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Gene cloning of a thaumatin-like (PR-5) protein of western white pine (*Pinus monticola* D. Don) and expression studies of members of the PR-5 group

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Abstract

To understand the molecular defence response of western white pine to the blister rust pathogen *Cronartium ribicola*, we have endeavoured to isolate and characterise pathogenesis-related (PR) proteins from western white pine. A full-length cDNA (*Pin m*TLP) was isolated from a cDNA library constructed from inoculated foliage. BLASTX search results indicated that the sequence shared a high degree of identity with the thaumatin-like proteins (TLPs) of the PR-5 family, and in particular with those of the 'small-TLP' subgroup mainly identified in monocots. The mature protein predicted from the cDNA sequence has a molecular mass of 16 kDa and pI of 4.1. Southern blot analysis showed that *Pin m*TLP is a single-copy gene member of a multi-gene family.

Two-dimensional Western blot analysis of total western white pine protein extracts probed with a Douglas-fir anti-TLP antibody detected a major and a minor protein spot of 23 and 17 kDa, respectively. Both 23 and 17-kDa proteins were identified as TLPs by Nanospray MS/MS analysis. The 23-kDa protein was shown to accumulate in canker margins in the bark of infected seedlings indicating that it is locally induced in response to fungal invasion. Both local wounding and methyl jasmonate treatments induced expression of the protein, whereas salicylic acid treatment did not. These results suggest that the 23-kDa TLP plays a role in the molecular defence response of western white pine to wounding and pathogen attack.

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1. Introduction

Pathogenesis-related (PR) proteins are described as proteins encoded by a host plant's genome that are 'induced specifically in pathological or related situations' [23]. They were first found in tobacco reacting to infection by the tobacco mosaic virus [22]. Since then, 17 families of PR proteins have been characterised and found in a diverse range of plant taxa [20,22] including conifers [14,25].

The PR-5 family of proteins is also referred to as the thaumatin-like proteins (TLP) due to their homology with the sweet-tasting protein found in the fruit of the West African shrub *Thaumatococcus daniellii* [24]. TLPs have been identified in numerous plant species, and often multiple isoforms are found, particularly in crop species such as *Hordeum vulgare* [16] *Orysa sativa* [17] and *Avena sativa* [12]. TLPs are found in different organs, including seed, fruit [21,11], leaves [6,7], and in conducting tissues. They have been implicated as allergens [14,11] and have been shown to have anti-freeze activity [8]. Some TLPs were also shown to have anti-fungal properties in that they are able to degrade fungal cell walls [26].

TLPs can be subgrouped into acidic, extracellular proteins that are characterised by the presence of a N-terminal signal peptide, or basic proteins with additional C-terminal extension targeted to vacuoles. They can be further subdivided into large TLPs, with molecular masses of 21–26 kDa and 16 conserved cysteine residues (these form eight disulfide bonds to stabilise the tertiary structure of the proteins), or small TLPs, with molecular masses of 15–18 kDa and, because of an intramolecular deletion of approximately 58 amino acids, only 10 conserved cysteine residues. While the more common large TLPs have been

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identified in moncots, dicots and conifers, small TLPs have primarily been described in monocot crop species. Recently, Hanselle et al. [7] identified a small TLP in the dicot *Cicer arietinum* (chickpea). X-ray crystallography of thaumatins revealed a common structure of three domains. A negatively charged surface cleft at the boundary between Domains I and II may be responsible for anti-fungal activity [10].

Cronartium ribicola Fisch. is the causal agent of white pine blister rust. Basidiospores that are produced on leaves of infected *Ribes*, the alternate host, infect pine needles through the stomata. Mycelia grows down the conducting tissues of the needle and into the bark of the stem, eventually producing aecial blisters and cankers. Cankers are invaded by secondary organisms, which often results in the death of the tree [9].

In studying the host–pathogen interactions of this pathosystem, we have focused on the role that PR proteins may play in the host defence response.

We previously identified cold and pathogen-induced PR-10 proteins in western white pine (*Pinus monticola* D. Don) [4,13]. Recently, an antibody raised against a conserved, antigenic region of a Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) TLP peptide [25] was found to immuno-react with two proteins of approximately 23 and 17 kDa in infected western white pine needles.

The objective of this study was to clone the gene(s) encoding TLP proteins and characterise the expression of TLPs in western white pine as an initial step in determining the functional role of these proteins in the molecular defence response of this species. In this paper, we report the molecular cloning and characterisation of one *Pin m*TLP gene encoding a protein homologous to the TLPs of the PR-5 protein family. Expression analysis of TLPs was performed using Douglas-fir TLP antibodies as a probe. Induction response of infection, treatment with signalling molecules (methyl jasmonate and salicylic acid) and mechanical wounding were investigated.

2. Methods

2.1. Plant material and treatment

Infected needles. One-year-old western white pine seedlings for cDNA library material, were placed beneath *C. ribcola*-infected *Ribes* plants for one month at the Saanichton Ribes garden (Saanichton, BC, Canada), while basiodospores were being released. Needles from four individual trees were collected on a weekly basis before and during this period and stored at −20 °C. A small number of samples were pooled for RNA extraction. *Stem tissues*. Cankered stem samples were collected from 4-year-old western white pines seedlings growing at the Cowichan Lake Research Station, BC, Canada, that had been infected with *C. ribicola* at age 2, as described by Ekramoddoullah et al. [4].

Western white pine seedlings used in all other experiments were grown in styrofoam blocks in shadehouses at the Pacific Forestry Centre, Victoria, BC, Canada.

Wounded needles. Two local wounding treatments were used on fully developed current year needles. The first method (single wounding) was to cut needles near their bases. The second method (multiple wounding) was to crush needles at 3- to 4-mm intervals with fine forceps. Both lots of needles were held in Petri dishes. For chemical treatments with defense-related signal chemicals, the wounded needles were put into solutions of 100 mM salicylic acid (SA), 1 mM methyl jasmonate (MeJA), or water controls for 3 s then transferred to Petri dishes. Each dish had 3 MM Whatman paper dampened with the corresponding test solution and was held at room temperature (22 °C) with 16 h of photoperiod. For the systemic wounding treatment, current year branches were cut from the tree, their lower needles were cut in the middle, and each branch was maintained by placing the cut end in water. Needles were taken periodically over 8 days from the Petri dishes and unwounded needles were collected from the top of the systemically treated branches. For each experiment, samples were taken from three different seedlings and frozen in liquid nitrogen and stored at −80 °C until proteins were extracted. For analysis, time samples were pooled.

2.2. Protein extraction

Total proteins were extracted from needles as outlined in Ekramoddoullah and Davidson [3]. Briefly, samples were lyophilised and ground to a fine powder using a mortar and pestle with liquid nitrogen. Proteins were extracted with a buffer containing 5% sucrose, 5% β-mercaptoethanol, and 4% SDS. Samples were vortexed, boiled and centrifuged; supernatant was precipitated with chilled acetone and the resulting concentrated protein pellet was resuspended in extraction buffer.

Apoplastic fluids were extracted from needles by vacuum infiltration of 50 mM Tris–HCl, pH 6.8 for approximately 10 min. Needles were then surface-dried, enclosed within a 5-cm^3 syringe barrel and centrifuged at 6000*g*. Fluids were collected and concentrated using PlusOne™ 2D Clean-Up kit (Amersham Biosciences, California, USA).

2.3. Western blot analysis

Proteins extracts from infected, wounded and chemically treated samples were separated on a 1D SDS-PAGE gel with a 12% acrylamide separating segment and a 4% stacking segment using a Biorad Protean II gel apparatus (BioRad Laboratories Ltd, Mississauga, Ont., Canada). Proteins were transferred to PVDF membrane (Millipore, Mississauga,

Ont., Canada) and incubated in a 1:500 dilution of anti-*Pm*TLP (*P. menziesii* TLP) antibody.

The immunoblots were developed using Biorad goat–anti-rabbit IgG-AP conjugate secondary antibody followed by exposure to AP substrate. Two-dimensional electrophoresis of concentrated apoplastic fluids was performed as described by Ekramoddoullah and Tan [5] using a Biorad Mini-Protean®II 2D Cell. Western blots were scanned using a 420oe™ Optically Enhanced light scanner and analysed using Quantity One software (BioRad).

Following 2D gel electrophoresis and western blot, two protein spots were located and digested with trypsin. The nanospray MS/MS analysis of tryptic digest was then performed on an Applied Biosystems (Foster City, CA) QStar pulsar i mass spectrometer. The data was collected using Analyst and the sequencing was performed using BioAnalyst (Foster City, CA).

2.4. Construction of cDNA library and isolation of Pin mTLP

Total RNA was extracted from *C. ribicola*-inoculated western white pine needles (pooled from time-course collection) according to Schultz et al. [19] and further purified with an RNeasy plant mini kit (Qiagen Inc., Mississauga, Ont., Canada). A cDNA library was constructed using a SMART cDNA library kit (Clontech Inc., Palo Alto, CA, USA) according to manufacturer's instructions. To screen the library for TLP cDNAs, a DIG-labelled Douglas-fir partial TLP cDNA fragment previously cloned in our lab [25] was used as a probe. Resulting positive clones were converted from λTriplEx2 to pTriplEx2 following the SMART cDNA library protocol, and were transformed into DH5α competent cells (Invitrogen life Technologies, Carlsbad, Burlington, CA, USA).

```
atggggccaacaacgatttatgttacagcaatagtagcgttaatatttaacgtactttat    60
 M  G  P  T  T  I  Y  V  T  A  I  V  A  L  I  F  N  V  L  Y     20
cttcaagggggttatgggtgcgacgtttgcgatccagaaccagtgttcatacacggtatgg   120
 L  Q  G  V  M  G  A  T  F  A  I  Q  N  Q  C  S  Y  T  V  W     40
gccgcaggaattcccgtaggtggcggtcaagcgcttgggcagggtcaatcatggagcgtc   180
 A  A  G  I  P  V  G  G  G  Q  A  L  G  Q  G  Q  S  W  S  V     60
tacgtgcccgcaggaacaagctcaggcagattttggggacgtactggatgctccttcgat   240
 D  V  P  A  G  T  S  S  G  R  F  W  G  R  T  G  C  S  F  D     80
gcctctggtaaaggaagctgcagcactggtgactgcggcggcgtgctcagctgtacactc   300
 A  S  G  K  G  S  C  S  T  G  D  C  G  G  V  L  S  C  T  L    100
tcgggacagcctcctacaacgctggttgaatatactctgaatgggggcaacaatcaagat   360
 S  G  Q  P  P  T  T  L  V  E  Y  T  L  N  G  G  N  N  Q  D    120
ttctatgacatctctgtaattgatggttacaatgtgcctctctccctaactccatcggac   420
 F  Y  D  I  S  V  I  D  G  Y  N  V  P  L  S  L  T  P  S  D    140
gctagctgccaagcactgacttgcaaaatggaccagtgcccagatgcctacctatatccc   480
 A  S  C  Q  A  L  T  C  K  M  D  Q  C  P  D  A  Y  L  Y  P    160
actgatgacactaaaacccacgcttgcccttctggtgctaattacaacattgtgttctgc   540
 T  D  D  T  K  T  H  A  C  P  S  G  A  N  Y  N  I  V  F  C    180
ccttga                                                         546
 P  *                                                          181
```

Fig. 1. *Pin m*TLP cDNA sequence (GeneBank Accession # AY500845). The underlined portion of the N-terminal indicates predicted signal peptide and the lightly shaded region spans the conserved, antigenic region of the protein. The intron position between G^{67} and G^{68} is marked with an arrow. The amino acid sequences confirmed by Nanospray MS/MS are boxed.

2.5. Isolation of genomic sequence encoding Pin mTLP

Genomic DNA was extracted from western white pine needles using a Qiagen DNeasy plant maxi kit (Qiagen). The genomic sequence of *Pin m*TLP was amplified from genomic DNA using primers designed according to the *Pin m*TLP cDNA ORF. Primer sequences are as follows: The forward primer was 5′-caagcatATGGGGCCAACAACGATTTATG-3′ and the reverse primer sequence was 5′-cccgggatcctcgagTCAAGGGCAGAACACAATGTTG-3′. PCR was performed with a Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer Canada Ltd, Montreal, Que.). The resulting PCR fragments were cloned with a TOPO TA cloning kit (Invitrogen life technologies, Carlsbad, CA, USA) and plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen Inc.)

2.6. DNA sequencing and analysis

cDNA and genomic clones were sequenced on an LI-COR 4200 Global addition IR DNA sequencer with λTriplEx2 and T7, SP6 sequencing primers at the Centre

```
Pinus       -----MGPTTIYVTAIVALIFNVLYLQGVMGATFAIQNQCSYTVWAAGIPVGGGQALGQG 55
triticum    -----MASSHLAAAASMVLFLAVFAAS-TNAATFNIKNNCPYTVWPAATPIGGGRQLNTG 54
orysa       -----MASPATSSAVLVVVLVATLAAGGANAATFTITNRCSFTVWPAATPVGGGVQLSPG 55
cicer       -----MSLIKICLSMLAFCLIS------AQGARFDIVNQCSYNVWPAAIPSGGGRQLNPR 49
juniperus   -----MARVSELAFLLAATLAISLHMQEAGVVKFDIKNQCGYTVWAAGLP-GGGKRLDQG 54
cupressus   ------------------------------VKFDIKNQCGYTVWAAGLP-GGGKEFDQG 28
Pseudotsuga MGGRTVAAASIWIAATATLLALNAYMQGVEGVTMTVKNQCSYTVWAAGSP-GGGKQLGQG 59
vitis       ---MFSSKLPSMSDLRILFIFFLCFISSIHAATFQITNQCSYTVWAAASP-GGGRRLDRG 56
                                          . : : *.* :.**.*. * ***  :.

Pinus       QSWSVDVPAGTSSGRFWGRTGCSFDASGKGSCSTGDCGGVLSCTLSGQPPTTLVEYTLNG 115
triticum    ETWTLDVPANTPSGRVWGRTGCNFNGNS-GSCQTADCGGALSCTLSGQPPLTLAEFTIGN 101
orysa       QTWTINVPAGTSSGRVWGRTGCSFDGSGRGSCATGDCAGALSCTLSGQKPLTLAEFTIGG 103
cicer       ETWGLDIPAGTQSARIWGRTGCNFDGSGRGSCQTGDCGGDLSCHLSGQPPTTLAEFSLNG 97
juniperus   QTWTVNLAAGTASARFWGRTGCTFDASGKGSCQTGDCGGQLSCTVSGAVPATLAEYTQ-- 102
cupressus   QTWTVNLAAGTASARFWGRTGCTFDASGKGSCRSGDCGGQLSCTVSGAVPATLAEYTQ-- 76
Pseudotsuga ETWSFDVAADTTGGRIWGRTGCSFDATGKGTCNTGDCGGLLSCQGYGTVPATLFEYGLNK 107
vitis       S-WTLNVPAGTKMARIWGRTNCHFDASGRGRCDTGDCGGVLNCQGWGSPPNTLAEYALNQ 104
            ::* .::.*.*  .*.****.* *:... * * :.**.* *.*   *  * ** *:

Pinus       GNNQDFYDISVIDGYNVPLSLTPSDASC-QALTCKM----------------------- 162
triticum    G--QDFYDISVIDGFNVPLSFSCSNG---PNLVCQA----------------------- 144
orysa       S--QDFYDLSVIDGYNVAMSFSCSSG---VTVTCRD----------------------- 146
cicer       GNNQDYFDISVIDGFNIPMQFSSTTNNCNRVLTCKD----------------------- 145
juniperus   -SDQDYYDVSLVDGFNIPLAINPTNAQC-TAPACKADINAVCPSELKVDGGCNSACNVFK 158
cupressus   -SDQDYYDVSLVDGFNIPLAINPTNTKC-TAPACKADINAVCPSELKVDGGCNSACNVLQ 132
Pseudotsuga YMNLDFLDISLVDGFNLPLTVTPSNSAC-KTISCTSDINAVCPSELKVTDGCKSACAEFN 166
vitis       FGNKDFFDISLVDGFNIPMDFSPTSGGC-RGIKCTANINGQCPQALKASGGCNNPCTVFK 163
                *: *:*::**:*:.: .. :         *

Pinus       ------------------------DQCPDAYLYPTDD-TKTHACPSGAN-YNIVFCP--- 185
triticum    ------------------------DKCPDAYLFPTDD-TKNHACNGNNNTYQVTFCP--- 167
orysa       ------------------------SRCPDAYLFPEDN-TKTHACSGNSN-YQVVFCP--- 169
cicer       ------------------------SSCPDAYHQP-ND-VKTVSCPGGTN-YRIVFCP--- 167
juniperus   TDQYCCRNAYVDNCPATNYSKIFKNQCPQAYSYAKDD-TATFACASGTD-YSIVFCP--- 217
cupressus   TDQYCCRNAYVNNCPATNYSKIFKNQCPQAYSYAKDD-TATFACASGTD-YSIVFCP--- 191
Pseudotsuga TPQYCCTGDYLNNCSPSDYSKFFKAQCPQAYSYAKDDATSTFTCATGSN-YNVVFCG--- 226
vitis       TPQYCCNN---IKCGPTDYSRFFKTRCPDAYSYPQDDPTSTFTCPGGAN-YRVVFCPTGS 220
                                    **:**  . :: . . :*  . : * :.**

Pinus       ---------------- 193
triticum    ---------------- 176
orysa       ---------------- 177
cicer       ---------------- 175
juniperus   ---------------- 225
cupressus   ---------------- 199
Pseudotsuga ---------------- 234
vitis       SNNIFPLEMYGSDSEE 247
```

Fig. 2. Sequence alignment of predicted *Pin m*TLP peptide sequence with other TLP proteins exhibiting homology from BLASTX search. BLASTX search results indicate that *Pin m*TLP has higher similarity (57–61% identities) with the small TLPs of *C. arietinum* PR-5a (accession no. AJ010502.1), *T. aestivum* TLP (accession no. X97687.1) and *O. sativa* TLP (accession no. X68197) rather than with the conifers (45–47% identities), *Juniperus ashei* Jun a 3 (accession no. AF121776.1); *Cupressus arizonica* Cup a 3 (accession no. AJ294411.1); and *P. menziesii* TLP (accession no. AJ131731.1). The dicot *Vitis riparia* (accession no. AF178653.1) has a large TLP with homology to *Pin m*TLP. The 16 conserved cysteine residues are highlighted in bold.

for Biomedical Research, University of Victoria, BC, Canada. Nucleotide and predicted amino acid sequences were analysed using BLAST [1], Signal P V1.1 [15], and LASERGENE (DNAstar Inc, Madison, WI, USA). ClustalW was used for multiple sequence alignment analysis.

2.7. Southern blot analysis

Genomic DNA was digested with restriction enzymes. The resulting fragments were separated on a 0.8% (w/v) agarose gel and blotted onto a Hybond-N nylon membrane (Amersham Pharmacia biotech, UK) following a protocol outlined in Sambrook and Russell [18]. The blot was hybridised overnight at 42 °C with DIG-labelled *EcoR1* insert of the *Pin m* TLP cDNA clone. Following hybridisation, the blot was washed twice for 20 min at room temperature with 0.5 × SSC; 0.1% SDS and twice for 20 min at 45 °C with 0.1 × SSC; 0.1% SDS. Hybridised fragments were detected using a DIG luminescent detection kit (Roche Diagnostic, Mannheim, Germany).

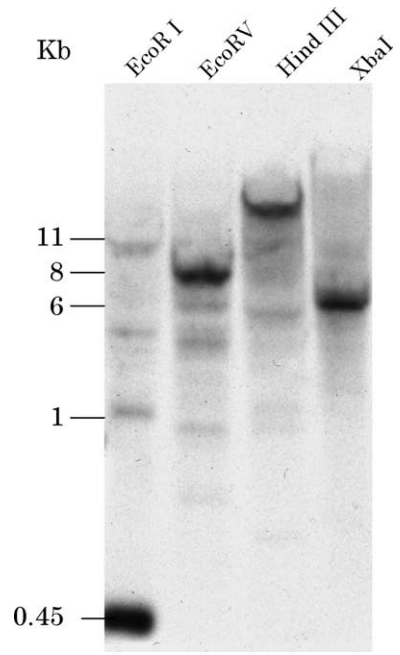

Fig. 3. Southern blot of western white pine genomic DNA (20 μg per lane) digested with (a) *EcoRI*, (b) *EcoRV*, (c) *Hind* III, (d) *Xba* I. Digested fragments were separated on a 0.8% agarose gel, blotted to a nylon membrane and hybridised with the DIG-labelled *Pin*mTLP cDNA coding region. Scale, indicated at right is in kilobase.

3. Results

3.1. Isolation and characterisation of Pin mTLP cDNA

A cDNA library constructed from *C. ribicola*-infected western white pine needles was screened using a DIG-labelled partial Douglas-fir TLP cDNA as a probe. After three rounds of screening, five clones exhibiting a strong hybridisation signal were excised and sequenced. Of these five, only one clone yielded a full-length cDNA sequence while the other four clones yielded partial cDNAs sequences identical to portions of the full-length cDNA sequence in the C-terminal region. The full-length cDNA (Fig. 1) had an ORF of 546 bp encoding a predicted polypeptide of 181 amino acids with an estimated molecular mass of 19 kDa. The first 26 amino acids in the N-terminal region were predicted by Signal P [15] to comprise a signal peptide and the lack of a C-terminal extension in the ORF, suggesting an extracellular location of the mature protein (16.05 kDa) with a theoretical pI of 4.1. The cloned full-length cDNA was termed *Pin m*TLP for *P. monticola* TLP.

A BLASTX (1) search conducted with the putative protein sequences demonstrated more similarity with other identified TLPs. Most notably, the protein predicted by *Pin m*TLP has similarity of 74, 72 and 71% with small TLPs of *C. arietinum*, *O. sativa* and *Triticum aestivum*, respectively (Fig. 2). *Pin m*TLP had more homology with these small TLPs than with other conifer TLP sequences in Genbank. This suggests that *Pin m*TLP encodes the first putative small TLP identified in a conifer genome. Of the homologous PR-5 proteins, only those with anti-fungal activity had a common motif, a negatively charged surface cleft. This cleft is at the boundary between Domains I and II, with a bottom part consisting of a three-stranded anti-parallel beta-sheet in Domain I (10). Like other identified small TLPs, *Pin m*TLP has an intra-molecular deletion of approximately one-fourth of its total amino acids occurring in Domain II at the C-terminal region (Fig. 2) as compared to large TLPs. This deleted region contained 6 of the 16 conserved cysteine residues found in large TLPs which play an important role in the tertiary structure of the protein. There are two potential O-linked GalNAc glycosylation sites at amino acid positions Threonine^{106} and Threonine^{161}, and an O-linked GlcNAc glycosylation site at amino acid position Serine^{139} (Fig. 1).

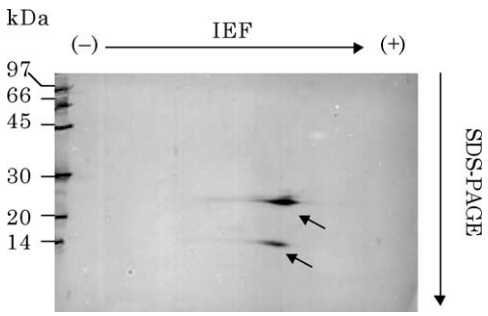

Fig. 4. Two-dimensional blot of western white pine infected bark total protein extract (20 μg) immuno-detected with anti-*Pm* TLP (Douglas-fir) antibodies. The first dimension was IEF (pH 3-10) and the second dimension was SDS-PAGE. Arrows indicate two identified spots of approximately 17 and 23 kDa and pI 5, estimated by the relative migration pattern of protein standards.

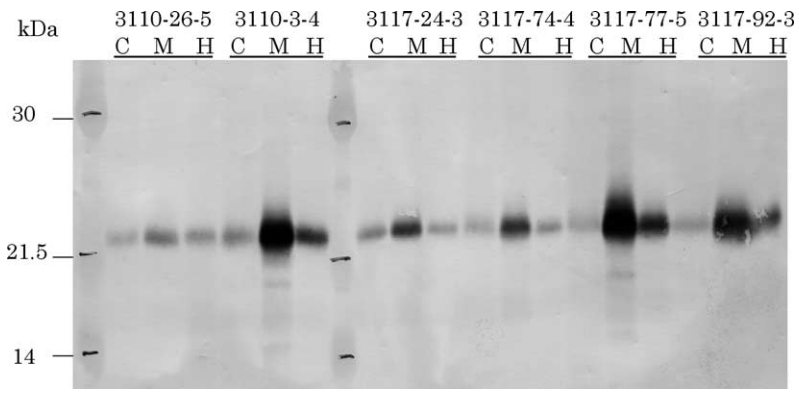

Fig. 5. Western blot of cankered bark samples. Each lane represents protein extracted from cankered tissue (C), canker margin tissue (M), and healthy tissue (H). Samples from six individual trees, identified by above numbers, were analysed. Forty micrograms of total protein extracts were separated on 12% SDS-PAGE gels, transferred to PVDF membrane and probed with anti-*Pm*TLP antibodies.

3.2. Genomic sequence and southern blot analysis

The genomic *Pin m*TLP sequence was isolated from the western white pine genome using PCR. Forward and reverse primers were designed based upon the N-terminal and C-terminal regions of the full-length cDNA, respectively. The resulting sequence indicated the presence of a single intron of 109 bp located in the signal peptide coding region (Fig. 1). Southern blot analysis was performed using DIG-labelled *Pin m*TLP as a probe (Fig. 3). Each lane of digested DNA showed one strong band and several weaker bands, suggesting that *Pin m*TLP is a single-copy gene, but belonging to a multi-gene family. PCR was performed on several other genomic isolates representing individual western white pine seedlings to identify polymorphisms within the gene. Only small single nucleotide polymorphisms were identified.

3.3. 2D Gel analysis

Two-dimensional PAGE was performed on proteins extracted from the apoplastic space of infected western white pine needles. Western blotting with a Douglas-fir TLP antibody detected two spots with apparent masses of 23 and 17 kDa, and an estimated isoelectric point of 5.0 (Fig. 4). On MS/MS analysis, one peptide (NGCTVDLNAVCAPELK) of 23 kDa protein spot matched with *P. menziesii* TLP (accession no. Q9ZRS9) whereas two peptides (peptide1: TGCSFDASGK; peptide 2: QCPDAYLFPTDDTK) of 17 kDa matched with PmTLP cDNA at amino acid sequence from 75 to 84 and at amino acid sequence from 152 to 165, respectively, with the exception that at position 159 the cDNA has Y while the peptide 2 has F (cf. Fig. 1). These two peptides also matched a small *T. aestivum* TLP2 (accession no. Q8LLM3) suggesting that PmTLP cDNA encodes of 17 kDa protein.

3.4. Western blot analysis of TLP induction

Analysis of cankered bark (Fig. 5) showed that, while TLP is present in the healthy tissues away from the canker, it is induced in the marginal tissues of the canker. 2D western blot analysis showed that the TLP pattern from canker margins was similar to that from the apoplastic space of infected western white pine needles (data not shown). This finding suggests a local induction of TLP proteins around necrotic regions of infected plants, potentially functioning to limit the proliferation of infection. Lower levels of the TLP were found within the cankered region itself, probably due to the general degradation of tissue in the region.

Multiple wounding of western white pine needles resulted in dramatic local induction of the 23-kDa TLP after day 2 but there was little increase in systemically and single locally wounded samples (Fig. 6).

Foliage treated with methyl jasmonate, known as a wounding signal messenger, also caused induction of the protein, whereas salicylic acid, implicated in systemic acquired resistance, did not (Fig. 7).

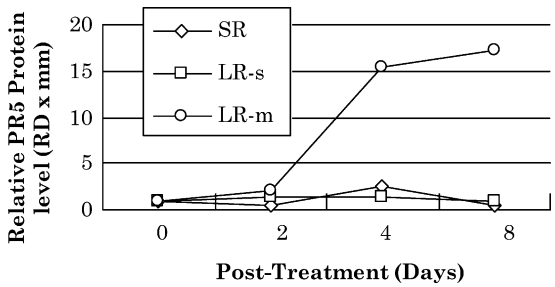

Fig. 6. *Pin m*TLP accumulation in wounded needles. A western blot of total protein extracted from each wounding treatment sample was scanned and analysed using Quantity one software. *Y*-axis indicates relative density of the 23-kDa immuno-detected band in each sample. SR, systemic wounding response; LR-s, local response, single wound; LR-m, local response, multiple wound. Forty micrograms of protein of each sample were run on a SDS-PAGE gel, blotted on PVDF membrane and probed with anti-*Pm*-TLP antibodies.

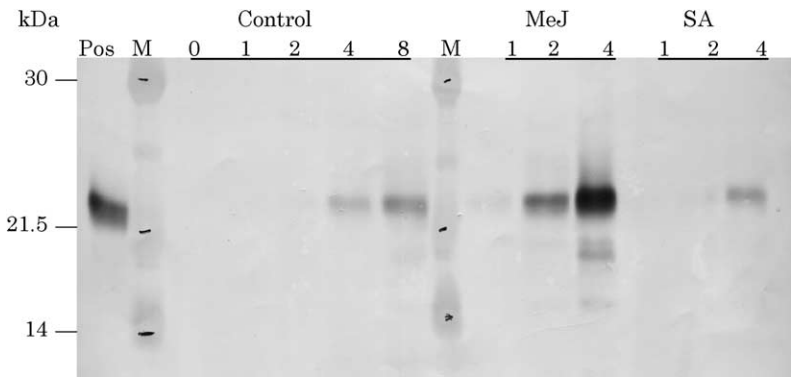

Fig. 7. Western blot of chemically treated healthy western white pine needles. Foliage was removed from branches, placed in Petri dishes with Whatman filter paper and treated with chemicals associated with PR protein induction. Control (distilled water only), MeJ, methyl jasmonate treatment, SA, salicylic acid treatment. Foliage samples were collected 1, 2 and 4 days after initial treatment. Forty micrograms of protein of each sample were run on a SDS-PAGE gel, blotted on PVDF membrane and probed with anti-*Pm*-TLP antibodies.

4. Discussion

We report the isolation of the first western white pine TLP cDNA. Of the five cDNA clones that were isolated and sequenced from a western white pine cDNA library, only one contained the full-length ORF. The sequences of four partial cDNAs contained premature stop codons, possibly introduced during PCR amplification, as they were identical to portions of the complete sequence in the C-terminal end. Excluding the 109-bp intron located in the signal peptide region of the genomic sequence, the cDNA and genomic sequence were identical. BLASTX search results indicated that the predicted *Pin m*TLP had a higher homology with 'small TLPs' commonly found in monocot species. Small TLPs and *Pin m*TLP have an intramolecular deletion of about one-quarter of the length of large TLPs. This deletion is conserved among small TLPs and occurs in Domain II of the C-terminal region of the protein, resulting in the loss of 6 of the 16 conserved cysteine residues found in the large TLPs. The effect of this deletion on biological activity is unknown. Koiwa et al. [10] compared the crystal structure of both tobacco PR-5d and zeamatin and revealed the presence of a common structural motif among the anti-fungal PR-5 proteins. They found that an acidic cleft is formed between Domains I and II of the anti-fungal PR-5 proteins and suggested that this cleft may be necessary for anti-fungal activity. Since the large intramolecular deletion found in the small TLPs occurs in Domain II of the protein, it is possible that the formation of the acidic cleft may be altered. However, zeamatin mutated in Domain II was shown to retain anti-fungal activity [10].

An immunoblot prepared from a 2-PAGE separation of apopalastic proteins clearly indicated the presence of a 23-kDa and a 17-kDa protein. Both the acidic nature and apoplastic location of the proteins concur with *Pin m*TLP cDNA data. The larger, 23-kDa spot was considered the major band, due to the consistent and higher level of expressions exhibited during induction. The presence of both higher and lower molecular mass forms of secreted proteins is common and was described for other PR-5 proteins [16]. The molecular mass of the major form (23 kDa) may be due to post-translational modifications, such as glycosylation. Indeed the protein predicted from *Pin m*TLP cDNA has three glycosylation sites. Previous glycoprotein analysis of apoplastic fluid collected from infected western white pine needles indicated a glycoprotein of the same approximate mass (data not shown) as the major isoform identified. In addition, sequence alignment of the synthetic, antigenic peptide against which the Douglas-fir TLP antibody was made and the corresponding region in the *Pin m*TLP peptide show high homology. Furthermore, genomic sequences from individual western white pine seedlings are identical to cDNA sequences of *Pin m*TLP and showed only single nucleotide polymorphisms. However, limited protein sequencing data of the two identified western white pine TLPs (23- and 17-kDa) would suggest that *Pin m*TLP cDNA encodes 17-kDa protein and the presence of a different gene coding for 23 kDa protein in western white pine genome.

Western blot analysis demonstrated that pathogen infection, mechanical wounding and methyl jasmonate all induce local expression of the 23-kDa TLP in western white pine. The 17-kDa protein was evident in samples induced by methyl jasmonate, but not in those induced by salicylic acid.

Although the effects of the signalling molecules salicylic acid and methyl jasmonate have not been studied extensively in conifers as they have been in flowering plants, some evidence has shown that they may have a role in the induction of PR proteins in pine. Davis et al. [2] demonstrated that certain pine chitinases were induced differentially with salicylic acid and methyl jasmonate, and Liu et al. [13] showed that the expression of PR-10 proteins in pine was induced by exogenous application of methyl jasmonate. These results suggest that the 23-kDa protein identified is involved in locally induced defence response in western white pine, and supports its classification as a PR-protein.

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