# Antifungal activity of a *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) and its accumulation in western white pine infected with *Cronartium ribicola*

**Arezoo Zamany, Jun-Jun Liu, Abul Ekramoddoullah, and Richard Sniezko**



**Abstract:** *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) was expressed and purified from bacterial cell lysate and its identity and purity confirmed by Western blot analysis using the Pm-AMP1 antibody. Application of Pm-AMP1 resulted in visible hyphal growth inhibition of *Cronartium ribicola*, *Phellinus sulphurascens*, *Ophiostoma montium*, and *Ophiostoma clavigerum* 3–12 days post-treatment. Pm-AMP1 also inhibited spore germination of several other phytopathogenic fungi by 32%–84% 5 days post-treatment. Microscopic examination of *C. ribicola* hyphae in contact with Pm-AMP1 showed distinct morphological changes. Seven western white pine (*Pinus monticola* Douglas ex D. Don) families (Nos. 1, 2, 5, 6, 7, 8, 10) showing partial resistance to *C. ribicola* in the form of bark reaction (BR) were assessed by Western immunoblot for associations between Pm-AMP1 accumulation and family, phenotype, canker number, and virulence of *C. ribicola*. There was a significant difference ($p < 0.001$) in mean Pm-AMP1 protein accumulation between families, with higher levels detected in the full-sib BR families (Nos. 1, 2, 5) than the half-sib BR families (Nos. 6, 7). Family 8, previously described as a Mechanism 'X' BR family, had the highest number of BR seedlings and displayed high Pm-AMP1 levels, whereas the susceptible family (No. 10) showed the lowest levels ($p < 0.05$). Family 1 showed a significant association between Pm-AMP1 accumulation and overall seedling health ($p < 0.01$, $R = 0.533$), with higher protein levels observed in healthy versus severely infected seedlings. In general, low Pm-AMP1 levels were observed with an increase in the number of cankers per seedling ($p < 0.05$), and seedlings inoculated with the avirulent source of *C. ribicola* showed significantly higher Pm-AMP1 levels ($p < 0.05$) in the majority of BR families. Cis-acting regulatory elements, such as CCAAT binding factors, and an AG-motif binding protein were identified in the Pm-AMP1 promoter region. Multiple polymorphic sites were identified within the 5′ untranslated region and promoter regions. Our results suggest that Pm-AMP1 is involved in the western white pine defense response to fungal infection, as observed by its antifungal activity on *C. ribicola* and a range of phytopathogens as well as through its association with different indicators of resistance to *C. ribicola*.

*Key words:* Pm-AMP1, bark reaction, partial resistance, phytopathogen, fungal inhibition.

**Résumé :** Le peptide antimicrobien Pm-AMP1 (« *Pinus monticola* antimicrobial peptide 1 ») a été exprimé et purifié à partir d'un lysat cellulaire bactérien, et son identité et sa pureté ont été confirmées par buvardage Western à l'aide d'un anticorps anti-Pm-AMP1. L'application de Pm-AMP1 a résulté en une inhibition visible de la croissance des hyphes de *Cronartium ribicola*, *Phellinus sulphurascens*, *Ophiostoma montium* et *Ophiostoma clavigerum*, 3 à 12 jours après le traitement. Pm-AMP1 inhibait aussi la germination des spores de plusieurs autres champignons phytopathogènes de 32 % à 84 %, 5 jours après le traitement. L'examen en microscopie des hyphes de *C. ribicola* mis en contact avec Pm-AMP1 montrait des changements morphologiques distincts. Sept familles de Pin argenté de l'ouest (*Pinus monticola*) (N° 1, 2, 5, 6, 7, 8, 10) montrant une résistance partielle à *C. ribicola* sous la forme d'une réaction de l'écorce (« Bark reaction », BR) ont été examinées par buvardage Western visant à évaluer l'association entre l'accumulation de Pm-AMP1 et la famille, le phénotype, le nombre de broussins et la virulence de *C. ribicola*. Il y avait une différence significative ($p < 0{,}001$) dans l'accumulation moyenne de Pm-AMP1 entre les familles, les niveaux les plus élevés étant détectés chez les familles BR à descendance biparentale (N° 1, 2, 5) comparativement aux familles BR à descendance uniparentale (N° 6, 7). La famille 8, décrite précédemment comme famille BR à mécanisme 'X', possédait le nombre le plus élevé de plants BR et les niveaux les plus élevés de Pm-AMP1, alors que la famille susceptible à l'infection (famille 10) montrait les niveaux les plus faibles ($p < 0{,}05$). La famille 1 montrait une association significative entre l'accumulation de Pm-AMP1 et la santé globale des plants ($p < 0{,}01$, $R = 0{,}533$), avec des niveaux de protéines plus élevés chez les individus sains *versus* les individus malades. En général, des niveaux faibles de Pm-AMP1 étaient observés lors d'une augmentation du nombre de broussins par plant ($p < 0{,}05$), et les plants inoculés avec la souche avirulente de *C. ribicola* montrait des niveaux significativement plus

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élevés de Pm-AMP1 ($p < 0,05$) chez la majorité des familles BR. Les éléments de régulation cis-actifs comme les facteurs de liaison de CCAAT et une protéine liant les motifs AG ont été identifiés dans la région du promoteur de Pm-AMP1. Des sites polymorphiques multiples ont été identifiés à l'intérieur de la région 5′ non transcrite et celle du promoteur. Nos résultats suggèrent que Pm-AMP1 est impliqué dans la réponse défensive du Pin argenté de l'ouest envers l'infection fongique tel qu'observé par son activité antifongique sur *C. ribicola* et sur un vaste spectre de phytopathogènes, ainsi que par son association à différents indicateurs de résistance à *C. ribicola.*

*Mots-clés :* Pm-AMP1, réaction de l'écorce, résistance partielle, phytopathogène, inhibition fongique.

[Traduit par la Rédaction]



## Introduction

Fungal phytopathogens challenge geneticists and forest tree breeders to devise new solutions to enable forest trees to survive and thrive in their habitat. In nature, the local and systemic induction of antimicrobial peptides, such as pathogenesis-related (PR) proteins, enzymes, and small peptides (<10 kDa), such as lipid-transfer proteins, thionins, and plant defensins (Broekaert et al. 1997), enhances a tree's ability to overcome the detrimental effects of phytopathogenic fungi. Using plant transformation techniques, it may be possible to improve forest tree resistance to pathogens by introducing antimicrobial peptides at the molecular level.

Introduction of the blister rust fungus *Cronartium ribicola* from Europe (Gussow 1923) has caused severe mortality in stands of five-needle *Pinus* spp. throughout North America (Hunt 1983; Scharpf 1993). Western white pine (*Pinus monticola* Douglas ex D. Don; WWP) is a valuable timber resource both economically and silviculturally owing to its resistance to root rot (Nelson and Sturrock 1993); however, it is highly susceptible to blister rust (Hunt 1983).

Major gene resistance (MGR) of WWP to *C. ribicola* involving the resistance gene *Cr2* and its interaction with a specific avirulence gene, *AVCr2*, results in a hypersensitive response (HR) that has been well characterized (Kinloch et al. 1999; Liu et al. 2006; Liu and Ekramoddoullah 2007, 2008). The virulent *vcr2* strain of the *C. ribicola* pathotype neutralizes the *Cr2* gene in WWP and overcomes the HR (Kinloch et al. 1999). Partial resistance of WWP to *C. ribicola* has also been identified with quantitative resistance phenotypes often referred to as bark reaction (BR; Hoff 1986; Kegley and Sniezko 2004) or slow canker growth (SCG; Hunt 1997) types. Partial resistance is a desirable trait in breeding programs because it is likely controlled by a number of different genes (Hoff 1986). The number and types of stem symptoms are important traits used to assess the degree of partial resistance, which is classified as complete BR, partial BR (PBR), normal canker (NC), and sometimes Mechanism 'X' (Sniezko et al. 2004). In general, BR manifests itself as a sunken necrotic lesion, often occurring at the base of a needle fascicle on stem tissue (Thiesen 1988; Kegley and Sniezko 2004). If the spread of infection is restricted and the stem heals, it is considered a complete BR and the tree would be expected to live (Kegley and Sniezko 2004). If *C. ribicola* continues to spread, it is considered a PBR, which can manifest itself as slow-growing cankers that may become inactive, become BR, or cause tree mortality over time (Kegley and Sniezko 2004). Trees with NCs develop orange discoloration of the bark with active mycelial growth around the periphery of diamond-shaped swollen cankers (Hunt 1997). Mechanism 'X' occurs in low frequency in open-pollinated, non-*Cr2* WWP families, characterized by a high incidence of stem-symptom free and BR-resistant seedlings where trees perform as well as with the HR but the type of resistance is difficult to categorize in phenotyping (Sniezko et al. 2004).

BR can be an effective long-term defense strategy owing to its stability and durability in the presence of a variable rust (Hoff and McDonald 1980). Although not all BR or PBR resistances can completely halt fungal growth, these phenotypes impede *C. ribicola* from producing aeciospores thus preventing completion of its life cycle. The current seedling screening assay for confirmation of BR resistance takes at least 5-6 years, so development of molecular markers that could identify SCG and BR in early stages of growth would be an invaluable tool.

We have identified several PR proteins, including PR3 chitinases (Robinson et al. 2000; Liu et al. 2005) and PR5 thaumatin-like proteins (Zamani et al. 2004; Piggott et al. 2004; Liu et al. 2010), in both the WWP – *C. ribicola* and the Douglas-fir – *Phellinus weirii* pathosystems and shown them to be upregulated as a result of pathogen infection. The accumulation of a WWP 10.6 kDa basic protein, later termed *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1), in SCG-resistant trees (Davidson and Ekramoddoullah 1997) prompted its cloning and genetic characterization (Ekramoddoullah et al. 2006). Pm-AMP1 exhibited considerable homology to other antimicrobial agents like MiAMP1 in Macadamia nut (Marcus et al. 1997) and Sp-AMP in Scots pine (Asiegbu et al. 2003).

The wide-ranging effect of MiAMP1 on numerous microorganisms (Marcus et al. 1997) makes the WWP homologue of this gene a suitable candidate to test for inhibitory activity not only against *C. ribicola* but also other fungal pathogens affecting British Columbia's forests. Examples include the basidiomycete *Phellinus sulphurascens*, a root pathogen causing extensive growth loss and mortality of Douglas-fir (*Pseudotsuga menziesii*) (Theis and Sturrock 1995), and the ascomycetes *Ophiostoma montium* and *Ophiostoma clavigerum*, pathogenic blue stain fungi symbiotically associated with the mountain pine beetle (*Dendroctonus ponderosae*; Robinson 1962 and others).

We overexpressed Pm-AMP1 in an *Escherichia coli* expression system and used affinity chromatography to purify the recombinant Pm-AMP1 to assess its inhibitory activity against WWP's natural aggressor *C. ribicola* as well as other important fungal pathogens. We also quantified Pm-AMP1 across seven WWP families showing partial resistance to de-

**Table 1.** List of primers and PCR conditions used for recombinant *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) vector construction and single nucleotide polymorphism analysis.

| Primer name | Primer direction | Primer sequence (5′→3′) | Annealing temp. (°C) | No. of cycles |
|---|---|---|---|---|
| 10.6K-T | Forward | CACCATGAGTTATTTCTCTGCGTGG | 52 | 30 |
| 10.6K-B | Reverse | AAGGATCCTCACAACACTCAGCACTGGATG | | |
| 10KR1 | Forward | AGTGCTGAGTGTTGTGA | 50 | 30 |
| 10KF1 | Reverse | TGTAACAATCATAATGCGCGATAC | | |
| 10KR2 | Forward | TTTTGTGTGCACTGATATTCCAGC | 50 | 30 |
| 10KF2 | Reverse | ACTCACACCTTAATATCCTGATC | | |
| 10KPR6 | Forward | CAAACTTTCACCAATTCAGATCTA | 60 | 30 |
| 10KBAM | Reverse | CATCCAGTGCTGAGTGTTGTGA | | |



termine whether associations exist between phenotypic traits and Pm-AMP1 accumulation. At the gene level, nucleotide polymorphisms were examined in the 5′ untranslated region (UTR) and promoter regions of Pm-AMP1.

## Materials and methods

### Fungal propagation

*Cronartium ribicola* basidiospores needed for in vitro culture were generated. First, *Ribes nigrum* potted plants were artificially inoculated with *C. ribicola* by rubbing aeciospores, collected from cankered WWP trees on Vancouver Island, British Columbia, on the underside of the leaves. Inside a temperature controlled growth chamber, infected leaves were initially covered by plastic and maintained in a moist environment at 24 °C for 48 h, then plastic bags were removed and plants were maintained at 24 °C for 10 days or until light orange blisters (urediniospores) were evident on stems. The temperature was lowered to 16 °C during the day and 12 °C at night to mimic early fall conditions for teliospore formation. After 3 weeks telial stalks bearing basidiospores were visible. A modified version of the nutrient-rich basal media of Gresshoff and Doy (1972), as described in Kinloch and Dupper (1996), was used for *C. ribicola* in vitro propagation. Infected *R. nigrim* leaves attached to the underside lids of Petri plates allowed for basidiospore shed on the nutrient-rich media as outlined in Kinloch and Dupper (1996) with the exception that our cultures were derived from multiple cast basidiospores. *Cronartium ribicola* is slow-growing in culture; by cycling the primary culture from solid to liquid media and back again, colonies grew faster. White, fluffy *C. ribicola* mycelia were visible on plates 3 months post-inoculation.

Agar plugs of the basidiomycete *P. sulphurascens* (provided by R. Sturrock, Natural Resources Canada) and the ascomycetes *O. montium* and *O. clavigerum* (provided by Dr. C. Breuil, The University of British Columbia) were grown on 2% malt agar for plate inhibition assays. The deuteromycetes *Fusarium oxysporum*, *Alternaria cucumerina*, and and the ascomycetes *Colletotrichum lagenarium* and *Thielaviopsis basicola* (provided by Dr. Z. Punja, Simon Fraser University) were grown on potato dextrose agar until colonies grew to cover the plate for spore collection. Once collected, spores were counted using a haemocytometer and applied to wells of a 96-well microplate for growth inhibition assays.

### Production of recombinant Pm-AMP1 in *Escherichia coli*

The DNA region encoding the mature form of Pm-AMP1 was amplified by PCR by using primer pairs 10.6K-T and 10.6K-B (Table 1) in an Eppendorf Mastercycler (Hamburg, Germany). The PCR amplified a cDNA fragment of 264 bp and introduced a new start codon ATG at the beginning of the mature Pm-AMP1 protein and a *Bam*HI site after the second stop codon. The pET TOPO expression system (Invitrogen, Carlsbad, California, USA) was used for directional cloning of *Taq*-amplified PCR fragments containing the Pm-AMP1 coding sequence. Presence of the recombinant plasmid was confirmed by DNA sequence analysis. Expression of a recombinant N-terminal His-tagged protein was performed in BL21 Star *E. coli* strain (Invitrogen) according to the manufacturer's instructions. Carbenicillin-resistant transformants were selected and grown in Lennox L broth supplemented with carbenicillin (50 μg/mL) overnight at 37 °C with shaking at 250 r/min to establish a starter culture (20 mL). The starter culture was added to 1 L of Lennox L broth – carbenicillin (50 μg/mL) the following day and grown to an optical density (OD) of 0.5 at 600 nm. Cultures were induced with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C.

Up to 20 L of cells were harvested by centrifugation at 4500*g* for 10 min at 4 °C, and the pellet was processed in such a way as to effectively solubilize misfolded, recombinant protein aggregates sequestered as inclusion bodies. The procedure outlined in Amersham Pharmacia Biotech's Recombinant Protein handbook (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA) was followed. Briefly, the pellet was resuspended in 15 mL of resuspension buffer (20 mmol/L Tris–HCl, pH 8.0), vortexed, and disrupted using a sonicator (Vibra Cell, Sonics and Materials Inc., Danbury, Connecticut) at constant power for four 10 s bursts. Cells were spun at 10 000*g* for 10 min at 4 °C and the pellet resuspended in 10 mL isolation buffer (2 mol/L urea, 20 mmol/L Tris–HCl, 0.5 mol/L NaCl, 2% Triton X-100, pH 8.0). Cells were again sonicated and the process repeated. The final pellet was resuspended in 5 mL of binding buffer (6 mol/L guanidine hydrochloride, 20 mmol/L Tris–HCl, 0.5 mol/L NaCl, 5 mmol/L imidazole, 1 mmol/L 2-mercaptoethanol, pH 8.0) and stirred for 30 min at room temperature. The suspension was centrifuged at maximum speed (15 min, 4 °C), and the final supernatant was clarified using a 0.45 μm filter.

### Purification of the His-tagged fusion protein

Protein purification was carried out using an ÄKTAprime chromatography system (GE Healthcare Bio-Sciences Corp.) and a 5 mL HiTrap Chelating column for on-column refolding and elution of the recombinant Pm-AMP1 following manufacturer's protocols. On-column refolding was achieved by use of a linear 6–0 mol/L urea gradient. Recombinant Pm-AMP1 was eluted off the column using 20 mmol/L Tris–HCl, 0.5 mol/L NaCl, 0.5 mol/L imidazole, 1 mmol/L 2-mercaptoethanol, pH 8.0, under a linear gradient starting with 0.02 mol/L imidazole in the refolding buffer and finishing with 0.5 mol/L imidazole in the elution buffer. Purified protein fractions were subjected to one-dimensional sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS–PAGE) using 3 μg of each protein fraction per lane. The gel was cut so that one half was used for Coomassie brilliant blue R-250 staining, while the other half used for Western blot analysis as previously described (Ekramoddoullah et al. 2006) to verify the presence of the 10.6 kDa Pm-AMP1. Fractions containing the purified peptide were further processed by desalting and removing low-molecular-mass materials with the HiTrap desalting column using ÄKTAprime. Purified Pm-AMP1 was suspended in a desalt buffer of 20 mmol/L Tris–HCl, 0.15 mol/L NaCl, pH 7, and stored at –80 °C. Aliquots were removed as needed for antifungal assays.

### Antifungal assay

The effect of recombinant Pm-AMP1 on the growth of *C. ribicola*, *P. sulphurascens*, *O. montium*, and *O. clavigerum* was tested in vitro on agar plates. Once subcultured colonies of these fungi had reached 2–5 cm in diameter, recombinant Pm-AMP1 (3–9 μg) and appropriate controls were applied adjacent to the growing margin of the colony. The application method involved either using sterile filter discs saturated with the treatment or the treatment being applied directly into wells created at different locations next to the colony margin. Both techniques produced similar results. Sterile water and desalt buffer were used as blanks, and bovine serum albumin (BSA; 10 μg) in desalt buffer was used as a protein control. The plates were observed daily for signs of inhibition by Pm-AMP1 and photographed. Owing to the slow-growing nature of *C. ribicola*, all treatments were applied every 3 days (3 μg of Pm-AMP1 each time) over a 12 day period to maintain protein activity.

Spores of *F. oxysporum*, *A. cucumerina*, *B. cinerea*, *C. lagenarium*, and *T. basicola* were collected and grown in 96-well microtitre plates at a density of 1000 spores per well in potato dextrose broth. Duplicate wells were prepared for each fungus and for each treatment. Purified, recombinant Pm-AMP1 was applied (15 μg/mL) as the inhibitory agent, and $H_2O$ and desalt buffer were used as blank controls. At 18, 64, 71, 88, 94, 111, and 118 h post-treatment, absorbance measurements were read at 595 nm using an Emax plate reader and SoftMax Pro software (Molecular Devices, Sunnyvale, California, USA). Because Pm-AMP1 was resuspended in desalt buffer, the relative inhibitory effect of Pm-AMP1 on fungal spore germination at each time point was calculated against the desalt buffer control as follows:

$$\%\ \text{Inhibition} = [(\text{Desalt Abs}_{\text{Xh}} - \text{Pathogen Abs}_{\text{Xh}})/\text{Desalt Abs}_{\text{Xh}}] \times 100$$

ANOVA was carried out using SigmaPlot 11 scientific data analysis and graphing software package (Systat software, Inc., San Jose, California, USA) to determine significant differences in % inhibition for each fungus and between different fungi.

### Microscopic examination of *C. ribicola*

Blocks of $H_2O$ and Pm-AMP1-treated mycelia were cut from prepared agar plates and fixed with formaldehyde for 48 h at room temperature. Following fixation, dehydration was carried out in 70% ethanol for 2 h at room temperature, then 96% ethanol for 2 h at room temperature, and finally in 100% ethanol for 1 h at room temperature. Dehydrated blocks were immersed in 100% ethanol and Technovit 7100 (equal parts) for 2 h at room temperature. Blocks were then infiltrated with 100 mL Technovit 7100 and Hardener I (dibenzoyl peroxide, $H_2O$ content 20%) for 1 h at room temperature, then at 37 °C for 1 h. After infiltration the tissues were placed into the embedding pan containing about 10 mL of embedding solution B (15 parts Infiltration Solution A, and one part Hardener II (accelerator–barbituric acid derivative)). Following polymerization, samples were sectioned on a pyramitome to 5 μm in thickness, and sections were then mounted on glass slides in preparation for staining in 0.05% aqueous toludine blue for 1 min, rinsed with water, and air-dried prior to coverslipping.

### Plant material and fungal strains used to inoculate plants for Pm-AMP1 analysis

Foliage tissue from seven WWP families sown in 2003, inoculated with *C. ribicola* in 2004, and sampled in 2006 at Dorena Genetic Resource Centre (USFS, Cottage Grove, Oregon, USA) were shipped under cold conditions to the Pacific Forestry Centre where they were stored at –20 °C until needed for evaluating Pm-AMP1 accumulation. Seeds had originally been selected from long-term breeding programs used to identify vigorous trees showing BR resistance. In this study, three seed families (Nos. 1, 2, 5) were progenies of a known BR-seed parent and a known BR-pollen parent (categorized as a full-sib BR family); two seed families (Nos. 6, 7) only had a BR-seed parent, which was wind-pollinated (half-sib BR family); one half-sib family (No. 8) showed mechanism 'X' resistance; and one full-sib family (No. 10) had two susceptible parents so that all but one seedling showed active cankers (Table 2).

*Ribes nigrim* leaves bearing *C. ribicola* basidiospores on telial stalks were harvested from different sites in Oregon for inoculation purposes. Leaves collected locally from the Dorena *R. nigrim* garden or from Champion Mine in the Cottage Grove Ranger District carried *C. ribicola* basidiospores with the virulent *vcr2* pathotype, whereas the *AVCr2* wild type was collected a distance away from any known plantings of *Cr2* trees (Kegley and Sniezko 2004). Half of the seedlings

**Table 2.** Phenotypic summary for seven western white pine families inoculated with *Cronartium ribicola* in their second growing season and sampled as 3-year-old seedlings for bark reaction resistance screening.

| Family ID No. | Parentage[b] | Total samples | Phenotype classification[a] | | | | | Mortality at 27 months post-inoculation (%) | Overall mortality 4.5 years postinoculation (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | | SS-free, BR resistant | PBR, no aecia | NC, PBR, no aecia | NC, PBR, aecia | Rust dead | | |
| 1 | Full-sib BR | 50 | 6 | 8 | 13 | 16 | 7 | 14.0 | 64 |
| 2 | Full-sib BR | 59 | 8 | 28 | 7 | 9 | 8 | 13.5 | 58 |
| 5 | Full-sib BR | 57 | 16 | 18 | 6 | 11 | 5 | 9.0 | 43 |
| 6 | Half-sib BR | 39 | 2 | 16 | 6 | 3 | 12 | 31.0 | 61 |
| 7 | Half-sib BR | 47 | 7 | 12 | 11 | 3 | 16 | 34.0 | 71 |
| 8 | Half-sib Mechanism 'X' | 54 | 44 | 8 | 2 | 0 | 1 | 2.0 | 11 |
| 10 | Full-sib Susceptible | 58 | 1 | 0 | 12 | 18 | 27 | 46.5 | 97 |

[a]Phenotype observations are based on a 27 month postinoculation inspection with the exception of the rust dead, which shows overall mortality rates 4.5 years following infection. SS-free, seedlings free from stem symptoms; BR resistant, seedlings with complete bark reaction; PBR, seedlings with partial bark reaction; NC, seedlings with normal canker.
[b]Full-sib seedlings have a known seed parent and a pollen parent both displaying bark reaction (BR), while the half-sib seedlings only have a known seed parent.

in each family were artificially inoculated with the *vcr2* strain of *C. ribicola* and the other half were inoculated with the *AVCr2* strain as outlined in Kegley and Sniezko (2004). Phenotypic traits were observed for each seedling annually for up to 5 years postinoculation; however, our study focused on traits observed 27 months postinoculation. Several traits, including number and types of stem symptoms (for example, clean and symptom-free stems, BR or PBR, NCs) and presence of aecia were used to assess seedlings for degrees of partial resistance (Kegley and Sniezko 2004). Since sampling occurred in June and the inspection was done in December, there were seedlings that were classified as rust dead in December but that were still alive in June for foliage collection to assess Pm-AMP1 accumulation.

### Pm-AMP1 quantification

Total protein was extracted and quantified from a total of 364 WWP needle samples representing seven WWP families as described by Ekramoddoullah and Davidson (1995). Equal protein (50 μg) was loaded per lane and an internal control was applied to each gel and factored in to account for blot-to-blot variability. Western blot analysis was performed using Pm-AMP1 antibody (Ekramoddoullah et al. 2006), and blots were scanned and processed using a GS-800 Imaging Densitometer (Bio-Rad Laboratories, Mississauga, Ontario, Canada) interfaced with Quantity One software (version 4.4, Bio-Rad Laboratories). Pm-AMP1 was quantified based on the OD of all pixels within the band boundary. OD unit values were used for statistical analysis by one-way ANOVA and linear regression using SigmaPlot 11. For linear regression analysis, the independent variables were the known or predictor variables, such as phenotype, canker number, or virulence, whereas the dependant variable was Pm-AMP1 OD values.

### Pm-AMP1 5′ UTR and promoter analysis

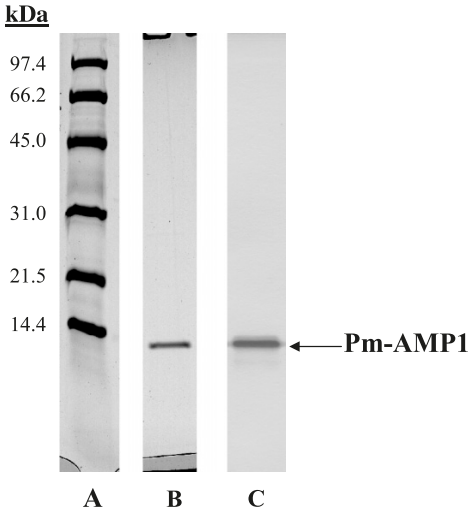
WWP genomic DNA was recircularized and used as template in a two-step, nested, inverse PCR approach to characterize the upstream 5′ UTR and promoter regions of Pm-AMP1. Based on the Pm-AMP1 cDNA coding region sequence (Ekramoddoullah et al. 2006), two sets of gene-specific primers were designed (Table 1). Primer pair 10KR1 and 10KF1 was used in the first-round PCR. The product of this reaction was used as template in a second-round PCR using primer pair 10KR2 and 10KF2 (Supplementary Figure 1[1]). Nonoverlapping regions between amplicons made the characterization of a longer fragment difficult. The final product was 392 bp long and contained both the 5′ UTR and partial promoter region. A promoter prediction program available from http://www.fruitfly.org/seq_tools/promoter.html was used to predict the Pm-AMP1 transcription start site (Supplementary Figure 1[1]).

Seedlings used to identify nucleotide polymorphism of the Pm-AMP1 promoter were grown and inoculated with *C. ribicola* as outlined in Ekramoddoullah et al. (1998). A total of six seedlings from family 3111 displaying SCG and eight susceptible seedlings from family 3115 showing normal bark cankers were used for genomic PCR cloning.

PCR was performed on all samples using primer pair

[1]Supplementary data are available with the article through the journal Web site at www.nrcresearchpress.com/cjm.

**Fig. 1.** One-dimensional SDS polyacrylamide gel electrophoresis of purified recombinant samples of *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1). (A) Molecular mass standard stained with Coomassie brilliant blue R-250. (B) Purified Pm-AMP1 fraction stained with Coomassie brilliant blue R-250. (C) Purified Pm-AMP1 fraction Western blotted and probed with the Pm-AMP1 antibody.




10KPR6 and 10KBAM (Table 1, Supplementary Figure 1[1]) and the 661 bp Pm-AMP1 containing the 5′ UTR and promoter region was cloned into the pGEM-T easy vector (Promega, Madison, Wisconsin, USA). A few recombinant clones were selected from each seedling sample and subjected to nucleotide sequence analysis. Replicate clones of the same PCR reaction were used to account for individual differences that may have indicated PCR artifacts. Alignment analysis was performed online with Clustal W network service at the European Bioinformatics Institute (EBI, Cambridge, UK). Cis-acting regulatory elements were identified using PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

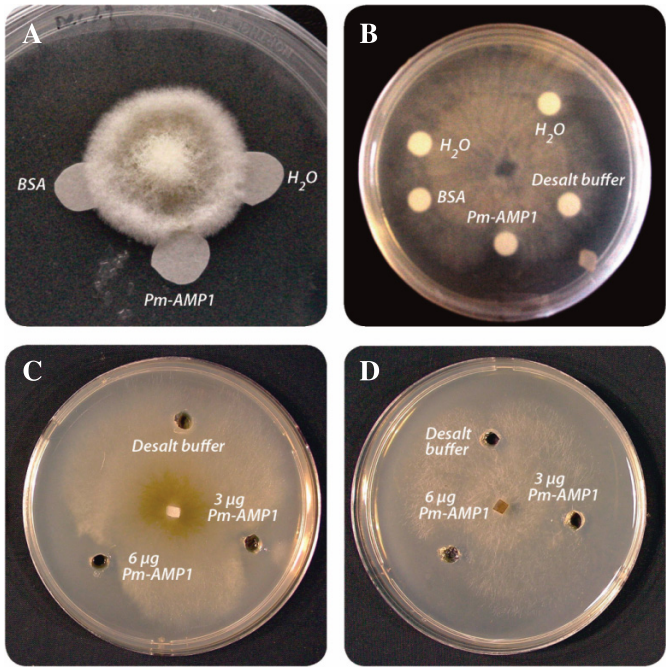
## Results

### In vitro antifungal activity

Following affinity column purification, recombinant Pm-AMP1 fractions were analyzed by 1-dimensional gel electrophoresis to confirm the presence of the 10.6 kDa Pm-AMP1 (Fig. 1). A molecular mass standard was run to determine protein size (Fig. 1, panel A). When the gel was stained with Coomassie brilliant blue R-250, a single band was detected at the 10.6 kDa range (Fig. 1, panel B), confirming the presence of a 10.6 kDa peptide of high purity in each fraction. Western blot analysis using the Pm-AMP1 antibody specifically detected the same-sized band, confirming the identity of the eluted 10.6 kDa protein as Pm-AMP1 (Fig. 1, panel C).

Application of purified Pm-AMP1 to the growing hyphal margins of *C. ribicola* resulted in visible hyphal inhibition 12 days post-treatment as observed by the halt in mycelial progression once it came into contact with the Pm-AMP1 saturated filter disc (Fig. 2A). In comparison, *C. ribicola* continued to grow over the $H_2O$ and BSA protein control filter

**Fig. 2.** Antifungal effect of *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) on the growing hyphal margins of (A) a 3-month-old *Cronartium ribicola* fungal colony 12 days post-treatment with 3 µg of purified Pm-AMP1 applied to the filter disc every 3 days for a total application of 9 µg of Pm-AMP1; (B) a 2-week-old culture of *Phellinus sulphurascens*, the causal agent of laminated root rot, 3 days post-treatment with 3 µg of purified Pm-AMP1 added to the filter disc; 2-week-old cultures of (C) *Ophiostoma montium* and (D) *Ophiostoma clavigerum*, fungal associates of the mountain pine beetle, 3 days post-treatment with Pm-AMP1. Purified Pm-AMP1 in 3 and 6 µg quantities was placed in holes next to the growing edge of the fungal mycelia. BSA, bovine serum albumin protein control.




discs (Fig. 2A). Similarly, *P. sulphurascens* grew around the Pm-AMP1 filter disc, whereas it grew over the control discs saturated with $H_2O$, desalt buffer, and BSA protein controls at only 3 days post-treatment (Fig. 2B). Pm-AMP1 was applied at two concentrations (3 and 6 µg) to the growing hyphal margin of *O. montium* (Fig. 2C) and *O. clavigerum* (Fig. 2D), and although both concentrations resulted in growth inhibition, there was visibly less growth around the periphery of the 6 µg Pm-AMP1 treated area of both blue stain fungi at 3 days post-treatment (Fig. 2C).

Pm-AMP1 had an inhibitory effect on spore germination of five phytopathogens. *Thielaviopsis basicola* and *B. cinerea* showed the highest growth inhibition at 84% and 78%, respectively (Fig. 3). The effect of Pm-AMP1 on *F. oxysporum* and *C. lagenarium* was more pronounced than on *A. cucumerina*, with a spike in inhibition to 45% at 64 h post-treatment (Fig. 3). Growth of these three fungi was inhibited 32%–34% at 118 h post-treatment (Fig. 3). There was a significant difference ($p \leq 0.005$) in percent inhibition for each fungus between the time 0 (control) and the various treatment times postPm-AMP1 application, with the exception of *A. cucumerina* where a significant difference in percent inhibition was not observed at 18 and 64 h postPm-AMP1 application.

**Fig. 3.** Inhibitory effect of *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) (15 µg/mL) on fungal spore germination in five phytopathogenic fungi. Data points represent mean Pm-AMP1±SE.

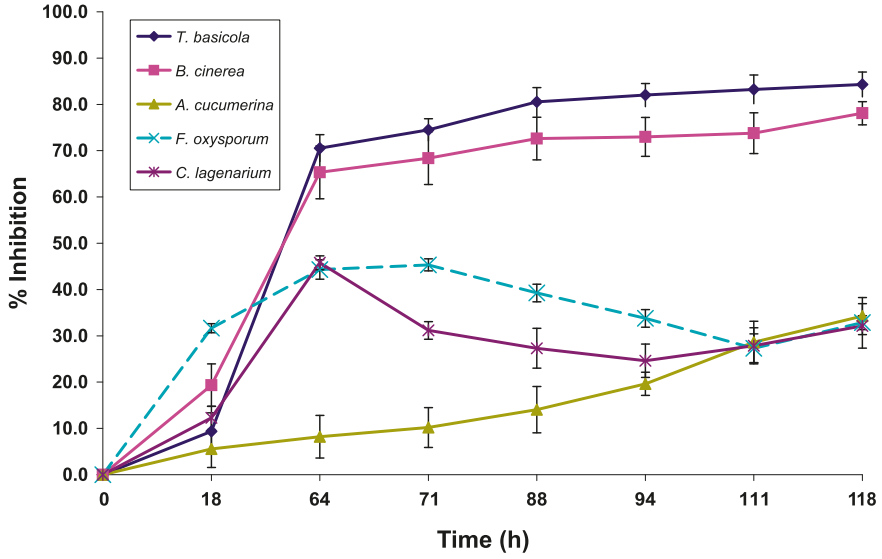
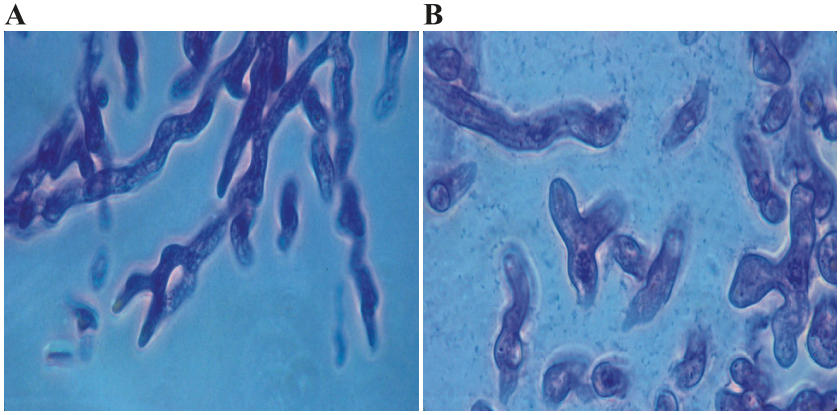



**Fig. 4.** Examination of *Cronartium ribicola* hyphal strands under a light microscope at 1250× magnification. Morphology of *C. ribicola* mycelia hyphal strands following 12 days contact with (A) *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) and (B) $H_2O$ (control).




Between the five fungal species, the most significant ($p < 0.001$) differences in percent inhibition were observed during the assay midpoint between 64 and 94 h postPm-AMP1 application where *T. basicola* and *B. cinerea* showed significantly higher percent inhibition rates (Fig. 3).

### Morphological change of *C. ribicola* hyphae caused by Pm-AMP1

Since Pm-AMP1 inhibited growth of *C. ribicola* mycelia, agar sections were removed from mycelial edges exposed to Pm-AMP1 and microscopically examined for effects of this antimicrobial peptide on hyphae morphology. Following three repeated applications of Pm-AMP1 over a 12 day period, the *C. ribicola* hyphal mycelia directly exposed to Pm-AMP1 showed long, slender strands (Fig. 4A), while those exposed to the $H_2O$ control showed more robust hyphal strands (Fig. 4B).

### Correlation between Pm-AMP1 abundance and the level of the BR resistance in WWP families

Phenotypic traits of seedlings from seven WWP families infected with *C. ribicola* were recorded 27 months postinoculation according to the number of BRs, PBRs, NCs, and aecia. The number of seedlings that fell into each of five phenotypic classifications within each family is shown in Table 2.

Assessing overall Pm-AMP1 accumulation across all seven families, we found a significant difference in mean Pm-AMP1 accumulation ($p < 0.001$), with BR family 1 showing the highest mean Pm-AMP1 level and susceptible family 10 showing the lowest (Fig. 5). The three full-sib BR families showed higher Pm-AMP1 means as compared with the half-sib BR families (Fig. 5). Family 8 was characterized by high vigor and low mortality more than 4 years postinoculation and was categorized as a Mechanism 'X' family (Table 2). This family had the highest number of BR seedlings, rela-

**Fig. 5.** Mean *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) accumulation in all seven western white pine families showing partial resistance. Means with different lowercase letters are significantly different from each other using one-way ANOVA ($p < 0.001$).

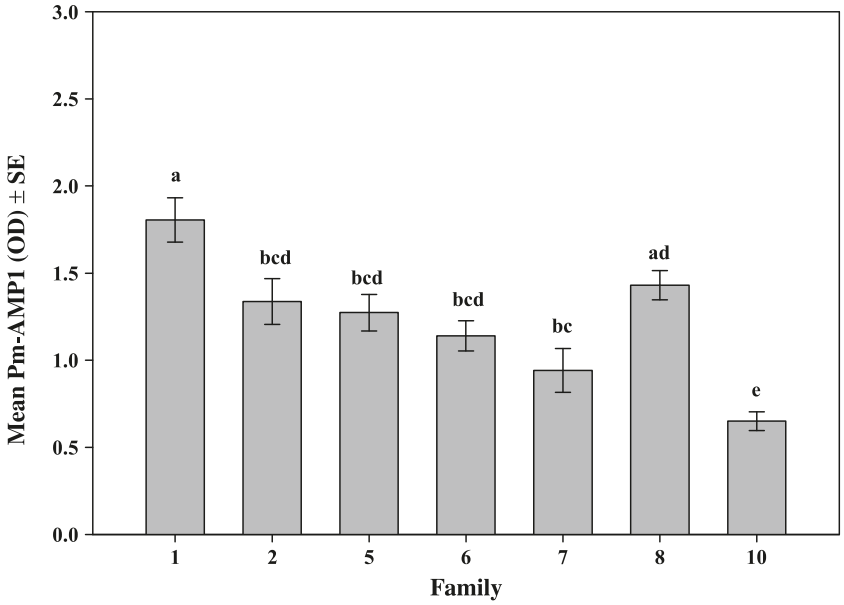
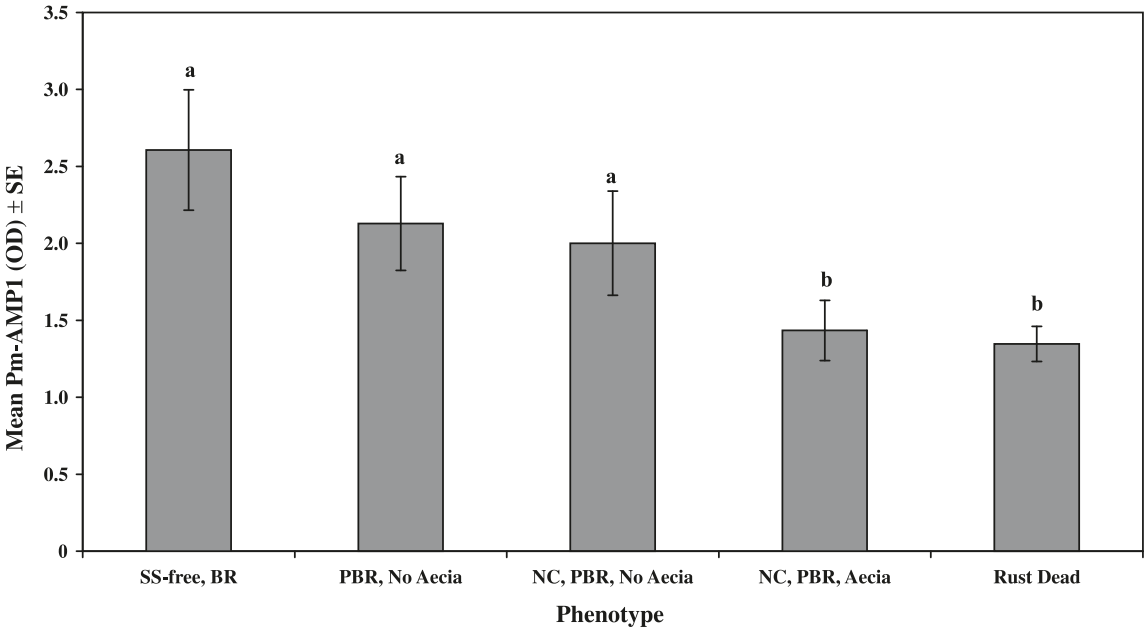



**Fig. 6.** Mean *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) accumulation by phenotype category for family 1. Means with different lowercase letters are significantly different from each other using one-way ANOVA ($p < 0.01$). SS-free, stem symptom free; BR, bark reaction; PBR, partial bark reaction; NC, normal canker.




tively few with any stem symptoms (Table 2), and showed significantly high Pm-AMP1 levels ($p < 0.05$) as compared with all other families except family 1 (Fig. 5). Families 2, 5, 6, and 7 had a higher proportion of seedlings with PBR, relative to the other three families (Table 2). This phenotypic trait was reflected in lower mean Pm-AMP1 levels for these four families, with significantly lower protein expression observed for family 7 ($p < 0.05$) as compared with the other families (Fig. 5). Within family 1 a significant correlation ($p < 0.01$, $R = 0.533$) was observed between Pm-AMP1 levels and the five phenotype categories (Fig. 6). Levels were significantly higher in stem-symptom-free (SS-free), BR-resistant seedlings and gradually declined with the occurrence of PBR, NCs, aecia, and eventual tree death due to rust.

The seven families showed variable Pm-AMP1 accumulation between the five phenotype categories (Fig. 7). At the time of sampling, seedlings in family 10 were severely infected; nearly half had died 27 months postinoculation (Table 2), only one SS-free, BR-resistant seedling remained, and no PBR, no aecia seedlings were observed; therefore, there was not sufficient data to graph the latter two categories in family 10 (Fig. 7). Families 5, 8, and 10 showed a constant level of

**Fig. 7.** Trends in mean *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) accumulation in each western white pine (WWP) family according to phenotype category. Family 10 had only one SS-free, BR-resistant seedling and no PBR, no aecia seedlings; therefore, these groups have not been graphed for family 10. SS-free, stem symptom free; BR, bark reaction; PBR, partial bark reaction; NC, normal canker.

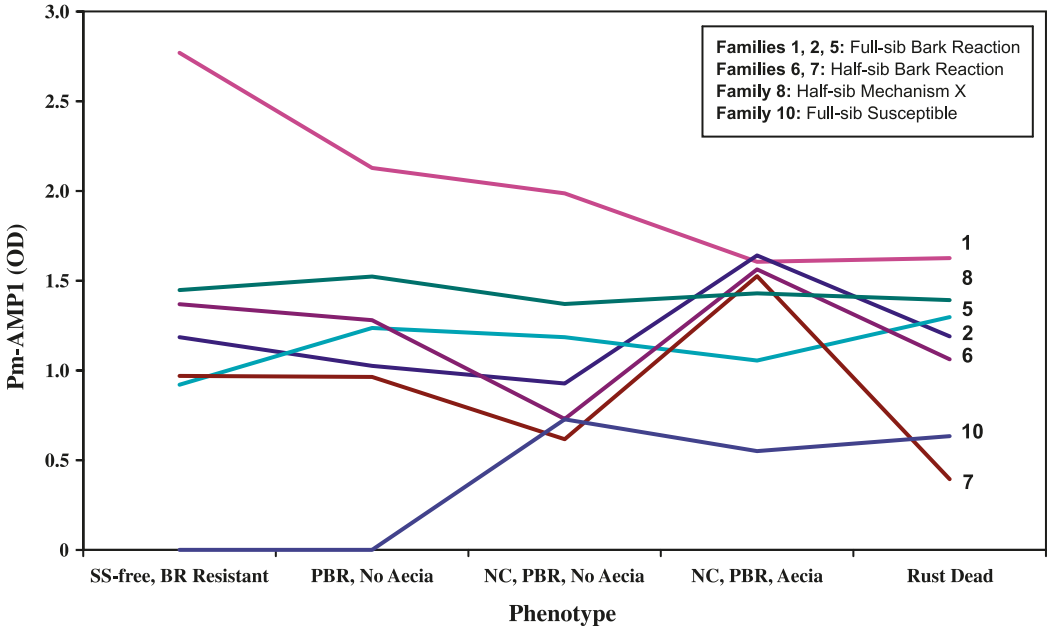
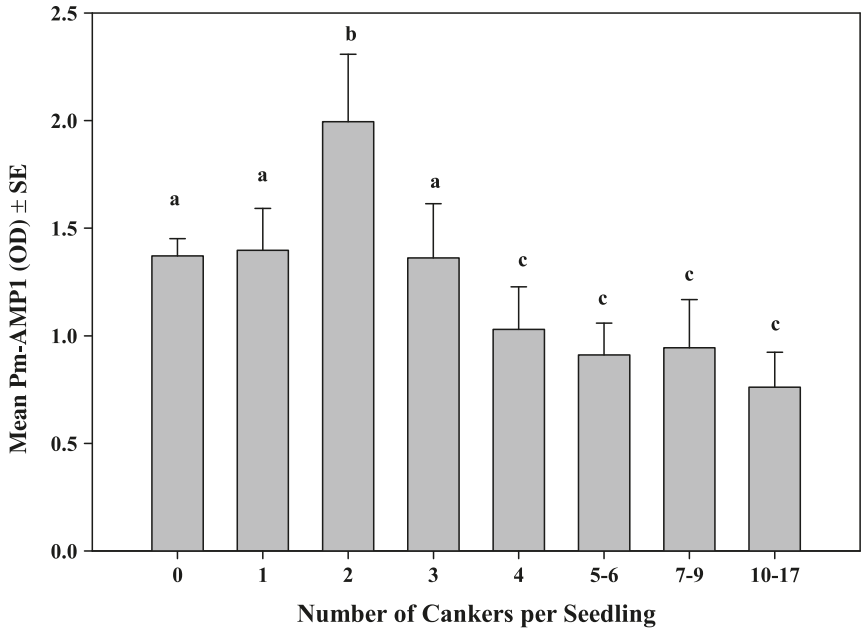



**Fig. 8.** Mean *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) accumulation across all families in association with number of normal cankers per seedling. Means with different lowercase letters are significantly different from each other using one-way ANOVA ($p < 0.05$).

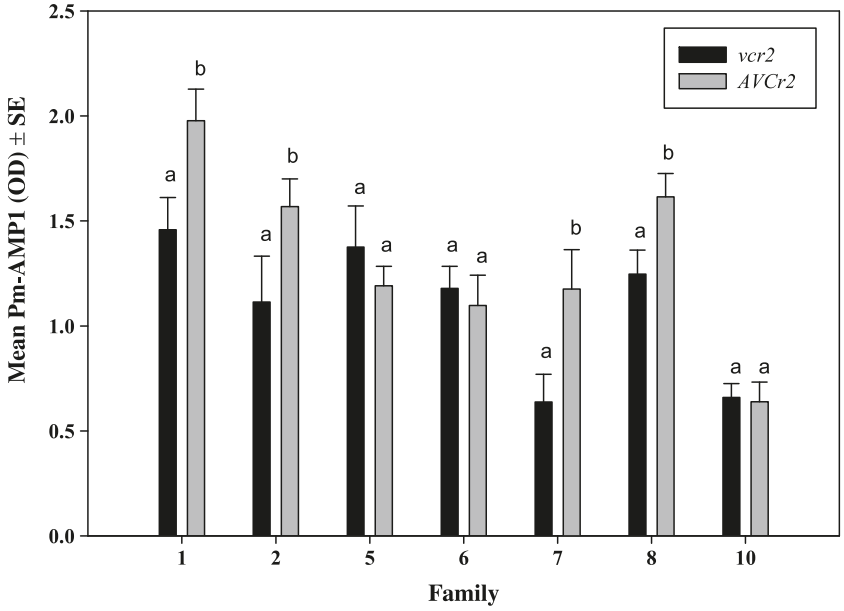



Pm-AMP1 across their respective phenotype categories, with family 8 at the higher end and family 10 at the lower end of this range (Fig. 7). Interestingly, as disease symptoms escalated and aecia became visible, some families with the largest numbers of PBR (Nos. 2, 6, 7) showed a sharp increase in Pm-AMP1 levels, which then declined with seedling death (Fig. 7).

Of the five phenotype categories, NC formation was associated with PBR development, either with or without aeciospores (Table 2). We grouped seedlings based on the number of NCs per seedling (Fig. 8) and observed a significant difference in mean Pm-AMP1 between cankered seedlings ($p < 0.05$; $R = 0.339$). Seedlings with four or more cankers showed similarly low levels of Pm-AMP1 (Fig. 8). A spike in Pm-AMP1was observed in seedlings with two cankers; whereas, the seedlings with the highest number of cankers (10–17) showed the lowest levels of mean Pm-AMP1 (Fig. 8).

It has been observed that *vcr2*, beyond its ability to overcome *Cr2*, is more aggressive and faster growing on PBR seedlings, causing higher mortality (McDonald et al. 1984;

**Fig. 9.** *Pinus monticola* antimicrobial peptide 1 (Pm-AMP1) accumulation (mean ± SE) in western white pine (WWP) seedlings inoculated with virulent (*vcr2*) and avirulent (*AVCr2*) sources of *Cronartium ribicola*. Within each family, means with different lowercase letters indicate a significant difference in Pm-AMP1 between *vcr2*- and *AVCr2*-infected seedlings using one-way ANOVA ($p < 0.05$).






R. Sniezko, USDA Forest Service, personal communication). Since half of all seedlings in each family were inoculated with *vcr2* and the other half with *AVCr2*, this provided an opportunity to look for associations among Pm-AMP1, inoculum source, and partial resistance in WWP. A significant difference in mean Pm-AMP1 was observed between *vcr2* and *AVCr2* seedlings in families 1 ($p = 0.03$, $R = 0.332$), 2 ($p = 0.011$, $R = 0.348$), 7 ($p = 0.032$; $R = 0.316$), and 8 ($p = 0.031$, $R = 0.302$) (Fig. 9). In all four families, mean needle Pm-AMP1 levels were higher when inoculated with the *AVCr2* inoculum source, which is associated with an incompatible interaction where the host tree survives. Three of these four families were either full-sib BR or Mechanism 'X'. Susceptible family 10 showed nearly identical Pm-AMP1 levels for both *vcr2* and *AVCr2* inoculated seedlings (Fig. 9).

### DNA polymorphism of the Pm-AMP1 5′ UTR and promoter

A predicted transcription start site was identified at –282 bp upstream of the translation start codon designating an upstream promoter fragment and a downstream 5′ UTR region. CCAAT box motifs were identified at positions –301 and –382, and a typical AG element was identified at position –375 within the promoter region (Supplementary Figure 1[1]). After cloning and nucleotide sequence analysis of a few recombinant clones from each seedling, we detected SNPs within the 392 bp 5′ UTR and promoter region using 14 individual samples from two seed families (Supplementary Table 1[1]). Roughly one in 40 bp exhibited a polymorphism. We identified nine polymorphic sites: three SNPs in the promoter region and six SNPs in the 5′ UTR (Supplementary Table 1[1]).

## Discussion

In this study we successfully overexpressed and purified the recombinant form of Pm-AMP1 (Fig. 1) and carried out mycelial bioassays that showed it to have inhibitory action on *C. ribicola*, *P. sulphurascens*, *O. montium*, and *O. clavigerum* (Fig. 2). The challenge in maintaining *C. ribicola*, a biotrophic rust fungus, in vitro and in conducting a bioassay makes this an important study showing antifungal activity of a host conifer protein against its natural aggressor. Inhibition was enhanced with increased Pm-AMP1 concentration; however, as little as 3 µg of Pm-AMP1 was sufficient to inhibit mycelial growth, making Pm-AMP1 a potent antifungal peptide. In vitro antifungal activity against a root rot fungus and two sap-staining fungi associated with the mountain pine beetle suggest that Pm-AMP1 may be an effective element for biotechnological control of various forest diseases.

Pm-AMP1 inhibited spore growth of some other commonly occurring phytopathogens, supporting wide-ranging antifungal activity for this peptide. The effectiveness of Pm-AMP1 ranged from moderately to highly inhibitory (32%–84%) depending on the phytopathogen (Fig. 3). With nearly 78% or greater inhibition observed, *T. basicola* and *B. cinerea* were highly affected by Pm-AMP1 exposure at 15 µg/mL. Statistical analysis also showed the inhibitory activity of Pm-AMP1 to be significant for each fungus as well as between the five fungal species tested over the 118 h course of the experiment (Fig. 3). The broad-spectrum inhibitory effects of Pm-AMP1 is in agreement with earlier reports of in vitro inhibitory activity of MiAMP1 (Marcus et al. 1997) and of enhanced quantitative resistance to *Leptosphaeria maculans* by overexpressing in transgenic canola (Kazan et al. 2002). These antifungal assay results demonstrate that Pm-AMP1 can impede hyphal development within three major fungal taxonomic divisions.

Unlike the MiAMP1 where no obvious alteration in hyphal morphology was observed with the inhibition of a filamentous fungal pathogen (Marcus et al. 1997), here *C. ribicola* showed morphological changes as a result of interaction with

Pm-AMP1 (Fig. 4). The thinning and extension of the hyphal strands in comparison with the robust hyphal ends in the control treatment is evidence of membrane permeability changes. Pm-AMP1 and MiAMP1 share 51% amino acid identity with similar 3-D structures (McManus et al. 1999; Ekramoddoullah et al. 2006). It appears likely that the mode of action of these AMPs may involve interaction with negatively charged membrane surfaces, but fungal receptors for these AMPs are as yet unknown. It is speculated that the structural resemblance between Pm-AMP1 and MiAMP1 probably reflects a robust structural framework and that the mode of action may be determined by specific protein motifs.

Genomics information from several plants has established that Pm-AMP1, MiAMP1, and other homologous proteins represent a conserved plant protein family (Manners 2009). The accumulation of Pm-AMP1 in WWP when challenged with *C. ribicola* infection was first observed in bark proteins of SCG-resistant trees (Davidson and Ekramoddoullah 1997). Protein or transcript of this AMP family was also found to be induced by fungal pathogen attack in gymnosperms *Pinus sylvestris* (Adomas et al. 2007) and *Pseudotsuga menziesii* (Sturrock et al. 2007), suggesting that it is a common component in plant defense against fungal pathogens.

Partial resistance is a continuum of phenotypic expression in WWP, ranging from SS-free seedlings to those where the onset and severity of disease symptoms may be delayed or reduced from the fastest dying seedlings, resulting in variable survival rates over time (Kegley and Sniezko 2004). BR occurs in the stem and is masked by the *Cr2* resistance mechanism, which first occurs in the needles (Kinloch et al. 1999); therefore, it is ideal to develop molecular tools to help identify its occurrence for breeding purposes. As with all plant–pathogen interactions there are numerous gene families encoding antimicrobial peptides, PR proteins, enzymes, and other regulatory molecules that are involved in complex series of events to aid in plant defense. Our Western blot results show that Pm-AMP1 is one such component associated with BR phenotypes in the WWP – blister rust pathosystem. When assessing the systemic accumulation of Pm-AMP1 in WWP needles, significant associations occurred with some families and traits. Significant trends in Pm-AMP1 accumulation in BR family 1 and Mechanism 'X' family 8 were in agreement with the hypothesis that expressed protein levels of Pm-AMP1 are related to resistant traits among these seed families (Figs. 5, 6, and 7). Susceptible family 10 was an ideal control family to compare the six partial resistant families to and to show how trees with partial resistance can have significantly higher mean Pm-AMP1 levels (Fig. 5). The downward trend in Pm-AMP1 protein expression as fungal colonization advances in tissues and trees experience more disease symptoms is clearly shown for the full-sib BR family 1 (Figs. 6 and 7). The fairly high and steady level of Pm-AMP1 observed in family 8 (Fig. 7) indicates a durable resistance type, as observed by the relatively low mortality rate of only 11%, 4.5 years postinoculation (Table 2). Interestingly, as disease symptoms escalated and aecia became visible, families with the largest number of seedlings with PBR phenotypes (Nos. 2, 6, 7) reached a maximum Pm-AMP1 level in the range of 1.5 OD (Fig. 7).

Development of NCs is symptomatic of mycelial growth within the bark and indicates disease susceptibility. Seedlings with multiple cankers are generally expected to die sooner, and we observed an association between this phenotypic trait and low Pm-AMP1 levels (Fig. 8). In fact, the lowest Pm-AMP1 levels were observed in susceptible family 10 (Fig. 5) where seedlings were cankered, sickly, and dying. Seedlings with two cankers showed a significant surge in mean Pm-AMP1 (Fig. 8), which may indicate a natural trigger to accumulate defense related compounds as the plant begins to experience pathogen stress.

Resistance mechanisms not controlled by a dominant gene, such as *Cr2* in a classic HR reaction, may still be vulnerable to virulent factors in the blister rust inoculum. Our results indicate there may be an underlying impact on Pm-AMP1 production due to the source of inoculum, even though partial resistance is believed to have polygenic heritability and not a gene for gene response (Hoff 1986). In four families (Nos. 1, 2, 7, 8), higher Pm-AMP1 levels were associated with the *AVCr2* source of blister rust (Fig. 9), which in a typical HR would lead to disease resistance. Perhaps in young seedlings, partial resistance is less effective when inoculated with the more vigorous and aggressive *vcr2* strain of *C. ribicola*. This finding is in accordance with early reports on variation in pathogen virulence and WWP performance (McDonald et al. 1984) as well as recent observations on the effect of *vcr2* and *AVCr2* strains on WWP and various on-going trials (R. Sniezko, USDA Forest Service, unpublished data).

Association genetics is a powerful strategy used to dissect molecular mechanisms underlying a complex plant phenotypic trait, such as quantitative resistance (Rafalski 2002). DNA variation among seed lots provides breeders with important tools for selecting plants with desirable traits. We cloned the Pm-AMP1 5′ UTR and partial promoter region and found regulatory elements and SNPs at multiple loci. The significance of these SNPs in the Pm-AMP1 5′ UTR and promoter fragment must be further investigated to determine their usefulness in WWP improvement. Future research should include a larger population size to explore whether there are associations between Pm-AMP1 SNPs and partial resistance.

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