resistance against *P. syringae* pv. *tabaci* 11528R by dip inoculation where the whole seedlings were immersed in bacterial inocula (Table 3.3). After inoculation, seedlings displayed water-soaked lesions within 1 to 2 days, that later turned yellow by 1 week following inoculation. The chlorotic areas eventually expanded to include the whole plants, and the plants then died.

None of the lines were significantly more resistant than both the wild type and DC1 controls (Table 3.3). Plants from lines 24-6 and 12-7 had the lowest amount of disease and were also tested by infiltration of *P. syringae* pv. *tabaci* 11528R into the leaf. Symptoms started with small lesions within the infiltrated area by 4 dpi, by which time bacterial populations were approximately $10^6$ CFU/cm² (Figure 3.8). In some cases, the lesions became surrounded by chlorotic areas. The necrosis and/or chlorosis often expanded to cover the whole infiltrated area by 7 dpi. However, line 12-7 had a significantly lower bacterial population than line DC1 at 3 dpi. It also had a significantly lower bacterial population than line 24-6 at 1, 2 and 3 dpi.
Table 3.3 Resistance of *N. tabacum* cv. Xanthi transformed with *po2+pbd-1* (7-5, 9-8, 14-5, 22-6, 24-6) or *pbd-1+pbd-1* (9-4, 11-3, 12-7, 13-4, 15-1) against *P. syringae pv. tabaci* 11528R. Three to four-week old seedlings were infected with *P. syringae pv. tabaci* 11528R by dip inoculation in a $1.2 \times 10^8$ CFU/mL suspension. Numbers of dead seedlings were assessed after 4 weeks. The tissue culture regenerated line (DC1) and wild-type cv. Xanthi served as controls.

<table>
<thead>
<tr>
<th>Line ¹</th>
<th>Mortality rate (%) ²³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthi</td>
<td>67 ab</td>
</tr>
<tr>
<td>DC1</td>
<td>81 a</td>
</tr>
<tr>
<td>7-5</td>
<td>61 ab</td>
</tr>
<tr>
<td>9-8</td>
<td>N/A</td>
</tr>
<tr>
<td>14-5</td>
<td>81 a</td>
</tr>
<tr>
<td>22-6</td>
<td>72 ab</td>
</tr>
<tr>
<td>24-6</td>
<td>42 b</td>
</tr>
<tr>
<td>9-4</td>
<td>64 ab</td>
</tr>
<tr>
<td>11-3</td>
<td>69 ab</td>
</tr>
<tr>
<td>12-7</td>
<td>47 b</td>
</tr>
<tr>
<td>13-4</td>
<td>72 ab</td>
</tr>
<tr>
<td>15-1</td>
<td>69 ab</td>
</tr>
</tbody>
</table>

¹ Lines 7-5, 9-8, 14-5, 22-6, and 24-6 are the T₂ homozygous lines of *N. tabacum* cv. Xanthi containing the fusion *po2+pbd-1* construct.

² Lines 9-4, 11-3, 12-7, 13-4, and 15-1 are the T₂ homozygous lines of *N. tabacum* cv. Xanthi containing the *pbd-1+pbd-1* construct.

³ Mortality rate was measured as a as a percentage of the total 12 inoculated seedlings.

Data represent the average of three trials. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at $p = 0.05$.

N/A - not available due to low germination rate.
Figure 3.8 Populations of *P. syringae* pv. *tabaci* 11528R in lines of *N. tabacum* cv. Xanthi transformed with *po2+pbd-1* (line 24-6) and *pbd-1+pbd-1* (line 12-7), following inoculation by the dip inoculation assay. Five-week-old plants were infected with *P. syringae* pv. *tabaci* 11528R by infiltration with a $1.2 \times 10^4$ CFU/mL culture. Bacterial populations inside the infiltrated area were assessed daily. The tissue culture regenerated line (DC1) served as the control. Standard error bars were calculated from five replications. (*---* = DC1, *---* = 24-6, *---* = 12-7)
3.3 Evaluation of resistance of the homozygous lines to *C. destructivum* N150 and *C. orbiculare* ATCC 20767

Among the T2 lines expressing the po2+pbd-1 construct, none was found to have a significantly lower number of lesions/cm² than the wild type or DC1 controls (Table 3.4). The same conclusion was drawn when data were expressed as % of lesions/cm² of wild-type plants (Table 3.4). This was true for both 5- and 6-week-old plants, although the 6-week-old plants appeared to be less susceptible to infection by *C. destructivum* N150. The only exception was 6-week-old line 7-5, which displayed fewer symptoms when data were expressed as severity (lesions/cm²) compared to wild-type plants.

For plants expressing the pbd-1+pbd-1 fusion, only line 11-3 had significantly fewer symptoms on 5-week-old plants. However, 6-week-old plants of this line did not show significantly fewer symptoms compared to wild type and DC1 controls. For *C. orbiculare* ATCC 20767, the lines with po2+pbd-1 or native pbd-1+pbd-1 fusion displayed the same number of lesions/cm² as the two controls following infection (Table 3.5). For both *C. destructivum* N150 and *C. orbiculare* ATCC 20767, lesions appeared as small water-soaked spots at 2 to 3 days on the wild-type, line DC1, and the transgenic lines.

3.4 Transformation of *Pichia pastoris* X-33 with pbd-1

Transformation of *P. pastoris* X-33 with po2+pbd-1 or with pbd-1+pbd-1 yielded a low recovery rate of two to eight colonies per transformation event, although the frequency of positives for most colonies was often 100% as any colonies that grew on Zeocin selection plates contained the transformed constructs. Transformation of *P. pastoris* X-33 was repeated four times to yield seven colonies for the po2+pbd-1 construct.
Table 3.4 Resistance of *N. tabacum* cv. Xanthi transformed with *po2+pbd-1* (7-5, 9-8, 14-5, 22-6, 24-6) or *pbd-1+pbd-1* (9-4, 11-3, 12-7, 13-4, 15-1) against *C. destructivum* N150. Five or six weeks old plants were sprayed with 5x 10^4 spores/mL of *C. destructivum* N150. The number of lesions/cm² leaf area was assessed 72 hr after the infection. The tissue culture regenerated line (DC1) and wild-type cv. Xanthi served as controls.

<table>
<thead>
<tr>
<th>Line</th>
<th>Number of lesions/cm² on 5-wk-old plants</th>
<th>Severity (%) compared to Xanthi for 5-wk-old plants</th>
<th>Number of lesions/cm² on 6-wk-old plants</th>
<th>Severity (%) compared to Xanthi for 6-wk-old plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthi</td>
<td>6.8 a</td>
<td>100</td>
<td>2.7 abc</td>
<td>100</td>
</tr>
<tr>
<td>DC1</td>
<td>6.7 a</td>
<td>100 a</td>
<td>2.8 abc</td>
<td>111 ab</td>
</tr>
<tr>
<td>7-5</td>
<td>6.8 a</td>
<td>102 a</td>
<td>2.1 c</td>
<td>80 c</td>
</tr>
<tr>
<td>9-8</td>
<td>6.0 ab</td>
<td>89 ab</td>
<td>2.3 bc</td>
<td>92 abc</td>
</tr>
<tr>
<td>14-5</td>
<td>6.8 a</td>
<td>101 a</td>
<td>2.6 abc</td>
<td>105 abc</td>
</tr>
<tr>
<td>22-6</td>
<td>5.9 ab</td>
<td>88 ab</td>
<td>3.0 ab</td>
<td>117 a</td>
</tr>
<tr>
<td>24-6</td>
<td>5.7 ab</td>
<td>85 ab</td>
<td>2.2 c</td>
<td>86 bc</td>
</tr>
<tr>
<td>9-4</td>
<td>7.1 a</td>
<td>105 a</td>
<td>3.0 ab</td>
<td>116 ab</td>
</tr>
<tr>
<td>11-3</td>
<td>4.4 b</td>
<td>66 b</td>
<td>2.4 abc</td>
<td>98 abc</td>
</tr>
<tr>
<td>12-7</td>
<td>5.2 ab</td>
<td>78 ab</td>
<td>2.6 abc</td>
<td>102 abc</td>
</tr>
<tr>
<td>13-4</td>
<td>6.0 ab</td>
<td>88 ab</td>
<td>3.1 a</td>
<td>121 a</td>
</tr>
<tr>
<td>15-1</td>
<td>6.6 a</td>
<td>100 a</td>
<td>2.8 abc</td>
<td>115 ab</td>
</tr>
</tbody>
</table>

1 Lines 7-5, 9-8, 14-5, 22-6, and 24-6 are the T₂ homozygous lines of *N. tabacum* cv. Xanthi containing the *po2+pbd-1* fusion construct.

2 Disease severity was measured as the number of lesions/cm².

3 Disease severity was expressed as the percentage of lesions/cm² vs. untransformed *N. tabacum* cv. Xanthi control.

4 Data represent the average of four trials for 6 weeks and three trials for 5 weeks plants. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at *p* = 0.05.
Table 3.5 Resistance of *N. tabacum* cv. Xanthi transformed with *po2+pbd-1* (7-5, 9-8, 14-5, 22-6, 24-6) or *pbd-1+pbd-1* (9-4, 11-3, 12-7, 13-4, 15-1) against *C. orbiculare* ATCC 20767. Five weeks old plants were sprayed with 1x 10⁵ spores/mL of *C. orbiculare* ATCC 20767. The number of lesions per cm² leaf area was assessed 72 h after infection. The tissue culture regenerated line (DC1) and wild-type cv. Xanthi served as controls.

<table>
<thead>
<tr>
<th>Line ¹</th>
<th>Number of lesions/cm² on 5 wk old plants ², 4</th>
<th>Severity (%) compared to Xanthi for 5 wk old plants ³, 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthi</td>
<td>5.4 abc</td>
<td>100</td>
</tr>
<tr>
<td>DC1</td>
<td>4.8 abc</td>
<td>95 ab</td>
</tr>
<tr>
<td>7-5</td>
<td>4.1 bc</td>
<td>84 ab</td>
</tr>
<tr>
<td>9-8</td>
<td>4.4 bc</td>
<td>87 b</td>
</tr>
<tr>
<td>14-5</td>
<td>5.4 abc</td>
<td>93 ab</td>
</tr>
<tr>
<td>22-6</td>
<td>4.9 abc</td>
<td>101 ab</td>
</tr>
<tr>
<td>24-6</td>
<td>5.1 abc</td>
<td>103 ab</td>
</tr>
<tr>
<td>9-4</td>
<td>6.9 a</td>
<td>139 a</td>
</tr>
<tr>
<td>11-3</td>
<td>6.1 abc</td>
<td>122 ab</td>
</tr>
<tr>
<td>12-7</td>
<td>6.0 abc</td>
<td>120 ab</td>
</tr>
<tr>
<td>13-4</td>
<td>4.9 abc</td>
<td>109 ab</td>
</tr>
<tr>
<td>15-1</td>
<td>6.4 ab</td>
<td>127 ab</td>
</tr>
</tbody>
</table>

¹ Lines 7-5, 9-8, 14-5, 22-6, and 24-6 are the T₂ homozygous lines of *N. tabacum* cv. Xanthi containing the *po2+pbd-1* fusion construct.
² Lines 9-4, 11-3, 12-7, 13-4, and 15-1 are the T₂ homozygous lines of *N. tabacum* cv. Xanthi containing the *pbd-1+pbd-1* construct.
³ Disease severity was measured as the number of lesions/cm².
⁴ Disease severity was expressed as the percentage of lesions/cm² vs. untransformed *N. tabacum* cv. Xanthi control.
⁵ Data represent the average of six trials. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at \( p = 0.05 \).

125
(Figure 3.4), and 14 colonies for the \( pbd-1+pbd-1 \) construct (Figure 3.5). Clones transformed with the \( po2+pbd-1 \) construct were screened by PCR using the PDPiFo and AOX1Re primers, and all clones gave the expected product of 493 bp (Figure 3.9A). Clones transformed with the \( pbd-1+pbd-1 \) construct were also screened by PCR using the SDPiFo and AOX1Re primers, and all clones gave the expected product of 331 bp (Figure 3.9B).

3.5 Expression of PBD-1 protein in \textit{Pichia pastoris} X-33

Tricine SDS-PAGE and western analysis were performed to assess the presence of PBD-1 secreted by \textit{P. pastoris} X-33 in YPD broth. However, the expected bands of 11.7 kDa for PO2+PBD-1 or 5.9 kDa for PBD-1+PBD-1 were not visible on silver-stained tricine SDS-PAGE gels or western blots using the antibody to the histidine tag. As it appeared that the level of PBD-1 in the media was insufficient for detection by those two techniques, a slot blot method was utilized, which allowed for a larger volume of YPD broth to be concentrated onto a membrane. Using slot blots, clones 1, 3 and 7 of the \( po2+pbd-1 \) construct and clones 1, 11 and 13 of \( pbd-1+pbd-1 \) construct were found to have the strongest staining and were selected for further synthesis of these proteins.

Small scale Ni-NTA agarose purification of secreted PBD-1 produced from clones 1, 3 and 7 of the \( po2+pbd-1 \) construct did not yield a His-tagged protein based on western results. None of the other PCR positive clones for this construct gave a His-tagged protein on western blot in any trials. As a result, no further work was done on the \( po2+pbd-1 \) construct in \textit{P. pastoris} X-33.

Small scale Ni-NTA agarose purification of PBD-1 from clones 1, 11 and 13 of the \( pbd-1+pbd-1 \) construct all yielded His-tagged protein detected in western blots.
Figure 3.9 The presence of *pbd-1* transgenes in clones of *Pichia pastoris* X-33 as determined by PCR.  

**A.** PCR analysis of *P. pastoris* X-33 clones transformed with *po2+pbd-1* construct. PCR with primers PDPiFo and AOX1Re produces an expected product of 493 bp indicated by an arrow.  

**B.** PCR analysis of *P. pastoris* X-33 clones transformed with *pbd-1+pbd-1* construct. PCR with primers SDPiFo and AOX1Re produces an expected product of 331 bp indicated by an arrow. *P. pastoris* (X-33) is a negative control, and the *P. pastoris* expression plasmid contained either *po2+pbd-1* (A) or *pbd-1+pbd-1* (B) was positive controls (+ve). M indicates O'GeneRuler 100 bp DNA Ladder Plus.
indicating that a fusion with the native \textit{pbd-1} instead of \textit{po2} yielded more protein. Clone 1 was chosen for large scale production of PBD-1 because it produced a prominent band between 6 to 10 kDa on both protein gels and western blots. The His-tagged PBD-1 protein was not detected in YPD broth after 2 to 3 days of growth by \textit{P. pastoris} before purification with Ni-NTA agarose, but was detected in the eluates after purification (Figure 3.10). In the eluates, there was one major band of 6.5 kDa with two minor additional bands of approximately 6 kDa and 4 kDa. The major band of 6.5 kDa was found in all purification batches, while the minor bands were not present in all batches and often not visible in diluted samples.

Two bands at 6.5 kDa and 4 kDa were excised from the protein gel and sent for identification by liquid chromatography-tandem mass spectrometry (LC MS/MS). LC MS/MS results were compared to the \textit{P. pastoris} database as well as the expected PBD-1 protein to validate the protein identity. Both analyzed bands were identified as PBD-1, and no peptide shared similarity to any \textit{P. pastoris} peptide. Although, the α-factor signal peptide appeared to be removed from PBD-1, this process seemed to be happening at a Kex2 signal cleavage site four amino acids upstream of the expected Ste13 signal cleavage site (Figure 3.8). These additional four amino acids at the N-terminus increase the calculated estimate molecular weight of PBD-1 made by \textit{P. pastoris} to 6.3 kDa and pI 9.42 similar to the visual estimate from the SDS-PAGE gel at 6.5 kDa.

### 3.6 Antifungal activity of PBD-1

Application of PBD-1 produced by \textit{P. pastoris} X-33 showed significant inhibition of \textit{C. destructivum} N150 growth at the lowest concentration tested, 12.5 µg/mL, and fungal growth progressively declined with increasing protein concentrations up to 50
Figure 3.10 Detection of secreted PBD-1 by tricine-SDS-PAGE (top) and western blot (bottom) during purification. 6xHis PBD-1 protein was collected from growth media (lane 1) of a P. pastoris clone expressing PBD-1+PBD-1 using Ni-NTA resin. His-tag protein bound to the Ni-NTA resin while the unbound growth media passed through (lane 2). The resin was washed to remove proteins bound non-specifically (lane 3). Then the bound proteins were eluted out 4 times (lanes 4-7) and these solutions were collected for further purification. His-tag antibody was used to detect the presence of 6xHis protein throughout the collection process. Samples were separated on a 16% acrylamide gel. M indicates Page Ruler Plus Prestained Protein (top), and the 6xHis Protein Ladder is shown on lane 8.
µg/mL, but then changed relatively little from 50 to 200 µg/mL (Figure 3.11A). The 50% effective concentration (EC50) for PBD-1 activity against *C. destructivum* N150 was 72 µg/mL (Table 3.6). PBD-1 produced by *P. pastoris* X-33 also showed significant inhibition of *C. orbiculare* ATCC 20767 with significant growth reduction first observed at 25 µg/mL and a lower EC50 value of 22 µg/mL (Figure 3.11B; Table 3.6). Fungal growth was almost completely inhibited at 100 µg/mL of PBD-1. These measurements were taken at 40 to 50 h of incubation, but some growth of *C. orbiculare* ATCC 20767 in 100 µg/mL and 200 µg/mL of protein was later observed at approximately 7 days of incubation.

3.7 Antibacterial activity of PBD-1

Near 100% lethality of PBD-1 was observed for three strains at concentrations of 300 µg/mL to 500 µg/mL (Figures 3.12A, 3.12B, 3.13A). PBD-1 was most effective against a gram-positive bacterium, *B. subtilis* strain GB03 (EC50 = 86 µg/mL) (Figure 3.13A; Table 3.6) followed by two gram-negative bacteria, *P. syringae* pv. *tomato* strain 06T2 (EC50 = 191 µg/mL) and *P. syringae* pv. *tabaci* strain 11528R (EC50 = 106 µg/mL) (Figure 3.12A and 3.12B; Table 3.6). Other bacteria showed sensitivity to PBD-1 but were never showed near 100% lethality in the concentrations tested. These were *E. coli* strain UB1005 (EC50 = 127 µg/mL) (Figure 3.14A; Table 3.6) and *C. michiganensis* subsp. *michiganensis* (EC50 = 174 µg/mL) (Figure 3.13A; Table 3.6). All EC50 values were calculated based only on concentrations where a dose response was observed.

There was no effect of PBD-1 in the doses applied to other tested bacteria (Figure 3.14 to 3.17), which included *E. carotovora* subsp. *carotovora* (Figure 3.14B),
Figure 3.11 Antifungal activity of PBD-1 produced by *P. pastoris* X-33 against *C. destructivum* N150 (A) and *C. orbiculare* ATCC 20767 (B). Fungal spores were incubated in 200, 100, 50, 25, 12.5 µg/mL of PBD-1 at 25 °C until the water control reached the OD$_{595}$ of 0.2 when all readings were taken, which was 40 to 45 h for *C. destructivum* N150 and 48 to 52 h for *C. orbiculare* ATCC 20767. Standard error bars were calculated from three replications.
Table 3.6 Antifungal and antibacterial activity of PBD-1-6xHis produced by *P. pastoris* X-33 as determined by 50% effective concentration (EC$_{50}$).

<table>
<thead>
<tr>
<th>Species</th>
<th>(EC$_{50}$) $^1$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. orbiculare</em> ATTC 20767</td>
<td>22</td>
</tr>
<tr>
<td><em>C. destructivum</em> N150</td>
<td>72</td>
</tr>
<tr>
<td><em>B. subtilis</em> GB03</td>
<td>86</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tabaci</em> 11528R</td>
<td>106</td>
</tr>
<tr>
<td><em>E. coli</em> UB1005 $^2$</td>
<td>127</td>
</tr>
<tr>
<td><em>C. michiganensis</em> Cmm $^2$</td>
<td>174</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tomato</em> 06T2</td>
<td>191</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> K799</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>E. carotovora</em> Ecc</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>K. pneumonia</em> 342</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>A. tumefaciens</em> A348</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tabaci</em> ZA Patrick</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>X. axonopodis</em> pv. <em>phaseoli</em> UG18</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. chlororaphis</em> B21</td>
<td>No dose response</td>
</tr>
</tbody>
</table>

$^1$ EC$_{50}$ values were calculated by SAS proc Probit
$^2$ EC$_{50}$ values of these bacteria were calculated only from the dose response range between 0 and 100 µg/mL.
Figure 3.12 Antibacterial activity of PBD-1 produced by *P. pastoris* X-33 against *P. syringae* pv. *tomato* strain 06T2 (A) *P. syringae* pv. *tabaci* strain 11528R (B), and *P. syringae* pv. *tabaci* strain ZA Patrick (C). Bacteria were incubated in 500, 300, 100, 25 µg/mL of PBD-1 for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications. Note: the average cell counts of 0 were replaced by 1 to make them valid for a log scale graph.
Figure 3.13 Antibacterial activity of PBD-1 produced by *P. pastoris* X-33 against *B. subtilis* strain GB03 (A) and against *C. michiganensis* subsp. *michiganensis* Cmm (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of PBD-1 for 3 h at 28 ºC before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications. Note: the average cell counts of 0 were replaced by 1 to make them valid for a log scale graph.
Figure 3.14 Antibacterial activity of PBD-1 produced by *P. pastoris* X-33 against *E. coli* strain UB1005 (A) and *E. carotovora* subsp. *carotovora* Ecc (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of PBD-1 for 3 h at 37 ºC for UB1005 and 28 ºC for Ecc before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 3.15 Antibacterial activity of PBD-1 produced by *P. pastoris* X-33 against *P. aeruginosa* strain K799 (A) and against *P. aeruginosa* strain PA01 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of PBD-1 for 3 h at 37 ºC for K799 and 28 ºC for PA01 before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 3.16 Antibacterial activity of PBD-1 produced by *P. pastoris* X-33 against *K. pneumonia* strain 342 (A) and against *P. chlororaphis* strain B21 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of PBD-1 for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
**Figure 3.17** Antibacterial activity of PBD-1 produced by *P. pastoris* X-33 against *X. axonopodis* pv. *phaseoli* strain UG18 (A) and against *A. tumefaciens* strain A348 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of PBD-1 for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
4. Discussion

Recombinant porcine β-defensin (PBD-1) was previously produced by an methanol-inducible *P. pastoris* expression system that was active against the animal pathogen, *S. aureus* (Jiang et al., 2006), and by a baculovirus-insect (*Trichoplusia ni*) expression system that was active against the animal pathogens, *E. coli, S. typhimurium, L. monocytogenes* and *C. albicans* (Shi et al., 1999). PDB-1 has also been produced by chemical synthesis showing activity against a human pathogen, *B. pertussis* (Elahi et al., 2006). Atnaseo (2003) produced transgenic *N. tabacum* cv. Xanthi plants containing two versions of *pdb-1*, one with the native porcine PBD-1 signal peptide (*pdb-1+pdb-1*) and another with the secretion signal peptide and a portion of the negatively charged domain of the mature PO2 peptide (*po2+pbd-1*). The PO2 peptide is encoded by an alfalfa plant pollen-specific gene (Qiu et al., 1997). The T₀ transformants containing either construct appeared to have resistance following infiltration with *P. syringae pv. tabaci* PBIC4 based on visual disease severity scoring (Atnaseo, 2003). This was the first attempt to produce PDB-1 in plants and show activity against a plant pathogen. The current study
aimed to test resistance to *P. syringae* pv. *tabaci* as well as two fungal foliar pathogens of tobacco on T₂ homozygous *N. tabacum* cv. Xanthi plants as they may display higher levels of resistance due to the additional copies of the *pbd-1* transgene. In addition, recombinant PBD-1 was produced from a *P. pastoris* expression system to test for *in vitro* activity against several animal pathogens, including *E. coli*, and plant pathogens, including *P. syringae* pv. *tabaci*.

In contrast to the observation that the original T₀ plants had some degree of resistance to *P. syringae* pv. *tabaci* PBIC4 based on symptoms, the T₂ homozygous plants showed no evidence of resistance to *P. syringae* pv. *tabaci* 11528R based on mortality rates following dip inoculation. A comparison of the bacterial populations following infiltration showed that line 24-6 with the *po2+pbd-1* construct had equal or greater populations than the control, but line 12-7 containing the *pbd-1+pbd-1* construct always had lower populations than the control. However, the latter was only significant at 3 dpi where the population was slightly reduced to 9.63 x 10⁵ CFU/mL in line 12-7 from 1.89 x 10⁶ CFU/mL in the control. Thus, there was a small enhancement of resistance to *P. syringae* pv. *tabaci* 11528R using the *pbd-1+pbd-1* construct. For *C. destructivum* N150 and *C. orbiculare* ATCC 20767, the T₂ lines showed no enhanced resistance over the non-transgenic controls.

Although the PCR and RT-PCR results of the T₂ plants verified the presence of *pbd-1* and transcripts from it in all T₂ lines, PBD-1 protein was not detected by 6xHis antibodies on western blots of extracts from these plants. The level of transgenic proteins can be higher in homozygotes than the heterozygotes, but they can also be the same or lower, possibly in the latter case due to silencing (James et al., 2002; Hobbs et al., 1993).
However, it is unlikely that the lack of detectable PBD-1 protein was a result of transgene silencing in homozygous lines since pbd-1 expression was detected at the mRNA level.

Another explanation for the lack of detection of PBD-1 protein may be due to post-translation events. Breakdown by plant proteases has been reported as a major obstacle to the production of AMPs in plants (Owens and Heutte, 1997). For example, mRNA of the animal AMP cecropin B gene was detected in potato (Allefs et al., 1996) and tobacco (Hightower et al. 1994; Florack et al., 1995), but no cecropin B protein could be detected by western blots. Similar to this study, transgenic plants with undetectable levels of cecropin B protein did not show enhanced disease resistance. Incubation of cecropin B protein in tobacco intercellular fluid led to faster degradation than incubation in potato or rice intercellular fluid, suggesting that secretions by tobacco cells might rapidly degrade the AMP and tobacco might not be a suitable plant for expression of cecropin B (Owens and Heutte, 1997; Sharma et al., 2000). Other studies did not report AMP protein detection but instead reported antimicrobial activity of the peptides either in vitro or in planta, thereby indirectly confirming the presence of the transgenic peptides (Cary et al., 2000; DeGray et al., 2001; Li et al., 2001; and Liu et al., 2001).

Improper processing or folding of PBD-1 in plants could also render the protein undetectable using antibodies as well as result in a loss of biological activity. Misfolding of foreign proteins has been reported in many plant expression systems and often leads to degradation of foreign proteins by plant proteases resulting in low level of protein accumulation (Doran, 2006). Production of animal protein in plant can also result in incomplete/incorrect post-translational modification resulting in degradation. For example, expression of bovine follicle-stimulating hormone in N. benthamiana resulted
in a protein with much lower biological activity than the native form of the protein when tested in mice, which believed to be due to rapid clearance of the hormone from the system as a result of incorrect post-translational modification rather than altered affinity toward a receptor (Dirnberger et al., 2001).

Two strategies were adopted for expressing \textit{pbd-1} in plants, both of which contained the codon-modified version of \textit{pbd-1} encoding the mature peptide. The first strategy was to mimic the secretion of the peptide into the extracellular space of the pig intestinal tissue by using the native porcine signal peptide. For this construct, the 20 AA PBD-1 signal sequence and 6 AA PBD-1 propeptide were fused with the codon-modified PBD-1 mature peptide. However, the porcine signal peptide may not be recognized by plants and so might not be secreted or the mature peptide incorrectly processed. Florack et al. (1995) showed that fusing cecropin B with the signal peptide of the barley thionin gene improved its expression in tobacco compared to using cecropin B native signal peptide from the giant silkmoth. Therefore, a second strategy was adopted where the PBD-1 signal sequence and PBD-1 propeptide sequences were replaced, and the codon-modified PBD-1 mature peptide sequence was fused to a sequence from the secreted alfalfa pollen protein, PO2, that included both a 20 AA signal peptide as well as the first 56 AA of PO2 mature peptide. It was expected that this fusion would enhance the secretion and stability of mature PBD-1 in the extracellular environment of the plant. Okamoto et al. (1998) showed that fusion of flesh fly AMP, sarcotoxin IA, to GUS yielded higher production of sarcotoxin IA in tobacco than expression without GUS fusion, and Hondred et al. (1999) found that fusion of ubiquitin to reporter proteins, such as GUS and LUC, enhanced their production in tobacco.
Although PBD-1 protein could not be detected in the transgenic plants carrying either construct, the pbd-1+pbd-1 construct was somewhat better in limiting growth of *P. syringae* pv. *tabaci* 11528R. The pbd-1+pbd-1 approach being more effective is supported by the expression of these two constructs in yeast, where PBD-1 protein was detected with the pbd-1+pbd-1 construct, but not with po2+pbd-1 construct.

Although several invertebrate AMPs, including heliomicin and drosomycin, have been expressed in plants (Table 1.1), thus far, only two vertebrate defensin genes have been reported to be expressed in plants, namely the rabbit α-defensin (NP-1) (Fu et al., 1998; Zhao et al., 1999; Zhang et al., 2000) and human β-defensin 2 (hBD-2) (Aerts et al., 2007). NP-1 was expressed in tobacco and provided a slight increase in resistance against wilt disease caused by *Ralstonia solanacearum*, although the level of protein produced was not reported (Fu et al., 1998). NP-1 was also expressed in poplar and tomato, and the plant extract from tomato expressing NP-1 had *in vitro* activity against *E. coli* and *Fusarium oxysporum* (Zhang et al., 2000), while protein extract from poplar was also reported to have *in vitro* antimicrobial activity against *B. subtilis* and *A. tumefaciens* (Zhao et al., 1999). For hBD-1, the mature peptide was fused with the N-terminus signal peptide sequence of the dahlia defensin defensin (DmAMP1) for secretion and then expressed constitutively in *A. thaliana* under the control of the CaMV35S promoter. To boost expression, the whole construct was also placed between a Matrix Attachment Region (MAR) sequence of the chicken lysozyme gene, and the sgs2 mutant of *A. thaliana*, which is deficient in post transcriptional gene silencing, was used (Aerts et al., 2007). With this combination of strategies, termed as the PTGS-MAR expression system, the level of hBD-2 was between 0.05 to 0.65% of total soluble protein in leaves,
and the level of increased resistance to \textit{B. cinerea} corresponded to the level of hBD-2 expression. Although this level of AMP expression is relatively high compared to most animal AMPs expressed in plants (Table 1.1), it is still far below those reported for other transgenes expressed with the PTGS-MAR expression system. In \textit{A. thaliana}, the PTGS-MAR expression system yielded the plant defensin, PDF1.3, at 2.86\% of the total soluble protein (Sels et al., 2007) and the GUS protein at 10 \% of the total soluble protein (Butaye et al., 2004). This suggests that there are possibly inherent difficulties in achieving high levels of expression of animal defensin proteins in plants.

If 0.65\% of total soluble protein reported by Aerts et al. (2007) is the maximum possible yield for a vertebrate defensin in plants, then it appears unlikely that plants can be used to deliver therapeutic proteins, such as vertebrate defensins, to livestock animals, either in extracts or intact plant material. It has been proposed that a minimum accumulation of 1\% of total soluble protein is required for a therapeutic protein to be commercially practical for purification from transgenic plants (Kusnadi et al., 1997). More changes in construct engineering and host plant selection may be required to reach that level.

As transgenic tobacco plants did not provide adequate amounts of PBD-1 for \textit{in vitro} tests against a broad range of microbes, an alternative expression system involving \textit{P. pastoris} was utilized. PBD-1 was produced in \textit{P. pastoris} with a pGAPZ\textalpha{} vector system (Invitrogen), which is a constitutive expression system controlled by the promoter of the glyceraldehyde-3-phosphate dehydrogenase (GAP) gene and terminator of the alcohol oxidase 1 (\textit{AOX}) gene, both from \textit{P. pastoris}. Unlike the plant expression constructs, the \textit{P. pastoris} constructs did not contain either the PO2 or native PBD-1
signal peptide as they were replaced by the α-factor secretion peptide from *S. cerevisiae*
within the pGAPZα vector. The α-factor secretion peptide sequence was fused with
either the first 56 AA of the PO2 mature peptide followed by the codon-modified 38 AA
of the PBD-1 mature peptide, known as the po2+pbd-1 construct, or fused with 6 AA
native PBD-1 propeptide followed by the codon-modified 38 AA of the PBD-1 mature
peptide, known as the pbd-1+pbd-1 construct.

Despite the presence of pbd-1 in all selected *P. pastoris* cells transformed with the
po2+pbd-1 construct, no fusion protein was detected in the growth media of these cells
before or after purification with Ni-NTA agarose. It is possible that the fusion protein
was not being produced or secreted by *P. pastoris* as the presence of the PO2 sequence
might prevent *P. pastoris* from recognizing and processing the signal peptide. However,
the possible presence of PO2+PBD-1 retained inside the *P. pastoris* cells was not
investigated in this work. Additionally, lack of detection of the PO2+PBD-1 fusion
protein might be due to mis-folding of the protein in such a way that the 6xhis site that
had been added to the C-terminus was inaccessible for purification with Ni-NTA agarose
and detection with the 6xhis-tag antibody.

In contrast, the pbd-1+pbd-1 construct resulted in the protein being produced and
secreted by *P. pastoris* into the growth media. Secreted PBD-1+PBD-1 protein was not
detected in the growth media prior to purification with Ni-NTA agarose suggesting that
level of protein production was below the detection limit of 0.67 μg/mL. After
purification with Ni-NTA agarose, the 6xhis-tagged protein was detected both on silver
stained protein gels and by western blot. As multiple bands were detected with the 6xhis-
tag antibody, it is likely that *P. pastoris* produced or processed PBD-1 in different forms.
Identification of the two prominent bands at 6.5 kDa and 4 kDa showed that both bands contained the expected PBD-1 sequence and did not share a significant match with any *P. pastoris* protein. The less abundant 4 kDa band may be a shorter version of PBD-1 without the N-terminal PBD-1 prosequence as the LC MS/MS data of this fragment gave no match to the region beyond the mature peptide at the N-terminus. The LC MS/MS data suggested that the more abundant 6.5 kDa band may be a version of PBD-1 where the yeast α-factor signal peptide was cleaved at the Kex2 site rather than the Ste13 cleavage site as expected. Cleavage at the Kex2 site would result in 12 additional AAs at the N-terminus of the mature peptide with 6 of extra AAs from the expression vector followed by 6 AAs of the PBD-1 prosequence. Including the 6xhis tag, this fusion protein would have a theoretical molecular weight of 6.3 kDa and a pI 9.42, which is in agreement with the visual estimation from the SDS-PAGE gel of 6.5 kDa.

PBD-1 produced by baculovirus-infected *T. ni* cell line expression system yielded two forms of PBD-1 with the shorter one containing the mature peptide of PBD-1 and the longer one containing an additional 4 out of the total 6 AAs of the native porcine prosequence as determined by N-terminus peptide sequencing (Shi et al., 1999). These 4 additional AA at the N-terminus would match the last 4 of the 12 extra AAs at the N-terminus in our 6.5 kDa *P. pastoris* produced PBD-1. Thus, both a mature peptide and a mature peptide with additional AAs at the N-terminus were produced in two very different constitutive expression systems, *T. ni* cell lines and *P. pastoris*. However, in the case of the methanol-inducible *P. pastoris* expression system, a 4.5-kDa protein was observed, which would contain the last 4 AA of the prosequence based on the design of the expression vector (Jiang et al., 2006). Therefore, there would be an extra 8 AA added to
the N-terminus if the signal peptide was cleaved at the correct site, but this is an estimate as LC MS/MS analysis was not reported and so the actual AA sequence is not known.

PBD-1 produced by *P. pastoris* inhibited growth of *E. coli* UB1005 by 98% at 100 μg/mL. By comparison, both the longer and shorter forms of PBD-1 produced by the baculovirus-*T. ni* expression system inhibited growth of *E. coli* ATCC 35218 by 100% at 40 μg/mL (Shi et al., 1999). A chemically synthesized PBD-1 was reported to inhibit growth of *E. coli* JG280 at a compatible level as that of Shi et al. (1999), but the results and concentrations were not reported (Elahi et al., 2006).

The lower activity of PBD-1 produced by *P. pastoris* compared to the chemically synthesized PBD-1 or PBD-1 from the baculovirus-*T. ni* expression system could be due to several factors. Purification of the material in this study was done by Ni-NTA agarose, desalting and sterile filtration, and there were at least 10 bands visible on the gel and three to four bands were stained in western blot suggesting that not all bands present in the gel contain PBD-1 or they would be visible on western blot using anti-his antibody, although these other bands were at a very low concentration compared to the two bands that was verified to contained PBD-1 by LC MS/MS. These other materials would lower the level of purity of PDB-1 and affect the concentration that was based on a total protein determination. Another factor is that different strains of *E. coli* were used in the different reports, *E. coli* UB1005 (this report), *E. coli* ATCC 35218 (Shi et al., 1999) and *E. coli* JG280 (Elahi et al., 2006), and they could have different sensitivities to PDB-1.

Differences among bacterial strains to sensitivity to β-defensin have been reported for the human β-defensins, hBD-2 and hBD-3 (Joly et al., 2004). The assay method may also have been a factor. The activity of PBD-1 is greatly affected by the pH with greater
activity at higher pH up to pH 8.5, which was the highest tested. The activity of PBD-1 was also affected by salt concentration with activity becoming dramatically reduced as NaCl concentration increased up to 50 to100 mM (Shi et al., 1999). The assay conditions for this study and Shi et al. (1999) both used 10 mM sodium phosphate at pH 7.4, but for our assay, 0.1% of TSB was also added because the bacteria did not grow well without nutrients included. The TSB would have increased the salt concentration reducing activity, but TSB did not significantly affect the pH (data not shown). Finally, activity was detected using a combination of the longer and shorter forms of PBD-1, and it is unknown if the additional 12 AAs attached to the mature PBD-1 may have had an effect on activity. Of the two forms of PBD-1 produced by the baculovirus-\textit{T. ni} expression system, the longer form with the 4 AAs of native porcine prosequence was 30% more active against \textit{L. monocytogenes} than the shorter one, but a difference between those forms of PBD-1 was not observed for \textit{E. coli} (Shi et al., 1999). This was not determined in this study, and therefore, it is unknown whether the additional of 8 AA at the N-terminus increased or decreased antimicrobial activity.

Although different forms of PBD-1 were synthesized (this report, Shi et al., 1999; Elahi et al., 2006), they were all able to inhibit the growth of \textit{E. coli}. This indicates that PBD-1 can tolerate some additional AAs (part of the propeptide at the N-terminus and 6xhis tag at the C-terminus) without losing activity, and the PBD-1 produced by \textit{P. pastoris} expression system has comparable activity to earlier reported activity of PBD-1 extracted from \textit{T. ni} expression system (Shi et al., 1999). Despite the limitations, these studies show that PBD-1 can be produced by several different expression systems, and retain antimicrobial activity.
In addition to activity against the gram-negative bacterium, *E. coli*, PBD-1 also has activity against gram-positive bacteria, *L. monocytogenes* EGD with approximately 92% and 62% growth inhibition with the long and short versions of PBD-1, respectively (Shi et al., 1999). Elahi et al. (2006) tested chemically synthesized PBD-1 against two gram-positive animal pathogens, *B. pertussis* and *B. bronchiseptica*, but only *B. pertussis* was sensitive to PBD-1 with 100% growth inhibition at 5 μg/mL. With the methanol-inducible *P. pastoris* expression system, unpurified PBD-1 obtained from the culture supernatant showed an unspecified level of growth inhibition of the gram-positive bacterium, *S. aureus* (Jiang et al., 2006).

In this study, *P. pastoris* produced PBD-1 showed a wide range of antimicrobial activity against plant and animal bacterial pathogens. The most susceptible bacteria were *P. syringae* pv. *tabaci* strain 11528R, *P. syringae* pv. *tomato* strain 06T2 and *B. subtilis* strain GB03, which were completely inhibited by PBD-1. The growth of *E. carotovora* subsp. *carotovora*, *P. aeruginosa* K799, *K. pneumonia* 342 and *C. michiganensis* subsp. *michiganensis* were inhibited similarly to that of *E. coli*, while *P. aeruginosa* strain PA01, *P. syringae* pv. *tabaci* strain ZA Patrick, *X. axonopodis* pv. *phaseoli* strain UG18, *A. tumefaciens* strain A348, and *P. chlororaphis* strain B21, were not susceptible to PBD-1. Thus, there was variation in sensitivity between bacterial genera (e.g., *Pseudomonas* versus *Xanthomonas*), between species with a genus (e.g., *P. chlororaphis* versus *P. aeruginosa*), between subspecies within a species (e.g., *P. syringae* pv. *tabaci* strain ZA Patrick versus *P. syringae* pv. *tomato*) and between strains of the same pathovar (e.g. *P. syringae* pv. *tabaci* strain ZA Patrick versus *P. syringae* pv. *tabaci* strain 11528R).
In addition to antibacterial activity, PBD-1 was also reported to have in vitro antifungal activity. The long version of the baculovirus-T. ni produced PBD-1 completely inhibited C. albicans at 40 μg/mL, but that was the only concentration tested and therefore it is possible that C. albicans is sensitive to PBD-1 at a lower concentration (Shi et al., 1999). The short version of PBD-1 was not tested against C. albicans. There are no other reports of antifungal activity of PBD-1, except for this study showing that PBD-1 had activity against two filamentous fungal plant pathogens, C. destructivum N150 and C. orbiculare ATTC 20767 with C. orbiculare ATTC 20767 being much more sensitive.

The current study, Shi et al. (1999), Elahi et al. (2006) and Jiang et al. (2006) all indicated that transgenic PBD-1, regardless of the production system, can display a broad range of antibacterial and antifungal activity against animal and plant pathogens. However, the level of sensitivity to PBD-1 may vary considerably. The antimicrobial activity of AMPs, including β-defensins, is believed to be due to disruption of microbial membranes by taking advantage of the interaction between the positively charged AMPs with negatively charged microbial phospholipid membranes (Ganz, 2003a). Microbial pathogens can develop resistance to AMPs through modification of their membranes by reducing the negative charge (Ganz, 2001). Elahi et al. (2006) suggested that the lack of activity of PBD-1 against the pig pathogen B. bronchiseptica was because its membranes had adapted to become resistant to PBD-1. On the other hand, B. pertussis was sensitive to PBD-1 because it is an obligate human pathogen, and so its membranes have not co-evolved with pigs as a host. However, different oral bacteria and fungi that would have co-evolved with humans displayed different degrees of sensitivity to the human β-
defensins, hBD-2 and hBD-3, even at strain level (Joly et al., 2004). In our case, PBD-1-sensitive or -resistant plant pathogens are not known to infect pigs, and so co-evolution with pigs cannot be an explanation. This suggests that microbes in the environment naturally have a range of membrane components that are affected differently by PBD-1, even within a single bacterial subspecies. In addition to modification of membrane components, microbes may also become resistant to AMPs by production of extracellular proteases to breakdown AMPs (Devine et al., 1999). Thus, the different levels of sensitivity to PBD-1 could also be due to secretion of various proteases. Regardless of the source of resistance, the variable degree of sensitivity of microbes to a particular AMP makes the use of AMPs for treatment of broad spectrum disease questionable because there may be selection for resistant species or resistant strains of the same species. The same concern also applies about using AMPs for production of disease resistant plant against broad spectrum of pathogens.

Although PBD-1 produced by P. pastoris exhibited strong activity against the two fungal pathogens, C. destructivum N150 and C. orbiculare ATTC 20767, and the bacterial pathogen P. syringae pv. tabaci strain 11528R, transgenic tobacco plants expressing PBD-1 showed little to no increased resistance to these pathogens compared to the control plants. This may be explained by differences in the conditions when the microbes are exposed to PBD-1 in the in vitro system compared to the conditions in the apoplast of tobacco, such as the presence of proteases, pH or salt (Shi et al., 1999), which might reduce or eliminate PBD-1 activity. Such an assumption might not apply for all β-defensins as Aerts et al. (2007) reported biological activity for hBD-1 in A. thaliana.
leaves against \textit{B. cinerea}, suggesting that at least for hBD-1 could survive and be active in the apoplast.

Another hypothesis for the failure of the transgenic tobacco plants to show increased resistance is that PBD-1 accumulation in the apoplast was too low to be active against these pathogens. This is supported by the lack of detection of PBD-1 in extracts from the transgenic plants. Based on numerous extractions of soluble proteins from tobacco leaves in these experiments, it is estimated that 1 mL of liquid leaf material contains approximately 8000 μg of total soluble protein. If the antifungal activity of PBD-1 was first significant in the \textit{in vitro} test at 25 μg/mL, then the equivalent volume of leaf tissue containing 8,000 μg of soluble protein would be 0.31% of the total soluble protein to contain 25 μg of PBD-1. Similarly, the activity of PBD-1 against \textit{P. syringae pv. tabaci} strain 11528R was first significant in the \textit{in vitro} test at 100 μg/mL, and so the equivalent volume of leaf tissue containing 8,000 μg of soluble protein would be 1.25% of the total soluble protein to contain 100 μg of PBD-1. As western blots of extracts from plants containing the \textit{pbd-1} constructs did not show any PBD-1 protein, then one can assume that the concentration of PBD-1 was below the detection limit of 0.03-0.01% total soluble protein. Thus, no activity \textit{in planta} should be expected. However, if a method could be employed to increase the expression levels in tobacco, then the \textit{in vitro} inhibition results of \textit{pbd-1} expressed from \textit{P. pastoris} shows that PBD-1 could be effective against a broad range of microbes including plant pathogens. Therefore to make use of PBD-1 to improve plant disease resistance, strategies to greatly enhance expression of this gene will need to be employed, even beyond strategies like the PTGS-MAR expression system (Sels et al., 2007).
Chapter 4. Transgenic expression of tobacco defensin 1 (Ntdef1) in Nicotiana tabacum and Pichia pastoris and its antimicrobial activity

1. Introduction

Plant defensins are a class of AMPs that have structural similarities to mammal and insect defensins (Terras et al., 1995). They are small cysteine-rich peptides of about 45-54 amino acids, and despite sharing very little amino acid homology, all plant defensins have four conserved intramolecular disulphide bonds (Epplle et al., 1997a; Garcia-Olmedo et al., 1998). Within a plant genome, defensins are encoded by gene families. For example, the Nicotiana tabacum and Arabidopsis thaliana Gene Indices (http://compbio.dfci.harvard.edu/tgi/; accessed July 2011) list 12 and 14 sequences, respectively, annotated as encoding for defensin or defensin-like proteins. Most plant defensins appear to have a role in innate immunity, a basic defense mechanism that is present in all kinds of organisms (Thomma et al., 2002; Nürnberger et al., 2004).

Some defensins act as inhibitors of insect α-amylases (Zhang et al., 1997) and bovine proteases (Wijaya et al., 2000) that could have roles in protection against herbivorous animals. Other defensins are inhibitors of plant, animal and microbial protein translation (Mendez et al, 1990; Mendez et al, 1996), which could have roles in defense against animal pests or microbial pathogens (Stotz et al., 2009b). In addition some defensins can inhibit plant root growth, which could have roles in regulation of plant growth and development (Allen et al., 2008; Stotz et al., 2009b). Finally, some plant defensins may act as an ion channel blockers in mammals, such as a rat pituitary epithelial-like tumour cell line, GH3 (Kushmerick et al., 1998). This diversity underlines how limited our knowledge is about plant defensins.
Plant defensins are active against a broad range of fungi, and a few are active against bacteria (Broekaert et al., 1997; Thomma et al., 2002). For example, a radish defensin (RsAFP2) is active against the fungus, *Fusarium culmorum* (Terras et al., 1992), while a *Clitoria ternatea* defensin (CtAMP1) is active against the bacterium, *Bacillus subtilis* (Osborn et al., 1995). In addition, some plant defensins, such as RsAFP2 and dahlia defensin (DmAMP1) can be active against fungal pathogens of animals, such as *Candida* spp. and *Aspergillus* spp. (Thevissen et al., 2007). Unlike animal defensins, plant defensins do not interact with membrane phospholipids directly (Stotz et al., 2009b). Although not well understood, the antifungal activity of plant defensins is believed to be due to the interaction of the defensin protein with a specific receptor on fungal membrane components, such as sphingolipids (Trevissen et al., 2003) or glucosylceramides (Trevissen et al., 2004) leading to membrane permeabilization and inhibition of fungal growth.

With such a broad range of antimicrobial activities, plant defensins are good candidates for the development of transgenic disease resistant plants and molecular farming of antimicrobial peptides against animal pathogens. Transgenic plants expressing ectopic plant defensin genes have been generated in different plant species (Table 1.2) including tobacco with increased resistance to *Alternaria longipes* (Terras et al., 1995), *Phytophthora parasitica* and *Peronospora hyoscyami* (Portieles et al., 2010), rice with increased resistance to *Magnaporthe grisea* (Kanzaki et al., 2002), canola with increased resistance to *Leptosphaeria maculans* (Wang et al., 1999), potato with increased resistance to *Verticillium dahliae* (Gao et al., 2000), melon to *F. oxysporum* and *A. solani* (Ntui et al., 2010) and tomato to *F. oxysporum* (Abdallah et al., 2010).
Thus far no plant defensin has been reported to be used in molecular farming, but there are reports suggesting plant defensins could be used in mammals because of their antifungal activity (Thomma et al., 2003; Thevissen et al., 2007).

Atnaseo (2003) identified three defensin genes from *N. tabacum* cv. Xanthi by PCR of genomic DNA using primers designed from known defensin sequences from plants in the Solanaceae. One of these is *N. tabacum* defensin 1 (*Ntdef1*) that has an identical amino acid sequence to thionin-like protein (TLP) of *N. tabacum* (GenBank accession number AB034956; DFCI Gene ID NP917113) (Takemoto and Kawakita, 1999, direct submission). The only information provided about its expression, function or antimicrobial activity is that the GenBank accession states that it was inducible by a fungal elicitor, which implies a role in defense.

Atnaseo (2003) overexpressed *Ntdef1* in *N. tabacum* cv. Xanthi using a pCAMBIA1301 binary vector (Center for the Application of Molecular Biology to International Agriculture, Canberra, AUS) with the CaMV enhanced 35S promoter and 35S terminator to give constitutive expression. Expression of *Ntdef1* could be detected in transformed plants, but Ntdef1 protein was not detected by western blots probing for a polyhistidine tag that had been added to the 3’ end of *Ntdef1*. Transgenic T₀ tobacco appeared to have enhanced resistance against *Pseudomonas syringae pv. tabaci* based on reduced severity of visual disease symptoms (Atnaseo, 2003).

To advance the work of Atnaseo (2003), homozygous progeny were generated from T₀ *Ntdef1*–transformed plants to test for increased disease resistance, and the presence of Ntdef1 protein was tested using the polyhistidine tag. In addition, the protein was expressed in a *Pichia pastoris* expression system (Invitrogen). *P. pastoris* has been
used for the synthesis of different plant defensins, such as corn defensin (Kant et al., 2009) and peach defensin (Wisniewski et al., 2003). Ntdef1 protein extracts from *P. pastoris* were used to test the biological activity of the Ntdef1 protein against various microbial pathogens, including those that affect animal health.

2. Materials and methods

2.1 Generation of transgenic plants homozygous for constitutive expression of *Ntdef1*

*Nicotiana tabacum* cv. Xanthi plants transformed with *Ntdef1* were previously generated by Atnaseo (2003) (Figure 4.1). Five T₀ plants were selected on the basis of a preliminary screen for resistance to *P. syringae* pv. *tabaci* strain PBIC4 (Atnaseo, 2003). Selection of the homozygous lines was performed as per Chapter 3 except that the antibiotic used for selection was 30 µg/mL of hygromycin. Analyses of the selected T₂ lines by PCR, RT-PCR and Western blot were also performed as described in Chapter 3. Preparation of the homozygous T₂ seedlings was as described in Chapter 3.

2.2 GUS assay

Five T₂ lines were tested for transgene expression. The expression cassette containing *Ntdef1* had been inserted between *Hind* III and *Eco*RI sites of pCAMBIA1301 (Center for the Application of Molecular Biology to International Agriculture, Canberra, AUS, Figure 4.1A). As the pCAMBIA1301 binary vector also contains a *gus* gene on a separate expression cassette, *gus* expression was assayed using this co-transformed gene. Leaf discs obtained from transformed and control plants grown in the greenhouse were incubated in 1 mL GUS assay buffer (10 mM EDTA, 100 mM NaH₂PO₄·H₂O, 0.5 mM
A. Components of the expression cassette of Ntdef1 gene construct and location of primers are indicated by arrows. This vector was used as a template for amplification of the Ntdef1 expression in P. pastoris X-33. The 6xH indicates the six histidine tag fused to the end of the mature peptide.

B. The DNA and amino acid sequences of the Ntdef1 gene. Restriction sites are in bold letters, underlined amino acid sequence represents putative NTDef1 signal peptide, amino acid sequence with bold letters represents the putative NTDef1 mature peptide, DNA sequence with upper case letters represent exon, and DNA sequence with lower case letters represents intron.

Figure 4.1 Construction of the expression cassette of Ntdef1 gene for expression in N. tabacum cv. Xanthi. A. Components of the expression cassette of Ntdef1 gene construct and location of primers are indicated by arrows. This vector was used as a template for amplification of the Ntdef1 expression in P. pastoris X-33. The 6xH indicates the six histidine tag fused to the end of the mature peptide. B. The DNA and amino acid sequences of the Ntdef1 gene. Restriction sites are in bold letters, underlined amino acid sequence represents putative NTDef1 signal peptide, amino acid sequence with bold letters represents the putative NTDef1 mature peptide, DNA sequence with upper case letters represent exon, and DNA sequence with lower case letters represents intron.
K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆·3H₂O, 0.4 mg/mL X-glucuronide (Labscientific, Livingston, NJ) at 37°C for 3 days (Jefferson, 1987). Leaf discs were then incubated with 95% ethanol to remove green pigment for 1 to 2 days after which the fresh 95% ethanol was removed and replaced, and the process was repeated until all green pigment was removed. The leaf discs were then observed for the blue colour that is characteristic of gus gene expression.

2.3 Detection of Ntdef1 DNA, RNA and encoded protein in homozygous T₂ lines

Genomic DNA was extracted according to Edwards et al. (1991). PCR using genomic DNA from homozygous T₂ lines was performed with a sense primer NtDef1F0 (5’- GCATTGCTTTGTACGGCT -3’) from Ntdef1 and an antisense primer 19-3 (5’- CAACACATGAGCGAAACC-3’) from the CaMV35S terminator, which gave a 1262 bp product. PCR was done in a 20 μL reaction containing of 500 ng genomic DNA, 0.8 units of Taq DNA polymerase (New England BioLabs), 1xThermoPol Reaction Buffer with 2 mM MgSO₄ (New England BioLabs), 0.2 mM dNTPs (Promega), and 0.2 μM of each primer. All PCRs were done with a Tpersonal thermocycler (Biometra) with the lid temperature set at 104°C, and the PCR products were separated in 1% agarose TAE gels.

Following RNA extraction and cDNA synthesis as described in Chapter 3, RT-PCR was performed with a sense primer JapanF0 (5’- TCTAGAGACGCGAAATGGGCAACTCC -3’) from Ntdef1 and the antisense primer 19-3 from the CaMV35S terminator, which gave a 365 bp product. Each 20 μL PCR reaction consisted of 250 ng of cDNA, 0.8 units of Taq DNA Polymerase (New England BioLabs), 1 mM MgCl₂, 0.2 mM dNTPs (Promega) and 0.2 μM of each primer in 1x PCR buffer (Invitrogen). A denaturing step of 95°C for 2 min, followed by 30 cycles of
95°C for 15 s, 55°C for 30 s, 72°C for 30 s, and a final elongation step at 72°C for 5 min was used. Protein extraction from the homozygous T2 lines as well as western blot analysis was performed using antibody against Histidine tag as described previously (Chapter 3).

2.4 Bacterial inoculations

Inoculations of T2 plants potentially expressing Ntdef1 with P. syringae pv. tabaci was performed as described in Chapter 3. For dip inoculation, 3-leaf stage T2 seedlings were submerged in a suspension of 1.2 x 10^8 CFU/mL of P. syringae pv. tabaci isolate 11528R in water with 0.02% Silwet L-77 (Witco Corp.) for 5 min before being transferred to soil and evaluated weekly for survival rate. For leaf infiltration, the first fully mature leaf of a 6 week-old T2 plants was infiltrated with 1.2 x 10^4 CFU/mL of P. syringae pv. tabaci 11528R in 10 mM sodium phosphate buffer pH 7 according to the method of DeGray et al. (2001). The number of bacteria inside the infiltrated area was estimated daily by excising two leaf discs (1.27 cm^2) from two infiltrated plants and homogenizing in 300 μL 10 mM MgCl₂ in the 1.5 mL microfuge tube. Then, 5 mL of 10 mM sodium phosphate buffer pH 7 was added to the homogenate before 10-fold serial dilutions to a dilution of 1:1000 using the same buffer. 100 μL of each dilution was plated on King’s B media (King et al., 1954) and incubated at 28°C overnight before counts were taken from plates that yielded 30 to 300 CFU. Two plants were used per time point per experiment and the experiment was repeated five times.

2.5 Fungal inoculations

Inoculations of T2 plants expressing Ntdef1 with C. destructivum or C. orbiculare was performed as described in Chapter 3. Briefly, C. destructivum strain N150 and C.
*orbelculare* strain ATCC 20767 were grown on SYAS medium (Manandhar et al., 1986) for 10 to 14 days in constant fluorescent light to produce spores. Spore suspensions were adjusted to $5 \times 10^4$ spores/mL *C. destructivum* strain N150, and $1 \times 10^5$ spores/mL for *C. orbelculare* strain ATTC 20767. Spore suspension was sprayed on the 5 to 6 weeks old seedlings using an air pressure sprayer, and then incubated under high humidity in the dark for 72 h. The number of lesions was counted on the two first fully expanded leaves from each plant, and two plants were used for each line. Leaf area was measured by a LI-3100 area meter (Li-Cor Inc.), and the number of lesions/cm$^2$ was calculated. The experiment was repeated 3 to 6 times.

2.6 Construction of *Pichia pastoris* expression vectors

Two constructs of *Ntdef1* were created for expression in *P. pastoris*. Both utilized the *Saccharomyces cerevisiae* α-factor secretion sequence contained in the pGAPZα A vector (Invitrogen) for secretion from *P. pastoris*. The first construct (designated *Ntdef1*-6xHis) contained the *S. cerevisiae* α-factor secretion peptide sequence fused in-frame with the sequence of the first codon of the *Ntdef1* mature peptide (Arg32), where the last codon of the mature *Ntdef1* peptide (Cys78) was immediately followed by the sequence for a 6xHis tag (Figure 4.2). This construct was designed to produce the same protein as those produced in the transgenic plant. To make this construct, the mature peptide sequence of *Ntdef1* was amplified from the plasmid that had been used to transform *N. tabacum* cv. Xanthi (Figure 4.1) using the sense primer (Nt1PiFo) 5’-GAATTCAAGAACTTGTGAGTCCAGAGC-3’ located in the 5’ end of *Ntdef1* mature peptide sequence and an antisense primer (Nt1PiRe1) 5’-TCTAGATTAGTGATGATGATGTTG-3’ located in the 6xHis-tag region. The
A. Components of the NtDef1-6xHis construct and location of primers. Sequence for NtDef1 prosequence and mature peptide were amplified from the existing binary vector used for tobacco transformation using Nt1PiFo and Nt1PiRe1 primers, which contained EcoRI and XbaI sites to accommodate insertion into pGAPZα expression vector in frame with the α-factor signal peptide.

B. The DNA and amino acid sequences of the NtDef1-6xHis construct. Restriction sites are in bold letters, underlined DNA sequences are primer sites. Amino acid sequence in bold letters is the putative NtDef1-6xHis peptide. Arrows indicate possible signal peptide cleavage sites.

Figure 4.2 Plasmid construct for tobacco defensin 1 (Ntdef1-6xHis) expression in P. pastoris X-33. A. Components of the Ntdef1-6xHis construct and location of primers. Sequence for Ntdef1 prosequence and mature peptide were amplified from the existing binary vector used for tobacco transformation using Nt1PiFo and Nt1PiRe1 primers, which contained EcoRI and XbaI sites to accommodate insertion into pGAPZα expression vector in frame with the α-factor signal peptide. B. The DNA and amino acid sequences of the Ntdef1-6xHis construct. Restriction sites are in bold letters, underlined DNA sequences are primer sites. Amino acid sequence in bold letters is the putative NtDef1-6xHis peptide. Arrows indicate possible signal peptide cleavage sites.
underlined sequences in primers Nt1PiFo and Nt1PiRe1 indicate EcoRI and XbaI restriction sites, respectively. *Ntdef1* contains a single intron located within the signal peptide sequence at the sequence for the Glu22 codon (Atnaseo, 2003) (Figure 4.1). Therefore, the DNA sequence for the putative mature peptide does not contain the intron, and processing of the intron by *P. pastoris* is not a concern.

The second construct (designated *Ntdef1*-en-myc-6xHis) was identical to the *Ntdef1*-6xHis construct, except that the codon for Cys78 was immediately followed by sequences for an enterokinase cleavage site, a myc epitope and a 6XHis tag (Figure 4.3). The enterokinase cleavage site between the Ntdef1 mature peptide and the two tags allowed for cleavage of the tags if required (Figure 4.3). To make this construct, the nucleotide sequence of the mature *Ntdef1* peptide was amplified from the plasmid that had been used to transform *N. tabacum* cv. Xanthi (Figure 4.1) using the sense primer (Nt1PiFo) located in the 5’ end of the *Ntdef1* mature peptide sequence and an antisense primer (Nt1PiRe1-EN) 5’-

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GGGCCCTCTAGAGCCCTTATCATCACGTACGCAAGGCCTGGTACAGAAAC
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located at the 3’ end of the *Ntdef1* mature peptide sequence. The underlined sequence indicates an *XbaI* restriction site, and the italicized sequence indicates an enterokinase cleavage site incorporated into the primer. The primers were designed so that the myc epitope tag and 6xHis tag located after the enterokinase cleavage site would be in frame as these tags were features already present on the pGAPZA A vector.
Figure 4.3 Plasmid construct for tobacco defensin 1 (Ntdef1-en-myc-6xHis) expression in P. pastoris X-33. A. Components of the Ntdef1-en-myc-6xHis construct and location of primers. Sequence for Ntdef1 mature peptide was amplified from the existing binary vector used for tobacco transformation using Nt1PiFo and Nt1PiReEN primers, which contained EcoRI and XbaI sites to accommodate insertion into pGAPZα expression vector in frame with the α-factor signal peptide. B. The DNA and amino acid sequences of the Ntdef1-en-myc-6xHis construct. Restriction sites are in bold letters, underlined DNA sequences are primer sites. Amino acid sequence in bold letters is the putative NtDef1-en-myc-6xHis peptide. Arrows indicate possible signal peptide cleavage sites and enterokinase cleavage site.
PCR was performed in a 50 μL reaction mixture of 1x High Fidelity PCR buffer, 0.2 mM dNTPs (Promega), 2 mM MgSO₄, 0.2 μM of each primer, 1 unit of Platinum® Taq DNA polymerase High Fidelity (Invitrogen) and 50 ng of template DNA. PCR was done with a denaturing step of 95°C for 2 min, followed by 25 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. PCR products were visualized and then purified with the QIAquick gel extraction kit (Qiagen). To incorporate the PCR fragment into the pGAPZα A vector, both the PCR fragment and the vector were digested with EcoRI and XbaI restriction enzymes. The digested vector and insert fragments were purified with the QIAquick gel extraction kit (Qiagen) and ligated as explained in Chapter 3. Transformation of E. coli strain DH5α with the ligated product as well as the selection of the positive colonies was performed as per Chapter 3, following instruction provided with pGAPZ products (Invitrogen).

E. coli colonies were screen by PCR using the pGAP forward primer located upstream of the insertion site (5’-GTCCCTATTTCAATCAATTGAA-3’) and AOX1 reverse primer located downstream from the insertion site (5’-GCAAATGGCATTCTGACATCC-3’). Cells from each colony were added to a PCR reaction mixture composed of 1 unit of Taq DNA polymerase, 1xThermoPol reaction buffer including 2mM MgSO₄ (New England BioLabs), 0.2 mM dNTPs (Promega), and 0.2 μM of each primer. PCR was performed with a denaturing step of 95°C for 2 min, followed by 30 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. The PCR positive clones were verified by sequencing of the insert (Laboratory Services, University of Guelph).
2.7 *Pichia pastoris* transformation

Plasmid DNA was extracted from positive *E. coli* clones and linearized with *Avr*II (Fermentas) and then introduced into *P. pastoris* X-33 following the instructions provided for pGAPZ products (Invitrogen), by performing electroporation with a MicroPulser Electroporator (BioRad). Colony selection was done on YPD plates supplemented with 100 μg/mL Zeocin (Invitrogen) as described in Chapter 3. Screening for Zeocin resistant *P. pastoris* colonies was done by colony PCR using the Nt1PiFo and AOX1 primers for both constructs under the PCR conditions explained above for screening of *E. coli* colonies.

2.8 Production, purification and identification of Ntdef1 protein from *P. pastoris*

Cultures of *P. pastoris* were grown as per Chapter 3, and the supernatants were collected by centrifugation at 3000 xg for 5 to 10 min after 3 days in culture. The supernatant was mixed with the Ni-NTA agarose (Qiagen) and incubated overnight at 4°C. The bound proteins were eluted from the Ni-NTA agarose and detected by Tricine SDS-PAGE as per Chapter 3. Eluates containing proteins of interest were combined and added to Amicon ultra centrifugal filters with a 3000 MW cutoff (Milipore) to remove salt and concentrate the protein. The concentrated protein was sterilized by filtering through a 0.22 μm Ultrafree-MC centrifugal filter (Milipore). Protein concentration was estimated with a Bio-Rad Protein assay kit using bovine γ-globulin as a standard (BioRad). Protein analysis by slot blot and western blot were done using anti-Histidine antibody for both the *Ntdef1*-6xHis and *Ntdef1*-en-myc-6xHis constructs following the protocol described in Chapter 3. Identification of the purified 6xHis-tag protein was done
with LC MS/MS at The Advanced Protein Technology Centre (APTC) of The Hospital for Sick Children (appendix A).

2.9 Antifungal and antibacterial activities of Ntdef1 protein produced in \( P. \) \textit{pastoris}

For the antifungal assay, spores from \( C. \) \textit{destructivum} strain N150 and \( C. \) \textit{orbiculare} strain ATTC 20767 were grown on SYAS agar, prepared and adjusted to \( 1 \times 10^5 \) spores/mL in liquid SYAS broth as described in Chapter 3. Then, \( 50 \) \( \mu \)L of Ntdef1-6xHis extract or Ntdef1-en-myc-6xHis extract from \( P. \) \textit{pastoris} was adjusted to 25, 50, 100, 200 and 400 \( \mu \)g/mL of total protein in the NtDef1 extract in sterile water. This was combined with \( 50 \) \( \mu \)L of spore suspension in 96-well microtitre plates (Sarstedt). As a control, sterile water was used instead of protein solution. Fungal growth was assessed by spectrophotometry at the moment that the control reached an OD\textsubscript{595} value of 0.2 as explained in Chapter 3.

For the antibacterial assay, bacterial strains (Table 3.1) were grown overnight in 3% tryptic soy broth and prepared as per Chapter 3. The bacterial concentration was adjusted to approximately \( 10^6 \) CFU/mL in 10 mM sodium phosphate buffer pH 7.4 with 1% tryptic soy broth. In a 500 \( \mu \)L microtube, 3 \( \mu \)L of bacteria was mixed with 27 \( \mu \)L of Ntdef1-6xHis extract or Ntdef1-en-myc-6xHis extract from \( P. \) \textit{pastoris} to give a final concentration of 25, 100, 300 and 500 \( \mu \)g/mL total protein. After 3 h incubation at 28 or 37\textdegree C, depending on the strains, the assay mixture was diluted in 10 mM sodium phosphate buffer pH 7.4, and plated onto TSA for assessment of bacterial growth as detailed in Chapter 3.
3. Results

3.1 Development of *N. tabacum* T2 homozygous for *Ntdef1* transgenes

The five T0 *N. tabacum* cv. Xanthi transformed lines that were most resistant to *P. syringae* pv. *tabaci* PBIC4 previously identified by Atnaseo (2003) were used to generate T1 seedlings by selfing each T0 plant and testing 48 T1 seedlings per line for resistance to hygromycin. Also, the presence of the transgenic version of *Ntdef1* was determined by PCR with DNA from 10 surviving seedlings from each line and a primer located within the gene and a primer located within the 35S terminator. These 10 T1 seedlings, which were both hygromycin resistance and PCR positive, were selected for each of the five original T0 transformants and then were self fertilized to produce T2 seeds. The T1 lines were determined to be homozygous if 80 out of 80 T2 seedlings tested were resistant to hygromycin. The selected lines were 6-9, 8-1, 20-10, 25-8, and 30-6. To verify the presence of the transgene, two randomly selected T2 seedlings from each line were tested with PCR and RT-PCR for *Ntdef1* presence and expression, respectively (Table 4.1; Figures 4.4). Based on band intensity, lines 6-9, 8-1, 20-10, 25-8 appeared to have the same level of transcripts, while line 30-6 appeared to have less transcripts. In addition, GUS activity derived from the *gus* gene co-transformed with *Ntdef1* was also observed in all selected T2 seedlings, with all lines appearing to have similar levels of GUS activity. However, the RT-PCR and GUS activity results were estimates rather than quantitative results (Table 4.1). All T2 plants appeared morphologically normal compared to the non-transgenic plant, and T2 seedlings were used in all subsequent work in this chapter.
Table 4.1 Screening of the five selected homozygous lines of *N. tabaccum* Xanthi expressing *Ntdef1* for the presence of transgene by PCR and its expression by RT-PCR. PCR was performed on genomic DNA using NtDef1Fo and 19-3 primers. For RT-PCR, cDNA was synthesized from oligo (dT)$_{18}$ and PCR was performed using primers JapanFo and 19-3.

<table>
<thead>
<tr>
<th>Line</th>
<th>Presence of <em>Ntdef1</em> tested by PCR</th>
<th>Expression of <em>Ntdef1</em> tested by RT-PCR</th>
<th>Expression of <em>gus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>6-9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20-10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25-8</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30-6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DC1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Xanthi</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 The specific tobacco lines were selected on the basis of reduced visual disease symptoms in the T$_0$ plants inoculated with *P. syringae* pv. *tabaci* strain PBIC4. The five T$_0$ lines showing the least symptoms were selected. The T$_2$ homozygous tobacco plants were selected on the basis of hygromycin resistance. DC1 is a line which has gone through the transformation and tissue culture selection process but does not contain the transgenic construct.

2 The GUS gene was co-transformed with *Ntdef1*, and GUS expression was evaluated as additional indication of transformation event.
Figure 4.4 The presence and expression of Ntdef1 transgenes in selected T2 homozygous N. tabacum cv. Xanthi as determined by PCR and RT-PCR. A. PCR analysis of tobacco plants transformed with Ntdef1. PCR with primers NtDef1Fo and 19-3 produced an expected product of 1262 bp as indicated by an arrow. B. RT-PCR with primers JapanFo and 19-3 gave the expected product of 365 bp indicated by an arrow. Wild type cv. Xanthi (Xan) and a transformed rescued line (DC1) were negative controls, and the Ntdef1 plasmid was a positive control (+). M indicates the Low DNA Mass Ladder for PCR, and M indicates the O’GeneRuler Low Range DNA Ladder for RT-PCR. Selected plants were lines 6-9, 8-1, 20-10, 25-8, and 30-6 of the T2 homozygous lines of N. tabacum cv. Xanthi containing Ntdef1 gene.
Western blot analysis using anti-His tag antibodies could not detect Ntdef1 protein in plant extracts in two different experiments. Total soluble protein extracts at 50-150 μg was used per lane, and the western blot analysis should have been able to detect approximately 10 ng of protein as determined by the detection of His-tag ladder. This suggested that any Ntdef1 protein in these plants is at a level lower than 0.02 to 0.007% of total soluble leaf protein.

3.2 Evaluation of resistance of T2 plants to *P. syringae pv. tabaci*

Dip inoculated seedlings developed water soaked areas by 1 to 2 dpi that developed into necrotic spots by 3 dpi that were often surrounded by chlorosis. The chlorosis sometimes expanded to cover the entire seedling, which then became necrotic. Dip inoculation showed that line 30-6 had 44% less mortality than the control DC1 line, which was significant (Table 4.2). However, when bacterial numbers were determined, the bacterial populations in line 30-6 were not significantly different from that found in line DC1 (Figure 4.5) indicating that the increased resistance was related to resistance to the damage caused by *P. syringae pv. tabaci* rather than affecting the population of the bacteria.

3.3 Evaluation of resistance of T2 plants to *C. destructivum* and *C. orbiculare*

Fungal inoculated 6-week-old plants had fewer lesions/cm² than 5-week-old plants, but the transgenic plants did not have a significant reduction in disease severity by *C. destructivum* or *C. orbiculare* compared to the controls (Table 4.3 and 4.4). This was consistent for *C. destructivum* either when analyzed by direct lesions/cm² or % lesions/cm² of the control, but when expressed as % lesions/cm² of the control, line 25-8 had significantly less symptoms to *C. orbiculare*.
Table 4.2 Resistance of *N. tabacum* cv. Xanthi transformed with *Ntdef1* gene against *P. syringae* pv. *tabaci* 11528R. Three to four weeks old seedlings were infected with *P. syringae* pv. *tabaci* 11528R by dip inoculation in a $1.2 \times 10^8$ CFU/mL suspension. Numbers of dead seedling were assessed after 4 weeks. The tissue culture regenerated line (DC1) and wild-type cv. Xanthi served as controls.

<table>
<thead>
<tr>
<th>Line 1</th>
<th>Mortality rate (%) 2,3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthi</td>
<td>85 a</td>
</tr>
<tr>
<td>DC1</td>
<td>69 a</td>
</tr>
<tr>
<td>6-9</td>
<td>78 a</td>
</tr>
<tr>
<td>8-1</td>
<td>69 a</td>
</tr>
<tr>
<td>20-10</td>
<td>64 a</td>
</tr>
<tr>
<td>25-8</td>
<td>64 a</td>
</tr>
<tr>
<td>30-6</td>
<td>25 b</td>
</tr>
</tbody>
</table>

1 Lines 6-9, 8-1, 20-10, 25-8, and 30-6 are the T2 homozygous lines of *N. tabacum* cv. Xanthi containing *Ntdef1*.

2 Mortality rate was measured as a percentage of the total 12 inoculated seedlings.

3 Data represent the average of three trials. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at $p = 0.05$. 
Figure 4.5 Population of *P. syringae* pv. *tabaci* 11528R in *N. tabacum* cv. Xanthi transformed with *Ntdef1* gene (line 30-6) following inoculation by the dip inoculation assay. Five weeks old plants were infected with *P. syringae* pv. *tabaci* 11528R by infiltration with a $1.2 \times 10^4$ CFU/mL culture. Bacteria populations inside the infiltrated area were assessed daily. The tissue culture regenerated line (DC1) served as the control. Standard error bars were calculated from five replications. (■ = DC1, ■ = 30-6)
Table 4.3 Resistance of *N. tabacum* cv. Xanthi lines transformed with *Ntdef1* against *C. destructivum* N150. Five or six weeks old plants were sprayed with 5x 10^4 spores/mL of *C. destructivum* N150. The number of lesions/cm^2^ leaf area was assessed 72 hr after the infection. The tissue culture regenerated line (DC1) and wild-type cv. Xanthi served as controls.

<table>
<thead>
<tr>
<th>Line</th>
<th>Number of lesions/cm^2^ on 5-wk-old plants</th>
<th>Severity (%) compared to Xanthi for 5-wk-old plants</th>
<th>Number of lesions/cm^2^ on 6-wk-old plants</th>
<th>Severity (%) compared to Xanthi for 6-wk-old plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthi</td>
<td>4.2 b</td>
<td>100</td>
<td>3.3 a</td>
<td>100</td>
</tr>
<tr>
<td>DC1</td>
<td>5.0 ab</td>
<td>115 a</td>
<td>3.2 ab</td>
<td>99 a</td>
</tr>
<tr>
<td>6-9</td>
<td>6.0 a</td>
<td>145 a</td>
<td>3.3 a</td>
<td>98 a</td>
</tr>
<tr>
<td>8-1</td>
<td>5.1 ab</td>
<td>121 a</td>
<td>2.2 b</td>
<td>67 a</td>
</tr>
<tr>
<td>20-10</td>
<td>5.4 ab</td>
<td>129 a</td>
<td>2.8 ab</td>
<td>81 a</td>
</tr>
<tr>
<td>25-8</td>
<td>5.5 ab</td>
<td>131 a</td>
<td>3.4 a</td>
<td>101 a</td>
</tr>
<tr>
<td>30-6</td>
<td>4.7 ab</td>
<td>110 a</td>
<td>3.3 a</td>
<td>102 a</td>
</tr>
</tbody>
</table>

1 Lines 6-9, 8-1, 20-10, 25-8, and 30-6 are the T2 homozygous lines of *N. tabacum* cv. Xanthi containing *Ntdef1*.
2 Disease severity was measured as the number of lesions/cm^2^.
3 Disease severity was expressed as the percentage of lesions/cm^2^ vs. untransformed *N. tabacum* cv. Xanthi control.
4 Data represent the average of five trials for 6 weeks and three trials for 5 weeks plants. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at *p* = 0.05.
Table 4.4 Resistance of *N. tabacum* cv. Xanthi lines transformed with *Ntdef1* against *C. orbiculare* ATTC 20767. Five weeks old plants were sprayed with 1x 10⁵ spores/mL of *C. orbiculare* ATTC 20767. The number of lesions per cm² leaf area was assessed 72 hr after the infection. The tissue culture regenerated line (DC1) and wild-type cv. Xanthi served as controls.

<table>
<thead>
<tr>
<th>Line ¹</th>
<th>Number of lesions/cm² on 5-wk-old plants ², ³</th>
<th>Severity (%) compared to Xanthi for 5-wk-old plants ³, ⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthi</td>
<td>3.7 ab</td>
<td>100</td>
</tr>
<tr>
<td>DC1</td>
<td>4.2 a</td>
<td>121 ab</td>
</tr>
<tr>
<td>6-9</td>
<td>4.1 ab</td>
<td>120 ab</td>
</tr>
<tr>
<td>8-1</td>
<td>4.1 ab</td>
<td>129 a</td>
</tr>
<tr>
<td>20-10</td>
<td>3.2 ab</td>
<td>100 abc</td>
</tr>
<tr>
<td>25-8</td>
<td>3.1 b</td>
<td>85 c</td>
</tr>
<tr>
<td>30-6</td>
<td>3.3 ab</td>
<td>95 bc</td>
</tr>
</tbody>
</table>

¹ Lines 6-9, 8-1, 20-10, 25-8, and 30-6 are the T₂ homozygous lines of *N. tabacum* cv. Xanthi containing *Ntdef1*.
² Disease severity was measured as the number of lesions/cm².
³ Disease severity was expressed as the percentage of lesions/cm² vs. untransformed *N. tabacum* cv. Xanthi control.
⁴ Data represent the average of six trials. Numbers designated with different letters were significantly different from each other according to Fisher’s protected LSD test as calculated by SAS Proc GLM at *p* = 0.05.
3.4 Transformation of *Pichia pastoris* X-33 with *Ntdef1*

Transformation of *P. pastoris* X-33 yielded eight Zeocin resistant colonies with *Ntdef1*-6xHis, and all these colonies contained the *Ntdef1*-6xHis insert giving the expected product of 340 bp (Figure 4.6A). Transformation of *P. pastoris* X-33 also yielded ten Zeocin resistant colonies of *Ntdef1*-en-myc-6xHis, and all these colonies contained the *Ntdef1*-en-myc-6xHis insert giving the expected PCR product of 336 bp (Figure 4.6B).

3.5 Expression of *Ntdef1* in *Pichia pastoris* X-33

Based on small scale purification with Ni-NTA agarose of all 18 clones, clone 1 was selected for a high level of *Ntdef1*-6xHis production and clone 6 was selected for a high level of *Ntdef1*-en-myc-6xHis production. The secreted *Ntdef1*-en-myc-6xHis, but not the *Ntdef1*-6xHis, could be detected directly in *P. pastoris* growing media by silver stained tricine SDS-PAGE gels and western analysis (Figure 4.7; 4.8, lane 1). Following purification with Ni-NTA agarose, both *Ntdef1*-6xHis and *Ntdef1*-en-myc-6xHis could be detected by both silver stained tricine SDS-PAGE gels and western blots (Figures 4.7 and 4.8, lanes 4-7). *Ntdef1*-6xHis gave a major band at about 8 kDa and a minor band at about 13 kDa, both of which were larger than the expected product of 6.4 kDa. *Ntdef1*-en-myc-6xHis gave multiple bands with the most prominent ones at approximately 13 kDa and 25 kDa, which once again were larger than the expected size of 8.8 kDa.

Two protein bands from each construct were excised from the gel and sent for identification by LC MS/MS. When compared with the sequence of the *Ntdef1* protein, all tested gel fragments were found to contain *Ntdef1* protein. There was no difference between the larger and smaller bands for both constructs except for the degree of peptide
Figure 4.6 The presence of Ntdef1 transgenes in clones of P. pastoris X-33 as determined by PCR.  
A. PCR analysis of P. pastoris X-33 clones transformed with Ntdef1-6xHis construct. PCR with primers Nt1PiFo and AOX1Re produced an expected product of 340 bp as indicated by an arrow.  
B. PCR analysis of P. pastoris X-33 clones transformed with Ntdef1-en-myc-6xHis construct. PCR with primers Nt1PiFo and AOX1Re produced an expected product of 336 bp as indicated by an arrow. P. pastoris (X-33) and a reaction with no template DNA (H2O) were negative controls, and the P. pastoris expression plasmid contained either Ntdef1-6xHis (A) or Ntdef1-en-myc-6xHis (B) was positive controls (+ve). M indicates O’GeneRuler 100 bp DNA Ladder Plus.
Figure 4.7 Detection of secreted Ntdef1-6xHis by tricine-SDS-PAGE (top) and western blot (bottom) during purification. 6xHis PBD-1 protein was collected from growth media (lane 1) of a *P. pastoris* clone expressing *Ntdef1*-6xHis using Ni-NTA resin. His-tag protein bound to the Ni-NTA resin while the unbound growth media passed through (lane 2). The resin was washed to remove proteins bound non-specifically (lane 3). Then the bound proteins were eluted out 4 times (lanes 4-7) and these solutions were collected for further purification. His-tag antibody was used to detect the presence of 6xHis protein throughout the collection process. Samples were separated on a 16% acrylamide gel. M indicates Page Ruler Plus Prestained Protein (top), and the 6xHis Protein Ladder is shown on lane 8.
Figure 4.8 Detection of secreted Ntdef1-en-myc-6xHis by tricine-SDS-PAGE (top) and western blot (bottom) during purification. 6xHis PBD-1 protein was collected from growth media (lane 1) of a P. pastoris clone expressing Ntdef1-en-myc-6xHis using Ni-NTA resin. His-tag protein bound to the Ni-NTA resin while the unbound growth media passed through (lane 2). The resin was washed to remove proteins bound non-specifically (lane 3). Then the bound proteins were eluted out 4 times (lanes 4-7) and these solutions were collected for further purification. His-tag antibody was used to detect the presence of 6xHis protein throughout the collection process. Samples were separated on a 16% acrylamide gel. M indicates Page Ruler Plus Prestained Protein (top), and the 6xHis Protein Ladder is shown on lane 8.
coverage due to different protein concentrations of each band. The secreted proteins appeared to have the signal peptide cleavage occurring at four amino acids upstream of the expected cleavage site. This was previously observed also with PBD-1 using this expression system (Chapter 3). Recalculation of the molecular weight of the Ntdef1-6xHis with the additional four amino acids resulted in a protein of approximately 6.8 kDa and pI 8.26 compared to 6.4 kDa and pI 8.78 without the four additional amino acids. Recalculation of the molecular weight with the additional four amino acids for the Ntdef1-en-myc-6xHis construct resulted in a protein of 9.2 kDa and pI 5.74 compared to 8.8 kDa and pI 6.14 without the four additional amino acids. However, none of these recalculated values matched the sizes in the silver stained gels and western blots.

Comparison of the LC MS/MS data to the *P. pastoris* protein database indicated that other *P. pastoris* proteins were also present in the samples. The Ntdef1-6xHis samples appeared to be mixed with *P. pastoris* helicase, and the Ntdef1-en-myc-6xHis samples were mixed with *P. pastoris* helicase (identical to that in the Ntdef1-6xHis sample), glyceraldehydes-3-phosphate dehydrogenase, protein components for the large (60S) and small (40S) ribosomal subunit, enolase I, pyridoxine phosphate oxidase, mitochondrial alcohol dehydrogenase isozyme III and an unidentified hypothetical protein. The presence of these proteins in the bands may indicate that they form a complex with NtDef1 inhibiting it from properly migrating through the gel. This may explain the larger than expected calculated size of the two proteins on the SDS-PAGE gel and western blot.
3.6 Antifungal activity of Ntdef1

For Ntdef1-6xHis, growth of *C. destructivum* N150 first significantly increased at a protein concentration of 12.5 µg/mL but then became significantly inhibited at a protein concentration of 100 µg/mL (Figure 4.9A). Further increases in protein concentration up to 200 µg/mL did not increase the antifungal activity. Ntdef1-6xHis also significantly increased *C. orbiculare* ATTC 20767 growth at the lowest concentration tested, 12.5 µg/mL (Figure 4.9B). Growth was first significantly decreased at 50 µg/mL. Fungal growth showed maximum inhibition at 100 µg/mL (Figure 4.9). The EC₅₀ value for Ntdef1-6xHis against *C. destructivum* N150 and *C. orbiculare* ATTC 20767 were 175 and 104 µg/mL, respectively (Table 4.5).

For the Ntdef1-en-myc-6xHis, growth of *C. destructivum* N150 never increased as when exposed to Ntdef1-6xHis (Figure 4.10A). Ntdef1-en-myc-6xHis first significantly inhibited *C. destructivum* N150 at protein concentration of 25 µg/mL (Figure 4.10A). Ntdef1-en-myc-6xHis activity against *C. destructivum* N150 showed maximum inhibition at 50 µg/mL. Ntdef1-en-myc-6xHis against *C. orbiculare* ATTC 20767 was first detectable at protein concentration of 25 µg/mL and showed maximum inhibition at 200 µg/mL (Figure 4.10B). The EC₅₀ value for Ntdef1-en-myc-6xHis against *C. destructivum* N150 and *C. orbiculare* ATTC 20767 were 109 and 200 µg/mL, respectively (Table 4.5).

3.7 Antibacterial activity of Ntdef1

All eleven gram-negative bacteria tested showed no lethal effect due to Ntdef1-6xHis or the Ntdef1-en-myc-6xHis, even at 500 µg/mL (Figures 4.11 to 4.20; Table 4.5). The two gram-positive bacteria, *B. subtilis* strain GB03 and *C. michiganensis* subsp.
Figure 4.9 Antifungal activity of Ntdef1-6xHis produced by \textit{P. pastoris} X-33 against \textit{C. destructivum} N150 (A) and \textit{C. orbiculare} ATTC 20767 (B). Fungal spores were incubated in 200, 100, 50, 25, 12.5 µg/mL of Ntdef1-6xHis at 25 °C until the water control reached the OD_{595} of 0.2 when all readings were taken, which was 40 to 45 h for \textit{C. destructivum} N150 and 48 to 52 h for \textit{C. orbiculare} ATTC 20767. Standard error bars were calculated from four replications for \textit{C. destructivum} N150 and three replications for \textit{C. orbiculare} ATTC 20767.
Table 4.5 Antifungal and antibacterial activity of Ntdef1-6xHis and Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 as determined by 50% effective concentration (EC$_{50}$).

<table>
<thead>
<tr>
<th>Species</th>
<th>EC$_{50}$ $^1$ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ntdef1-6xHis</td>
</tr>
<tr>
<td><em>C. orbiculare</em> ATTC 20767$^2$</td>
<td>104</td>
</tr>
<tr>
<td><em>C. destructivum</em> N150$^2$</td>
<td>175</td>
</tr>
<tr>
<td><em>C. michiganensis</em> Cmm</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>B. subtilis</em> GB03</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>K. pneumonia</em> 342</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tabaci</em> ZA Patrick</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>E. coli</em> UB1005</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>E. carotovora</em> Ecc</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> K799</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PA01</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tomato</em> 06T2</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. syringae</em> pv. <em>tabaci</em> 11528R</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>X. axonopodis</em> pv. <em>phaseoli</em> UG18</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>A. tumefaciens</em> A348</td>
<td>No dose response</td>
</tr>
<tr>
<td><em>P. chlororaphis</em> B21</td>
<td>No dose response</td>
</tr>
</tbody>
</table>

$^1$ EC$_{50}$ values were calculated by SAS proc Probit

$^2$ EC$_{50}$ values for the two fungi were calculated from the dose response range between 0 and 100 µg/mL.
Figure 4.10 Antifungal activity of Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *C. destructivum* N150 (A) and *C. orbiculare* ATTC 20767 (B). Fungal spores were incubated in 200, 100, 50, 25, 12.5 µg/mL of Ntdef1-en-myc-6xHis at 25 °C until the water control reached the OD$_{595}$ of 0.2 when all readings were taken, which was 40 to 45 h for *C. destructivum* N150 and 48 to 52 h for *C. orbiculare* ATTC 20767. Standard error bars were calculated from three replications.
Figure 4.11 Antibacterial activity of Ntdef1-6xHis produced by *P. pastoris* X-33 against *E. coli* strain UB1005 (A) and *E. carotovora* subsp. *carotovora* Ecc (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-6xHis for 3 h at 37 °C for UB1005 and 28 °C for Ecc before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.12 Antibacterial activity of Ntdef1-6xHis produced by \textit{P. pastoris} X-33 against \textit{P. aeruginosa} strain K799 (A) and against \textit{P. aeruginosa} strain PA01 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-6xHis for 3 h at 37 °C for K799 and 28 °C for PA01 before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.13 Antibacterial activity of Ntdef1-6xHis produced by \( P. \) \textit{pastoris} X-33 against 
against \( P. \) \textit{syringae} pv. tomato strain 06T2 (A) \( P. \) \textit{syringae} pv. \textit{tabaci} strain 11528R (B) and \( P. \) \textit{syringae} pv. \textit{tabaci} strain ZA Patrick (C). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-6xHis for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.14 Antibacterial activity of Ntdef1-6xHis produced by *P. pastoris* X-33 against *X. axonopodis* pv. *phaseoli* strain UG18 (A) and against *A. tumefaciens* strain A348 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-6xHis for 3 h at 28 ºC before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.15 Antibacterial activity of Ntdef1-6xHis produced by *P. pastoris* X-33 against *K. pneumonia* 342 (A) and against *P. chlororaphis* strain B21 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-6xHis for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.16 Antibacterial activity of Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *E. coli* strain UB1005 (A) and *E. carotovora* subsp. *carotovora* Ecc (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-en-myc-6xHis for 3 h at 37 ºC for UB1005 and 28 ºC for Ecc before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.17 Antibacterial activity of Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *P. aeruginosa* strain K799 (A) and against *P. aeruginosa* strain PA01 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-en-myc-6xHis for 3 h at 37 ºC for K799 and 28 ºC for PA01 before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.18 Antibacterial activity of Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *P. syringae* pv. tomato strain 06T2 (A) *P. syringae* pv. *tabaci* strain 11528R (B) and *P. syringae* pv. *tabaci* strain ZA Patrick (C). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-en-myc-6xHis for 3 h at 28 ºC before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.19 Antibacterial activity Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *X. axonopodis* pv. *phaseoli* strain UG18 (A) and against *A. tumefaciens* strain A348 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-en-myc-6xHis for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
**Figure 4.20** Antibacterial activity of Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *K. pneumonia* 342 (A) and against *P. chlororaphis* strain B21 (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-en-myc-6xHis for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.21 Antibacterial activity Ntdef1-6xHis produced by *P. pastoris* X-33 against *B. subtilis* strain GB03 (A) and against *C. michiganensis* subsp. *michiganensis* Cmm (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-6xHis for 3 h at 28 ºC before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
Figure 4.22 Antibacterial activity of Ntdef1-en-myc-6xHis produced by *P. pastoris* X-33 against *B. subtilis* strain GB03 (A) and against *C. michiganensis* subsp. *michiganensis* Cmm (B). Bacteria were incubated in 500, 300, 100, 25 µg/mL of Ntdef1-en-myc-6xHis for 3 h at 28 °C before being plated on TSA to determine cell viability. Standard error bars were calculated from three replications.
michiganensis showed a small but significant decrease in viability to Ntdef1-6xHis at a protein concentration of 300-500 µg/mL (Figure 4.21). However, the EC₅₀ values for these bacteria were higher than 500 µg/mL (Table 4.5). Neither B. subtilis strain GB03 nor C. michiganensis subsp. michiganensis was affected by Ntdef1-en-myc-6xHis (Figures 4.22A and 4.22B).

4. Discussion

Ntdef1 is a 1230-bp gene composed of two exons separated by a 954 bp intron in the signal peptide (Atnaseo, 2003). The putative coding region of Ntdef1 codes for a 78 amino acid peptide, which includes a 31-amino acid signal peptide located at the N-terminus followed by a 47-amino acid mature peptide. The 47-amino acid mature peptide contains the eight conserved cysteine sites of plant defensins, and has a molecular weight of 5.3 kDa at a pI of 8.99. RT-PCR analysis of healthy leaves and flowers of N. tabacum cv. Xanthi plants indicated that Ntdef1 was constitutively expressed in these tissues. The only other information about the biological activity of Ntdef1 is that an identical gene encoding thionin-like protein (TLP) cloned by Takemoto and Kawakita (1999) was induced by fungal elicitor. This suggests that overexpression of Ntdef1 may improve plant disease resistance in tobacco.

In previous work, transgenic N. tabacum cv. Xanthi plants containing Ntdef1 with a 35S promoter were created, and based on the disease severity scoring of the T₀ lines, these plants appeared to be more resistant to P. syringae pv. tabaci PBIC4 (Atnaseo, 2003). Homozygous T₂ progeny of these transgenic N. tabacum were generated for the current study in an attempt to increase level of protein production and disease resistance.
and were tested against *P. syringae* pv. *tabaci* 11528R, *C. destructivum* N150 and *C. orbiculare* ATCC 20767. In addition, Ntdef1 produced by *P. pastoris* expression system was also tested for antimicrobial activity against several plant and animal pathogens.

The PCR and RT-PCR results of the T2 plants verified the presence of Ntdef1 and its expression in the five selected lines. However, Ntdef1 protein was not detected in these plants. Seedlings of line 30-6 had lower mortality rates due to *P. syringae* pv. *tabaci* 11528R than the control plants when dip-inoculated, but the bacterial populations in line 30-6 were not significantly lower than the control. This indicates that greater resistance in line 30-6 is related to its ability to limit the negative effects caused by pathogen rather than its ability to limit pathogen growth. It appears that line 30-6 may have increased disease tolerance, which is defined as the ability of a plant cultivar to endure substantial effects of disease without substantial yield or quality loss in comparison with other cultivars (Schafer, 1971). None of the transgenic plants had enhanced resistance against *C. destructivum* N150, or *C. orbiculare* ATCC 20767 compared to the controls plants.

Since the Ntdef1 protein was not detected, it is possible that the plants do not contain sufficient Ntdef1 protein to increase resistance to the tested pathogens. Such a result could be due to homology-dependent gene silencing, i.e. suppression both the transgene and the endogenous copy of the gene. A well-known example of this phenomenon was the overexpression of the petunia chalcone synthase gene which resulted in unexpected white patches in some flowers (Napoli et al., 1990). However, the presence of Ntdef1 mRNA suggests that expression of Ntdef1 was not silenced, but there could have been a partial down regulation of the transgene expression leading to reduced
levels of Ntdef1 protein in the T2 homozygous lines. Another possible explanation is that the homozygous lines do not have higher expression despite having at least two copies of the gene and may even have lower levels. For example, homozygous progeny of high expressor parents of CaMV35S promoter-gus fusions in *N. tabacum* yielded plants with double the GUS activity compared to the parental lines, while homozygous progeny from low expressor parents did not have GUS activity that was not different from the heterozygous parents (Hobbs et al., 1993). Homozygous progeny of high expressor parents of a CaMV35S promoter-gus fusion in rice yielded plants with 1.7 fold higher GUS activity compared to the parental lines, while homozygous progeny from low expressor parents yielded plant with 0.64 fold lower GUS activity compared to the heterozygous parents indicating that gus expression could be either higher or lower with multiple copies of the transgene in homozygous plants (James et al., 2002). A third possible explanation for low protein levels is that there was degradation of the transgenic version of Ntdef1 protein. The lifespan of Ntdef1 protein in tobacco is not known. However, plant defensins are described as highly stable proteins (Broekaert et al., 1995), and so it is likely that Ntdef1 would not be rapidly degraded by tobacco proteases present in the intercellular space.

Expression of genes coding for antimicrobial peptides has been suggested as a method for enhancing plant disease resistance (Salmeron and Vernooij, 1998; Punja, 2001). Plant defensin genes have been widely used to create transgenic plants with different degrees of resistance (Table1.2). Almost all attempts to express plant defensin genes in transgenic plants to enhance disease resistance have utilized heterologous plant defensin genes rather than the endogenous genes. An exception is the overexpression of
endogenous *A. thaliana* defensins, *AtPDF1.1* and *AtPDF1.2a* (De Coninck et al., 2010). Although both defensins purified from *A. thaliana* extract had *in vitro* activity against *B. cinerea* and *AtPDF1.1* and *AtPDF1.2a* were induced upon inoculation with *B. cinerea*, the transgenic plants displayed no enhanced resistance against the *A. thaliana* pathogens, *B. cinerea*, *Alternaria brassicicola* and *Plectosphaerella cucumerina*. The authors suggested that the limited success in alteration of disease resistance could be due to the weak antimicrobial activity of these proteins and the aggressive nature of the pathogens, such that they could overcome the effect of expressing a single transgene. Moreover, the use of endogenous antimicrobial peptide genes to improve resistance against pathogens of that host plant may not be effective as the pathogens have already developed resistance to them. Indeed, most reports of successful attempts to create disease resistant plants utilizing AMPs either use animal AMPs (Table 1.1) or heterologous plant genes (Table 1.2). In contrast to this study and the study by De Coninck et al. (2010), overexpression of tomato defensin gene (*DEF2*) in tomato resulted in both *in vitro* and *in planta* activities against *B. cinerea* (Stotz et al., 2009a). Therefore, it is possible to produce transgenic disease resistant plant overexpressing an endogenous defensin gene, but the success may depend on the activity of the selected gene and its level of expression. Overexpression of *DEF2* also resulted in pleiotropic effects on growth and development of tomato plants making it unsuitable for practical use (Stotz et al., 2009a). In contrast, no pleiotropic effects were observed in this study.

Although De Coninck et al. (2010) did not report the level of defensin protein in the transgenic plants, earlier work by this group indicated that overexpression of *AtPDF1.1* in wild-type *A. thaliana* plants amounted to 0.25% of total soluble protein
(TSP) in leaves (Sels et al., 2007), but protein levels were not reported for AtPDF1.2a. De Coninck et al. (2010) and Sels et al. (2007) employed the PTGS-MAR expression system, which uses a MAR sequence of the chicken lysozyme gene to enhance expression combined with the sgs2 mutant of A. thaliana, which is deficient in PTGS to block any possible PTGS in the transgenic plant. However, the PTGS component of the system would not function in wild-type A. thaliana because it is capable of PTGS. When AtPDF1.1 was overexpressed in the sgs2 A. thaliana so that the PTGS-MAR expression system was fully functional, AtPDF1.1 production increased to 1% TSP (Sels et al., 2007). However, the sgs2 plants of A. thaliana expressing AtPDF1.1 were not tested directly against pathogens, and therefore, it is not known if AtPDF1.1 at 1% TSP increased disease resistance in planta. Overexpression of DEF2 in tomato resulted in DEF2 in tomato leaves at 0.9% TSP, which was sufficient to increase resistance against B. cinerea (Stotz et al., 2009a). This suggests that 0.25% TSP may not be sufficient in A. thaliana leaves to have in planta activity against B. cinerea, although the lack of in planta activity could be due to other differences between the two defensins or the two plant systems.

If defensin overexpression must reach close to 0.9% TSP for increased resistance in planta (Stotz et al., 2009a), then the transgenic tobacco containing Ntdef1 construct used in this study would not likely be more resistant, as the concentration is below the detection limit of 0.02% TSP in western blot. However, heterologous plant defensin expression can give protein production as low as 0.05% TSP for Dahlia merckii defensin, Dm-AMP1, in papaya and still increase disease resistance (Zhu et al., 2007). High levels
of defensin overexpression might not be required if that defensin protein is highly active against a particular pathogen.

As protein levels of the Ntdef1 gene were too low in the transgenic plants to evaluate if Ntdef1 protein is highly active against *P. syringae pv. tabaci* 11528R, *C. destructivum* N150, *C. orbiculare* ATTC 20767 or other microbes, a *P. pastoris* expression system was utilized to produce the protein. Two versions of Ntdef1 were expressed in *P. pastoris*. The first construct, Ntdef1-6xHis, would essentially yield the same protein product as the construct in the transgenic plants. The second construct, Ntdef1-en-myc-6xHis, would yield the native Ntdef1 protein if the C-terminal tag was removed by digestion at the enterokinase cleavage site preceding the myc epitope tag and 6xHis tag. The presence of the 6xHis tag at the C-terminus of both constructs enables protein purification by Ni-NTA agarose and detection with the anti-Histidine antibody.

Both *P. pastoris* expression constructs yielded secreted Ntdef1 protein in the growth media. For the Ntdef1-6xHis construct, the protein was not detected directly in the growth media, suggesting a concentration below 0.67 μg/mL, but it was detected after purification with Ni-NTA agarose on both silver-stained protein gels and western blots. For the Ntdef1-en-myc-6xHis construct, the presence of the 6xHis-tagged proteins was detected directly in *P. pastoris* growth media prior to purification with Ni-NTA agarose. Both constructs yielded Ntdef1 mixed with proteins from *P. pastoris* suggesting that the protein formed complexes with these *P. pastoris* proteins in the media, which could not be removed in the purification process and might negatively affect their antimicrobial activity. Another issue with Ntdef1 produced by *P. pastoris* was that the yeast α-factor secretion peptide was processed at the Kex2 signal cleavage site adding six amino acids
to the N-terminus of the Ntdef1 mature peptide. This could also negatively affect antimicrobial activity. The additional AAs due to the C-terminus tags also could affect activity. For the Ntdef1-6xHis construct, this only caused slight decrease to the pI, but for Ntdef1-en-myc-6xHis construct, this changed the pI from basic pI of native Ntdef1 to acidic pI. This is important because it has been suggested that the antifungal activity of defensins is related, in part, to their net charge based on the observation that of the two Raphanus sativus defensins, Rs-AFP2 with a higher net positive charge is more active than Rs-AFP1 (Terras et al., 1992). A similar difference is observed between A. thaliana defensins AtPDF1.1 and AtPDF1.3, where AtPDF1.1 with a higher pI displayed higher antifungal activity (Sels et al., 2007). Therefore, the change in pI could alter the activity of both versions of Ntdef, particularly the Ntdef1-en-myc-6xHis, which is why removal of the myc and 6xHis tags is desirable. Removal of the myc and 6xHis tags was not feasible in these experiments, as attempts to remove these tags with enterokinase resulted in a prohibitively large loss of protein.

For the Ntdef1-6xHis and Ntdef1-en-myc-6xHis constructs, there was no activity detected against P. syringae pv. tabaci 11528R. The lack of in vitro activity of Ntdef1 against P. syringae pv. tabaci 11528R suggests that overexpression in planta should not affect its resistance. Ntdef1-6xHis protein showed weak activity against C. destructivum N150, where the maximum growth inhibition was only 20%. However, C. orbiculare ATTC 20767 was more sensitive to Ntdef1-6xHis, where maximum growth inhibition was 43% at 100 µg/mL. Ntdef1-en-myc-6xHis displayed stronger activity against these two fungi, where growth of C. destructivum N150 reached a maximum of 45% inhibition at 50µg/mL. Sensitivity of C. orbiculare ATTC 20767 to Ntdef1-en-myc-6xHis was
lower with a maximum of 44% growth inhibition at 200µg/mL. The stronger antifungal activity by Ntdef1-en-myc-6xHis compared to Ntdef1-6xHis indicates that the changes in molecular weight and pI due to additional AA increased rather than decreased its antifungal activity.

If the levels of Ntdef1 required for increased resistance to *C. destructivum* N150 and *C. orbiculare* ATCC 20767 *in planta* are similar to those for the *in vitro* test with the purified proteins from *P. pastoris*, then the transgenic tobacco plant overexpressing *Ntdef1* would not likely be more resistant. These results are similar to those reported from overexpression of genes encoding *AtPDF1.1* and *AtPDF1.2a* in wild-type *A. thaliana*, where the protein appeared to have antifungal activity *in vitro* at 5 µg/mL for 50% growth inhibition, but there was no increase in resistance *in planta*, at least at the levels expressed in those plants (De Coninck et al., 2010).

To further investigate the antimicrobial activity of Ntdef1 proteins, ten gram-negative bacteria were tested, but none displayed sensitivity to the protein. On the other hand, two gram-positive bacteria were found to be sensitive to Ntdef1-6xHis specifically. Ntdef1-6xHis reduced the growth of *B. subtilis* strain GB03 by 75% and *C. michiganensis* subsp. *michiganensis* by 91% both at 500 µg/mL of protein. In contrast, these pathogens were not sensitive to Ntdef1-en-myc-6xHis. This lack of activity of Ntdef1-en-myc-6xHis could be due to the shift from a basic to an acidic pI resulting from the tags on Ntdef1-en-myc-6xHis. In addition, the presence of the *P. pastoris* proteins in the extract of Ntdef1-en-myc-6xHis could also alter its activity.

This is the first report of a study on the antimicrobial activity of a defensin from tobacco. As the antimicrobial activity appeared to be weak and higher protein
concentrations up to 500 µg/mL in the assay did not improve antimicrobial activity, it is possible that Ntdef1 does not have antimicrobial activity and may serve other purposes in the plant although these were not tested in this study. Vigna radiata defensin (VrD1) (Chen et al., 2004) and V. angularis defensin (VaD1) (Chen et al., 2005) can inhibit plant protein translation. A sunflower defensin, HaDef1, inhibited root growth of the parasitic plant, Orobanche cumana (de Zélicourt et al., 2007), and Medicago sativa defensin (MsDef1, MsDef2) and R. sativus defensins (Rs-AFP2) inhibited root growth of A. thaliana (Allen et al., 2008). Plant defensins also show activity against animals. Vigna unguiculata defensin (VuD1) (Pelegrini et al., 2008), Hordeum vulgare defensins (B1α1 and B1α2) (Zhang et al., 1997) and Sorghum bicolor defensins (S1α1, S1α2 and S1α3) inhibit insect α-amylase (Bloch and Richardson, 1991), and Vigna radiata defensin (VrCRP) has insecticidal activity (Chen et al., 2002). Cassia fistula defensin (CfD2) (Wijaya et al., 2000) and V. unguiculata Cp-thionin (Melo et al., 2002) can act as animal protease inhibitors as shown by in vitro activity against the model substrate, bovine trypsin, and H. vulgare γ1-hordothionin (Mendez et al, 1990), and ω-hordothionin (Mendez et al, 1996) display vertebrate and bacteria protein translation inhibitor activity. Finally, Zea mays γ1-zeathionin and γ2-zeathionin (Kushmerick et al., 1998) can function as ion channel blockers in rat pituitary epithelial-like tumour cell line. Thus, a plant defensin may exhibit more than one of these functions, such as, VrD1, which has antifungal activity, insecticidal activity as well as ability to inhibit plant protein translation (Chen et al., 2004). Therefore, Ntdef1 could be involved in protection against herbivorous insects, parasitic plants or regulation of plant growth and development.
In conclusion, transgenic plants overexpressing *Ntdef1* did not display increased resistance to a range of microbial pathogens. *Ntdef1* protein could not be detected in the transgenic plants, and *Ntdef1* produced by *P. pastoris* showed that *Ntdef1* had no activity against gram-negative pathogens, and relatively weak activity against fungal and gram-positive bacterial pathogens. These results suggest that the lack of resistance displayed by plants expressing *Ntdef1* may be due to not only low levels of expression of the transgene, but also to the lack of antimicrobial activity of *Ntdef1*. Based on the results from this study, *Ntdef1* might not be a good candidate gene for the generation of disease resistant plants or molecular farming. In this regard, expression systems such as *P. pastoris* provide a useful tool for screening potential antimicrobial proteins and determining their biological activity before creating transgenic plants. However, the use of *P. pastoris* maybe restricted for some plant defensins as they may be active against *P. pastoris*. Therefore, proper expression system will have to be used for the different plant defensins, and in some cases, direct extraction from plant tissue may be the preferred approach.
Chapter 5. General Discussion

Defensins constitute a class of cationic peptides with a broad range of antimicrobial activities, a feature which makes defensin genes promising candidates for engineering disease resistance in plants. Plants have also been promoted as a cost-effective means for producing therapeutic proteins, such as defensins and other AMPs (Daniell et al., 2001). The only reports of attempts to utilize vertebrate defensin genes to create plants more resistant to plant pathogens involve the rabbit α-defensin (NP-1) (Fu et al., 1998; Zhao et al., 1999; Zhang et al., 2000) and human β-defensin 2 (hBD-2) (Aerts et al., 2007), and the only reports of attempts to overexpress an endogenous plant defensin gene to create plants more resistant to plant pathogens involve the A. thaliana defensins, AtPDF1.1 and AtPDF1.2a (De Coninck et al., 2010) and tomato defensin gene (DEF2) (Stotz et al., 2009a). The research presented in this thesis documents the attempt to increase disease resistance in plants through expression of defensin genes from widely divergent organisms, a porcine β-defensin, pbd-1, and a tobacco defensin 1, Ntdef1.

Agroinfiltration is a transient expression system often used for testing the activity of foreign genes in plants because it allows for a protein to be synthesized inside the plant by a transgene without the need to create transgenic plants. Thus, it was a promising approach to express pbd-1 and Ntdef1 in order to study their potential use for increased plant disease resistance. However, it was found that the process of agroinfiltration, when the leaves are inoculated with Agrobacterium tumefaciens alone, with or without a transgene, already induced a defense response in N. benthamiana rendering the infiltrated leaves much more resistant to C. destructivum. As C. destructivum is a hemibiotroph, it is unknown if this resistance is a response to the biotrophic or the necrotrophic phase of
its life cycle. Therefore, additional pathogens of *N. benthamiana*, such as the biotroph, TMV (Shew and Lucas, 1991) or the necrotroph *Botrytis cinerea* (El Oirdi and Bouarab, 2007), could also be tested to determine the range of the resistance induced.

The resistance to *C. destructivum* induced by agroinfiltration in *N. benthamiana* was triggered by T4SS-dependent effectors necessary for plant infection by *A. tumefaciens*. However, of the non-pilus *vir* genes only VirE2 was examined in this study. Mutants in *virD2* (Howard et al., 1992), *virE3* (Lacroix et al., 2005), *virF* (Schrammeijer et al., 2001) and *virD5* (Schrammeijer et al., 2000), which are also required for translocation by T4SS, could be tested to determine if they also play a role in agroinfiltration induced resistance.

Agroinfiltration also induced resistance in *N. tabacum*, but the response was mediated by both PAMPs and T4SS-dependent effectors, unlike in *N. benthamiana* where only T4SS-dependent effectors can induce the resistance. It was not expected that a plant of the same family would perceive PAMPs differently, although it has been reported that Ax21, a PAMP from a *Xanthomonas* species, can only be perceived by specific rice cultivars (Lee et al., 2009b; Zipfel and Robatzek 2010), which indicated that certain PAMPs may have genotype-specific recognition. However, it is unknown if such a narrow range of PAMP recognition exists between *A. tumefaciens* and different *Nicotiana* species. To date only a limited number of plant species have been characterized for their abilities to perceive different PAMPs, so the possible variation for this trait across a broader range of plants could be a fruitful area for further research. For this purpose *A. thaliana*, which has a variety of mutants in different signaling pathways would be an attractive candidate.
Agroinfiltration-induced resistance in *N. tabacum* required the ET-dependent pathway with the SA-dependent pathway playing a minor but non-essential role. As mutant plants for the ET and SA pathways are not available in *N. benthamiana*, the possible roles of these pathways could not be determined in that species. However, other plant species, such as *A. thaliana*, have an SA mutant (Delaney et al., 1994), an ET mutant (Bleecker et al., 1988) and a JA mutant (Staswick et al., 1998), which could be tested for the effect on those signaling molecules in agroinfiltration-induced resistance. To further understand the role of the ET and SA pathways, mutants such as *nahG* and *etr-1* in *N. benthamiana* will need to be created and tested. In our study the expression patterns of four genes associated with either the ET or SA pathway were studied, and although differential expression was observed, these patterns could not explain the agroinfiltration-induced resistance in *N. benthamiana*. Therefore, other defense related genes may be responsible for this phenomenon in *N. benthamiana*, such as genes involved in cell wall deposition of callose. A broader scale examination of gene expression through microarrays, such as that done by Ditt et al. (2006) for *A. tumefaciens*-infected *A. thaliana* cells, or RNA-seq, which can give an RNA profile of the agroinfiltrated leaf (Wang et al., 2009), could help to identify genes involved in agroinfiltration-induced resistance.

This study has demonstrated that agroinfiltration, a technique commonly used to study plant-microbe interactions, can have ancillary effects on plant defense mechanisms, due simply to the activities of *A. tumefaciens*. This phenomenon has now been reported in at least three commonly used model plant systems, including *N. benthamiana* (this work), *N. tabacum* (Pruss et al., 2008; Rico et al., 2010) and *A. thaliana* (Zipfel et al., 2008).
The use of this technique for evaluating genes for disease resistance in other plant species should, therefore, be undertaken with caution and only after preliminary tests such as those used in this study.

Agroinfiltration, however, could be a particularly simple and rapid system for expressing proteins of interest in plants, from which they could then be purified and characterized. Although plant proteases might result in degradation of such proteins, low protease transgenic plants could be developed and grown in closed systems. Thus far, the use of low protease transgenic plants has not been reported. In addition, plants expressing a MAR border sequence in combination with inactivation of PTGS may be used for agroinfiltration; such a system has been used successfully for expression of defensins in stably transformed *A. thaliana* (Butaye et al., 2004; Sels et al., 2007; Aerts et al., 2007). The use of MARs enhances transgene expression in stable transformants by reducing position effects (Spiker and Thompson, 1996; Butaye et al., 2005), although this may be of minor importance in transient systems as expression can occur from non-integrated T-strands as well as those integrated in the genome. Of more value perhaps for this purpose is the PTGS mutant plants in which a reduction of transgene silencing allows for higher levels of transgene expression (Butaye et al., 2004; Butaye et al., 2005). A similar strategy for enhancing gene expression with agroinfiltration is to co-infiltrate with a PTGS suppressor gene, such as the gene coding for P19 from the tomato bushy stunt virus (Scholthof et al., 1995). This approach was found to be effective for enhancing transient expressions of green fluorescent protein (GFP) and disease resistance genes (*Cf-9, Cf-4*) in *Nicotiana benthamiana* (Voinnet et al., 2003).
Transgenic tobacco plants containing \textit{pbd-1} displayed slightly increased resistance against \textit{P. syringae} pv. \textit{tabaci} 11528R based on bacterial population counts, but not against \textit{C. destructivum} N150 and \textit{C. orbiculare} ATTC 20767, while transgenic plants containing \textit{Ntdef1} displayed no resistance against these pathogens. Transcription of both transgenes was detected, but the corresponding proteins were not detectable in total plant extracts. The lack of increased disease resistance, therefore, could be due to the low levels of expression, low levels of defensin protein accumulation, no or low antimicrobial activity against these pathogens, or a combination of these factors.

To evaluate the antimicrobial activity of PBD-1, a \textit{P. pastoris} expression system was employed to synthesize the protein and then test this protein for \textit{in vitro} activity. \textit{P. pastoris} produced PBD-1 that displayed high activity against \textit{P. syringae} pv. \textit{tabaci} 11528R and \textit{C. orbiculare} ATTC 20767 and lower activity against \textit{C. destructivum} N150 suggesting that the lack of \textit{in planta} activity in tobacco plants expressing \textit{pbd-1} was not likely due to a lack of antimicrobial activity. PBD-1 also displayed activity against a broad range of other bacterial plant and animal pathogens, including both gram-negative and gram-positive strains. However, PBD-1 had different levels of antimicrobial activity depending on the bacterial species. This suggests that specific microbial membrane components are targets of PBD-1 that could vary even between isolates, affecting the ability of PBD-1 to interact with the microbe. Therefore, a wider range of microbes should be tested. An unexpected result was the variation in the level of antimicrobial activity for different strains of the same bacterial species. This would imply that bacteria may be able to mutate and lose the target site for PBD-1 activity or develop counteracting mechanism to suppress the effect of PBD-1. To test for this, bacterial cultures could be
treated with gradually increasing concentration of PBD-1 and observe the development of resistant strains. Similar experiments have been done to observe the development of bacterial strains resistant to the antibiotic, pexiganan (Perron et al., 2006). This raises the possibility that widespread use of transgenic plants expressing PBD-1 could result in PBD-1 resistant strains arising after a period of time with sufficient selection pressure.

Expression of Ntdef1 in the P. pastoris system was also successful enabling tests for in vitro activity. However, Ntdef1 protein produced by P. pastoris displayed no activity against P. syringae pv. tabaci 11528R, and weak activity against C. destructivum N150 and C. orbiculare ATTC 20767. When tested against different bacterial plant and animal pathogen species, Ntdef1 only showed weak activity against gram-positive bacteria. Ntdef1 may lack significant antimicrobial activity or it may show specificity against microbes that were not tested in this study. If it plays a role in disease resistance, then it would most logically be against pathogens of tobacco. Other tobacco pathogens that could be tested would be the gram-positive bacterium Rhodococcus fascians, the fungus Fusarium oxysporum, and the oomycete Peronospora tabacina (Shew and Lucas, 1991). Since plant defensins may have other roles than disease resistance, future work could look at anti-insect activity (Pelegrini et al., 2008; Zhang et al., 1997; Bloch and Richardson, 1991), root growth inhibition (de Zélicourt et al, 2007) and developmental control (Allen et al., 2008; Stotz et al., 2009a) in tobacco plants by methods such as gene silencing..

This study provides a direct comparison of two quite different systems for expressing defensin proteins. The P. pastoris system produced different levels of foreign protein depending on the gene and/or gene construct. Of the four gene constructs
transferred to *P. pastoris*, only *Ntdef1*-en-myc-6xHis provided sufficient protein for direct detection in the media, and was readily purified in sufficient quantities for extensive testing *in vitro*. Proteins from both the *pdb-1+pbd-1* construct, which had the native PBD-1 prosequence fused with PBD-1 mature peptide, and the *Ntdef1*-6xHis construct were at a much lower level in the media and required larger cultures and laborious purification for the assays. Protein from the *po2+pbd-1* construct, which had PO2 sequence fused with the PBD-1 mature peptide, was not detectable in the media and could not be purified from it, even though the gene was present in *P. pastoris*. Thus, it appears that the en-myc-6xHis fusion tag was superior to the 6xHis tag for expression and/or secretion of *Ntdef1*, and the choice of fusion tag may be critical for protein stability, the ability of *P. pastoris* to secrete the protein, or both.

The *P. pastoris* and other microbial expression systems are much more rapid than transgenic plant systems and generally provide high yields of protein that are also more easily purified as they are secreted into the media thus bypassing the cell disruption process. However, *P. pastoris* is sensitive to some defensins, such as radish defensin, Rs-AFP2, and *A. thaliana* defensins, *AtPDF1* (Sels et al., 2007), and thus would not be suitable for these and possibly other AMP genes. In addition, the *P. pastoris* system introduces possible artifacts into the bioassays due to the extra amino acids included on the N-terminus of the mature peptide and tags on either end for purification or detection, all of which may interfere with biological activity. Removal of the 6xHis or other tags invariably resulted in considerable loss of protein in this study.

This research demonstrated the importance of assessing antimicrobial activity *in vitro* when selecting a gene to engineer disease resistance in plants. This would be
especially true in the case of putative plant defensins as they may perform other functions, such as inhibition of protein translation, insect α-amylases, and proteases, or as ion channel blockers (Carvalho and Gomes, 2009). Although a high level of antimicrobial activity *in vitro* would make a gene a promising candidate, the results from such assays may not necessarily translate into effective resistance to pathogens in plants. For example, the overexpression of *A. thaliana* defensins, *AtPDF1.1* or *AtPDF1.2a*, in *A. thaliana* did not improve resistance to *B. cinerea* despite its apparent *in vitro* antimicrobial activity against *B. cinerea* (De Coninck et al., 2010). An alternative approach to determine the importance of a gene in resistance to pathogens is to silence the gene and look for decreased resistance. This has been done for a pepper AMP gene (*CaAMP1*) (Lee et al., 2008b), a tomato defensin gene (*DEF2*) (Stotz et al., 2009a), and a *snakin-2* homolog in *N. benthamiana* (Balaji et al., 2011). Such an approach could be done for *Ntdef1* by using antisense RNA (Schuch, 1991) or RNAi (Smith et al., 2000).

Because of the other possible roles for plant defensins, such as inhibitors of protein translation, insect α-amylases, proteases, or ion channel blockers (Carvalho and Gomes, 2009), it is possible that overexpression of *Ntdef1* may affect other traits such as insect resistance. For example, expression of *Brassica rapa* defensin (BrD1) in rice conferred increased resistance to brown planthopper (*Nilaparvata lugens*) (Choi et al., 2009). Possible insect pests of tobacco to do feeding tests with the purified protein from *P. pastoris* or material from transgenic plants would be tobacco budworm (*Heliothis virescens*), tobacco hornworm (*Manduca sexta*) and green peach aphid (*Myzus persicae*) (Self et al., 1964).
Given that animal defensins broadly possess antimicrobial activity, they have potential for developing disease-resistant plants. Animal defensin genes have promise, since plant pathogens have not been exposed to animal defensins, and therefore should not have developed resistance to them. For example, two vertebrate defensins, rabbit $\alpha$-defensin ($NP-1$) (Fu et al., 1998; Zhao et al., 1999; Zhang et al., 2000) and human $\beta$-defensin 2 ($hBD-2$) (Aerts et al., 2007) conferred resistance to microbial pathogens in different plant species. However, the expression and stability of animal proteins in plant cells and tissues, as well as their activity, poses challenges for this strategy, which explains perhaps the lack of successful applications of vertebrate defensins for this purpose. Insect AMPs have shown potential, as well, for disease resistance in plants, but synthetic versions of these were necessary to withstand plant protease activity (Owen and Heutte, 1997).

Plant defensin genes have the advantage of having co-evolved with pathogens, and so may be adapted to attack pathogens of that plant. However, overexpression in the same species can result in silencing. Silencing may have been responsible for the lack of resistance against $B. \text{cinerea}$ observed in $A. \text{thaliana}$ plants overexpressing $A. \text{thaliana}$ defensins (De Coninck et al., 2010). This is possibly why most successful attempts to develop transgenic plants with disease resistance have utilized defensin genes from other plant species (Terras et al., 1995; Kanzaki et al., 2002; Gao et al., 2000; Table 1.2).

For a transgene to be effective in increasing disease resistance in plants, a minimum level of expression is required in the plant host. A number of methods have been developed to do this. Codon optimization involves modification of the DNA sequence to the preferred codons used for particular amino acids in the host species, and
has been used to increase expression of the cryIA gene from Bacillus thuringiensis in tobacco or tomato to increase the level of CryIA protein (Perlak et al., 1991). However, this approach did not improve the production of porcine epidermal growth factor (EGF) in tobacco (Snelgrove, 2004) or cecropin in tobacco (Hightower et al., 1994). Codon modification of pbd-1 in this study was not sufficient to provide detectable levels of the protein in tobacco. Another approach is to incorporate the transgene in the chloroplast genome, which has been used to express Bt toxin (De Cosa et al, 2001) and viral antigens (Molina et al., 2004). This was also done for an AMP analog of magainin 2, which resulted in increased disease resistance (DeGray et al., 2001). An additional complementary strategy is to target the foreign protein to a subcellular compartment, such as the endoplasmic reticulum (ER), a method that was successful for expressing an anti-Hepatitis B virus surface antigen (HBsAg) in tobacco (Ramirez et al., 2002). Similarly targeting the foreign protein to the vacuole resulted in increased levels of the Lt-B protein of enterotoxigenic E.coli in maize (Streatfield et al., 2003). However, such sequestration strategies may not improve disease resistance in the plant as the invading microbes may not be exposed to the protein. The PTGS-MAR expression system (Butaye et al., 2004), in which the transgene construct is bordered by MAR sequences and the construct transferred to a PTGS mutant plant, is another possibility for enhancing transgene expression as the presence of the MAR sequence reduces positional effects and the PTGS mutation reduces silencing of the transgene. This strategy has been used to achieve relatively high levels of plant and animal defensins in A. thaliana leaves (Sels et al., 2007; Aerts et al., 2007). However, it is suitable only for generating transgenic plants for protein extraction and not for developing disease resistant plants because PTGS
mutant plants are more susceptible to diseases, for example *A. thaliana* to cucumber mosaic virus (CMV) (Mourrain et al., 2000) and *A. thaliana* to *Verticillium dahliae* (Ellendorff et al., 2009).

The research reported in this thesis demonstrates the challenges faced by those attempting to express AMP genes in plants for therapeutic or plant disease purposes. It is clear that different approaches are required for different applications. For example, the *P. pastoris* expression system is more efficient for the extraction, purification, characterization and testing of AMPs. However, it is likely that for each AMP a specific secretion signal or fusion partner may need to be developed to achieve high protein levels, a daunting task if one desires to test several genes. Agroinfiltration may also be useful for AMP extraction and purification, especially if one desires to examine properties of the protein produced in a plant system. However, as shown in this thesis and elsewhere, agroinfiltration would not be useful for testing antimicrobial activity of a gene *in planta*. For the purpose of generating transgenic plants with increased disease resistance, a good starting point, especially for genes with only a putative function, would be to determine the antimicrobial activity of the AMP gene following production of the AMP in a *P. pastoris* expression system. The data from such experiments could be used to select the most promising candidate genes, and then AMP could be further tested for expression and stability in plants using agroinfiltration. If such tests are promising, then one could be even more confident of the decision to generate stably transformed plants and test the AMP for biological activity.
References


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syringae pv. phaseolicola which is physically linked to hrpY, a new hrp gene identified in the halo-blight bacterium. *Molecular Plant-Microbe Interactions* 7:726-739.


World Wide Web References

Appendix A

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK). Mascot was set up to search *P. pastoris* database (the NCBInr_20090719 database) and against the expected proteins database assuming digestion with trypsin. The Mascot search was conducted with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 3.0 Da. The iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and oxidation of methionine were specified in Mascot as variable modifications.

Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.)