EXPRESSION AND CHARACTERIZATION OF A NOVEL ANTIMICROBIAL PROTEIN FROM LOBLOLLY PINE (*Pinus taeda* L.)

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CHAPTER I

INTRODUCTION

1.1 Goals and Objectives

Plants and animals can survive a world laden with pathogenic bacteria and fungi as well as harmful insects. This ability may be attributed to their defense systems. Antimicrobial proteins (AMP), important components of the defense systems, play an important role in protecting plants and animals from infections caused by microorganisms. AMPs may exist widely among both plants and animals. As plants lack the immunoglobulin-based immunity system, AMPs play an important role in plant defenses (Broekaert et al., 1997).

Loblolly pine is the most important softwood species in the United States. Because loblolly pine has an extended life cycle compared with vegetable or sow crops, which results in a shortage of the multi-generation pedigreed materials for genetics research, very limited knowledge has accumulated on gene expression and regulation of the defense systems of loblolly pine. Recently, Zhang cloned a novel antimicrobial protein gene from the loblolly pine and named the gene PtAMP (Zhang et al., 1998). This gene shares 34% homology with the nucleotide sequence of the Mi-AMP gene (Marcus et al., 1997), which has a wide antimicrobial spectrum. To my knowledge, PtAMP is the first antimicrobial protein gene cloned from gymnosperms. This gene has been cloned into *E. coli* and its expression product appeared to strongly inhibit the growth of *E. coli* and

Fusarium oxysporium in vitro. The function of the gene in loblolly pine is not clear. The purpose of this research was first to characterize the *in vitro* effects of the gene product on microbes such as bacteria or fungi, then to find out the potential function of the gene in loblolly pine. To accomplish the objectives of the project, the following research activities were planed:

1. Identification of an efficient *E. coli* system to express the PtAMP gene.
2. Purification of the PtAMP protein from *E. coli* cells containing a recombinant PtAMP gene.
3. Determination of the antimicrobial activity spectrum of the recombinant PtAMP protein.
4. Testing the toxicity of the recombinant PtAMP protein to plant cells.
5. Examination of the expression patterns of the PtAMP gene in loblolly pine
6. Determination of the family size of the PtAMP gene in the genome of loblolly pine.

This research will provide important genetic information on the defense system of loblolly pine. The research will also provide direct data for the potential application of the gene in pest management.

1.2 Significance

During the history of the battle between human beings and pathogenic microorganisms, a large number of chemical bactericides and fungicides were invented. However, pests also evolved. Cases of chemical-resistant bacteria or fungi are reported every year. It is sometimes necessary to use increased amount of chemicals to control those bacteria and fungi, which brings about the problem of pollution to soil and water. To keep a healthy environment by reducing the application of chemicals, in the last 30 years, scientists have continued developing novel bactericides and fungicides or biochemical substitutes for chemical bactericides and fungicides. Antimicrobial peptides or their derivatives are considered to be ideal substitutes for chemical bactericides or fungicides, for several reasons. First, antimicrobial peptides are internal components of the host plant or animal cell and normally are harmless to the host cells. Second, as perishable proteins, AMPs may not pollute water and soil. Third, few cases of resistance from bacteria to AMPs have been reported todate. In addition to developing novel bactericides or fungicides, genetic engineering holds great potential to produce plants with enhanced resistance to pests. AMP genes are ideal candidate genes to improve the resistance of plants to infectious diseases through transformation.

About 30 years ago, the first reported member of the AMP family, thionin, was found to protect barley seeds and seedlings from infection by bacteria and fungi (Fernandez et al., 1972). Since then, numerous AMP genes have been identified and several AMPs have been isolated from plants. Some of these genes have already been used in transgenic

plants and improved resistance of those plants to bacteria or fungi or plant-feeding insects (Molina et al., 1997; Terras et al., 1995). However, the knowledge obtained to date does not yet provide enough theory for wide application of the AMPs in plant protection. Currently, research on AMPs is focused on finding new AMPs with stronger and wider antimicrobial activity, exploring the mechanism of action of the AMPs, synthesizing AMPs, and using the AMP genes in transgenic plants.

Loblolly pine is the most important economic timber species in the South. Since loblolly pine seldom catches a bacterial infection, it is supposed that some important resistance factors may exist in this species. The novel PtAMP gene recently cloned in our lab is the first gene reported in pines that is involved in the plant antimicrobial defense. Characterization of this gene and it gene product could provide important information on the antimicrobial defense system of loblolly pine.

Wide application of AMPs would largely depend on the breakthrough discovery of the mechanism of action of the AMPs. Many reported antimicrobial proteins are small cystein rich peptides that are less than 100 amino acid residues (Broekaert et al., 1997). To my knowledge, the PtAMP gene encodes the longest antimicrobial protein (105 amino acid residues) that has ever been reported. Although it is not clear if the small size of AMPs is a potential element that may attribute to membrane penetration, the relatively large size of the PtAMP protein may provide some new information on the mechanism of action of AMPs.

Antimicrobial proteins are usually purified from seeds. The AMPs obtained are usually only seed specific and the yield of AMPs is extremely low. Because most reported AMPs do not express in intact plant tissue, the possibility of isolation those

proteins directly from plant tissue is low. The process from gene to protein in this research, afforded by the cloned gene, is more efficient than the process from protein to gene. Part of our research will focus on developing an optimum system for efficient expression of the PtAMP in *E. coli*. This research should provide a good model for characterization of other genes and gene products of plant defense systems.

Mechanisms of plant defense against infectious diseases are very complicated. Clarification of the process depends heavily on the cloning and characterization of more genes involved in the defense activity. The PtAMP gene is possibly one of the key genes in the defense system of loblolly pine, because it strongly inhibits the growth of some pathogenic microbes *in vitro*. If the importance of the PtAMP gene in the defense system of loblolly pine is clarified, it may be possible to identify defense-related genes from loblolly pine or other plants by using a similar approach.

In summary, this research should make a significant contribution to the understanding of the defense mechanisms of pines and perhaps other plants. At the same time, data on the antimicrobial activity, toxicity and expression pattern of the PtAMP gene and its product will provide direct evidence of the potential application of PtAMP in plant protection.

1.3 Literature Review

1.3.1 Important roles of AMPs in plant defense

Plants and animals share a world with microoganisms such as bacteria and fungi. In most circumstances bacteria and fungi are non-pathogenic or even mutually beneficial

with plants and animals. There are also uncountable pathogenic bacteria and fungi that endanger their health and life. However, plants and animals seldom catch a serious infection. This resilience may be attributed to the presence of a repertoire of host defense systems.

In animals, defense may be mediated by events such as an immune response, complement activation, phagocytosis, and/or the release of small molecular weight antimicrobial peptides (AMPs) (Elsbach et al., 1990). In insects, amphibians, and other lower organisms, small molecular weight peptides play a major role in warding off infections (Kimbrell et al., 1991). In plants, the first barriers against pathogens are the cell wall, the cuticle and phenolic compounds. When these barriers are broken, plants show a fast response (the early defense), which is characterized by ion flux, hydrogen peroxide production, and phosphorylation. If pathogens are not killed and further threaten the plant, the plant will express some specific genes that permit synthesis of various antimicrobial substances such as hydrogen peroxide, digestive enzymes, and a number of specific proteins in response (Keen 1993). Such inducible secondary metabolites can either alter the properties of the extracellular matrix of the host (such as hydroxyproline-rich glycoproteins involved in reinforcement of the cell wall to delay the infection), or kill the invading pathogens directly (local defense). The invading pathogens may also induce a long distance signal so the whole plant can activate defense systems, which is characterized by antimicrobial peptides and phytoalexins production (system defense). The antimicrobial peptide is a main component in both the local and systematic defense activity of plants (Boeles et al., 1990; Vidhyasekaran et al., 1997).

Plant AMPs are small, highly basic, cysteine-rich proteins that are products of single genes. As plants lack immunoglobulin-based immunity, AMPs play an important role in plant defenses. It has been known for over 50 years that thionin, a member of the AMP family, inhibits the growth of bacteria and fungi *in vitro* (Stuart and Harris, 1942). Fernandez (1972) found that thionin could protect barley seeds and seedlings from infection by bacteria and fungi. Direct evidence of AMPs action in plant defenses could be demonstrated by developing an AMP deficient mutant that has much lower disease resistance. However, production of AMP-mutants may be very difficult because of the huge genome of most plants. At present, there are two methods commonly used to examine the protective function of AMPs in plants. The first method is to detect antimicrobial activity of AMPs *in vitro*. More than 40 kinds of plant AMPs have been purified and almost all of them have been proven to actively inhibit microbial pathogens *in vitro* (Bohlmann et al., 1994, Terras et al., 1992b, Collinge et al., 1993, Cammue et al., 1995, Segura et al., 1999). The second method is to transfer AMP genes into model plants. The improved resistance of the transgenic plant to microbial pathogen infection can provide evidence of the protective function of the AMP *in vivo*. There are already a number of such successfully transformed plants (Carmona et al., 1993; Terras et al., 1995; Epple et al., 1997; Molina and Garcia-Olmedo, 1997). In this research, the antimicrobial function of the PtAMP will at first be verified by *in vitro* inhibition tests. The potential for use of an *in vivo* test will depend on the information obtained from the *in vitro* test.

1.3.2 Purification of AMPs

Seed is a common source of antimicrobial proteins because it is well known that many antimicrobial proteins as well as other enzyme inhibitors exist in the seeds of plant species and serve as defensive agents against attacks from animals, insects, or microbial pests (Richardson et al., 1991). For example, Ib-AMP1, Ib-AMP2, Ib-AMP3 and Ib-AMP4 were isolated from the seeds of *Impatiens balsamina* (Tailor et al., 1997); MiAMP1 was isolated from the seeds of *Macadamia integrifolia* (Marcus et al., 1997); Mj-AMP1 and Mj-AMP2 were isolated from the seeds of *Mirabilis jalapa* (Miguel et al., 1995); and Rs-AFP1 and Rs-AFP2 were isolated from the seeds of radish (Terras et al., 1995). Some AMPs are also isolated from other parts of the plant, such as thionin isolated from the leaves barley (Bohlmann et al., 1988) and snakin-1 isolated from the tubers of potatoes (Segura et al., 1999).

Methods to isolate AMPs directly from plant tissue are similar, normally beginning with grinding of a large amount of the target plant part, followed by extraction of total proteins with a large amount of extraction buffer. Partially purified protein can be obtained by a series of precipitations and chromatographies. Totally purified protein is obtained by reverse-phase HPLC, cation-exchange HPLC, etc (Miguel et al., 1995; Marcus et al., 1997; Tailor et al., 1997). The whole process is very time consuming and the yield of AMP is very low. In some research, purification of certain kinds of AMP requires large quantities of seeds from resistant plants, while the supply of such seeds may be rather limited. This has hampered efforts to study the antimicrobial activity and the mechanism of the AMPs. Therefore, to express the AMP in microbial expression systems such as *E. coli* is an important step towards producing large amounts of protein.

Although more than 40 kinds of AMPs have been purified, there is only one report on the expression and purification of antimicrobial protein in *E.coli* (Chen et al., 1999). This is probably because most AMPs are small and soluble in water, and most of them are very toxic to the host cells used in the expression systems. The other reason may be most of the research on AMPs began with protein purification. Researchers usually obtain the gene sequence of their AMPs after they have sequenced the protein they isolated. In this research, however, PtAMP gene has been intentionally cloned. It is convenient to express this gene in *E. coli* and produce a large amount of protein to characterize its function in plants.

To date trypsin inhibitor (TI) is the only AMP reported expressed in *E. coli* (Chen et al., 1999). After it was cloned into *E. coli*, an inclusion body was formed, which prevented the protein from attacking the host cell. Consequently the TI gene could be overexpressed and purified efficiently. However, the purified protein required refolding to resume its original activity. It was impossible to observe the potential inhibitory activity of the protein to the cell *in vivo*. Also, the formation of an inclusion body prevented the use of traditional nickel ion affinity chromatography, which is a very convenient and efficient method for purifying His-tagged recombinant protein (Chen et al., 1999).

The amino acid sequence of the PtAMP indicated that it was a very soluble protein. This high solubility made it impossible to form inclusion bodies and get overexpression. However, the advantage was that direct *in vivo* effect of the protein to the host cell can be observed. A disadvantage was the resultant difficulty in producing quantities sufficient for purification, since host response was negative.

1.3.3 Antimicrobial spectrum and toxicity of AMPs

AMPs have been grouped into several families on the basis of their primary sequences. The groups include chitinases (Collinge et al., 1993), chitin-binding proteins (including hevein-like peptides, Bolle et al., 1993; Van Parijs et al., 1991; Broekaert et al., 1994), β-1,3-glucanases (Manners et al., 1973), thionins (Bohlmann et al., 1991; Caleya et al., 1972), permatins (Vigers et al., 1991), traumatin-like proteins (Woloshuk et al., 1991), PR-1 type proteins (Niderman et al., 1995), lipid-transfer proteins (LTP) (Cammue et al., 1995), plant defensins (Terras et al., 1995), and knottin-like peptides (Cammue et al., 1992). Some new types of AMP have been identified, which cannot be classified into any known group, such as snakin-1 (Segura et al., 1999) and MiAMP1 (McManus et al., 1999). All plant antimicrobial peptides characterized so far contain multiple disulfide bridges, which maintain a very stable structure of the AMP molecule. The amino acid sequences between different AMP groups are highly divergent. Their antimicrobial properties and other biological activities are also quite different (Broekaert et al., 1995).

The spectra of antimicrobial activity and toxicity of AMPs are of common interest to researchers because they are the critical factors in determining whether an AMP may be widely used as a therapeutic agent or as candidate gene resource in genetically engineering disease resistance plants. Thionins have been shown to inhibit several Gram-positive and Gram-negative plant pathogenic bacteria as well as 20 different phytopathogenic fungi (Cammue et al., 1992, Molina et al., 1993). Rs-AFP1 and Rs-AFP2, isolated from the radish seed, belong to plant defensin (Terras et al., 1992) and

have been proven to be the predominantly proteinaceous antifungal substances released from germinating radish seed. In general, plant defensins are less active against bacteria than other AMPs, with some exceptions such as Ct-AMP1, a plant defensin from *Clitoria ternatea*, which actively inhibits the growth of *Bacillus subtilis* (Osborn et al., 1995), and a potato tuber plant defensin, which inhibits the growth of *Psuedomonas solanacerum* and *Clavibacter michiganensis* (Moreno et al., 1994). Lipid-transfer proteins from different plant species present different antimicrobial activities. For example, an onion seed LTP is highly active against a broad range of fungi; wheras a radish seed LTP is only moderately active against most fungi. Maize and wheat seed LTPs are inactive against most fungi (Cammue et al., 1995).

Because loblolly pine is highly immune to bacterial infections, it is presumed that loblolly pine contains some special components that strongly inhibit the growth of bacteria. Previous experiments have shown that crude recombinant PtAMP protein can inhibit the growth of *E. coli* and *F. oxysporium*, thus it is possible that the PtAMP protein is one of several inhibitors, or, it may be the main inhibitor of bacteria in loblolly pine. It is also of interest to determine the spectrum of activity against plant pathogenic bacteria such as *Agrobacteria, Pseudomonas*, *Clavibactor*, and *Erwinia* and *Xanthomonas*. None of the AMPs isolated to date inhibit all the genera of plant pathogenic bacteria. It is therefore of interest to determine whether the recombinant PtAMP protein is inhibitory to the growth of these main phytopathogenic bacteria.

The safety of an AMP to plants is important in the potential application of the AMP. If the AMP is toxic to plants, it may not be useful as a bactericide or fungicide. In addition, if an AMP is used in plants that serve as food resources, the mammalian toxicity

of the AMP will be important. An ideal AMP for use as an antimicrobial substance should be nontoxic or non-allergic to plant and animals. Many kinds of plant AMPs discovered to date have been proven to be nontoxic to plant and animal cells, such as MiAMP1 (Marcus et al., 1997), however, thionins have been observed to be toxic to insects and mammals when injected into their body fluids (Kramer et al., 1979; Rosell et al., 1966; Evett et al., 1986). The α- and β-thionins from the Gramineae are the most toxic of the plant antimicrobial peptides isolated so far. They inhibit the growth of both gram-negative and gram-positive bacteria, fungi including Oomycetes, insect cells, and mammalian cells. Some thionins have even been shown to inhibit the growth of plant cells (Florack and Stiekema, 1994). The toxicity of thionins to animal and plant cells limits the use of thionin genes for genetic engineering in agriculture.

Inhibitory or killing concentration of an antimicrobial chemical does not describe the time course of the antimicrobial activity of the chemical against bacteria. Bacterial killing kinetics may provide information on the interaction of bacteria and the chemical. In many cases, antimicrobials which are inhibitors of protein and nucleic acid synthesis exhibit prolonged prevention of bacterial growth after antimicrobial exposure (Craig et al., 1990). Different AMPs also have different modes of action with bacteria. Generally, AMPs of similar structures have similar cell killing kinetics (Epand, 1999). Thus if the structure of an AMP is not clear, cell killing kinetics of the AMP may be used to deduce the potential structure of the protein as well as the mode of action of the AMP with bacteria.

1.3.4 Expression pattern of AMPs in plants

Plants utilize a variety of strategies to defend against pathogen attack. Cell walls, cuticles and phenolic compounds are their first barrier to the pathogens. If the first barrier is broken, plants can either strengthen their cell wall or synthesize antimicrobial compounds. The predominant strategy that plants use to defend against pathogens is the hypersensitive response (HR). At least some AMPs are involved in the HR. But to date, there is only indirect evidence of their involvement. Strain K60 of *Ralstonia solanacearum* attacks a wide range of plant species including potatoes and tomatoes because it has a high degree of natural resistance to thionins and lipid transfer proteins (LTPs). The *rfaF* gene of *R. solanacearum* K60 encodes a putative heptosyltransferase involved in lipopolysaccharide (LPS) biosynthesis. When the *rfaF* gene was mutagenized with the transponson Tn5, the mutants M2 and M88 became sensitive to purified LTPs and an LTP-enriched, cell wall extract from tobacco leaves. The mutants also became avirulent based on the phenomenon that they died rapidly *in planta,* failed to produce necrosis when infiltrated in tobacco leaves, and failed to cause wilting when injected in tobacco stems. However, when the mutants were complemented with a DNA fragment harboring gene *rfaF*, they became resistant or partially resistant to thionins and were fully virulent or partially virulent again (Titarenko et al., 1997).

Traditionally, a distinction in defensive expression in plants has been made between developmentally regulated (pre-existing) expression and inducible expression. The HR is an inducible expression. In the case of plant AMPs, this distinction is not absolute. Some antimicrobial peptides have been observed to exist mostly in peripheral cell layers surrounding plant organs. It is supposed that these AMPs take part in preformed defense mechanisms because they obviously form an antimicrobial shield around the plant organ

(Broekaert et al., 1997). Some other AMPs are encoded by multi-gene families in which some of the genes are constitutively expressed in storage and reproductive organs and some other genes are pathogen-induced in plant parts such as in leaves (Garcia-Olmedo et al., 1995; Moreno et al., 1994). Furthermore, some genes, such as the one encoding LTP4 in barley, expresses at a basal level that produces sufficient protein to reach inhibitory concentrations, while it also can be induced to a high level by some pathogens (Molina and Garcia-Olmedo, 1993; Garcia-Olmedo et al., 1996; Molina et al., 1996). In addition, the Rs-AFPs are expressed as a normal component in radish seeds and are expressed at a higher level when the seed coat is disrupted during germination or wounding.

Several kinds of AMPs may coexist in one organism. For example, two different kinds of AMP (SN1 and PTH1) with distinct activity spectra have been isolated from potato (Caaveiro et al., 1997; Segura et al., 1999). The simultaneous presence of two types of peptides in the same tissue would make the pre-existing barrier against pathogens more polyvalent.

Considerable research has focused on acquired resistance (AR), the phenomenon of induced expression of defense genes, because it is a promising strategy in plant protection. AR can be induced either by inoculation of the plant with an avirulent virus, bacteria or fungi (Ward et al., 1991, Uknes et al., 1992), or triggered by natural or synthetic compounds (Schneider et al., 1996, Kessmann et al., 1994). At present, the most commonly used synthetic inducers of systemic resistance are jasmonic acid (JA), salicylic acid, abscisic acid and 2,6-dichloroisonicotinic acid (INA) (Wendehenne et al., 1998, Schweizer et al., 1997, Yalpni et al., 1991, Schweizer et al., 1997). They can either be

used alone or in combination to enhance the expression of pathogenesis-related proteins in plants. Systemic induction of AMPs in plants with chemical inducers has not been well studied. Segura and his co-workers have isolated two kinds of AMPs from potato tubers, StPTH1 and StSN1. They treated the young potato plants with seven different kinds of plant hormones. It was found that the defensin gene StPTH1, but not the gene StSN1, was involved in the systemic defense response (Segura et al, 1999).

The high level of AMPs in seeds is probably important in protecting the embryos from potential microbe infections (Schrader-Fisher and Apel, 1994). Germination is a vulnerable stage of plant development because the seed coat is broken which makes the seeds especially vulnerable to wounding and infection. AMPs have been proven to express at higher levels to protect the seedlings at this stage. Rs-AFP1 was observed to release from the seeds of radish at the stage of germination. The germinating seeds can effectively inhibit the growth of fungal colonies on agar media. When seed germination was inhibited by addition of the plant hormone abscisic acid to the medium, no release of the Rs-AFP1 was observed. When a mechanical incision was applied to the seed coat, Rs-AFP1 was observed once again (Terras et al, 1992). The function of the PtAMP gene in this research is not yet clear; it is not known if it is a seed-specific protein. Thus both seeds and seedlings will be used to analyze the expression pattern of this gene.

Microbial infection is also a factor that can induce the HR in plants. The HR occurs when an avirulent microbial pathogen attacks a resistant plant. Perhaps because woody plants like loblolly pine have a long juvenile stage, little research has focused on the HR and the gene-for-gene interaction. Callus shares many characters with whole plants, so callus is often used in studies on cellular biology of pines to make efficient use of

experimental materials in short supply. It is reported that callus cells of pine from a "resistant" family of loblolly pine or shortleaf pine can be induced to express anti-fungal materials following infection by *Phytophthora cinnamomi*. The resistance to *P. cinnamomi* appeared to be regulated by physiological and biochemical mechanisms as no HR was observed in any inoculated callus tissues (Jiang, 1990). In this research, *Phytophthora cinnamomi* will be used to inoculate loblolly pine seedlings in order to observe possible induced expression of the PtAMP gene. *Cronartium quercuum* will be used as another pathogen in the treatment of loblolly pine. *Cronartium quercuum* is the cause of fusiform rust in loblolly pine, which leads to great loses in productivity. However, some loblolly pine families are resistant to *Cronartium quercuum* infection (Schultz, 1997). The expression pattern of the PtAMP gene following fungal infection might reveal the role of the PtAMP gene in protection of loblolly pine.

Because loblolly pine is commonly immune to bacterial infections, induction of expression by bacteria will not be studied in this research.

1.3.5 Gene families of AMPs in plants

A gene family is a group of genes that encode structurally or functionally related proteins. For studying the function of a single gene, knowledge of gene families may be not very important. However, knowledge of gene families is important to characterize gene evolution, genome evolution, and phylogenetics.

Duplication, recombination, and gene conversion are main strategies by which eukaryotes create redundancy and variability within a population. The redundancy provides an opportunity for novel functions. Redundancy also buffers the cell against

faults in metabolism, and otherwise permits a more versatile regulatory strategy. During the co-evolution of pathogens and plants, plants have to possess a mechanism flexible enough to ensure a large number of highly polymorphic disease resistance genes (Ronald, 1998). Because most virulence genes of pathogens are recessive, novel virulence genes can be obtained by pathogens by a simple loss-of-function mutation in the avirulence gene. It has long been speculated that gene rearrangements play a key role in the evolution of those polymorphic defense genes, so that plants could generate novel resistance to match the changing pattern of pathogen virulence (Pryor et al, 1987, 1993).

Antimicrobial peptides are host defense effector molecules broadly distributed throughout the plant kingdom. Many plant AMPs have been identified to be encoded by gene families of low complexity, such as the Mj-AMP1 and Mj-AMP2 from seeds of *Mirabilis jalapa* (Miguel et al., 1995), Rs-AFP1 and Rs-AFP2 from radish seeds (Terras et al., 1995) and Pa-AMP-1 from pokeweed seeds (Liu et al., 2000). These genes normally share high homology in their amino acid sequences as well as a similar spectrum of activity. The Mj-AMP1 and Mj-AMP2 are interrupted by a single intron in their coding sequences (380 bp for the Mj-AMP1 and 900 bp for the Mj-AMP2 respectively). This phenomenon suggests that during the evolution of these AMP genes, novel genes could form by duplications and further expansion of an existing gene to create clustered gene families (Richer et al., 2000). The precise evolutionary history of the various AMP families remains to be determined. Sequence conservation among the AMP gene families can yield important clues to the evolution and regulation of AMPs.

1.3.6 Mode of action of plant AMPs

Mechanisms of AMPs against pathogens have been most studied in one major group of animal AMPs, the short linear polypeptides. This group of AMPs can form amphipathic α-helices in solution and associate with lipid membranes of target cells and to transiently form ion channels (Duclohier et al., 1994, Matsuzaki et al., 1995). Plants do not have short linear α-helices AMPs. Most plant AMPs have an amphipathic structure. The mechanism of plant AMPs is well studied in thionin/*Saccharomyces cerevisiae* system. It was found a wheat seed thionin causes permeabilization of yeast cells and release of cellular components into the culture medium (Okada and Yoshizumi, 1973). Plant defensins can also cause increased membrane permeability through direct protein-lipid interactions. However, permeabilization does not appear to be the primary cause of inhibition of growth of fungi and bacteria. Plant defensin can cause a marked and sustained influx of Ca^{2+} and outflux of K^{+}, which are also observed in plant cells during defense responses (Thevissen et al., 1996).

Most plant antimicrobial peptides are cationic. The positive charge facilitates AMP interaction with negatively charged membranes, which have been found to occur in higher concentration in pathogenic cells than in normal eukaryotic cells (Oren et al., 1999, McManus et al., 1999). This provides a ready explanation for their specificity for bacterial membranes. In Gram-negative bacteria, both the outer leaflets of the plasma membrane as well as the outer membrane contain anionic molecules orientated toward the exterior of the cell, while plant or mammalian cell membranes are more positively charged. Hence, the cationic AMPs will preferentially bind to the exposed negative charges of bacterial membranes, but not to the zwitterionic amphiphiles present in the extracellular monolayer of plant or mammalian plasma membranes (Epand, 1999). The

activity of plant AMPs against most microbes is reduced in the presence of increased salt concentration, typically 1 mM Ca^{2+} and 50 mM KCl, further suggesting that the electrostatic interaction may be important in the mode of action of plant AMPs (Marcus et al., 1997, Tailor et al., 1997).

However, there is not always a correlation between the ability of peptides to permeabilize membranes and their antimicrobial activity. It is possible that the membrane effects of these peptides are not directly related to their mechanism of cytotoxic action but rather simply, by entering the cell and reaching an alternative target (Wu et al., 1999). An antimicrobial peptide from the Australian native legume, *Hardenbergia violacea* (HvAMP1) showed an inhibitory effect on the *in vitro* translation of a eukaryotic cell free system. It may be indirect evidence that some AMPs may be able to interfere with *in vivo* cell activity rather than just rupture the cell (Harrison et al., 1997).

Several kinds of AMPs can mediate the aggregation of artificial liposomes under low or high salt conditions. This aggregation may play a role *in vivo* through controlling the migration of the pathogen to unaffected areas. This aggregation may not be involved directly with antimicrobial activity of AMPs because the tested bacteria were not inhibited at the concentrations that caused aggregation (Caaveiro et al., 1997).

A biological activity common among cystein-rich peptides is the inhibition of enzymes via direct peptide-protein interactions. It has been found that α-amylases of animal origin can be inhibited by AMPs from the plant defensin family (Bloch and Richardson, 1991), LTP family (Campos and Richardson, 1984) and knottin-type peptide family (Chagolla-Lopez et al., 1994). This property may serve to protect plants against

herbivore damage. Because loblolly pine is seldom endangered by plant feeding insects, the interaction of PtAMP protein with amylases will not be studied in our research.

CHAPTER 2

MATERIALS AND METHODS

2.1 Selection of an efficient expression system

The PtAMP gene was cloned into an expression vector by Dr. Y. Huang. As observed early, *E. coli* strain have different capacity to express foreign genes, particularly those genes encoding toxic products. Thus we screened a number of *E. coli* strains in order to identify a good expression system for the recombinant PtAMP gene in vitro.

2.1.1 Transformation of the host cells

Nova-Blue and pLysS competent cells were purchased from the Novagen, Inc. and DH5α competent cells were purchased from Gibico BRL. These competent cells were grown in LB medium at 37°C with shaking at 250 rpm. Blank vector pET30c+ (purchased from Novagen) and recombinant plasmid pET30c+/PtAMP constructed by Dr. Huang were used to transform the host cells. The pET30c+/PtAMP construct contains an insert, a full length cDNA of the PtAMP gene. Transformation was carried out following the protocol provided by the Novagen. Briefly, 20 ul of competent cells were thaw on ice. Plasmid DNA 1.0 ul was added directly to the competent cell and mix by flicking. The cell suspension was put on ice for 5 minutes followed by heat shocking in 42°C water-bath for exactly 30 seconds then put on ice for 2 minutes. Eighty ul of room temperature SOC medium was added directly to the cell suspension and incubated with

shaking at 37°C for 1 hour. Transformed cells were screened on LB agar plates containing 30 ug/ml kanamycin after incubation at 37°C overnight.

2.1.2 Induction of protein expression

Induction of protein expression was performed according to the Novagen' s with some modifications. A single colony of the transformed bacteria was inoculated into 3 ml of LB medium containing 30 ug/ml of kanamycin. The bacteria cells were grown at 37°C with shaking at 250 rpm overnight and the culture was stored at 4 °C for 24 hours. Five ml of the cell suspension was used to inoculate 500 ml of LB medium containing 30 ug/ml of kanamycin. When growth of the bacteria reached a density of OD_{600} about 1.0, culture temperature was lowered to room temperature and the bacteria continued to grow for 30 minutes. Then IPTG solution was added into the culture to a final concentration of 1.0 mM to induce PtAMP protein expression.

2.1.3 Plotting of growth curve of transformed bacteria

While inducing expression, about 3 ml of bacterial culture was taken out at intervals of 15 ~ 30 minutes. Cell density was measured at OD_{600} with a spectrophotometry. OD_{600} of bacteria without induction was used as control. Growth curves were generated by plotting cell density versus time.

2.1.4 Extraction of total proteins

Forty minutes after induction, 1.0 ml of cell suspensions was harvested by centrifugation at 6,000 x g for 3 minutes. Cell pellets were resuspended in 100 ul of 2x

protein loading buffer [62.5mM Tris, pH 6.8, 10% glycerol (v/v),2% SDS (v/v), 5% 2-mercaptoethanol (v/v), 0.05% bromophenol blue (w/v)], boiled for 5 minutes followed by chilling on ice for 3 minutes. To reduce the viscosity, the protein solutions were centrifuged at 12,000 x g for 15 minutes and supernatants were removed to a fresh microtube. Protein samples were stored at –20°C before using.

2.1.5 SDS-PAGE

Expression levels of the recombinant PtAMP protein in the *E. coli* strains were assessed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the standard protocol in Molecular Cloning (Sambrook et al., 1987). Concentration of the stacking gel was 6% and concentration of the resolving gel was 15%. The gel was allowed to polymerize at 4°C for 24 to 48 hours to insure a complete polymerization. Ten ul of each protein sample was used to load the gel and electrophoresis was performed at 70 V until the dye reached the bottom of the gel. The gel was stained with 200 ml of Coomassie Brilliant Blue stain solution (50% methanol, 0.05% Coomassie Brilliant Blue-R, 10% acetic acid) for 5 hours and destained with 200 ml of destain buffer (5% methanol, 7% acetic acid) with gentle shaking overnight.

2.2 Purification of the recombinant PtAMP protein

Purification and identification of the recombinant PtAMP protein expressed in *E. coli* were performed according to the protocol provided by Novagen. All chemicals used in purification and identification were purchased from Novagen.

2.2.1 Cell extract preparation

Bacterial cells with the highest expression level of the recombinant PtAMP protein were grown and induced as described in section **2.1.2**. Cells were stored on ice for 5 minutes then harvested by centrifugation at 6,000 x g for 10 minutes. The cell pellets were frozen at –20°C overnight to completely breaking-up the cells, and then resuspended in 10 ml of ice-cold 1x binding buffer containing 10 ul NP-40. The resulted lysate was then sonicated in an ice-salt bath until the sample was no longer vicious. Cell debris was removed by centrifugation at 39,000 x g for 20 minutes. The supernatant was filtered through a 0.45-micron membrane and stored on ice before loading onto the chromatography column.

2.2.2 Resin preparation

Stocks of charge buffer, binding buffer, washing buffer and elute buffer were diluted to 1x with sterilized deionized water. His.Bind Resin was suspended by gentle inversion, then transferred to a small polypropylene column using a wide-mouth pipette. The resin was allowed to pack under gravity flow to get a bed volume of 2.5 ml. When the level of storage buffer drops to the top of the column bed, the column was charged and equilibrated in the following order:

3 volumes sterile deionized water

5 volumes 1x charge buffer

3 volumes 1x binding buffer

2.2.3 His·tag column chromatography

When the binding buffer drained to the top of the column bed, cell extract was loaded onto the column. The column was washed with 25 ml (10 volumes) of 1x binding buffer, followed with 15 ml (6 volumes) of 1x washing buffer. Bound protein was eluted with 15 ml (6 volumes) of 1x elute buffer. The elution was collected with microfuge tubes, stored 1.5 ml in each tube. Protein concentration of each collection was measured with Bradford method as described below. The elution with right concentration of protein was pooled and dialyzed against solid sucrose at 4 °C for 24 hours.

2.2.4 Measurement of protein concentrations

Bradford reagent was purchased from Sigma, St. Louis, MO. Standard protein solutions ranging from 50 ug/ml to 1,400 ug/ml were prepared using bovine serum albumin provided with the Bradford reagent. One hundred ul of each standard was added to each tube labeled with the appropriate concentration. In the tubes labeled blank, 100 ul of distilled water was added. The dialyzed PtAMP was unknowns. Unknowns were diluted to an approximate concentration between 100 ug/ml to 1,400 ug/ml and aliquoted to the labeled tubes. Exact 3.0 ml of Bradford Reagent was added to each tube and mixed well. Absorbence of standards and unknowns was measured according to the Bradford method by using the UV-VIS MBA2000 system (Perkin-Elmer Corp.). Concentrations of unknowns were calculated from the standard curve.

2.2.5 Western blot

Western blot was performed according to the S.Tag western blot protocol provided by Novagen. Briefly, crude proteins and purified proteins were separated by SDS-PAGE.

Protein bands were transferred electronically from the polyacrylamide gel onto a positive charged nylon membrane with transfer buffer (12 mM Tris base, 96 mM glycine, 20% methanol, pH 8.3) at 100v for 1.5 hours. The membrane was removed from the blotting apparatus and incubated in blocking buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20, 1% gelatin) for 15 minutes. The membrane was incubated with a 1/5000 dilution of S-portein Alkaline Phosphatase Conjugate in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 15 min then washed with 25 ml TBST for 4 times. Color was developed by incubating the membrane with 60 ul NBT and 60ul BCIP in 15 ml of 1x AP buffer.

2.3 Antimicrobial activity assay

The microplate-reading method was the main method used to determine the antimicrobial activity of the recombinant PtAMP protein to bacteria and fungi. The microplate-reading method used in this research was method from Marcus et al (1997) with some modifications. Except for calculation of 50% inhibition concentration (CI_{50}) of the recombinant PtAMP protein to each bacterial and fungal strain, comparison of microplate-reading method and plating method as well as bacteria killing kinetics were studied to get some information on the potential mode of action of the recombinant PtAMP protein.

2.3.1 Preparation of bacterial and fungal cultures

Bacteria or fungi were grown at 28°C for 48 hours except for *E. coli* and *Saccharomyces cerevisiae* which were grown at 37°C. The test bacteria were grown in

LB medium. Fungi were grown in synthetic medium (Marcus et al., 1997), K_2HPO_4 (2.5 mM), $MgSO_4$ (50 uM), $CaCl_2$ (50 uM), $FeSO_4$ (5 uM), $CoCl_2$ (0.1 uM), $CuSO_4$ (0.1 uM), Na_2MoO_4 (2 uM), H_3BO_3 (0.5 uM), KI (0.1 uM), $ZnSO_4$ (0.5 uM), MnSO4 (0.1uM), glucose (10 g/l), asparagine (1 g/l), methionine (20 mg/l), myo-inositol (2 mg/l), biotin (0.2 mg/l), thiamine-HCl (1 mg/l) and pyridoxine/HCl (0.2 mg/l). The test microoganism inoculum was either cell suspension, fungal hyphal fragments or fungal spores.

Bacterium from the Log phase of growth were diluted from overnight bacterial culture for 10^4~10^5 time with fresh LB and incubated at 250 rpm for 30 minutes with shaking. Fungi were originally grown on PCAL medium, then subcultured on 1.0% water agar plate to produce spores. When enough spores were produced, 10 ml of sterilized water was added to the plate and spores were removed from the plate by scraping the surface of the agar gently with a sterilized spatula. The spores were filtered through four layers of sterilized cheesecloth and counted under a microscope. Concentration of spores was adjusted to 4000 spores/ml with synthetic medium. Fungal hyphal fragments were produced by the following steps: break up fungal mycelium with a sterile forceps and resuspend in 10 ml fungal growth medium, force the hyphal fragments through 18G syringe needle for hundreds of times then force the hyphal fragments through 26G syringe needle for hundreds of times, filter the hyphal fragments with four layers of cheesecloth.

2.3.2 Antimicrobial assay with microplate-reading method

Purified recombinant PtAMP was diluted with LB or synthetic medium to appropriate concentrations. The protein solution was mixed well with an equal volume of

microoganism suspension by gentle vortexing. The suspensions were aliquoted into three wells of a microplate (100 ul/well). Microoganisms growing in either pure medium or 20 ug/ml of purified host cell extract were used as blank. Inhibition of the recombinant PtAMP to testing bacteria and fungi was calculated with the following formula:

$$\text{Percentage of growth inhibition} = \frac{(OD_{570nm} \text{ in control culture} - OD_{570nm} \text{ in test culture}) \times 100\%}{OD_{570nm} \text{ in control culture}}$$

2.3.3. Antimicrobial assay with the paper disc method

Several fungal strains were also assayed with the filter paper disc method. Briefly, a mycelium plug was placed on 1.0% water agar plate and incubated at room temperature overnight. Sterilized filter paper discs were soaked in the recombinant PtAMP solutions (100 ug/ml, 50 ug/ml, 25 ug/ml, respectively) or sterilized water as control for 2 minutes. The filter paper discs were put at spots 1 cm in front of the growing mycelium. The plates were incubated at room temperature for 24 – 48 hours to observe any inhibition zone of the mycelium growth.

2.3.4. Bacterial viability in the recombinant PtAMP protein

After the antimicrobial assay of *R.solanacearum* with the microplate-reading method, 50 ul of cell suspension was pipetted from each well containing 20 ug/ml, 15 ug/ml, 10 ug/ml, 8 ug/ml, 3 ug/ml and 0 ug/ml of the recombinant PtAMP protein. These cell suspensions were diluted appropriately and plated onto LB agar medium. Colony forming units (cfu) were counted after incubation of the plates at 28°C for 18-20 hours. Cell concentration deduced from the plating method represented the concentration of viable cells. Concentration of total cells (including both viable and dead cells) deduced from

absorbence were obtained from a standard curve of cfu versus OD_{600}. The following method was used to generate a standard curve: *R. solanacearum* was grown in LB medium to stationary phase, the bacterial suspension was made a series of dilution ranging from 10 to 1000 times and OD_{600} of each dilution was measured with a spectrophotometer, aliquots of each dilution were appropriately diluted again and plated onto LB agar medium, the cfu was counted after incubating the plates at 28°C for 18-20 hours, the standard curve was plotted as cfu versus OD_{600}. Living cells were calculated as 100 times the ratio of viable cells and total cells. Cell viability in the recombinant PtAMP protein was plotted as cell viability versus PtAMP protein concentrations.

2.3.5 Bacterial killing kinetics assay

R. solanacearum was used in the bacterial killing kinetics study. Log-phase bacteria (2.4×10^6 cfu/ml) or bacteria of high concentrations (10^8 cfu/ml) were incubated with 60ug/ml, 40 ug/ml, 20 ug/ml and 10 ug/ml of recombinant PtAMP protein in LB medium at 28°C with shaking at 250 rpm. Bacteria cells growing in pure LB medium were used as a control. Aliquots of the culture were removed at intervals of 30min, appropriately diluted and plated onto LB agar plate. Colony forming units were counted after incubation of the plates at 28°C for 18-20 hours. The bacterial killing kinetics curve was plotted as Log cfu/ml versus time.

2.4. Plant cell toxicity assay

A plant cell toxicity assay was performed by using the fluoresceint acetate plus phenosafranin stain method developed by Widholm (1972). Tobacco has been used as a

model plant in several studies to test the toxicity of AMPs to plant cells (Marcus et al., 1997, Harrison et al., 1997). In this research, both loblolly pine callus and tobacco callus were used to test the effect of the PtAMP protein on the host plant.

2.4.1 Cell suspension preparation

Fresh tobacco leaves were surface sterilized with 15% Clorox (commercial bleach) in hood for 15 minutes with occasional shaking. The leaves were rinsed with sterilized water for several times then blotted dry on a piece of sterilized filter paper. The leaves were cut to small pieces with a sterilized scalpel in a sterilized petri dish and placed upside down onto CIM plates, which consisted of KNO_3, 1900(mg/l); NH_4NO_3(mg/l), 1650(mg/l); $CuSO_4.5\ H_2O$, 0.025(mg/l); $MgSO_4.7\ H_2O$, 15.4(mg/l); $ZnSO_4.7H_2O$, 8.6(mg/l); H_3BO_3, 6.2(mg/l); KH_2PO_4, 170(mg/l); $NaMoO_4.2H_2O$, 0.25(mg/l); $CaCl.2H_2O$, 440(mg/l); $CoCl_2.6H_2O$, 0.025(mg/l); KI, 0.83(mg/l); $FeSO_4.7H_2O$, 27.8(mg/l); Na_2EDTA, 37.3(mg/l); inositol, 100(mg/l); thiamine-HCl, 1.0(mg/l); pyridoxine-HCl, 0.5(mg/l); nicotinic acid, 0.5(mg/l); glycine, 2.0(mg/l); indoleacetic acid (IAA), 15(mg/l); 0.5% casein hydrolysate; 3% sucrose; 0.8% agar, pH 5.8. The leave discs were incubated in the dark at room temperature for 2 weeks. Contaminated tissues were removed as soon as possible. When enough calli were developed, the calli were broken to small pieces with a pair of sterilized forceps and transferred to 50 ml of liquid CIM. The callus cells suspensions were incubated in dark at room temperature with shaking at 80 rpm for 2 weeks to obtain single cells. Loblolly pine callus was kindly provided by Dr. Y. Huang and directly used in the plant toxicity assay.

Pine callus suspension medium was prepared as $Ca(NO_3)_2.4H_2O$, 600(mg/l); NH_4NO_3, 200(mg/l); KNO_3, 900(mg/l); $CuSO_4.5H_2O$, 0.125(mg/l); $MgSO_4.7H_2O$, 180(mg/l); $MnSO_4$, 6.9(mg/l); $ZnSO_4.7H_2O$, 4.3(mg/l); H_3BO_3, 3.1(mg/l); KH_2PO_4, 135(mg/l); Na_2MoO_4, 0.1064(mg/l); $CoCl_2.6H_2O$, 0.0125(mg/l); KI, 0.04(mg/l); $FeSO_4$.7H2O, 15(mg/l); Na_2EDTA, 20(mg/l); Myo-Inositol, 1000(mg/l); Thiamine HCl, 0.4(mg/l); 3% Sucrose; BAP 2.5(mg/l); NAA, 10(mg/l); pH 5.5.

2.4.2. Plant cell toxicity assay

Phenosafranin (0.1% W/V) solution was dissolved in the suspension media described above for staining tobacco or pine cells. Fluorescein acetate solution was prepared by dissolving fluorescein acetate stock (5% W/V) in acetone then diluting to 0.01% in a final concentration with tobacco or pine callus suspension medium. Purified recombinant PtAMP protein was diluted to 200 ug/ml in CIM and filter-sterilized with a 0.45-micron membrane. Callus cell suspension was mixed with an equal volume of the recombinant PtAMP protein solution (200 ug/ml) in a 2-ml microfuge tube and incubated in dark with shaking at 80 rpm for 24 hours. Heat-killed callus cells with the same treatment were used as a control. Fifty ul of callus cells with the above treatment were pipetted onto a glass slide. Equal volume of 0.1% phenosafranin solution or 0.01% fluorescein acetate solution was added to the callus cells and mixed well. A cover slide was put over the cells. Five minutes later, the slides were observed under a microscope and cell viability was evaluated. The fluorescein acetate treated cells were examined with fluorescence techniques (Marcus et al, 1997).

2.5. Characterization of the gene expression pattern in loblolly pine

Since PtAMP is an endogenous defense gene, it is supposed to express at a higher level in loblolly pine at stress conditions. Northern blot was used to study the expression pattern of the PtAMP gene in both seeds and seedlings of loblolly pine. Loblolly pine (*Pinus taeda* L. family OSU 78) seeds were stratified in our lab at 4°C for about one month. The protocol of Northern blot was kindly provided by Dr. M. Patricek of Department of Biochemistry and Molecular Biology, OSU.

2.5.1 Treatment of seeds

Coats were removed of carefully to avoid wounding. The seeds were surface-sterilized with 15% Chlorox for 15 minutes followed by rinsing with sterilized water and blotting dry on sterile filter paper. To study the effect of chemical inducers on seeds, 50 mM of jasmonic acid or 100 mM of abscisic acid was sprayed onto the seeds; the seeds were incubated at room temperature for 12 or 24 hours then harvested for RNA isolation. To study the effect of germination on seeds, the seeds were incubated on sterile wet filter paper in dark at room temperature for 1 or 5 days then harvested for RNA isolation. RNA was also isolated from normal stratified seeds with seed coats as control.

2.5.2 Treatment of seedlings

Seed coats were removed and surface-sterilized as described above. The seeds were placed on 1% (W/V) water agar and incubated in the dark at room temperature for ten days to germinate. For chemical inducers treatment, 50 mM of jasmonic acid or 100 mM of abscisic acid was sprayed onto the seedlings. To prevent too much moisture, the lids of

plates were left uncovered. After spraying, samples were collected for RNA isolation at 12 and 24 hours respectively. For wounding treatment, sterilized flat forceps were used to punch, or sterile syringe needles were used to stab the seedlings to make multiple wounds on the needles. Wounded seedlings were collected at 4, 8, 12, 24 and 48 hours following wounding. For treatment with fungi, spores of *Cronartium quercuum* and *Phytophthora cinnamomi* were adjusted to a concentration of 4,000 spores/ml with water. The spore suspensions were daubed onto seedlings with a cotton swab. To avoid too much moisture, plates were left uncovered. The seedlings inoculated with fungal spores were incubated at room temperature in light for 1, 2 and 4 days then collected for RNA isolation.

2.5.3 Total RNA isolation

Harvested seeds or seedlings (0.5 – 2 g) were frozen in liquid nitrogen. The RNA extract buffer was composed of 1% (w/v) SDS, 1mM aurin tricarboxylic acid (ATA), 1% (w/v) tri-isopropylnapthalene-sulfonic acid (TPNS), 4% (w/v) p-aminosalicylic acid (PAS), 1x TE (10 mM Tris pH 7.5, 1 mM EDTA), 2% (v/v) B-mercaptoethanol (BME). Seven hundred and fifty ul of the RNA extract buffer and 750 ul of phenol (pH 4.5-5.5)/chloroform/Isoamyl alcohol (25:24:1) were added to the frozen tissues and ground for 1-2 minutes at high speed until they become a homologous emulsion. The emulsion was centrifuged at 9,000 x g at 4°C for 30 minutes and the supernatant was removed to a fresh microfuge tube containing 5 ul of 100mM ATA. LiCl (12 M) was added to this supernatant to a final concentration of 1.5 M. The samples were placed on ice bucket and stored at 4°C cold room overnight followed by centrifugation at 13,000 x g, 4°C for 30 minutes to pellet RNAs. After discarding the supernatant, residual phenol was removed

by pushing the pipet tip under the pellet to the bottom of the tube and slowly pipetting up. RNA pellets were resuspended in 200 ul of 100 uM ATA and precipitated once again with 100 ul of 7.5 M NH_4OAc and 600 ul of 95% cold ethanol at –70°C for at least 30 minutes. After centrifugation at 13,000 x g, 4°C for 30 min, the RNA pellet was briefly air-dried for 10 minutes and resuspend in 60 –80 ul 100 uM ATA. The RNA samples were stored at –70°C until use.

2.5.4 Northern blot analysis

RNA samples (30 ug) were adjusted with 100 uM ATA to a total volume of 11 ul. Forty three ul of fresh glyoxyl mix (242 ul glyoxal, 720 ul DMSO, 144 ul 0.1 M phosphate buffer, 179 ul 0.2% bromophenol blue) was added to the RNA samples and incubated in 50°C water bath for 1 hour. Electrophoresis was performed in 1.2% agarose gel in 0.5 M phosphate buffer (0.29 M Na2HPO4, 0.21 M NaH_2PO_4, pH 7.0). RNA bands were transferred from gel to positive charged nylon membrane with 25 mM phosphate buffer overnight by the capillar blotting method. The RNAs were UV cross-linked to the membrane at 1200 J with Stratagene. The membrane was incubated with prehybridization buffer [50% (V/V) formamide, 5x SSC, 1x PE*, 625 ug/ml CL RNA] in a rolling tube at 42°C for 2-4 hours. Probe was developed by labeling cDNA of PtAMP gene with ^{32}P. Briefly, 600 ng cDNA of the PtAMP gene was prepared in 11 ul of 1x TE (pH 8.0); the cDNA was denatured by heating at 95°C for 10 min and immediately quenched on ice; the denatured cDNA was mixed with 4 ul High Prime solution (Roche Diagnostic GmbH) and 5 ul ^{32}P-dCTP (3000 Ci/mMol) on ice; the mix was incubated at 37 °C for 30 ~ 40 min and the reaction was stopped by adding 2 ul of 0.2 M EDTA (pH 8.0); the

resulted probe was purified by chromatography on a Sephadex column. The labeled probe was added at a specific activity of at least 1x 10^6 cpm/ml of hybridization buffer and hybridized with the RNA bound on the membrane at 42°C overnight. The membrane was washed twice at 65°C in a big tray with 2 x SSC/0.1% SDS for 20 minutes followed by washing with 0.5 x SSC/0.1% SDS for 20 minutes twice. Signals were developed by autoradiography in cassettes with intensifying screens at –70°C for 24 – 72 hours.

* 5x PE

75 ml	1.0 M Tris (pH 7.5)
15 ml	0.5 M EDTA
1.5g	Na pyroPO_4
15g	SDS
3g	PVP (40,000)
3g	Ficoll
180 ml	H_2O

The above mix was autoclaved, once cooled, 30 ml of filter-sterilized 10% BSA was added.

2.6. Gene family analysis

The gene family analysis was conducted with Southern blot analysis of two randomly chosen family of loblolly pine: OSU78 and 133-11A. Genomic DNA isolation and Southern blot followed the protocols developed by Dr. Y. Huang of Department of Forestry, Oklahoma State University.

2.6.1 Genomic DNA isolation from loblolly pine

DNA extraction buffer was composed of 1.4 M NaCl, 100 mM Tris.HCl, pH 8.0, 2% (w/v) CTAB (hexadecyltrimethylammoniumbromide), 20 mM EDTA, 1% (w/v) PVP 40,000, 0.1% (w/v) sodium metabisulfite, 0.2% (v/v) 2-mercaptoethanol. Half to one g of loblolly pine seeds were ground in liquid nitrogen to a fine powder. The frozen tissue powder was extracted with 7.5 ml of the pre-heated extract buffer in a 60°C water bath for 30 minutes with occasional gentle swirling followed by one extraction with phenol (pH 8.0) /chloroform/isoamyl alcohol (25:24:1). The sample was centrifuged at 1,600 x g at room temperature for 10 minutes. The aqueous phase was transferred to a clean glass tube with a wide-bore pipet. Two-thirds volume of isopropanol was added to precipitate DNA. The resulted DNA was spooled out with a glass hook or centrifuge to pellet at 10,000 x g for 10 minutes. The DNA was washed in 75% ethanol/10 mM ammonium acetate then pelleted at 1,600 x g for 10 minutes. The DNA pellet was air-dried and resuspended in 500 ul of water.

2.6.2 Digestion of the genomic DNA

DNA samples (30 ug) were digested with EcoR I or Hind III. The total reaction volume was 200 ul. Digestion mixtures were incubated in a 37°C water bath for 3 hours then supplied with additional 2 ul of enzymes. Digestion was continued for 3 more hours. The reaction mixtures were precipitated with 1 ml absolute ethanol. DNA was collected by centrifugation and resuspended with 50 ul of water.

2.6.3 Southern blot

The digested DNA samples were separated by electrophoresis in 0.8% agarose gel in 1x TBE buffer. The gel was depurinated in 0.25 M HCl for 10 – 15 minutes with gentle shaking followed by denaturation with the denaturation buffer (1.4 M NaCl, 0.5 M NaOH) for 20 minutes twice with gentle shaking. The gel was neutralized twice with the neutralization solution (1.4 M NaCl, 1.0 M Tris.HCl, pH 8.0) for 20 minutes with gentle shaking. The DNA was transferred from the gel to a nylon membrane with 10x SSC (1 M NaCl, 0.15 M Sodium citrate, pH 7.0) using the capillar method for 18 hours. DNA was UV cross-linked to the membrane, then incubated in a rolling tube with prehybridization buffer [6x SSC, 5x Denhart's solution, 0.5% (w/v) SDS, 50% (v/v) formamide, 100 ug/ml denatured/sheared salmon sperm DNA] at 50°C for 2 – 4 hours. Radiolabeled cDNA probe was added at a concentration of at least 1x 10^6 cpm/ml of hybridization buffer and hybridized with the immobilized DNA on the membrane at 50°C for 12-24 hours. After hybridization, the membrane was washed in a big tray with 2x SSC/0.1% SDS at 68°C for 15 minutes twice, followed with 0.5x SSC/0.1% SDS at 68°C for 15 minutes twice. Signals were developed by autoradiography in a cassette with X-ray film against an intensifying screen at –70°C for 72 hours.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Identification of expression system for the pET30c+/PtAMP

In many cases, *E. coli* cells can be used as an expression system for foreign genes. However, this is not the case for antimicrobial proteins. Most AMPs are small and cationic. They cannot be folded into an inclusion body and overexpressed in *E. coli* cells. In addition, antimicrobial protein can effectively inhibit the growth of bacteria and fungi *in vitro*, and this may be toxic to the host cells. Consequently, AMPs lower the yield of desired protein from the host cells, or even kill them (Broekeart et al., 1997; Ganz et al., 1999).

In this research, the nucleotide sequence of the PtAMP gene was analyzed with MacVector 6.0 (Oxford Molecular Group) and the amino acid sequence was deduced (Figure 3.1). More than 60% of the amino acid of the recombinant PtAMP protein was polar amino acids or charged amino acids (Table 3.1). The hydrophobicity profile of the PtAMP protein indicated that most of the protein was hydrophilic and only a small fragment of the protein was hydrophobic (Figure 3.1). Therefore, the protein should be very soluble in water. The small hydrophobic segment might contribute to the amphipathic pattern of the protein. The calculated molecular weight of the recombinant protein was 20.0 kDa. In addition to the sequence encoding the PtAMP protein, the recombinant protein contained a short peptide encoded by the vector itself. The short

peptide contained a His.tag sequence and a S.tag sequence that had specific affinity for the Ni+ ion and aided in purification of the protein by affinity chromatography. The addition of the His.tag and S.tag sequence to the PtAMP might produce some delicate changes to the secondary structure of the protein. Firstly, the vector encoded sequence brought two additional cysteins into the recombinant protein, which might help to form another bisulfate bond or might change the original arrangement of cystein pairs that formed the bisulfate bond. Secondly, although the amino acid component of the vector encoded peptide was very close to the PtAMP protein itself, the recombinant PtAMP protein decreased the estimated pI of PtAMP protein from 9.31 to 9.13. It was probable that the delicate changes might not influence the biochemical properties of the PtAMP protein, because the protein was estimated to be still strongly cationic, which is characteristic for most AMPs (Marcus et al., 1995).

Figure 3.1 Predicted amino acid sequences (A) and hydrophobicity profile (B) of the recombinant PtAMP protein and the PtAMP protein. The amino acid sequence underlined is the PtAMP protein. The black peaks in the hydophilicity profile picture represent the PtAMP protein and the grey peaks represent the amino acid sequence encoded by the vector. The analysis was performed by MacVector 6.0.

A. Amino acid sequence

MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMGYLWIL

METKRLAYVLFVLVCLFLALAQPSEGSYFTAWAGPGCNNHAARYSKCGCPNIG

KDVHGGYEFVYQGQTAAAYNTDNCKGVAHTRFSGSVNQACSGFGWKSFFIQC

GSEFELRRQACGRTRAPPPPPLRSGC

(Figure continued)

B. Hydrophobicity profile

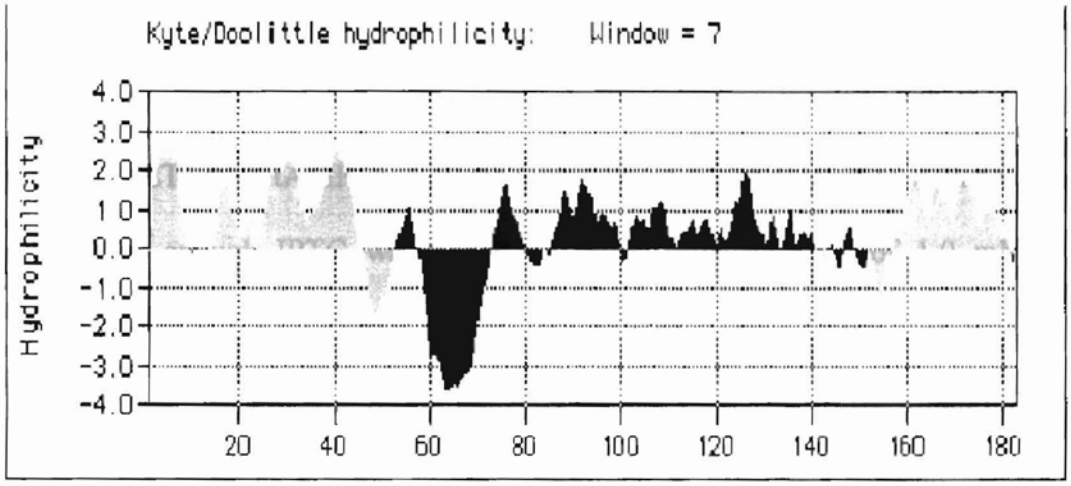


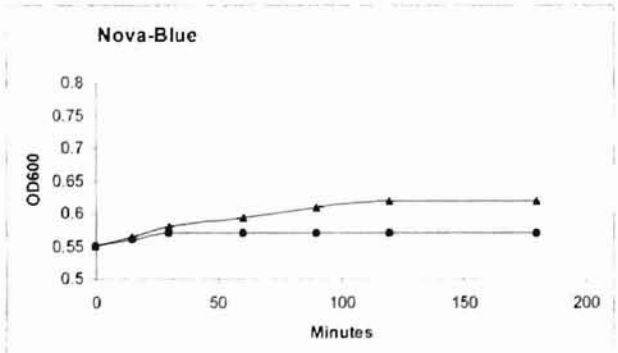
Table 3.1 Comparison of amino acid constitution, calculated molecular weight and estimated pI between the PtAMP protein and the recombinant PtAMP protein. The data was obtained from the analysis of the cDNA sequence of PtAMP gene and recombinant PtAMP gene with MacVector 6.0 software.

	Non-polar	Polar	Acidic	Basic	Cystein	MW kDa	pI
PtAMP protein	41 (39.0%)	48 (45.7%)	5 (4.8%)	11 (10.5%)	7	11.4	9.31
Recombinant PtAMP protein	69 (37.9%)	70 (38.4%)	15 (8.3%)	28 (15.4%)	9	20.0	9.13

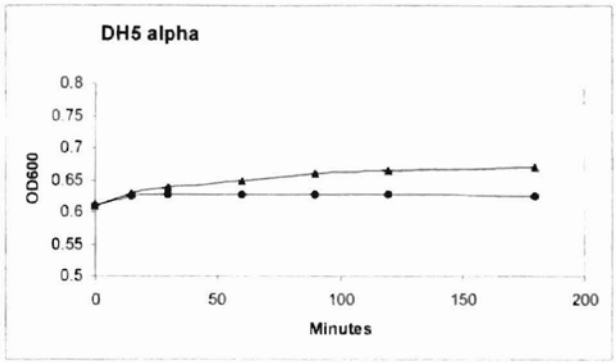
Different *E. coli* strains normally differ in their ability to express proteins. For a protein that may be toxic to the host, the ability to tolerate the toxicity is most important in expressing a sufficient amount of protein. Therefore, it was necessary to determine if

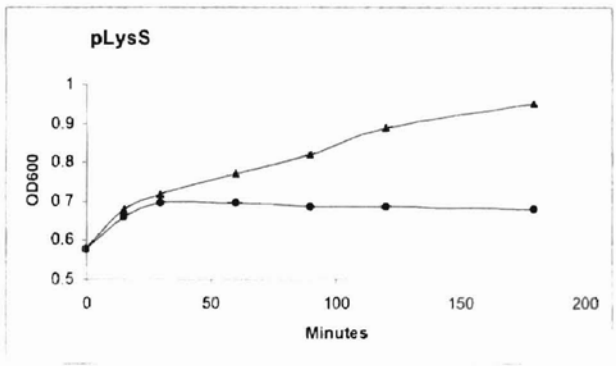
the recombinant PtAMP protein was toxic to *E. coli in vivo*. The growth curves of *E. coli* with and without the expression of the recombinant PtAMP protein were used to analyze the influence of the protein on the host cells *in vivo* (Figure 3.2). Competent cells of three *E. coli* strains, Nova-Blue, DH5α and pLysS were transformed with the recombinant plasmid pET30c+/PtAMP. When the transformed *E. coli* grew to a density of OD_{600} about 0.6, enough IPTG was added to a final concentration of 1.0 mM to induce expression of the recombinant protein. From these curves (Figure 3.2), it was observed that before addition of the IPTG, the *E. coli* grew logarithmically, which means there is no or very little expression of the PtAMP gene without induction. Thirty minutes following inducing, all three strains (Nova-Blue, DH5α and pLysS) stopped growth. However, the non-induced controls continued to grow logarithmically. Therefore, the recombinant PtAMP protein inhibited the growth of all three *E. coli* strains tested *in vivo*.

Figure 3.2 Growth curves of the transformed *E. coli* cells with and without expression of the recombinant PtAMP protein. Values of the OD_{600} measured at each time spot were the average value of three cultures grown and induced at the same time. All triplicate cultures had similar growth speed and similar responses to IPTG. With induction (●), whithout induction (▲).



(Figure continued)





To compare the tolerance level of the three tested strains to expression of the recombinant PtAMP protein, total protein was collected from the three strains 40 minutes after induction by IPTG. Total proteins of the same strains harboring only the vector pET30c+ were used as a control. The deduced molecular weight of the recombinant PtAMP protein was 20.0 kDa and the deduced molecular weight of the peptide encoded by the vector was 8.6 kDa. The SDS-PAGE gel (Figure 3.3) showed that the protein pattern of the Nova-Blue pET30c+ (lane 1), Nova-Blue pET30c+/PtAMP (lane 2), DH5α pET30c+ (lane 3) and DH5α pET30c+/PtAMP (lane 4) were very similar. There was no detectable band at either 20 kDa or 8.6 kDa position. The protein patterns of the pLysS pET30c+ (lane 5) and pLysS pET30c+/PtAMP (lane 6) were quite different. There was a strong band with a molecular weight of about 8.6 kDa in the protein sample from

the pLysS pET30c+, which should be the peptide encoded by the vector. This peptide should have no negative effect on the pLysS and it was allowed to express at high levels in the host cells. In the total protein profile of pLysS.pET30c+/PtAMP, there was a light but obvious band of about 20 kDa that did not exist in the pLysS.pET30c+, which should be the recombinant PtAMP protein. The band was light because the recombinant PtAMP protein inhibited the growth of the host cells, resulting in the reduced expression of the recombinant protein. Neither Nova-Blue nor DH5α had the unique 20.0 kDa in the total protein profiles.

Western blot was used to confirm the expression of the recombinant PtAMP protein in the three *E.coli* strains (picture is not shown). For Nova-Blue and DH5α, no visual signal appeared even several hours after color development. However, for pLysS pET30c+, 5 minutes after color development, a strong signal appeared at the 8.6-kDa position. Thus the 8.6 kDa band was the peptide with the His.tag and S.tag sequences encoded by the vector. For pLysS pET30c+/PtAMP, a light purple color signal appeared about 30 minutes after color development. The position of the signal was about 20 kDa, which was the expected molecular weight of the recombinant PtAMP protein. Because the concentration of the 20 kDa band was low in the total protein, the purple color appeared so slow that strong background also developed during the time.

Based on the total protein patterns of the Nova-Blue, DH5α and pLysS, the pLysS showed the greatest tolerance to expression of the recombinant PtAMP protein. Thus, the pLysS was chosen as the best expression system for the recombinant PtAMP gene.

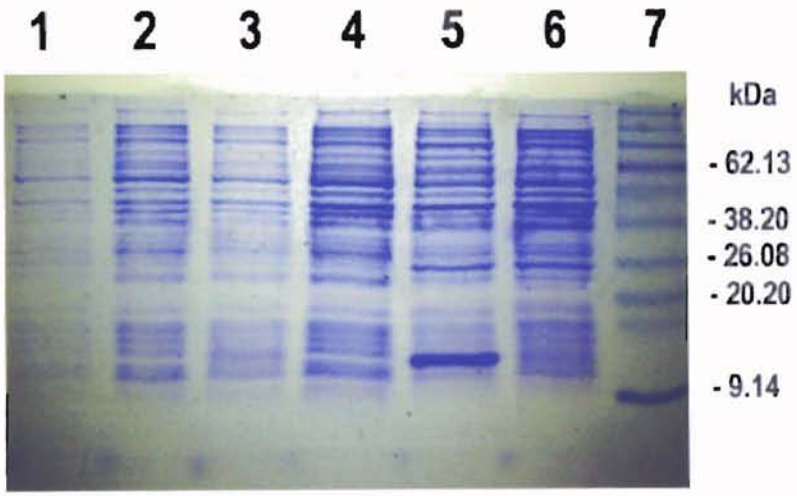


Figure 3.3 Total protein profiles of transformed *E. coli*: (1) Nova-Blue.pET30c+, (2) Nova-Blue.pET30c+/PtAMP, (3) DH5α.pET30c+, (4) DH5α.pET30c+/PtAMP, (5) pLysS.pET30c+, (6) pLysS.pET30c+/PtAMP, and (7) molecular marker.

3.2 Partial purification of the recombinant PtAMP protein

The expression of PtAMP in pLysS was expected to be low as shown by the growth curve and the total protein profile of the pLysS.pET30c+/PtAMP after induction. Thus, modifications were made in the protocol provided by Novagen to improve the expression of the PtAMP gene. Culture volume was increased from 100 ml to 500 ml and the induction point was increased from OD_{600} about 0.6 to 1.0 ~1.2. The purpose was to grow a larger amount of cells, which were induced to express the PtAMP protein. When the OD_{600} of the culture was close to 1.1, culture temperature was lowered from 37°C to room temperature. In this way, the *E. coli* could synthesize more soluble proteins when the growth temperature was reduced (Huang et al., 1998). It was also thought that when the growth medium is less rich in nutrition, the *E. coli* cells was able to produce more recombinant protein and less structural protein (Free et al., 1997). However, perhaps because the recombinant PtAMP gene had a strong negative influence on *E. coli* growth,

the transformed *E. coli* did not grow in poor medium. Therefore, this modification was abandoned.

Even with a 500 ml culture, the cell pellet of induced pLysS.pET30c+/PtAMP obtained was no bigger than those of pLysS.pET30c+ and pLysS without a vector. This result suggested that expression of the recombinant PtAMP protein in pLysS strongly inhibited its growth.

After purification of the recombinant PtAMP protein with affinity chromatography, a SDS-PAGE gel was run to check the pattern and the purity of the protein. Purified extract of pLysS without a vector and pLysS.pET30c+ were used as controls. In the gel stained with Coomassie Brilliant Blue (figure 3.4), it was observed that there was one band of about 30 kDa in the extract of pLysS, pLysS.pET30c+ and pLysS.pET30c+/PtAMP. This band was thought to be a nonspecific band and homologous to the His.tag or S.tag sequence. There was a specific band in the extract of pLysS.pET30c+, which was about 8.6 kDa and it was thought to be the peptide encoded by the blank vector. Its density was lower than before purification. Perhaps because of its small size, a large amount of the protein was trapped in the resin and could not be eluted out. The extract of pLysS.pET30c+/PtAMP had a specific band at the position of 20 kDa. The density of the band was not very strong. Perhaps because the content of this recombinant protein was low in the total protein of the pLysS.pET30c+/PtAMP. Also, nonspecific binding from proteins such as the 30 kDa protein might be a strong competitor than the recombinant PtAMP protein for the Ni+ on the resin. By comparing the relative density of each band on the SDS-PAGE gel, the approximate percentage of the recombinant PtAMP protein in the total protein was estimated to be 30%. Despite an imperfect purification system,

considerable effort compensated for this deficiency, and sufficient protein was obtained for the following studies.

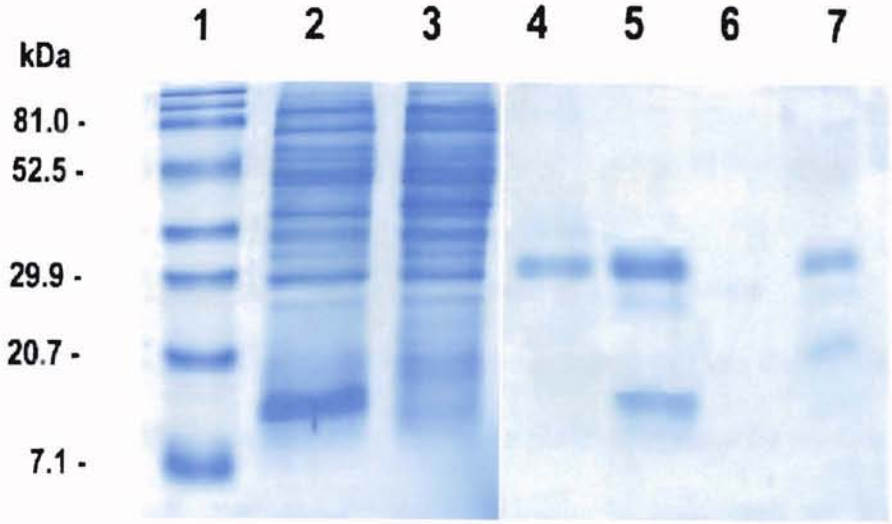


Figure 3.4 SDS-PAGE profiles of crude and purified *E. coli* extract. The gel was stained with Coomassie Brilliant Blue. To prevent overflow, lane (6) was left blank, (1) molecular marker, (2) total protein of pLysS.pET30c+, (3) total protein of pLysS.pET30c+/PtAMP, (4) purified pLysS extract, (5) purified pLysS.pET30c+ extract, (6) blank, (7) purified PtAMP.

The results from Western blot analysis (Figure 3.5) showed that the nonspecific 30 kDa band appeared first, followed by the 8.6 kDa band and then the 20 kDa band. Because the 30 kDa band was strong and appeared first, it may be highly homologous to the S.tag sequence. The molecular weight and the order of appearance of the 20 kDa band and the 8.6 kDa band were in accord with the expected molecular weight and the amount of the recombinant PtAMP protein and the vector peptide.

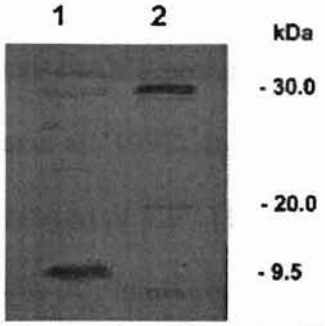


Figure 3.5 Western blot analysis of purified pLysS.pET30c+ extract (1) and recombinant PtAMP protein (2).

3.3 Antimicrobial spectrum of the recombinant PtAMP protein

The purified recombinant PtAMP protein was tested *in vitro* for its inhibitory effect on a wide range of bacteria and fungi. Because plant AMPs can be used as pesticide for plant disease control, the major phytopathogenic bacteria such as *Agrobacterium, Erwinia, Pseudomonas, Ralstonia,* and *Xanthomonas* were chosen for this study. Since *E. coli* was observed to be greatly inhibited by the recombinant PtAMP protein in *vivo*, the *in vitro* effect of this protein on *E. coli* was also of interest.

The recombinant PtAMP protein was found to strongly inhibit the growth of all the bacteria above (Table 3.2). The 50% inhibition concentration (CI_{50}) was used to evaluate the inhibition of the purified recombinant PtAMP protein to all the bacteria and fungi. CI_{50} is the concentration of the recombinant PtAMP protein, at which the average absorbance in test wells was 50% of the average absorbance in control wells, after incubation of the microorganisms for 48 hours.

The recombinant PtAMP protein is a cationic protein. Its antimicrobial activity may involve interaction of the protein with the anionic cell membranes of bacteria or fungi. If so, a high concentration of cations in the growth medium would compete with the recombinant PtAMP protein interaction with the anionic cell membranes, so the

inhibition of the recombinant PtAMP protein to the microorganisms would be diminished (Marcus et al., 1997; Tailor et al., 1997). In this research (Table 3.2), a second medium B, containing a high concentration of Ca^{++} (1 mM) and K^{+} (50 mM), was compared with medium A. For all six strains that inhibition in both media was tested, the CI_{50} in medium B was generally much higher than in medium A, except for *Collectotrichum obiculare*. These results suggest that the mechanism of the PtAMP protein may involve interaction of the cationic peptide with the anionic cell membranes.

Table 3.2 Inhibition of bacterial and fungal growth by the recombinant PtAMP protein Absorbance of tested microoganisms and controls was average values from triplicate wells. Medium B was medium A plus 1 mM $CaCl_2$ and 50 mM KCl. The inhibition curve was plotted as percentage of inhibition vs. recombinant PtAMP protein concentration. CI_{50} was the x-value from the inhibition curve when the Y-value was 50%.

Strains of bacteria or fungi	CI_{50} in medium A (ug/ml)	CI_{50} in medium B (ug/ml)	Inocula
Agrobacterium	7	----	Cell
Clavibacter michiganesis	1.9	14	Cell
E.coli	28	----	Cell
Erwinia chrysanthemi	11.2	----	Cell
Pseudomonas syringae	2.5	> 20	Cell
Ralstonia solanacearum	3	> 20	Cell
Collectotrichum obiculare	20	20	Mycelium

*Fusarium oxysporum**	1.7	3.6	Mycelium
Macrophthora phaseolina	17.3	----	Spore
Phytophthora citricola	16.4	----	Spore
Saccharomyces cerevisiae	17	----	Cell
Sclerotinia sclerotiorium	3	----	Mycelium
*Thielaviopois basicola***	25	> 30	Mycelium

* For *Fusarium oxysporium*, although the CI_{50} in medium A was close to the CI_{50} in medium B, the percentage of inhibitory effect at 15 ug/ml to *F.oxysporum* in medoum A was 100, while it was less than 60 in medium B.
** The inhibition of the recombinant PtAMP protein to *Thielaviopois basicola* was weak. When the recombinant PtAMP protein was 25 ug/ml, the inhibition to the *Thielaviopois basicola* was only 20%. So the CI_{20} was used here for both media.

Genebank database search found the PtAMP gene shared 43% homology in nucleotide sequence with MiAMP1, a recently published plant AMP gene (Zhang 1998; Marcus et al., 1997). Since the PtAMP protein might share a similarity with the MiAMP and since they may have a similar antimicrobial spectrum, eight strains of bacteria or fungi belonging to the same species or genera as tested by Marcus (1997) were selected for an antimicrobial assay. The inhibitory effects of the two proteins were found to be similar (Table 3.3); however, PtAMP showed stronger inhibition to bacteria. This result is compatible with the observation that loblolly pines seldom suffers bacterial infections (Schultz, 1997). The PtAMP protein might be an important component of loblolly pine in resistance to bacterial infection. It was also noticed that the MiAMP1 had almost no inhibition to microorganisms grown in medium B, while most microorganisms were still sensitive to the recombinant PtAMP protein in medium B. Probably, the main antimicrobial mechanism of the MiAMP1 protein is a cationic interaction with the cell

membranes, while the antimicrobial mechanism of the recombinant PtAMP protein includes a mechanism other than cationic interactions with cell membranes.

Table 3.3 Comparison of antimicrobial activity of the recombinant PtAMP protein with MiAMP1 protein

Strains	Inhibition by PtAMP*	Strains	Inhibition by MiAMP1
Fusarium oxysporum	Very strong	*Fusarium oxysporum*	Very strong
Macrophthora phaseolina	Moderate	*Macrophthora Phaseolina*	Moderate
Phytophthora citricola	Moderate	*Phytophthora crytogea*	Strong
Saccharomyces cerevisiae	Moderate	*Saccharomyces cerevisiae*	Very strong
Sclerotinia sclerotiorium	Very strong	*Sclerotinia sclerotiorium*	Strong
Collectotrichum obiculare	Moderate	*Collectotrichum falcatum*	No inhibition
Clavibacter michiganesis	Very strong	*Clavibacter michiganesis*	Strong
E.coli	Weak	*E.coli*	No inhibition

*There are no absolute criteria for the inhibition level of AMPs to microbes. The following criteria were defined for an easy comparison between the two AMP proteins. Very strong: CI_{50} <5 ug/ml; strong: CI_{50} 5-10 ug/ml; moderate: CI_{50} 10 – 20 ug/ml Weak: 20 – 50 ug/ml; no inhibition: > 50 ug/ml.

The filter paper disc method was also used to evaluate the inhibitory effects of the recombinant PtAMP protein to the growth of bacteria and fungi. However, because of the large molecular weight of the recombinant PtAMP protein, it did not diffuse well in the LB agar medium or water agar medium. No obvious inhibition zone was observed for bacteria or fungi in the filter paper disc assay.

The CI_{50} reported here were obtained from the microplate reading method. In the microplate reading method, living cells and dead cells are read equally, as they have the same absorbance. Therefore, the data may include a small deviation from the real inhibitory concentrations. The alternative, the plating method is more reliable, in which every colony formed on the plate is recovered from a living cell. Thus, the plating method was chosen to evaluate the antimicrobial activity of the recombinant PtAMP protein. Since the *Ralstonia solanacearum* gave the best inhibition curve and the inhibition effect was strong, *R. solanacearum* was chosen for the viability experiment. The control was bacteria growing in either pure LB medium or LB containing 20 ug/ml of purified pLysS extract.

Cations contained in growth medium B did not inhibit growth of the *Ralstonia solanacearum* (Figure 3.6). When purified pLysS extract was used as control, bacterial viability was 100%. Therefore, contaminations in the partially purified recombinant PtAMP protein had no negative effects on *Ralstonia solanacearum*. The bacterial viability was negatively related to the recombinant PtAMP concentration in either medium A or in medium B. And cell viability in medium A was always lower than in medium B When 20ug/ml of recombinant PtAMP protein was used to incubate *Ralstonia solanacearum*, the bacterial viability was 0 in medium A, however, the bacterial viability

was 10.73% in medium B. When 3 ug/ml of recombinant PtAMP protein was used to incubate *R. solanacearum*, the bacterial viability was 55.51% in medium A while 87.58% in medium B (Figure 3.6). These results indicated that the recombinant PtAMP protein can kill bacteria even at a concentration of a low as 3 ug/ml, and cations could interfere with the effect of the recombinant PtAMP protein. When comparing with colonies recovered on the control plates with most colonies recovered from bacteria incubated with recombinant PtAMP protein, the number and size of colonies from PtAMP plates were much smaller, suggesting that except for killing effects, the recombinant PtAMP protein inhibits growth of bacteria even after the inhibitor was get rid of.

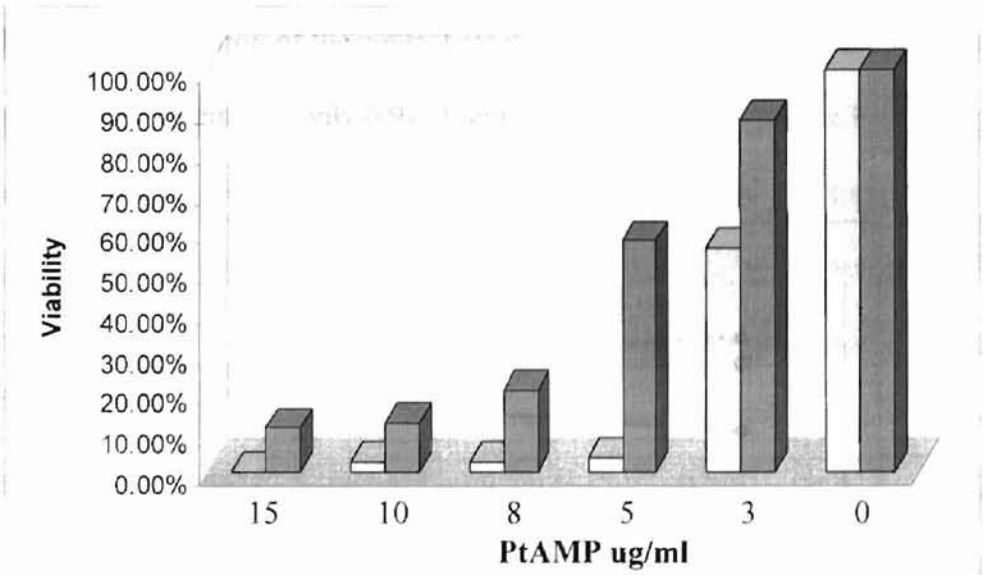


Figure 3.6 Viability of *Ralstonia solanacearum* in media with the recombinant PtAMP protein. The bacterial viability was calculated as 100 times the ratio of viable cell concentration and the deduced total cell concentration in the test well of the microplate. Both viable cell concentration and deduced total cell concentration were average value from three triplicate wells. The open bar represent viability in medium A and the hatched bar represents viability in medium B.

Cell killing kinetics may be used to illuminate the mode of action of AMPs (Gennaro, 1998). A number of studies indicate that bacterial membranes are primary targets for cationic antimicrobial peptides (Thevissen, 1996; Oren, 1999). Since the comparison between the microplate-reading method and the plating method showed that the PtAMP protein killed bacteria, an examination of the cell killing kinetics seemed appropriate. Aliquotes of *R. solanacearum* culture were removed at intervals of 30~60 min, appropriately diluted and plated onto LB medium. When there was 20 ug/ml of PtAMP protein contained in the medium, the log value of living bacteria dropped from 6.38 to 5.16 during six hours. When there was 10 ug/ml of PtAMP protein in the Log-phase bacteria, cell concentration increased slower than that of the control. After incubation for 6 hours, the log concentration of the control reached 7.65 while the log cell concentration in 10 ug/ml PtAMP protein was only 6.91 (Figure 3.7).

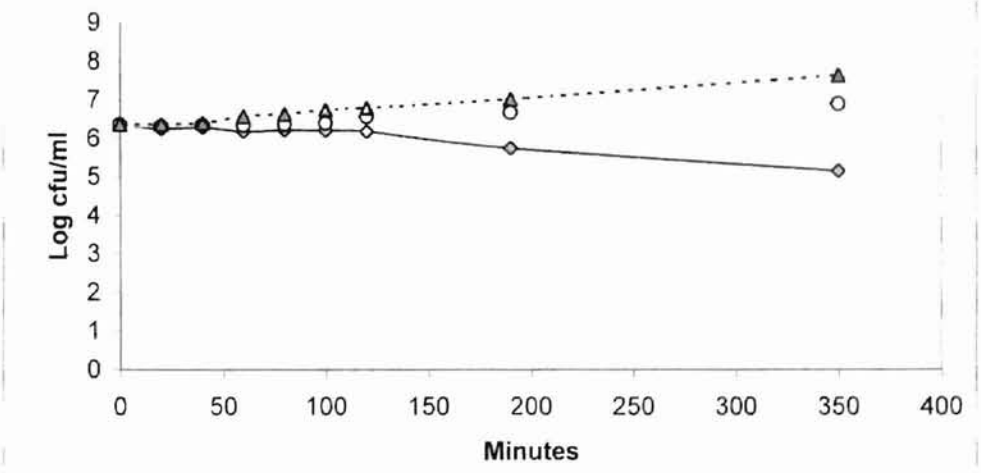


Figure 3.7 Killing kinetics of the recombinant PtAMP protein to Log-phase concentration of *R. solanacearum*. Bacteria were incubated at 37°C with shaking in LB medium containing the recombinant PtAMP protein at 20ug/ml (◇), PtAMP protein 10 ug/ml (○), or no PtAMP protein (△). Bacterial concentrations at each time were average values of colonies recovered from three LB plates.

The recombinant PtAMP protein had a much slower bacterial killing speed on *R. solanacearum* comparing with the BMAP-34, which caused a 2-3 log decrease in 80 minutes or BAMP-28, which caused a 2-3 log decrease in only 10 minutes on *E coli* (Gennaro, 1998). Thus the mechanism of the recombinant PtAMP protein may be much different from that of the BAMP-34 and BAMP-28. The mechanism of the BAMP-34 and BAMP-28 were considered to depend on a rapid permeabilization of the outer membrane and a relatively slow interaction with the inner membrane. However, two other factors should also be noted in explaining these data. First, Gennaro (1998) used *E. coli* while *R. solanacearum* was used in this research. Although both bacteria are gram negative and share similar membrane components and structures, they may have minor differences in membrane structure as well as membrane charge gradient. Second, the recombinant PtAMP protein used in this research was only a partially purified protein; the recombinant PtAMP protein was estimated to be only 30% of the total protein. If completely purified protein was used, the killing efficiency might be improved.

As noted, when the recombinant PtAMP protein was expressed *in vivo* in *E. coli* cells, the inhibition of *E. coli* by this protein was strong (Figure 3.2 and 3.3). However, when the antimicrobial assay was used against *E. coli in vitro*, the inhibition of the recombinant PtAMP protein to *E. coli* was only moderate. These results may suggest the mode of action of the recombinant PtAMP protein. There are two major hypotheses about how the disruption of membrane integrity kills target microbes. First, dissipation of ion gradients across the disrupted membrane leads to a loss of microbial viability owing to the cumulative effects of energy drain. Alternatively, antimicrobial peptides might enter target cells through the disrupted membranes, bind to as yet unknown intracellular

molecules or sites and interfere with their metabolic function. Either way, repairing processes might limit or reverse these lesions when peptide concentrations are low or when the target is exposed for only a short time. However, prolonged exposure to high concentrations of antimicrobial peptides would overwhelms the repairing capacity of the microbe and damages become irreversible (Ganz, 1999). In the interaction of the recombinant PtAMP protein with bacteria, the second explanation might be a dominant mechanism for inhibition or killing of bacteria.

Plant pathogens normally need to reach a concentration of 10^8 c.f.u/ml to be pathogenic. Therefore, the ability of the recombinant PtAMP protein to inhibit bacteria of such a high concentration was examined. *R. solanacearum* of a starting concentration of 10^8 c.f.u/ml was used in a time course experiment with varying concentrations of the recombinant PtAMP protein. It was found that bacteria were killed in 10 hours by protein concentration of 60 ug/ml, in 13 hours by protein concentration of 40 ug/ml, and in 20 hours by protein concentration of 20 ug/ml (Figure 3.8). These results indicate that the recombinant PtAMP protein is able to kill of high concentration bacteria. Thus it may be a candidate substitute for chemical bactericides such as cupric agents. Although the recombinant PtAMP protein cannot achieve such a high concentration *in vivo*, when applied onto the surface of a plant, even higher concentrations can be achieved. If the protein were used as a bactericide, it would probably be a surface spray agent.

For any bacteria, growth on the surface or inside of a plant is quite different from growth in liquid culture. Most bacteria reside on the leaf lower surface or hide in structures such as stromata where application of bactericide is difficult (Leben, 1981). Bacteria can also produce extra-cellular polysaccharides and other compounds to

interfere with the action of AMPs to cell membranes (Weiner, 1995). In liquid culture, there are no such problems. Thus to exploit the protein in developing a commercial bactericide, the efficiency of PtAMP protein against bacterial diseases should be determined on plants in greenhouse and the field.

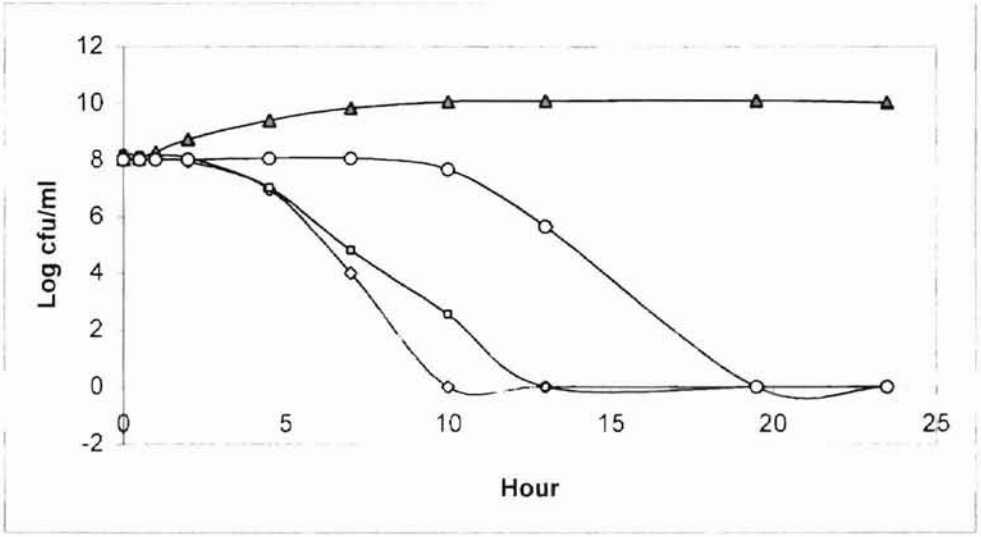


Figure 3.8 Killing kinetics of the recombinant PtAMP protein to *R. solanacearum* at a starting bacterial concentration of 10^8 cfu/ml. Bacteria were incubated at 37°C with shaking in LB medium containing 60 ug/ml (◇), 40 ug/ml (□) and 20 ug/ml (○) of PtAMP protein. LB without PtAMP protein (▲) was used as control. Bacterial concentrations at each time spot were average values of colonies recovered from three LB plates.

3.4 The recombinant PtAMP protein is not toxic to plant cells

The PtAMP can greatly inhibit the growth of bacteria and fungi. So it might be potentially useful in controlling plant infectious diseases. However, if it is toxic to plants, application will be greatly limited. Therefore, information on its toxicity to plant cells is needed.

Tobacco callus began to form about 3 days after incubation on callus induction medium (CIM) at room temperature. A large amount of callus formed in 10 more days. When callus cells were cultured in CIM liquid medium with shaking at 80 rpm for 2 weeks, suspension culture was obtained, which was used in the toxicity assay. Pine callus was provided by Dr. Huang and used directly in the toxicity assay.

Staining methods of Widholm (1972) has long been used to detect the viability of plant cells. This method is both rapid and efficient. Fluoresein diacetate only stains living cells. Cell walls and nuclei stain green. On the contrary, the second dye, phenosafranin only stains dead cells. Similarly, cell walls and nuclei stain red.

For the toxicity assay, the recombinant PtAMP protein at 100 ug/ml in plant culture suspension medium was used for incubation of the callus cells for 24 hours. This concentration was much higher than most CI_{50} in this research. Both the tobacco cells and the pine cells were found to be viable after incubation with the recombinant PtAMP protein for 24 hours, evidenced by their staining by the fluorescein acetate but not by the phenosafranin. On the contrary, heat killed tobacco or pine callus cells stained red by phenosafranin but not stained by fluorescein acetate. These results indicate that the recombinant PtAMP protein is not toxic to plant cells.

The pine callus used in this study was rather old. When stained with phenosafranin, it gave a pink appearance. However, compared with the dark red of the heat-killed cells, it was still safe to conclude that the recombinant PtAMP protein was not toxic to plant cells.

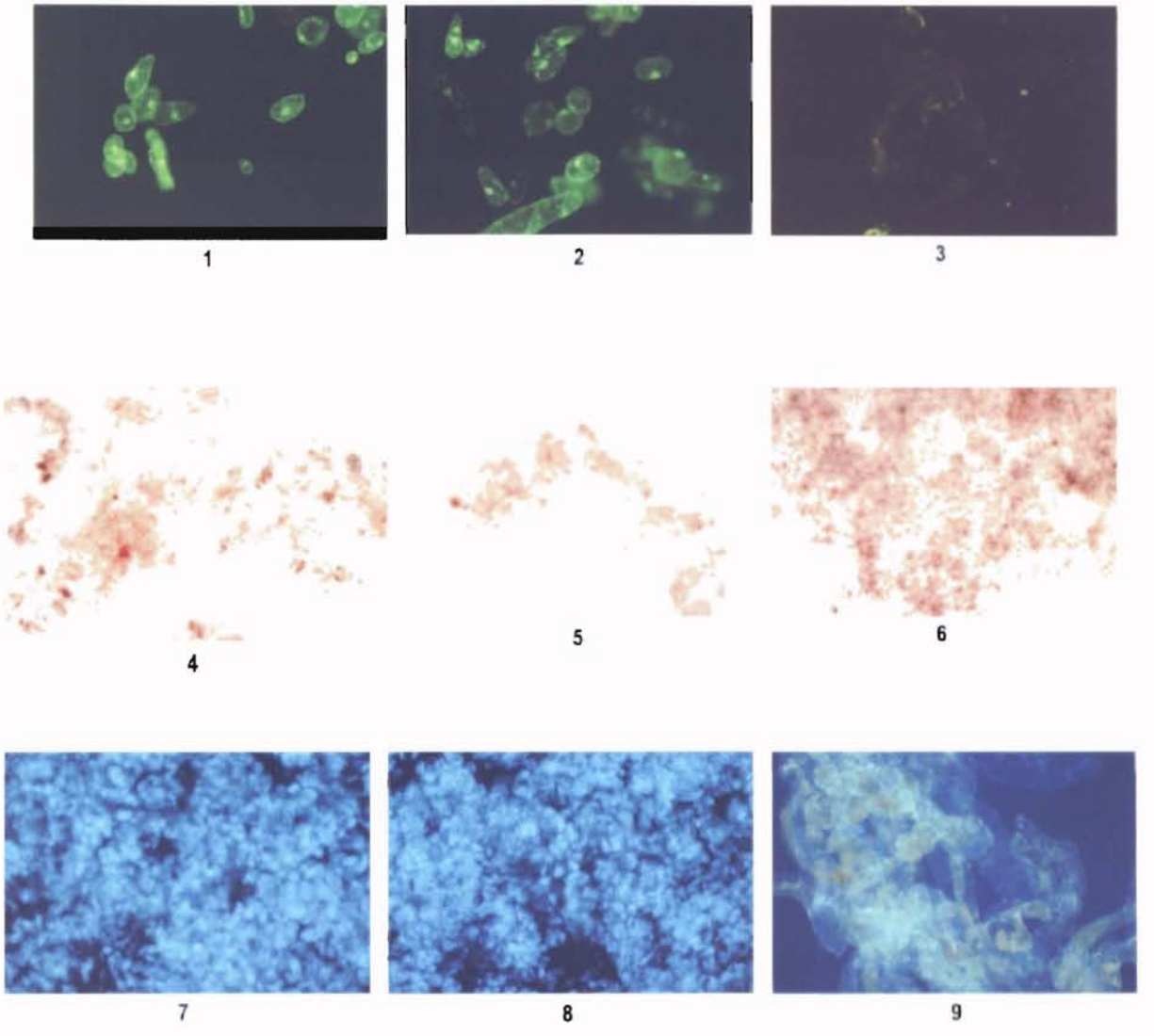


Figure 3.9 Plant cell viability examined with the staining methods. (1) normal tobacco cells stained with fluorescein acetate, (2) tobacco cells incubated in 100 uM recombinant PtAMP protein for 24 hours stained with fluorescein acetate, (3) heat-killed tobacco cells stained with fluorescein acetate, (4) normal tobacco cells stained with phenosafranin, (5) tobacco cells incubated in 100 uM recombinant PtAMP protein for 24 hours stained with phenosafranin, (6) heat-killed tobacco cells stained with phenosafranin, (7) normal pine cells stained with fluorescein acetate, (8) pine cells incubated with 100 uM recombinant PtAMP protein for 24 hours stained with fluorescein acetate, (9) heat-killed pine cells stained with fluorescein acetate.

3.5 Expression Pattern

AMPs can express in various types of plant organs such as seed, leaf, cotyledon, flower and rosette leaf (Garcia-Olmedo et al., 1992; Schrader-Fisher and Apel, 1994). In most cases, AMP genes express at higher levels in organ or tissue under stress conditions to protect plants (Broekaert et al., 1997). Thionins, plant defensins and LTP are all shown an induced expression under stresses such as drought, osmotic stress, fungal challenges and wounding (Penninckx et al., 1996; Pena-Cortes et al., 1990; Torres-Schumann et al., 1992). Jamonic acid and abscisic acid are endogenous stress plant hormones. External application of these chemical inducers can mimic stress and induce the accumulation of AMPs in plants (Penninchx et al., 1996).

The PtAMP gene was cloned from a cDNA library of loblolly pine callus by differential display (Zhang, 1998). Therefore, the PtAMP gene may be one of the genes that express in plants under stress, but may not express at normal physiological conditions. In this research, a series of stresses were applied to induce expression of the PtAMP gene. Stress conditions examined include: removing of seed coats and storing at low temperature, wounding, treatment with chemical inducers, germination and challenge with fungi. Since function and location of the PtAMP protein in loblolly pine were not clear, expression patterns of the PtAMP gene in both seeds and seedlings were studied. Stratified loblolly pine seeds were used for the stress treatments mentioned above. Normal dry seeds were not used in this research because normal dry seeds have been shown to be difficult to germinate and have low level of mRNA (Comai, 1990). Young seedlings germinated on water agar for ten days were used in the expression pattern study in seedlings. These seedlings gave high yields of total RNA.

In normal stratified seed no transcription of the PtAMP gene was detected with the PtAMP cDNA probe. When the stratified seeds were treated with jasmonic acid for 24 hours or with abscisic acid for 12 hours, transcript of the PtAMP gene was accumulated at higher levels (Figure 3.10). These results suggested that the PtAMP gene was activated in response to stress in loblolly pine seeds. In the germination treatment, after incubation of loblolly pine seeds on wet filter paper at room temperature for 12 hours, seeds imbibed enough water to become swollen. However, transcription of the PtAMP gene was not detected, which suggested that the PtAMP gene was not activated during the seed inbibition processes. After incubation of the seeds on wet filter paper at room temperature for 5 days, the seeds appeared ready to germinate. The PtAMP gene was induced to express at a much higher level at this stage (Figure 3.10). These results suggested that the PtAMP gene might play an important role in protection of seeds at germination.

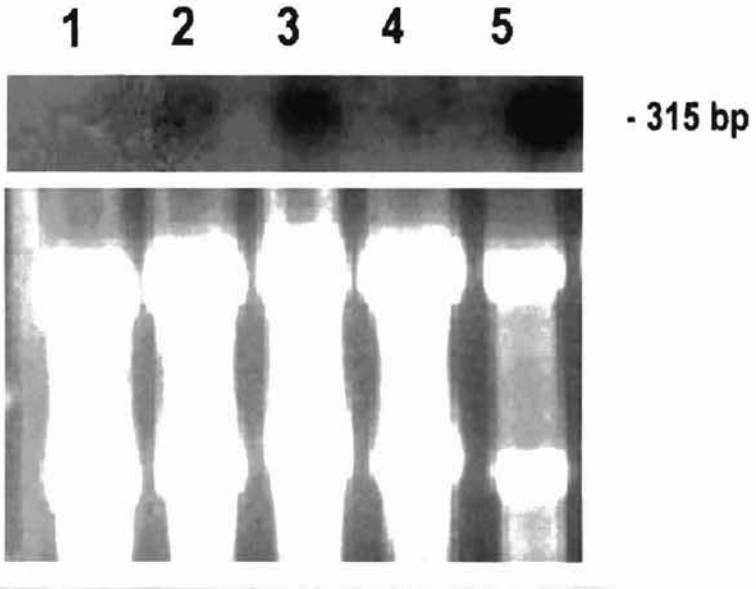


Figure 3.10 Northern blot analysis of the PtAMP gene expression in loblolly pine seeds. (1) Normal seeds, (2) seeds treated with 100 uM abscisic acid for 12 hours, (3) seeds treated with 50 uM jasmonic acid for 24 hours, (4) seeds incubated with water for 12 hours, (5) seeds incubated with water for 5 days.

After incubation of stratified loblolly pine seeds on water agar medium for ten days, most seedlings were 2-3 cm long with the megagametophyte still embracing the cotyledons of the seedlings. Jasmonic acid and abscisic acid did not efficiently induce transcription of the PtAMP gene in young seedlings. Only a faint signal of the expected size 315 bp was detected in the total RNA samples from seedlings that were treated with the chemical inducers for 12 hours or 24 hours (Figure 3.11). Wounding did not strongly effect transcription of the PtAMP gene efficiently either. Four hours after wounding of the seedlings, wounding sites became brown, which suggested a hypersensitive response (HR) took place (Heath, 2000). Eight hours after wounding, the HR symptoms became more obvious. And 12 hours after wounding, no additional change in symptoms was observed. Only a faint signal of the expected 315 bp was detected in the Northern blot for all wounding treatments (Figure 3.11). Thus, wounding may trigger a different pathway to protect young seedlings of loblolly pine. This result was concordant with that of Bogre (1997), who reported that in alfalfa, mechanical injury induced a rapid and transient mitogen-activated protein (MAP) kinase pathway which was independent of methyl jasmonic acid and abscisic acid signals (Bogre et al., 1997).

Normal ten-day old seedlings were used as control in this research; however, the PtAMP gene was shown to transcribe at a much higher level in these seedlings than in seedlings treated by wounding or with chemical inducers (Figure 3.11). Normal stratified seeds stored in refrigerator did not show a PtAMP gene transcript (Figure 3.10). However, when those seed coats were removed and stored in refrigerator for one week, the PtAMP gene showed a much higher level of transcription than in the normal 10-day old seedlings (Figure 3.11). These results suggested the PtAMP gene might play an

important role in protection of loblolly pine seeds and young seedlings at vulnerable germination and early growth stages.

Challenges from bacteria and fungi to plants are quite different from wounding or chemical treatment. The defense mechanism of plants against bacteria or fungi may involve with gene-for-gene interactions. Some bacteria or fungi may harbor specific avirulence genes that encode elicitors to induce HR in plants contain respondent resistance genes. No HR will happen if the pathogen is virulent or if the plant is susceptible to the pathogen (Heath, 2000). Some families of loblolly pine are resistant to *C. quercuum* and a gene involved with this resistance has been detected (McKeand et al., 1999; Wilcox et al., 1996). It is thus of interests to know if the PtAMP gene is involved in the HR in loblolly pine. *P. cinnamomi* was reported to induce defense response in pine callus (Jiang, 1990). Thus *P. cinnamomi* was also used to inoculate young seedlings to find out if PtAMP gene is involved in defense response of loblolly pine to *P. cinnamomi.* Loblolly pine family OSU 78 was a randomly chosen family in this research. Twenty-four hour after inoculation with either these two fungi, transcription of the PtAMP gene was not detected. Forty-eight hour after inoculation, the PtAMP gene transcribed at a higher level compared with the level 24 hours after inoculation (Figure 3.11). Ninety-six hours after inoculation, the PtAMP gene transcription level dropped to the same as 24 hour. Thus the PtAMP gene might play a role in protection of OSU 78 from fungal infection, at least for *C. quercuum* and *P. cinnamomi* infection. However, the protection may not be involved with the HR, because a HR normally happens within 24 hours of fungal inoculations (Heath, 2000). This result was also consistent with the result of Jiang

(1990), which indicated that the defense response elicited by *P. cinnamomi* in loblolly pine callus was not a hypersensitive response (Jiang, 1990).

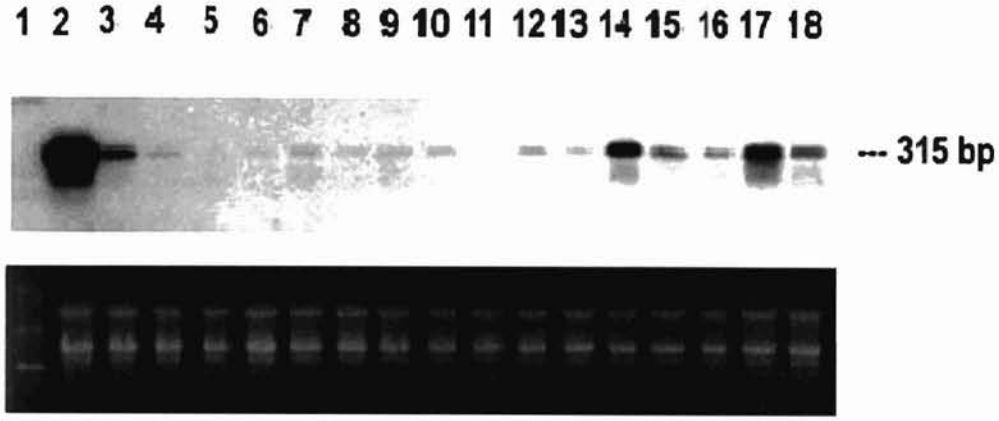


Figure 3.11 Northern blot analysis of the PtAMP gene expression in loblolly pine seeds and young seedlings. (1) Molecular marker, (2) seeds without coat in refrigerator for one week, (3) normal young seedling, (4) seedlings 4 hours after wounding, (5) seedlings 8 hours after wounding, (6) seedlings 12 hours after wounding, (7) seedlings 24 hours after wounding, (8) seedlings 48 hours after wounding, (9) seedlings 12 hours after treatment with 100 uM abscisic acid, (10) seedlings 24 hours after treatment with 100 uM abscisic acid, (11) seedlings 12 hours after treatment with 50 uM jasmonic acid, (12) seedlings 24 hours after treatment with 50 uM jasmonic acid, (13) seedlings 24 hours after inoculation with *P. cinnamomi*, (14) seedling 48 hours after inoculation with *P. cinnamomi*, (15) seedlings 96 hours after inoculation with *P. cinnamomi*, (16) seedlings 24 hours after inoculation with *C. quercuum*, (17) seedlings 48 hours after inoculation with *C. quercuum*, (18) seedlings 96 hours after inoculation with *C. quercuum*.

3.6 Analysis of the PtAMP gene family

Antimicrobial peptides often exist as small gene families. For example, the Pa-AMP from pokeweed and the Mj-AMP each contains two members in the gene family (Liu et al., 2000; Miguel et al., 1995). Four AMPs of very similar structure and biological character have been isolated from the seed of *Impatiens balsamina* (Tailor et al., 1997). Those family members may exert similar or different antimicrobial activities against invading microorganisms.

Southern blot analysis was conducted with 30 ug of genomic DNA from two different families of loblolly pine (OSU 78 and 133-11A) was digested and used in Southern blot. *EcoR* I and *Hind* III were used to digest the genomic DNA of loblolly pine. Southern blot result indicated that a gene family of small complexity encodes the PtAMP protein (Figure 3.12). When the genomic DNA from either family was digested with *Hind* III, three bands were detected by the Southern blot. Thus the PtAMP gene might be a single copy gene, since there are two *Hind* III cutting sites on the PtAMP gene. When the genomic DNA was digested with *EcoR* I, three bands were detected in 133-11A and four bands were detected in OSU78. These results indicate that some genes are homologous to the PtAMP gene in the loblolly pine genome.

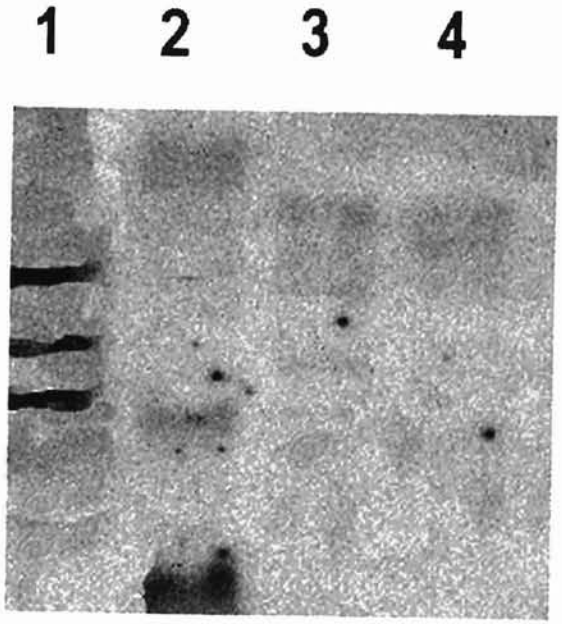


Figure 3.12 Southern blot analysis of the PtAMP gene family in loblolly pine (1) *EcoR* I digested 133-11A, (2) *Hind* III digested 133-11A, (3)*EcoR* I digested OSU 78, and (4) *Hind* III digested OSU 78.

Genomic DNA of yew was also digested with the same enzymes in the Southern blot analysis. Because yew is another conifer, and it may contain genes that are homologous to the genes of loblolly pine. It is very interesting to note that a gene was also detected with ^{32}P-labeled PtAMP DNA fragment in the genome of yew (data not shown). Therefore there is considerable possibility that this AMP gene may have evolved in other conifers.

CHAPTER 4

CONCLUSIONS

Antimicrobial proteins (AMP) represent a group of proteins that are widely dispersed in the plant and animal kingdoms. AMPs contain one to three intra-molecular bisulfate bonds that give the molecules very stable chemical structures and exert extended inhibition to invading microorganisms. The PtAMP gene was originally cloned from loblolly pine, was expressed in a heterologous host, *E. coli*. The strain pLysS is currently the best expression system we identified for *in vitro* expression of the PtAMP gene. However, because the recombinant PtAMP protein is soluble in *E. coli* and exhibited toxicity to *E. coli* cells, limiting its expression at a very low level. Direct *in vivo* inhibition of the recombinant PtAMP protein to the host cells was observed, probably due to an inhibitory mechanism beyond cationic interaction with cell membranes.

Partially purified recombinant PtAMP protein was obtained by affinity chromatography. This method is very easy and also convenient for detection of the purified protein by S.tag western blot. The purified recombinant PtAMP protein was estimated to be approximately 30% of the protein isolated from chromatography. During the antimicrobial assay, total proteins isolated from the host pLysS were used as control, had no detectable negative effect to the bacteria or fungi tested. Therefore, the only component that had antimicrobial activity was the recombinant PtAMP protein.

The purified recombinant PtAMP protein showed significant inhibition of all the bacteria and fungi tested. The inhibition of *Clavibacter michiganesis*, *Sclerotinia sclerotiorium*, *Ralstonia solanacearum* and *Fusarium oxysporum* was strong. The inhibition of *E. coli*, *Collectotrichum obiculare*, *Macrophthora Phaseolina*, *Phytophthora citricola* and *Saccharomyces cerevisiae* by the recombinant PtAMP protein was moderate. The recombinant PtAMP protein effectively inhibited some major bacterial phytopathogens such as *Agribacterium*, *Erwinia*, *Pseudomonas*, *Clavibactor* and *Ralstonia*, which suggested that the PtAMP gene has a potential for controlling those pathogens.

By comparing the plating method and the microplate reading method for calculating bacterial viability, it was found that the recombinant PtAMP protein killed bacteria even at a low concentration (3 ug/ml). Bacteria killing kinetics indicated that the recombinant PtAMP protein killed bacteria at a low speed; however, the killing effect was strong. High concentrations of PtAMP protein were able to kill bacteria of very high concentration, higher than pathogenic levels. Thus, it is possible to apply protein products as spray reagents directly to plants to control pests because of the stable chemical structures of AMPs.

The cell killing kinetics of PtAMP protein on *R. solanacearum* suggests that the mechanism of PtAMP protein in killing bacteria may not be a rapid bursting of cell membranes. The protein may enter the cell by some unknown mechanism and then interact with the interior components of the cell to kill the bacteria. By comparing the *in vivo* and *in vitro* effect of the PtAMP protein to *E. coli*, it is further suggested that the

PtAMP protein may have a mechanism to inhibit the growth of bacteria other than rapidly rupturing cell membranes.

The PtAMP gene may be a key defensive gene expressed in loblolly pine seeds. In intact seeds, the expression level was low and almost undetectable. When seed coats were removed and when seeds were near germination, the expression of the PtAMP gene increased to a high level, which suggested that PtAMP gene might play an important role in protection seeds at this vulnerable stage. Expression of PtAMP gene was also induced to higher levels in seeds with chemical inducers. In normal 10-day old seedlings, the expression level of the PtAMP gene was high, which suggested the PtAMP gene was also an important defensive gene in young seedlings. The PtAMP gene appeared to have no or very limited function in protection of young seedlings from wounding. The gene was not induced to express in young seedlings with the treatment of the chemical inducers. Forty-eight hours after inoculation with fungi, expression of the PtAMP gene moderately increased, however, the expression might not involve HR.

The PtAMP gene belongs to a single copy gene in the genome of both tested loblolly pine families. There may be some genes homologous to the PtAMP gene in the genome of these two families. There were also some genes homologous to the PtAMP gene in the genome of yew, which suggested that the gene might be conserved in other conifers.

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