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Cloning and Characterization of a Putative Antifungal Peptide Gene (*Pm-AMP1*) in *Pinus monticola*

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ABSTRACT

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We have been working on proteins that are involved in the defense response of western white pine (WWP) (*Pinus monticola*) to the blister rust fungus *Cronartium ribicola*. Our objective was to identify candidate genes that could be used for improving resistance of WWP to this rust pathogen. During proteomic analysis of bark proteins extracted from WWP trees exhibiting slow-canker-growth (SCG) resistance, a 10.6-kDa peptide, termed Pm-AMP1, was found to be enriched at the receding canker margin. The cDNA encoding this peptide was cloned and characterized. A BLASTX search revealed that the Pm-AMP1 encoded by its

cDNA has a 50% homology with MiAMP1, a broad-spectrum antifungal protein isolated from *Macadamia integrifolia*. Based on the deduced amino acid sequence, an antibody was produced against the Pm-AMP1. Immunochemical quantification of the Pm-AMP1 in bark samples of susceptible WWP trees revealed this protein to be barely detectable in the cankered tissues, but occurring in higher concentrations in healthy tissues away from canker margins. Foliage of SCG-resistant trees contained higher concentrations of the Pm-AMP1 than foliage from susceptible cankered trees. Both wounding and methyl jasmonate treatment of WWP needles induced the expression of this protein, further supporting its putative role as a defense response protein.

Additional keywords: electrophoresis, western immunoblot.

Western white pine (WWP) (*Pinus monticola* Dougl. Ex D. Don) is an economically important, fast-growing species that is widely distributed throughout the Pacific Northwest and California. The high stumpage value of this pine and its excellent genetic resistance to root rot by *Inonotus tomentosus*, *Armillaria ostoyae*, and *Phellinus weirii* make WWP an attractive species for use in reforestation. However, the potential for commercialization has not yet been realized, because of its susceptibility to infection by white pine blister rust (*Cronartium ribicola* J.C. Fisch.). This rust is an ecologically and economically significant disease, and has resulted in reduced plantation of WWP and other five-needle pines (15).

Because almost all North American WWP is susceptible to *C. ribicola*, obtaining genetic resistance to *C. ribicola* and maintaining genetic diversity among production populations used in WWP-breeding programs are important objectives. Genetic resistance to white pine blister rust varies widely among WWP populations. Several types of WWP genetic resistance to white pine blister rust, including slow-canker-growth (SCG) (11) and major dominant gene resistance (*Cr2*) (13), have been identified after multiyear genetic-breeding studies. SCG resistance is a durable resistance expressed in WWP seedling stems; it restricts the infection area, and cankers eventually heal (11). Different kinds of SCG resistance have been observed, suggesting multiple genes may control it (7).

In a dominant gene-mediated resistance of white pine, infection is restricted to the needle (which is the target of blister rust patho-

gen attack) by the hypersensitive response. In SCG resistance, infection spreads from the needle to the bark and develops into cankers, but with time these cankers heal. In our breeding program, four types of SCG based on canker size and shape are recognizable (7). Little is known about the physiology or biochemical nature of SCG mechanisms, although it has been shown that the delayed onset of this resistance is associated with longer field survival of infected seedlings. This mechanism has a heritability value of 0.21 to 0.46, making the phenotype attractive to tree breeders (9). Unfortunately, screening of seedlings for SCG resistance is a long-term task that takes 6 to 7 years. A WWP-breeding program requires increased understanding of the plant's defense mechanisms against *C. ribicola*, as well as a search for potential molecular markers for marker-assisted selection.

With advancements in molecular techniques, an increasing number of defense response genes have been characterized in several plant-pathogen systems including conifers (7). Proteins encoded by these genes are usually pathogenesis-related (PR) proteins or low-molecular-weight antimicrobial peptides (AMPs) that may act in concert to limit the growth of pathogens in infected hosts. In our laboratory, we have characterized several PR proteins that are up-regulated in the white pine blister rust pathosystem. While investigating bark proteins associated with SCG in WWP, we discovered a 10.6-kDa protein, termed Pm-AMP1, in SCG-resistant trees (3). A partial N-terminal sequence was determined. At the time, the sequence revealed no homology with known protein sequences in the databank. The N-terminal sequence was recently searched against a database and showed significant homology with AMP MiAMP1 isolated from *Macadamia integrifolia* (16). This highly basic AMP inhibited several major taxonomic groups of microbes and showed no toxic effects toward the host plant or animal cell, making it a potentially important candidate in the design of new fungicides (17). More recently, an antimicrobial gene (Sp-AMP) with homology to MiAMP1 has been isolated in Scots pine showing elevated mRNA transcript levels in necrotic root tissue (1).

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AMPs are present in most plant species (2). These peptides contain even numbers of cysteines (four, six, or eight) that form disulfide bridges, thus providing high stability to the peptides. Based on homologies at the primary structure level, plant AMPs can be classified into distinct families including thionins, defensins, lipid transfer proteins, and hevein- and knottin-type AMPs. Detailed three-dimensional structure information has been obtained for one or more members of these peptide families. All AMPs studied to date appear to exert their antimicrobial effects at the level of the plasma membrane of the target microorganism, but different peptide types probably act via different mechanisms. Plant defensins, thionin, and lipid transfer proteins have been observed to be induced upon infection of plants by pathogens, which suggests that they have an active role in host defense. In some cases, overexpression of these peptides can lead to enhanced resistance. Although MiAMP1 contains six cysteine residues, it has no homology with these distinct families of AMPs. In vitro studies showed MiAMP1 has broad-spectrum antimicrobial activity. Interestingly, the structure of MiAMP1 bears remarkable similarity to a yeast-killing toxin from *Wiliopsis mrakii*, which inhibits β -glucan synthesis (17). However, the role of MiAMP1 in host defense response remains unknown.

In the present study, we cloned and characterized *Pm-AMP1*. Using an anti-Pm-AMP1 antibody as a probe, we examined the levels of Pm-AMP1 in infected WWP trees.

MATERIALS AND METHODS

Plant materials and experimental treatments. Bark samples (all tissues outside the xylem, including periderm, phloem, and the cambial zone) were collected from resistant (i.e., canker-free) and susceptible (i.e., cankered) mature WWP grown in plantation 4/plot B (10) located at Lens Creek, Vancouver Island, British Columbia. Selection for resistance (clones G-8, G-161, and B643) was based upon so-called "mature resistance" (R. S. Hunt, Canadian Forest Service, Victoria, B.C., *personal communication*). Such clones were difficult to infect in a diseased garden (i.e., when grown with *C. ribicola*-infected *Ribes* sp. [W. A. Porter, 1960, Canada Department of Agriculture, Victoria, B.C., *unpublished data*]), and their ramets lacked cankers. Similarly tested susceptible clones (G-27 and 6149) were also sampled, and ramets from these had four or more cankers. Ramets were 31 to 33 years old in 1991. All samples were taken from healthy trunk bark in April 1991, February 1993, October 1993, and May 1994.

Two-year-old susceptible WWP seedlings (20 cankered individuals) and resistant seedlings, which displayed slow canker growth (23 individuals), were inoculated in August 1992 by suspending over the seedlings *Ribes* leaves (10) infected with a composite inoculum from six coastal British Columbia sources of the rust fungus. The seedlings were grown outdoors under natural day length and temperature conditions at Cowichan Lake Research Station, Vancouver Island. The following winter, the seedlings were transferred from Styrofoam blocks to 1-gallon (4.5 dm³) pots. Both susceptible and SCG seedlings were from families undergoing screening for blister rust resistance for coastal British Columbia (4). Samples of bark, twigs, and foliage were collected from these seedlings in summer (July 1995) and winter (February 1996).

The chemical treatment with methyl jasmonate and the wounding treatment were performed on fully developed, current-year needles collected from several seedlings grown from a resistant (Cr₂/cr₂) seed lot. The above treatments were carried out as described previously (14). Each treatment was repeated three times. Each time, tissue samples were taken from three individual seedlings, frozen in liquid nitrogen, and stored at -80°C until analyzed.

Cloning of WWP cDNA encoding Pm-AMP1. RNA extraction from WWP needle tissues was carried out using a standard

protocol (14). Reverse transcription-polymerase chain reaction (PCR) was used to clone WWP cDNA encoding Pm-AMP1. The first-strand cDNA was synthesized using a SMART cDNA Library Construction Kit (Clontech Laboratories, Inc., Palo Alto, CA) following the manufacturer's instructions. According to the N-terminal amino acid sequence of the 10.6-kDa protein (3; accession no. P83880), one reverse primer, 10.6K-A (5'-(A/G)TA (A/T/C/G)C(T/G) (A/T/C/G)GC (A/G)TT (A/G)TG (A/G)TT (A/G)TT-3'), was designed. With 10.6K-A primer and PCR 5' primer from the SMART cDNA Library Construction Kit (Clontech Laboratories, Inc.), 5'-rapid amplification of cDNA ends (RACE) was performed to clone the 5' end partial cDNA sequence of the 10.6-kDa protein including the 5'-untranslated region (5'-UTR) and a partial coding region of the N terminus. A forward primer, 10.6K-F (5'-ACT CAC ACC TTA ATA TCC TGA TCC-3'), was designed and synthesized based on 5'-UTR sequence from the above 5'-RACE. To clone the full-length cDNA of the 10.6-kDa protein, 3'-RACE was performed using primer 10.6K-F and oligo(dT)₃₀.

PCR was performed with a PCR Master Mix Kit (Qiagen Inc., Mississauga, ON, Canada) using first-strand cDNA as template in a final volume of 50 μ l on a Perkin-Elmer Thermocycler (Applied Biosystems, Foster City, CA). For 5'-RACE, thermal cycling conditions consisted of an initial denaturation step at 94°C for 30 s, followed by 35 cycles of denaturation at 94°C for 30 s, and primer annealing at 50°C for 1.5 min and primer extension at 72°C for 1.5 min with a final 10-min extension at 72°C. Thermal cycling conditions for 3'-RACE were the same as those for 5'-RACE, except that primer annealing occurred at 60°C for 1 min. After agarose gel fractionation, the amplified cDNA fragments with expected sizes were purified and cloned into pGEM-T easy vector (Promega, Madison, WI). Plasmid construction and manipulation were carried out using standard methods (18).

DNA sequencing and computer analysis. DNA sequencing of cDNA clones were determined on both strands on a DNA sequencer (ABI310; Applied Biosystems) using a Thermo-cycle sequence kit (Amersham Biosciences Corporation, Piscataway, NJ) with T7 and SP6 primers and other internal primers as needed, according to the manufacturer's instructions. DNA sequence data were assembled and analyzed using BLAST, ClustalW, and ORFfinder network services at the National Center for Biotechnology Information (NCBI).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western immunoblot analysis. Total proteins were extracted from plant organs and quantified according to procedures described elsewhere (5). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis described by Ekramoddoullah et al. (6), with equal protein loading per lane. For western blot analysis, rainbow molecular-weight standard markers (14.3 to 220 kDa) were used (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Separated proteins were transferred onto an immobilon-P membrane (Millipore Corporation, Bedford, MA) following a standard protocol (6). A polyclonal antibody was raised in rabbits against the synthetic polypeptide with an antigenic region (H-YNTDNC(acetamidomethyl) KGVAQTR-FSSSVNQAC-NH₂) within the Pm-AMP1 peptide. Immunological detection of protein blots was carried out as described previously (6). The scanning, detection, and quantification of the blots were performed using a GS-800 Imaging Densitometer (Bio-Rad Laboratories) with Quantity One software (version 4.4, Bio-Rad Laboratories) according to the instruction manual. The protein level detected by western immunoblot analysis was calculated based on the optical density of all pixels within the band boundary, and expressed in optical density units \times millimeters.

Statistical analysis. A two-way analysis of variance (ANOVA) for independent samples was used to assess significance in mean Pm-AMP1 content between various susceptible and SCG-resistant WWP tissues due to season and pathogen infection at $P <$

WWP blister rust pathosystem, an antibody probe was initially produced to a synthetic N-terminal peptide (3): this being the only Pm-AMP1 sequence information known at the time. Unfortunately, the antibody reacted poorly to bark protein samples that contained Pm-AMP1 (data not shown). Later, having obtained the full-length Pm-AMP1 cDNA sequence, the entire deduced amino acid sequence was analyzed for potential antigenic sites using the DNASTAR Program (DNASTAR Inc., Madison, WI) with the Jameson-Wolf module (12). This analysis revealed three regions along the primary sequence containing potential antigenic epitopes. A polyclonal antibody was produced against one of these sites, which is also mostly hydrophilic. The antigenic index analysis also showed the N-terminal region to be the least antigenic of Pm-AMP1. Figure 3 demonstrates that this antibody reacts primarily to a single band in crude bark protein extracts of WWP.

Detection of Pm-AMP1 in infected WWP trees. Quantities of Pm-AMP1 in bark samples of mature WWP that were collected in April 1991, February 1993, October 1993, and May 1994 from four resistant (free from cankers) and four susceptible (with active cankers) white pine trees are shown (Fig. 4). The level of Pm-AMP1 was generally higher in cankered, susceptible trees in all months except April. In May, Pm-AMP1 expression was significantly higher in susceptible trees compared with resistant trees ($P = 0.0001$), indicative of Pm-AMP1 elevation as a result of infection by the white pine blister rust fungus. Two-way ANOVAs for independent samples were performed to determine statistical significance between sample sets.

Two-year-old WWP seedlings from susceptible and SCG families were sampled in the summer. These showed mean Pm-AMP1 levels that were significantly higher in older needles and twigs and barely detectable in current-year tissues ($P < 0.001$ for each tissue type) (Fig. 5). Additionally, the level of Pm-AMP1 was consistently higher in both needles and twigs from SCG seedlings than in needles and twigs from susceptible seedlings. In needles (Fig. 5A and B), there was a significant difference in mean Pm-AMP1 content ($P = 0.004$) between 2-year-old susceptible and 2-year-old SCG-resistant samples. In twigs (Fig. 5C and D), the differences in mean Pm-AMP1 content between susceptible and SCG-resistant samples were significant for both 1- and 2-year-old tissues ($P = 0.0082$).

In winter samples of the same seedlings, Pm-AMP1 content in SCG-resistant needles was significantly higher than in susceptible

needles for each year of growth ($P < 0.0001$) (Fig. 6A). This elevated Pm-AMP1 expression in needles from SCG-resistant seedlings was more pronounced in the winter (Fig. 6A) than in the summer (Fig. 5B). Unlike the summer samples, Pm-AMP1 was detectable at high levels in current-year needles and twigs during the winter (Fig. 6A and B). In winter twigs, the only significant difference observed was between 1-year-old SCG-resistant and 1-year-old susceptible samples ($P = 0.02$) (Fig. 6B). These data show that the overall levels of Pm-AMP1 are generally higher in SCG-resistant tissues and that a seasonal and age-related effect on Pm-AMP1 expression also exists.

In summer and winter, stem bark tissue was sampled only from cankered, susceptible 2-year-old seedlings and not from resistant ones, as this would have destroyed seedlings saved for orchard propagation (Fig. 7). During the summer, there was no significant difference in Pm-AMP1 expression between cankered, marginal, and healthy bark ($P > 0.05$). However, in winter, we observed a gradual decline in the amount of Pm-AMP1 being synthesized in tissues sampled from healthy bark near the canker margin to within the cankered tissue (Fig. 7A). The decrease in mean Pm-AMP1 synthesis within the cankered tissue as opposed to that within the healthy bark was statistically significant ($P = 0.02$) (Fig. 7B).

Wounding and chemical treatment. Pm-AMP1 could also be induced (Fig. 8) by treatment with methyl jasmonate or by local wounding. Current-year needles collected from several resistant seedlings were subjected to these treatments indicating that Pm-AMP1 is a typical defense response protein.

DISCUSSION

Previous work on proteomic analysis of bark proteins from WWP revealed qualitative and quantitative differences between *C. ribicola*-resistant and -susceptible trees (3). One of these proteins, a 10.6-kDa polypeptide, was found enriched in receding canker margins of SCG-resistant trees. In this study, cloning and characterization of this protein (Pm-AMP1) revealed that it belongs to a new family of AMPs. Pm-AMP1 has significant homology to MiAMP1 isolated from *Macademia integrifolia* and putative AMPs from *Pinus sylvestris*. Not surprisingly, Pm-AMP1 has strongest homology to AMPs from a related *Pinus* species (1). All three AMPs belonging to this family have six conserved cysteine residues.

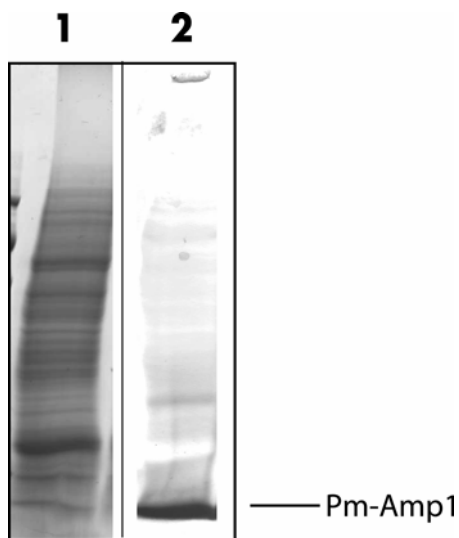


Fig. 3. Analysis of Pm-AMP1 antibody specificity. Coomassie stained gel of white pine crude protein extract (lane 1). Western immunoblot of white pine crude extract (lane 2) probed with Pm-AMP1 polyclonal antibody. Each lane was loaded with 50 µg of protein.

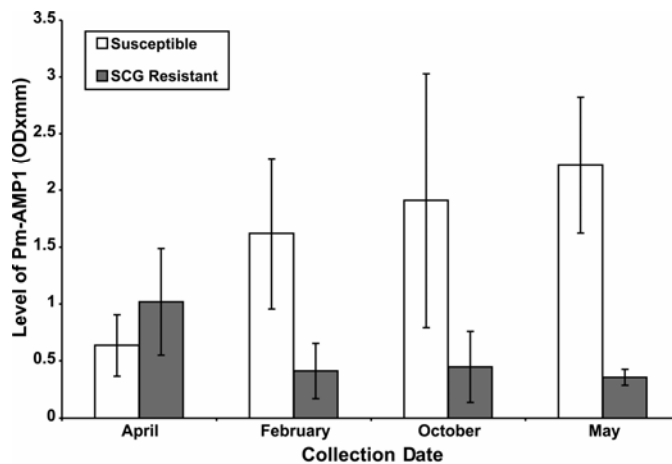


Fig. 4. Immuno-quantification of Pm-AMP1 content in susceptible and resistant tree bark. Relative amounts of Pm-AMP1 protein in mature (31 to 33 years old) bark tissue collected from susceptible (distal to cankered tissue) and resistant (devoid of canker) western white pine trees during April 1991, February 1993, October 1993, and May 1994. Bars represent mean Pm-AMP1 content (\pm SE) of four susceptible and four resistant samples.

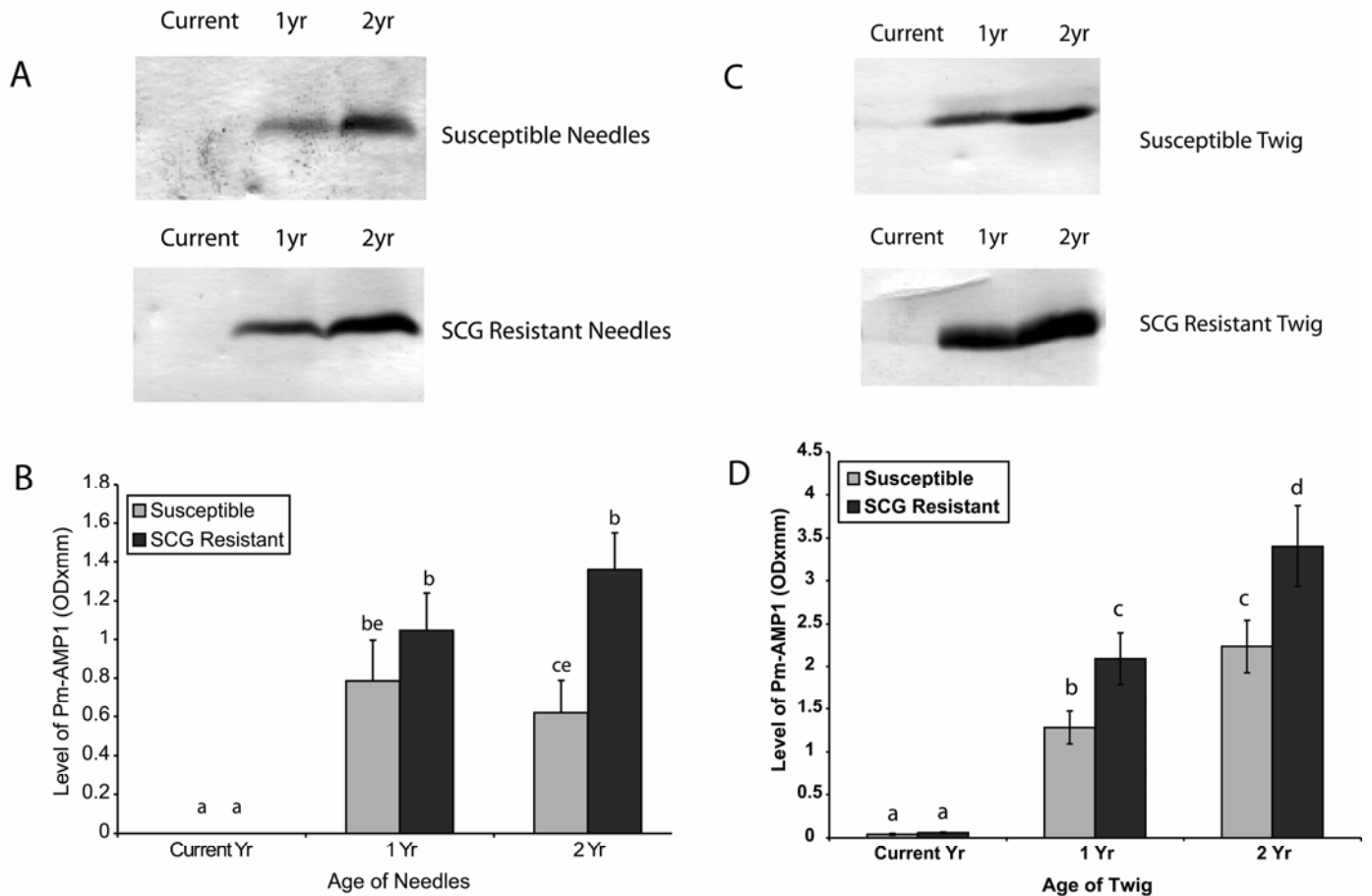


Fig. 5. Immuno-quantification of Pm-AMP1 content in susceptible and resistant needles and twigs during summer. Summer tissue samples of susceptible and slow-canker-growth (SCG)-resistant western white pine needles (A) and twigs (C) representing 3 years of growth were probed with Pm-AMP1 antibody. Quantitative analysis of Pm-AMP1 levels in A and C are shown in B and D, respectively. Significant differences ($P < 0.05$) in mean Pm-AMP1 accumulation (\pm SE) between susceptible and SCG tissues in each year of growth are indicated by different letters, according to Tukey's analysis.

Molecular characterization of this protein also revealed more reactive antigenic sites for antibody synthesis, and a new antibody was generated to the region between Y46 and C67 located in the second β -sheet of Pm-AMP1 (Fig. 2B). This new antibody reacted strongly to the target protein. Having an antibody specific to Pm-AMP1 made it possible to further investigate the role of Pm-AMP1 in the WWP-blister rust pathosystem. Western blot analysis of mature trees showed elevated Pm-AMP1 levels following infection with the fungus. In mature tree bark, a higher level of Pm-AMP1 was observed in susceptible bark tissues relative to resistant (i.e., devoid of cankers) bark tissues. However, in 2-year-old seedlings, a higher level of Pm-AMP1 was observed in SCG-resistant twig/needle tissues relative to tissues sampled from susceptible seedlings. This apparent contradictory finding may be due to the unknown nature of resistance in the mature trees while the resistant young seedlings are known to be SCG. These mature trees were classified as resistant because they were devoid of cankers, whereas mature trees with cankers were classified as susceptible. The observation of relatively high levels of Pm-AMP1 in foliage of SCG-resistant trees suggests regulation of the Pm-AMP1 gene may be linked to genetic elements that underlie this type of resistance and should be exploited to develop marker(s) for breeding this phenotype.

Ontogenic resistance is involved in needle tissues of varying maturity in the white pine blister rust pathosystem. Cotyledons and primary needles are extremely susceptible to blister rust pathogen infection (7). The role of Pm-AMP1 in this ontogenic resistance needs to be investigated. These factors may explain

why barely detectable levels of Pm-AMP1 were observed in current-year needles and twigs sampled in the summer (Fig. 5).

A thorough investigation would be required to see how season and age affect Pm-AMP1 expression.

As we have previously shown that a WWP PR-10 protein was also up-regulated during winter months (6), it was of interest to examine the difference in the level of Pm-AMP1 in infected WWPs in both summer and winter. The low level of Pm-AMP1 observed within cankers or near canker margins of samples collected from susceptible trees only in winter months (Fig. 7B) suggests seasonal factors such as low photoperiod and cooler temperatures in winter may contribute to the pathogen overcoming the host's defense response.

Both mechanical wounding and methyl jasmonate treatment of healthy white pine foliage also induced Pm-AMP1, much the same way as chemical treatment and wounding can induce other AMPs such as defensin and thionin (8). These results suggest that Pm-AMP1 plays a role in the host's defense response.

The mode of action of Pm-AMP1, however, remains unknown. Other research suggests that cationic AMPs may act on the fungal cell wall or on enzymes involved in the synthesis of cell wall components, that they may interact with the lipid bilayer of plasma membranes, creating pores that allow leakage of electrolytes, or that they simply may interact with intracellular targets (20). Based on its pI value, we assume that Pm-AMP1 has cationic properties under physiological conditions. Interestingly, the simulated structure of Pm-AMP1 resembled that of MiAMP1. A three-dimensional structure obtained by nuclear magnetic reso-

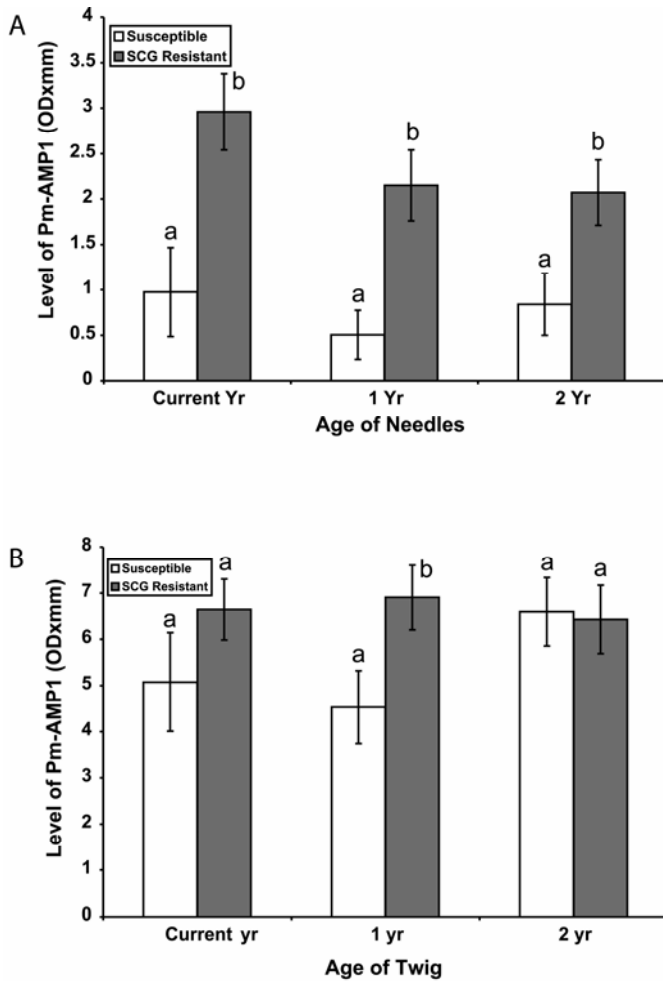


Fig. 6. Immuno-quantification of Pm-AMP1 content of Pm-AMP1 levels in susceptible and resistant needles and twigs during winter. Level of Pm-AMP1 in winter tissue samples of susceptible and slow-canker-growth (SCG)-resistant western white pine needles (A) and twigs (B) from 2-year-old seedlings. Significant differences ($P < 0.05$) in mean Pm-AMP1 accumulation (\pm SE) between susceptible and SCG tissues in each year of growth are indicated by different letters, according to Tukey's analysis.

nance spectroscopic data revealed that MiAMP1 is similar to a yeast-killing toxin from *Williopsis mrakii* that inhibits synthesis of glucan, a fungal cell wall component (17). A simulated three-dimensional structure of Pm-AMP1 revealed interesting features in common with the cationic properties of AMPs.

The structurally related MiAMP1 has shown in vitro inhibitory activity against a variety of fungal, oomycete, and bacterial phytopathogens as well as human mycopathogens (16). The high level of inhibition (95%) exhibited by MiAMP1 at low concentrations (5 μ g/ml) makes it a potent inhibitor of some microbial plant pathogens and highly comparable to other AMPs (16). To demonstrate antifungal properties of Pm-AMP1, work continues to express its recombinant form for use in *C. ribicola* inhibition assays.

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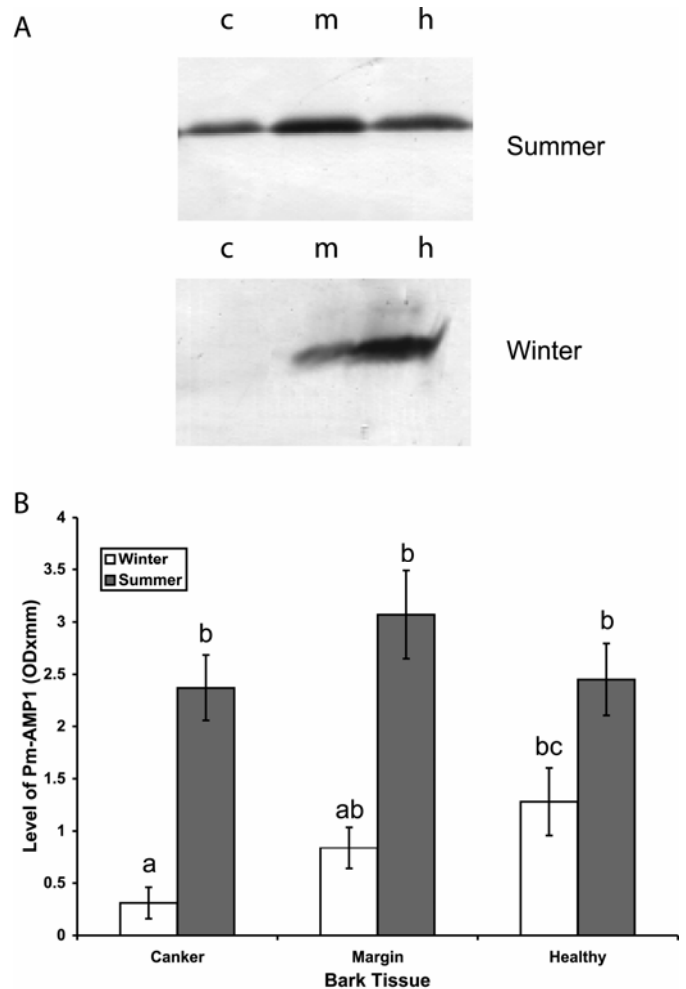


Fig. 7. Immuno-quantification of seasonal PmAMP-1 levels in cankers and surrounding tissue. A, PmAMP-1 protein content in cankered (c), marginal (m), and healthy (h) stem bark of susceptible western white pine sampled in summer and winter. B, Mean PmAMP-1 accumulation (\pm SE) in susceptible bark. Different letters indicate significant differences between bark tissues ($P < 0.05$), according to Tukey's analysis.

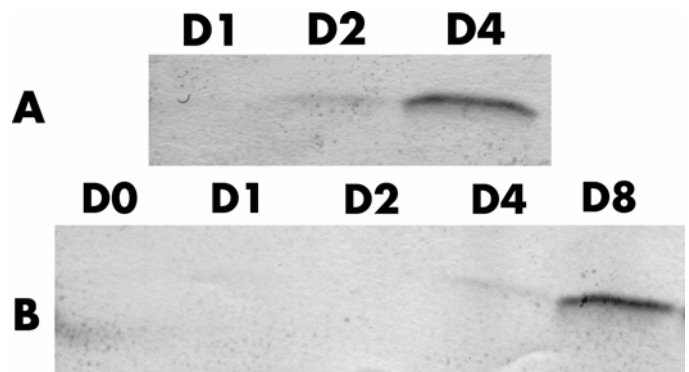


Fig. 8. Effect of methyl jasmonate and wounding treatments on Pm-AMP1 levels. Pm-AMP1 levels in needles after treatment with A, methyl jasmonate or B, local wounding 0 to 8 days (D0 to D8) posttreatment are shown. The Pm-AMP1 protein was detected by western immunoblot.

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