Genetic Analysis of a Disease Resistance Gene from Loblolly Pine

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Abstract: Rapid advances in molecular genetics provide great opportunities for studies of host defense mechanisms. Examination of plant responses to disease at the cellular and molecular level permits both discovery of changes in gene expression in the tissues attacked by pathogens, and identification of genetic components involved in the interaction between host and pathogens. Expression of specific proteins, which is one type of defense mechanism, may offer the host a weapon to protect it from invasion of pathogenic attack. Recently, we have isolated a novel antimicrobial protein gene (PtAMP) from loblolly pine during our gene screening effort. Studies of molecular characterization show that the PtAMP gene shares limited similarity with previously reported antimicrobial proteins in the amino acid sequences, but it contains some common features, e.g. DNA sequences and protein structure, with those antimicrobial proteins. The function of this novel antimicrobial gene has been analyzed at the *in vitro* and *in vivo* levels. Antimicrobial assay data showed that the purified PtAMP protein has strong inhibitory activities against a variety of pathogenic bacteria and fungi. Furthermore, the gene for the PtAMP was transferred into the tobacco genome via *Agrobacterium-mediated* transformation. Ectopic expression of the PtAMP protein in transgenic tobacco plants confers resistance to bacterial and fungal phytopathogens. Our data suggest that the PtAMP gene has the potential through genetic manipulation to protect plants from a wide range of plant pathogens. Analysis of its function provides further understanding of plant defense mechanisms in loblolly pine.

Keywords: Loblolly pine, disease resistance, antimicrobial protein, transgenic plant, forest biotechnology

INTRODUCTION

Due to continuous exposure to microbial pathogens, animals and plants have evolved various highly effective mechanisms to fend off microbial invaders. A widespread defense strategy in many living organisms involves the production of small peptides (i.e., proteins) that exert antimicrobial activity (Broekaert et al. 1997). Well-known examples of antimicrobial peptides (AMP) are the cecropins that accumulate in the hemolymph of many invertebrates in response to injury or infection and the magainins that occur in the

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skin and in the gastrointestinal system of amphibians to protect them from microbial invasion. Another class of antimicrobial proteins is formed by the cysteine-rich peptides, which in contrast to cecropins and magainins, have a complex disulfide bond-stabilized three dimensional folding pattern. Defensins, occurring in mammalian, insect and plant systems, are a major group among cysteine-rich antimicrobial peptides (Broekaert et al. 1995). In general, those defensins share some common features in their structural and functional properties, suggesting that this class of AMP peptides acts on molecules and/or cell structures common to a wide range of microbial organisms.

Cysteine-rich antimicrobial peptides play an important role in host defense against infectious agents. This type of protein exhibits basic and small molecules, and often possesses a broad spectrum of inhibitory activity against several major taxonomic groups of microbes. As products of single genes, antimicrobial peptides can be synthesized in a swift and flexible way. Also, because of their small size they can be produced by the host with a minimal input of energy and resources. More importantly, the simple genetic bases make it amenable for genetic manipulation and easier to develop antibiotic-like biotechnological products.

Unlike animals, plants do not have an innate immunity system. However, the antimicrobial proteins in plants form the defense system similar to the innate immunity in animals. It is believed that antimicrobial proteins are deployed by plants and play an important role in certain stages of plant development when a plant or plant tissue is under vulnerable conditions. In general, plant seeds are especially rich in antimicrobial proteins, and the level of antimicrobial proteins is several fold higher compared to the tissues such as leaves and flowers from a developing plant (Wang et al. 2001) as they could contribute to improved seed production and seed germination. Plant response to pathogen infection is known to result from the activation of a specific cellular program on the part of the plant and is frequently associated with a certain degree of cellular damage (Huang et al 1998). This finding was also supported by an early observation, inhibition of the fungus *(Cronartium fusiforme)* by loblolly pine (Jacobi, 1982). Resistance of plant callus to infection has been attributed to antimicrobial metabolites exuded by the host tissues (Campbell et al. 1965). In a similar manner, tissue culture is an active process, triggered in response to environmental and phytohormonal clues, that is also accompanied by a certain level of cell injury. We have observed that in addition to pathogen infection, the expression of various defense-related genes has been found to be activated during in vitro tissue culture (Huang et al 1998).

As part of an ongoing study of genetic mechanisms of host resistance in loblolly pine, we have begun a characterization of antimicrobial properties present in the tissues of loblolly pine. Several plant proteins with antibacterial or antifungal activity have been reported, but to date none has been reported from a gymnosperm. Using molecular biological approaches, we have recently isolated a loblolly pine gene encoding an antimicrobal protein (PtAMP). Based on the protein sequence, this AMP represents a novel type of plant antimicrobial peptide; however, it contains AMP conserved domains and multiple disulfide bridges and can therefore be considered as the counterpart of cysteine-rich AMPs from animals. Thus, the PtAMP may be a potent inhibitor of the growth of a wide

range of microbial pathogens. In order to determine the antimicrobial feature of the protein, this gene has been cloned and expressed in *E. coli* and in transgenic plants. In this report, we demonstrate that the PtAMP gene from loblolly pine expressed a novel antimicrobial protein, which has strong inhibition against a broad group of pathogenic microorganisms including bacteria and fungi. The PtAMP gene was able to contribute an enhanced disease resistance while expressing in the target plants. We believe that the PtAMP can be used to engineer plants to confer effective and durable resistance in plant against microbial pathogens.

MATERIALS AND METHODS

Plant materials and tissue culture

Loblolly pine *(Pinus taeda* L.) seeds were aseptically germinated *in vitro.* Then the shoot apices were excised from these germinating seedlings and placed on a modified LePoivre medium (LP) for callus induction using a modification of the method developed by Aitken-Christie et al. (1988). Thus, both the callus derived from these young seedlings and seedling tissues (i.e. control) from the same seed source were used in parallel for the studies of gene expression.

Gene identification and cloning

Total RNA was isolated from both apical meristematic cultures and normal seedling tissues (i.e., controls) according to Huang and Tauer (1996). A cDNA library representing all expressed genes in the callus cultures of loblolly pine was constructed in a X-ZAP vector (Stratagene, La Jolla, CA) using the poly(A) RNAs prepared from the total RNA. In order to identify the special group of genes expressed in the treated tissues, analysis of RNA (i.e. transcripts) differential display was performed using the differential display kit (Genhunter Corp., Nashville, TN). Similarities and differences in gene expression were uncovered by comparing the transcripts in the callus cultures to those from controls. Thus, distinguishing DNA fragments of co-migrating cDNA bands were separated through DNA sequencing gel electrophoresis and differential display (DD) fragments were excised from the differential display gels. Finally the resulted DD cDNA fragments were confirmed by reverse northern blotting analysis.

Several selected distinguishing cDNA fragments were used as the probes to screen the cDNA library of loblolly pine callus to obtain a full-length cDNA of the gene of interest. To recover the cDNA inserts, the pBluescript phagemids were excised with ExAssist helper phage and the host strain *E. coli* XL-1 Blue (Stratagene, La Jolla, CA). The nucleotide sequences were determined using thermal cycle sequencer with dye terminators and the ABI automatic sequencer (Applied Biosciences, Foster City, CA). Sequence analysis was performed using the MacVector program (Oxford Molecular Group). Similarity analysis to known sequences and to expressed sequences tag (EST) in GenBank was assessed by the BLAST program of the National Center for Biotechnology Information (NCBI) (Bethesda, MD).

Bioassay for antimicrobial activities

For the antimicrobial assay, the PtAMP protein was expressed in vitro and purified with affinity chromatography. The microplate-reading method was used to determine antimicrobial activities of the recombinant PtAMP protein against a selected group of bacteria and fungi as described by Jin (2001). The inhibitory effect was assessed by calculation of the 50% inhibition concentration (CIO of the PtAMP protein to each of the bacterial and fungal strains.

Functional studies of the gene in transgenic plants

For the construction of a plant transformation vector, the cDNA coding for the PtAMP protein was cloned into the plasmid pBI121 (Clontech), and the gene was placed under the control of the CaMV35S promoter. This gene fusion construct was introduced into the strain LBA4404 of *Agrobacterium tumefaciens,* which was then used for transformation of tobacco leaf explants according to the leaf-disc transformation method (Horsch et al. 1985). Transgenic tobacco plants were recovered in vitro and confirmed to contain the PtAMP gene using Southern blot analysis or PCR DNA amplification with the PtAMP specific primers. T_1 plants of transgenic tobacco were subjected to bioassay for plant protection. Non-transformed tobacco plants served as controls. All plants were inoculated with one of the two virulent strains of *Pseudomonas syringae* pv *tabaci* AT81 and AT2004 by infiltration under aseptic conditions. After inoculation, plants were kept at the appropriate humidity. Disease symptoms of those plants were evaluated at 4 and 12 days after infection.

RESULTS AND DISCUSSION

The cDNA library from loblolly pine callus was screened with the selected differential display cDNA fragments and several interesting cDNA clones were obtained. Sequence analysis indicated that one of the identified cDNA clones encodes a cysteine-rich protein, although its nucleotide sequences did not show a high degree of homology with any known genes in the Genbank database. Cysteine rich in this protein is an important feature as many other antimicrobial peptides isolated to date contain numerous cysteine residuals, which contribute a high stability to the proteins by forming disulfide bridges. Thus, this cDNA clone, named PtAMP (as isolated from loblolly pine, *Pinus taeda)* was chosen for further characterization.

First, we cloned the PtAMP gene into the expression vector pET30C+ in order to heterologously express the AMP protein *in vitro.* In general, any recombinant proteins can be effectively expressed in the model system, *E. coli* cell, which also facilitates subsequent purification of the target protein. However, expression of this antimicrobial protein in *E. coli* cells was a challenging task due to the nature of toxicity of the AMP protein to the microbial host. After testing a variety of expression systems, an *in vitro* expression system based on a combination of bacterium and bacterial phage was identified, which allowed us to isolate an adequate amount of the AMP protein. We also

identified the critical point in the growth phase of the host cells, which permitted the host cells to synthesize an optimal amount of the AMP protein before the host cells were inhibited by the toxic protein. Subsequently, the expression product was purified using affinity chromatography.

Purified PtAMP protein was tested *in vitro* for inhibitory activity against a variety of microorganisms. Preliminary results, utilizing a highly pathogenic strain of *Pseudomonas (Ralstonia) solanacearum* (a vascular pathogen that causes severe wilting and eventually death) indicated that the purified PtAMP protein had very strong inhibitory effect to bacterial growth. As shown in Fig. 1, the viability of the bacterial cells was 55.1% when *P. solanacearum* was grown in a medium supplemented with 3 ug/ml of purified PtAMP protein. Antimicrobial assay was also carried out with six fungal phytopathogens, five bacterial phytopathogens, and baker's yeast. Very strong inhibition (IC50 <10 ug/ml) was observed against six microorganisms and strong to moderate inhibitions against all other microorganisms tested (Table 1). However, the PtAMP protein was not toxic to plant cells (data not shown) when we conducted toxicity assays with both loblolly pine and tobacco cells *in vitro*. The addition of 100 ug/ml of purified PtAMP protein to plant cell suspensions showed no effect on plant cultures, which was evidenced by no decline in cell viability and proliferation rate.
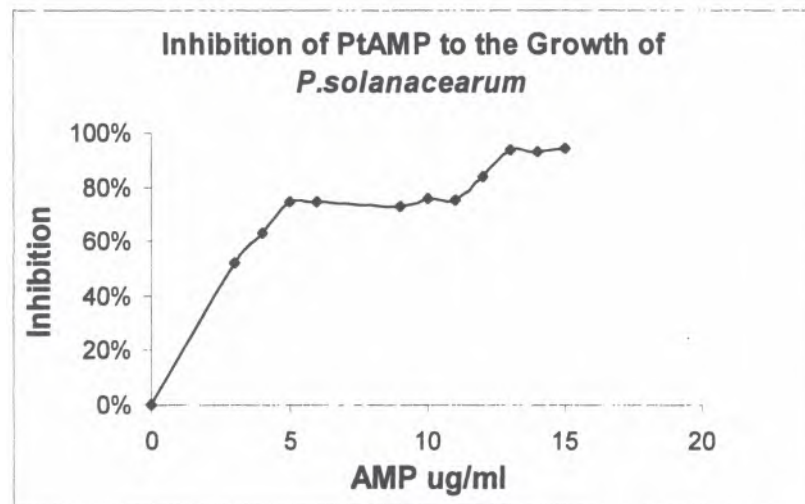

Figure 1. Bactericidal effect of the PtAMP protein on Pseudomonas solanacearum. The inhibition dynamics of various concentrations of the PtAMP on the pathogen grown *in vitro*.

In order to test *in vivo* expression of the antimicrobial gene in target plants, two expression vectors were constructed, containing the PtAMP gene and other regulatory elements such as enhancer sequences. The gene fusion was used to transform the

Bacteria or fungi	IC_{50} (ug/ml)*
Clavibacter michiganesis	<2
Pseudomonas syringae	<3
Pseudomonas solanacearum	<3
Fusarium oxysporum	<2
Collectotrichum obiculare	21
Saccharomyces cerevisiae	30
Thielaviopois basicola	75

Table 1. The PtAMP protein was tested for antimicrobial activity with a range of bacterial and fungal pathogens. Microbial growth inhibition was measured at the protein level (microgram per milliliter) when 50% of inhibition (IC_{50}) was observed.

leaf-disks of tobacco via the *Agrobacterium-mediated* transformation system. The transformants were selected by their resistance to the selection agent (kanamycin) and transgenic tobacco plants were regenerated from shootlets by transferring to a root-induction medium containing kanamycin (10 ug/ml). Thus, fertile transgenic plants were obtained, which appeared normal in phenotype but were confirmed to possess intact copies of the PtAMP gene by standard Southern blot analysis (data not shown). In the first assay, we tested in vitro antimicrobial activity against virulent strains of *Pseudomonas syringae* pv *tabaci* which causes wild-fire disease on tobacco plants. PtAMP protein was partially purified from the PtAMP-expressing plants and the extract from the transgenic plants was used in the in vitro antimicrobial assay. The expressed PtAMP protein from transgenic plants clearly inhibited bacterial growth (Fig. 2), whereas extracts from the control plant (non-transformed) showed no inhibition to the growth of bacteria (data not shown). This result also suggests that the recombinant PtAMP protein from transgenic plants was folded correctly and had its full function. To determine the effect of disease resistance, the plant expressing PtAMP and non-transgenic control plants were grown under aseptic conditions. Following this, the plants were inoculated by infiltration with two highly pathogenic strains of *Pseudomonas syringae* pv *tabaci* AT81 and AT2004. Seven days after infection, disease symptoms such as chlorsis and necrosis appeared at the inoculation sites of control plants but not on transgenic plants (data not shown). Fourteen days after infection, the control plants showed severe disease symptoms and eventually died with severe leaf wilting and stem rot after 21 days (data not shown). In contrast, little necrotic lesion was observed on leaves of PtAMP-
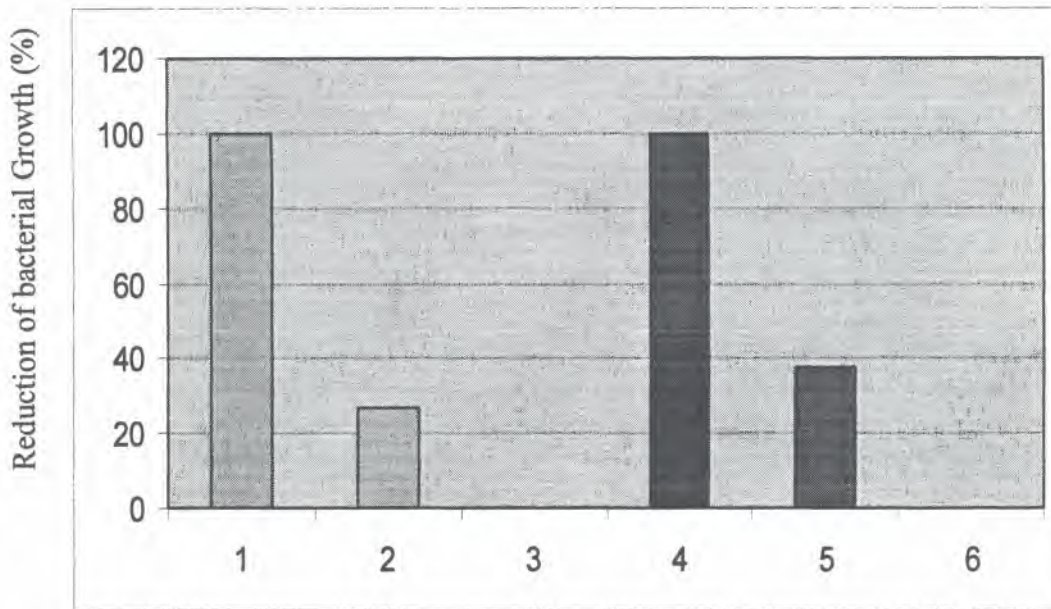
Figure 2. Inhibitory activity of the plant extract from the transgenic tobacco plant expressing the PtAMP. Plant extract was added into bacterial growth medium and bacterial growth rates were determined after 15 hours of incubation. Samples 1 and 2 represent the relative growth of *Pseudomonas syringae* pv *tabaci* PTBR2004, and samples 4 and 5 for the growth rate of *Pseudomonas syringae* pv *tabaci* PT81. Samples 2 and 5 were grown in the medium containing the plant extract from the transgenic plant, and samples 1 and 4 were grown in the medium containing the plant extract from non-transformed plant.

transgenic plants challenged by the same pathogenic organisms (data not shown). Taken together, the results clearly suggest that overexpression of the PtAMP gene in the host plants confers an enhanced host resistance to *Pseudomonas syringae* pv *tabaci* AT81 and AT2004, two major pathogens of tobacco.

CONCLUSIONS

In summary, the PtAMP gene appears to be a novel antimicrobial gene from loblolly pine and encodes a small protein with the molecular size of 11.7 kDa. Purified PtAMP protein shows strong antimicrobial activities against both fungal and bacterial phytopathogens but no toxicity to plant cells. Analysis by Southern blot with loblolly pine genomic DNA indicated that the PtAMP gene belongs to a single or low copy gene family. When its expression pattern was investigated by Northern blot analysis, expression of the PtAMP gene had strong correlations with the status of seed damage and attack of phytopathogens. Thus, the results from this study suggest that the PtAMP protein has great value for engineering disease-resistance in crop plants.

ACKNOWLEDGMENT

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