Differential expression of multiple PR10 proteins in western white pine following wounding, fungal infection and cold-hardening

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Herein the wound-induced expression of a set of PR10 genes (*PmPR10*) from *Pinus monticola* (Dougl. Ex D. Don) is described. Thirteen different PmPR10 cDNAs were isolated and their nucleotide sequences were determined from wounded needles. Northern blot analysis showed that PmPR10 gene expression was activated with both local and systemic responses after wounding, and the accumulation of PmPR10 transcript was much more abundant and rapid in wounded needles than in unwounded tissues. Western immunoblot analysis demonstrated that PmPR10 protein synthesis was activated upon wounding, suggesting that the expression of the wound-activated PmPR10 gene was regulated at the transcription level. In wounded needles, the PR10 protein level increased from day 1 to day 8 after treatment. Western immunoblot analysis following isoelectric focusing and two-dimensional electrophoresis revealed that nine PmPR10 proteins accumulated to different extents after wounding. These proteins have a molecular mass of about 18 kDa with different isoelectrical points ranging from 5.2 to 6.0. Wound-inducible PmPR10 proteins were differentially expressed in response to cold-hardening and fungal infection. The wound-induced PmPR10 protein accumulation was enhanced by the wound-signal compound methyl jasmonate and okadaic acid, a specific inhibitor of type 1 or type 2 A serine/threonine protein phosphatases. However, it was partially suppressed by salicylic acid and abscisic acid. These data provide an opportunity to elucidate further the signal transduction pathway involved in the activation of PmPR10 protein synthesis in the defence response of white pine against mechanical injury.

Introduction

Wounding of plants is one of the severest environmental stresses caused by herbivory and parasitism, or by mechanical injury from wind and fire. Wounding can result in the retardation of plant growth and reproduction and the easy penetration of pathogens into plant tissues. Plants respond to wounding with many distinctive biochemical changes through activation of a variety of genes to produce defence-related proteins either in the vicinity of the wounding site alone or in undamaged parts of the wounded plants as well (Bowles 1990, Ryan 1990). The wound-inducible defence-related proteins include pathogenesis-related (PR) proteins, proteinase inhibitors (Peña-Cortés et al. 1989, Farmer and Ryan 1990) and enzymes involved in phenylpropanoid metabolism (Dixon and Païva 1990). The mechanisms underlying the regulation of wound-induced gene expression in plants are complex and not well understood. In plants, jasmonates (Dixon and Païva 1990), abscisic acid (ABA) (Peña-Cortés et al. 1993) and ethylene (O'Donnell et al. 1996) have been shown to be signal compounds for wound-induced gene expression. In *Arabidopsis*, at least two signalling pathways are activated for wound-induced gene expression: jasmonic acid (JA)-independent and JA-dependent pathways (Titarenko et al. 1997). The JA-independent wound-signalling pathway is principally responsible for gene activation in the vicinity of the wounding site, whereas the JA-dependent pathway involves JA perception and activation of gene expression at the wounding site as well as systemically.

Among the wound-activated PR genes, the PR10 genes encode small, primarily acidic and protease-resistant intracellular proteins with molecular masses ranging from 15 to 19 kDa. PR10 proteins include intracellular PR proteins, tree pollen allergens and major food allergens. They have been reported in a variety of higher plant species, both angiosperms and gymnosperms. In angiosperms, PR10 proteins were found in numerous dicots, such as parsley (*Petroselinum crispum*) (Somssich et al. 1988), pea (*Pisum sativum*) (Fristensky et al. 1988), potato (*Solanum tuberosum*) (Matton and Brisson 1989), white birch (*Betula verrucosa*) (Breiteneder et al. 1989), bean (*Phaseolus vulgaris* L) (Walter et al. 1990), soybean (*Glycine max* cv Mandarin) (Crowell et al. 1992), apple (*Malus domestica*) (Vanek-Krebitz et al. 1995), celery (*Apium graveolens*) (Breiteneder et al. 1995) and alfalfa (*Medicago sativa*) (Breda et al. 1996), as well as several monocots, such as asparagus (*Asparagus officinalis*) (Warner et al. 1992), rice (*Oryza sativa*) (Midoh and Iwata 1996), lily (*Lilium longiflorum*) (Huang et al. 1997) and sorghum (*Sorghum bicolor*) (Lo et al. 1999). Among gymnosperms, PR10 protein was found in sugar pine (*Pinus lambertiana*), eastern white pine (*Pinus strobus*), western white pine (*Pinus monticola*) (Ekramoddoullah et al. 1995), Douglas-fir (*Pseudotsuga menziesii*) (Ekramoddoullah et al. 2000), white spruce (*Picea glauca*) (Matheus et al. 2000) and maritime pine (*Pinus pinaster*) (Dubos and Plomion 2001).

Many PR10 genes have been shown to be expressed during the defence response or after wounding, suggesting their involvement in a general defence mechanism. We have reported a Pin m III protein associated with cold-hardiness of western white pine (Ekramoddoullah et al. 1995) and subsequent characterization of its cDNA revealed that it belonged to the PR10 gene family (Yu et al. 2000). In the white pine blister rust pathosystem our previous work demonstrated that Pin m III protein was up-regulated following the infection of *Cronartium ribicola* (Ekramoddoullah et al. 1998). As Pin m III belongs to the PR10 multiple family, and in keeping with the established nomenclature of plant pathogenesis-related proteins (Van Loon et al. 1994), we hereafter refer to the western white pine genes homologous to PR10 as PmPR10 genes. Our previous work has shown that *Pin m III* is a member of a highly polymorphic family of PR10 gene in western white pine (Yu et al. 2000). To identify other PR10 genes and to investigate their differential expression in response to environmental stresses, here, we report on PR10 gene expression upon wounding in western white pine.

Materials and methods

Plant materials and experimental treatments

Western white pine (*Pinus monticola* Dougl. Ex D. Don) seedlings were grown in the greenhouse at the Pacific Forestry Centre, Victoria, British Columbia, Canada. Fully developed current-year needles were collected in July and August of 2000 and 2001. For local response after wounding treatments, two methods were used. The first method was to cut the needles from trees at their bottom position and this is referred to as single site wounding. The second method was to crush needles at 3–4 mm intervals with fine forceps and this is referred to as multiple-site wounding. The injured needles were transferred onto 3 MM-Whatman paper (Whatman International Ltd., Maidstone, UK) dampened with sterile water in Petri-dishes and incubated at room temperature (22°C) with normal daily light conditions. For systemic response after wounding treatment, current year branches were cut from the tree, their needles at the lower positions were cut in the middle and the branch was immersed in water. The top unwounded needles were collected to test systemic response. Wounded and unwounded samples were collected at time points indicated on the figures.

For treatments with defence-related signal chemicals and okadaic acid (OA), the multiple-wounded needles were put into test solutions without chemicals (control), or into solutions with chemicals for 30 s, then transferred to the Petri dishes and incubated on moist filter paper dampened with the corresponding test solutions. The following treatments: 100 μM salicylic acid (SA), 100 μM abscisic acid (ABA), 1 μM methyl jasmonate (MeJA), 1 μM OA were applied. For the low temperature (5°C) experiment, the seedlings in a growth chamber at 24°C with 16 h light and 8 h darkness were transferred to a growth chamber at 5°C with the same lighting conditions and tissue samples were collected at different times for protein extraction. For each experiment, tissue samples were taken from three different seedlings and frozen in liquid nitrogen and stored at −80°C until analysed.

cDNA cloning of PR10 genes

To clone PR10 cDNAs induced by wounding, total RNA was extracted from the wounded needles at 0 (control), 4 and 8 days post-treatment. Double-strand cDNA was synthesized using a SMART cDNA library construction kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) following the manufacturer's instructions. A PCR strategy was used for the cDNA cloning of PR10 genes. According to the PR10 cDNA sequence of *Pin m III* (Yu et al. 2000), two PR10 gene-specific primers (GSP1 and GSP2) were designed at the highly conserved P-loop region and at the C-terminal region, respectively. The primer sequences are: GSP1, 5′-GCC GAC GCC TCC ATC TCC TTG AAG TAA G-3′ and GSP2, 5′-C CTC TCC AAT CCC AAC TTA TAC TG-3′. With primer GSP1 and PCR 5′ primer from the SMART cDNA library construction kit (Clontech Laboratories, Inc.), a 5′-RACE was performed to clone the 5′-end partial sequences of PR10 cDNAs, including the 5′ untranslated region (5′UTR) and partial coding region of the N-terminal end. A 3′-RACE was performed to clone the 3′ region of the PR10 gene including the 3′ end untranslated region (3′ UTR) and poly (A) tail with primer GSP2 and oligo (dT)$_{30}$. Based on the sequences of 5′ UTR and 3′ UTR clones obtained from

above 5′- and 3′-RACE, new primers were designed and synthesized to clone the full length coding regions.

PCR was performed with a PCR Master Mix Kit (Qiagen Inc., Mississauga, ON, Canada) using double strand cDNA as template in a final volume of 50 μl on a Perkin-Elmer Themocycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s and primer annealing and extension at 68°C for 3 min, with a final 10-min extension at 72°C. After agarose gel fractionation, the amplified DNA fragments with expected sizes were purified and cloned into pGEM-T easy vector (Promega, Madison, WI, USA). Plasmid construction and manipulation were carried out using standard methods (Sambrook et al. 1989).

DNA sequencing and computer analysis

DNA sequences of PR10 cDNA clones were determined on both strands on an ABI310 DNA sequencer (Perkin-Elmer Applied Biosystems) using a Thermo-cycle sequence kit (Amersham, Baie d'urfe, Quebec, Canada) with T7, SP6 primer and other internal primers as needed according to the manufacturer's instructions. DNA sequence data were assembled and analysed using BLAST, CLUSTALW and ORF finder network services at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

RNA isolation and northern blot analysis

Total RNA was extracted from western white pine tissues after treatments. Each sample (approximately 0.5–1.0 g) was ground to a fine powder in liquid nitrogen, then homogenized in 10 ml of extraction buffer (0.1 M Tris-HCl, pH 7.4, 50 mM EDTA, 0.5 M NaCl, 2% SDS, 2% PVP and 10 mM β-mercaptoethanol). The mixture was incubated for 20 min at 65°C. Cellular debris was removed by centrifugation of 12 000 g for 15 min at room temperature. To the supernatant, 3.3 ml of 5 M KAc (pH 5.5) was added. The mixture was incubated on ice for 30 min, then centrifuged at 12 000 g for 10 min at 4°C. After phenol/chloroform extraction, the RNA was precipitated from supernatant by adding 1/3 volume of 8 M LiCl and incubating overnight at 4°C. The RNA was recovered by centrifugation at 12 000 g for 30 min and washed with 2 M LiCl and 70% ethanol. The air-dried RNA precipitate was re-suspended in 50 μl of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and kept at −80°C until use. The RNA concentration of individual extracts was determined by UV absorbency and the integrity was checked by agarose gel electrophoresis and ethidium bromide staining.

For northern blot analysis, RNA was denatured by heating at 65°C for 10 min in a formaldehyde gel-loading buffer, 10 μg of RNA was separated by electrophoresis on 1.2% agarose gels containing 7.4% formaldehyde in morpholinepropanesulphonic acid (MOPS) buffer and transferred to hybond N membranes (Amersham). The RNA was cross-linked on the blots by UV illumination.

The 3′-UTR region of *PmPR10-1.5* was amplified with primers GSP2 and oligo (dT)$_{30}$ to be used as probe. Our study showed that there were three subgroups of PmPR10 gene family in western white pine (Liu and Ekramoddoullah, unpublished data), *PmPR10* cDNAs cloned in this study belonged to a same subgroup. This probe was subgroup-specific and hybridized crossly to different *PmPR10* cDNAs cloned in this study, but it did not hybridize to the other two subgroups of the PR10 gene family in western white pine. The probe was labelled with digoxygenin (DIG) using a random primer labelling kit (Boehringer Mannheim, Laval, Quebec, Canada). Pre-hybridization and hybridization were performed at 65°C in 0.5 M sodium phosphate buffer (pH 7.0), 7% (w/v) sodium dodecyl sulphate (SDS) and 1 mM EDTA (pH 8.0). After hybridization, membranes were washed twice with 40 mM sodium phosphate (pH 7.0) and 1% SDS for 15 min at room temperature and finally twice at 65°C for 30 min. Membranes were exposed to X-ray film for autoradiography. Equal loading and the transfer of RNA were confirmed by monitoring the levels of ribosomal RNA (rRNA) stained with ethidium bromide in agarose gel or with methylene blue on the blots (Sambrook et al. 1989). All northern blot experiments were repeated at least twice.

Western immunoblot analyses

For western immunoblot analyses, total soluble proteins were extracted from tissues and separated by using SDS–polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, or two-dimensional PAGE. The molecular weight and IEF standard markers were obtained from Bio-Rad (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). In addition, the recombinant PR10 protein of *Pin m III* was produced in *Escherichia coli* and used as a reference marker to determine isoelectric points on IEF gels. After electrophoresis, the separated proteins were transferred onto membranes. The N-terminal regions of putative PmPR10 proteins deduced from cDNA clones of western white pine were almost identical to that of Pin l I of sugar pine. The blots were incubated with polyclonal antibodies raised against the N-terminal region of a PR10 protein (Pin l I) from sugar pine as reported previously (Ekramoddoullah et al. 1995). The scanning, detection and quantification of the blots were performed with PDI QUANTITY ONE software (Version 3.0, PDI, Huntington Station, NY, USA) according to the instruction manual. The protein level detected by western immunoblot analysis was calculated based on the optical density (OD) of all pixels within the band boundary and expressed in OD units × millimetre.

Results

Isolation and characterization of multiple members of PmPR10 gene family from wounded needles

To characterize PmPR10 gene expression after wounding of western white pine, 5′- and 3′-RACE was performed

to amplify the 5′-untranslated region (5′-UTR) and 3′-UTR segments of PR10 cDNA separately. PCR results shown on agarose gels indicated that distinct bands were produced from the tissues of 4 and 8 days post-wounding treatments, but only weak smear products were produced from the 0 days control (Data not shown). Because gene-specific primers (GSP1 and GSP2) were designed at conserved regions of conifer PR10 proteins, the 5′- and 3′-RACE cloning strategy allowed us to amplify the 5′-UTR or 3′-UTR fragments from multiple gene family members. The amplified fragment mixtures were then cloned for sequence analysis to identify individual PmPR10 gene fragments. DNA sequence analysis subsequently showed that more than 90% of all cloned fragments encoded an authentic PR10 protein as determined by the deduced amino acid sequence comparison. Using 5′ UTR primer combined with 3′-UTR primer or oligo (dT) $_{30}$, full-length coding regions of individual PmPR10 genes were cloned. We identified 13 different wound-inducible PmPR10 cDNAs from 59 clones, designated as *PmPR10-1.1* to *PmPR10-1.13*, indicating the

presence of multiple wound-inducible PR10 genes in western white pine (Fig. 1). Among them, the nucleotide sequence of *PmPR10-1.10* was identical to *Pin m III* cDNA except for the absence of 11 nucleotides at the beginning of *Pin m III* (Yu et al. 2000). We have cloned and sequenced the genomic DNAs of *PmPR10-1.10* and *PmPR10-1.13* (Liu and Ekramoddoullah, unpublished data) and found that they also lacked the sequence of these 11 nucleotides, suggesting that it might be a cloning artifact in *Pin m III*.

Southern blot analysis using probe of full-length cDNA of *Pin m III* (*PmPR10-1.10*) showed at least seven bands after digestion of genomic DNA with restriction enzyme *Xba* I (Yu et al. 2000). When a 3′-UTR probe of *PmPR10-1.5* was used on *Hin*d III-restricted DNA, at least six bands were detected (Data not shown). These results allow us to conclude that a multiple family of PmPR10 genes is present in the genome of western white pine and several genes or allelic variants of the PmPR10 gene family were expressed in the wounded needles of western white pine. All 13 cDNA

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PmPR10-1.11  MVAGTSTTEEVVQVDARRLWNATTKDSHNFLPKVFPEVFTSVTLLQGDGGVGSVKQFNIT  60
PmPR10-1.12  *******S****************************************************  60
PmPR10-1.5   **S*********E***********************L****************T***L*F*  60
PmPR10-1.1   **S***S*****E**********************L*G***************T***L*F*  60
PmPR10-1.7   **S***S*****E********A*************L*****************T***L*F*  60
PmPR10-1.3   **S***S*****E*********************L**A***************T***L*F*  60
PmPR10-1.6   **S***S*****E*********************L******************T***L*F*  60
PmPR10-1.2   **S***S*****E*******************AL*******************T***L*F*  60
PmPR10-1.4   **S***S*****E*********************L******************T***L*F*  60
PmPR10-1.8   **S***S*****E*********************L******************T***L*F*  60
PmPR10-1.9   **S***S*****E********R************L*****************T***L*F*  60
PmPR10-1.10  **S***S*****E*********************L******************T***L*F*  60
PmPR10-1.13  **S******D****E******************L****A**************T***L*F*  60

PmPR10-1.11  PGKKDFSFIKERVDELDEENFVYKYTAIEGGPLGKKLSSACFEVKLVPRKEGGCVARWTC  120
PmPR10-1.12  ************************************************************  120
PmPR10-1.5   ************************************************************  120
PmPR10-1.1   *******************************************************K***  120
PmPR10-1.7   *******************************************************K***  120
PmPR10-1.3   *******************************************************K***  120
PmPR10-1.6   *******************************************************K***  120
PmPR10-1.2   *******************************************************K***  120
PmPR10-1.4   ************************************************************  120
PmPR10-1.8   ******************Q**************Q*************************  120
PmPR10-1.9   ******************Q**************Q*************************  120
PmPR10-1.10  ******************Q**************Q**********I**************  120
PmPR10-1.13  ******************G**L****V*******N*****S*************S***  120

PmPR10-1.11  NYDTLPGFQPDEGKLKEIKEDSFGMLKKVEQYLLSNPNLYC  161
PmPR10-1.12  *******V*********************************  161
PmPR10-1.5   **E****V*********M***********************  161
PmPR10-1.1   **E****V*******F*************************  161
PmPR10-1.7   **E****V*******F*************************  161
PmPR10-1.3   **E****V*******F***********************F  161
PmPR10-1.6   **E****V*******F*************************  161
PmPR10-1.2   **G****V*******F*************************  161
PmPR10-1.4   **E****V*******F*************************  161
PmPR10-1.8   **E****V*********************************  161
PmPR10-1.9   **E****V*********************************  161
PmPR10-1.10  **E****V*********************************  161
PmPR10-1.13  **E****V*******I*******LAL***************  161
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Fig. 1. Alignment of the deduced amino acid sequences derived from 13 PmPR10 cDNAs from wounded needles of western white pine. The amino acid sequence PmPR10-1.10 is identical to that of the previously published Pin m III protein. Amino acids identical to the PmPR10-1.11 sequence are represented by asterisks. The positions of PCR primer GSP1 and GSP2 are indicated by arrows at the P-loop and C-terminal regions, respectively. The cDNA sequences have been deposited in GenBank (accession no. from AY064193 to AY064205).

clones contain a complete open reading frame encoding a polypeptide of 161 amino acids. The deduced amino acid sequences have significant homology to the PR10 proteins from angiosperms and other gymnosperms and contain the conserved amino acids characteristic of the PR10 gene family. The conserved P-loop region (GXGGXGXXK) is located at positions 47–55 (Fig. 1) and is considered as the possible phosphate-binding site necessary for ribonucleolytic activity (Bantignies et al. 2000). The differences among the 13 PmPR10 genes are found in both 5′- and 3′ UTR, as well as in the coding region. In the coding region some of the nucleotide changes appear to be silent, but some result in changes of amino acids (Fig. 1). The identities among the PmPR10 protein sequences deduced from these 13 cDNAs range from 87 to 99%. The molecular masses of the putative PmPR10 proteins are at about 18 kDa, but their theoretical isoelectric points (pI) ranged from 5.30 to 5.94, making it possible to distinguish them on IEF gel (Table 1).

Accumulation of the PmPR10 gene transcript in response to wounding

Northern blot analyses were conducted to monitor the expression of PmPR10 mRNA in a time course after wounding treatments. Both locally wounded needles with multiple injury sites and unwounded needles in the systemically wounded branches were harvested at various times for total RNA isolation. In the wounded needles for local response, a PmPR10 transcript, approximately 0.9 kb in size, was undetectable in the control needles and needles harvested 1 and 4 h after wounding, but began to accumulate 8 h after wounding (Fig. 2A). The transcripts of *PmPR10-1.5* and related *PmPR10* genes reached a maximum level after 1 day and remained steady up to 8 days after wounding.

Table 1. PR10 genes from wounded needles of western white pine. Data were calculated from the deduced amino acid sequences of the wound-inducible PR10 cDNAs using Expert Protein Analysis System (ExPASy) of the Swiss Institute of Bioinformatics (http://www.expasy.ch/tools/). [a]MM, predicted molecular mass of the polypeptide encoded by the PmPR10 cDNA clones. [b]pI, predicted isoelectric point of the polypeptide encoded by the PmPR10 cDNA clones. [c]Corresponding PmPR10 protein spots as detected in Fig. 4 based on pIs and MM.

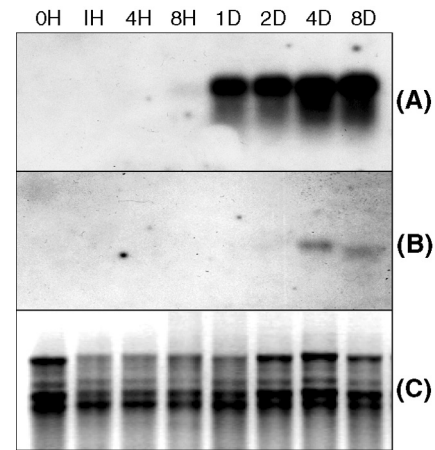| PmPR10 cDNA	MM (kDa)[a]	pI[b]	Protein spot[c]
PmPR10-1.1	17.9224	5.94	Group I (a, b, or c)
PmPR10-1.2	17.8944	5.94	
PmPR10-1.3	18.0105	5.58	Group II (d, e, f)
PmPR10-1.4	18.0225	5.58	
PmPR10-1.5	18.0205	5.58	
PmPR10-1.6	17.9945	5.58	
PmPR10-1.7	17.9645	5.58	
PmPR10-1.8	17.8975	5.56	
PmPR10-1.9	18.0155	5.56	
PmPR10-1.10	18.0015	5.56	
PmPR10-1.11	18.0265	5.55	
PmPR10-1.12	17.9644	5.55	
PmPR10-1.13	17.7912	5.30	Group III (g, h, or i)

Fig. 2. Northern blot analysis of PmPR10 gene expression in western white pine needles following wounding treatment. Total RNA was extracted from wounded needles with multiple sites and from unwounded needles from wound branches. The tissue samples were harvested at the indicated times post-wounding (H, hours; D, days). (A) and (B) X-ray film images of northern blots from wounded and unwounded needles to show local and systemic responses, respectively. Ten micrograms of total RNAs in each lane was hybridized with a 3′-UTR probe from *PmPR10-1.5*. (C) An ethidium bromide-stained gel is shown as a loading control.

In contrast to results from local response after wounding treatments, mRNA accumulation of *PmPR10-1.5* and related *PmPR10* genes in the unwounded needles from systemic wounded branches did not begin until 4 days after treatment and remained at very low levels 8 days after wounding (Fig. 2.B). A shorter PmPR10 transcript was observed in 8 days than in 4 days after treatment for an unknown reason. Activation of PmPR10 genes occurred much earlier and was much stronger for the local response in wounded needles than those for systemic response in the unwounded needles from wounded branches. Whereas the systemic wound response has been well characterized with respect to the production of defence-related products such as proteinase inhibitors, this, to the best of our knowledge, is the first report of systemic accumulation of a PmPR10 transcript in response to wounding.

Induction of PmPR10 proteins in response to wounding

To investigate the kinetics of PmPR10 protein induction following wounding, we performed time course experiments. Local versus systemic responses and single versus multiple-wounding treatments were evaluated. As shown in the western immunoblot analysis (Fig. 3), PmPR10 protein was not detected at time 0 (control), consistent with our previous report that PmPR10 protein was barely detectable in the summer months (Ekramoddoullah et al. 1995). In needles with multiple wounding, PmPR10 proteins were first detected at 1 day and displayed a progressive accumulation up to 8 days, the longest time point we tested (Fig. 3A). In needles with a single wounding, the profile of PmPR10 protein accumulation
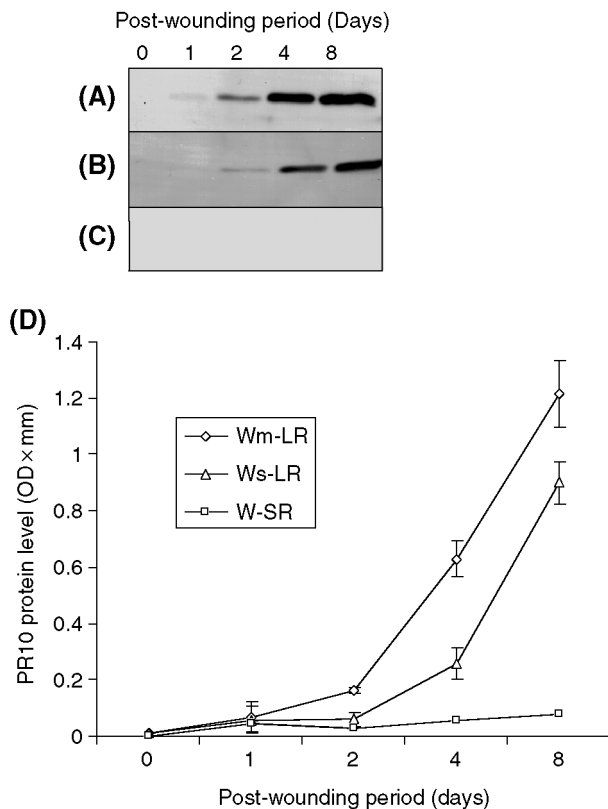
Fig. 3. Wound induction of PmPR10 protein accumulation in western white pine needles. Total soluble proteins were extracted from tissues collected on different days following wounding. Western immunoblot analyses were performed to show PmPR10 protein accumulation after different wounding treatments. (A) Wm-LR: local response after wounding treatment with multiple sites; (B) Ws-LR: local response after wounding treatment with single site; and (C) W-SR: systemic response after wounding treatment. Ten micrograms of total protein was loaded in each lane, separated by electrophoresis on SDS-polyacrylamide gels, blotted to PVDF membranes, and analysed using an antiserum against the N-terminal region of a PR10 protein (Pinl I) from sugar pine. (D) PR10 protein levels were quantified using Quantity One software. Each point represents the means ($\pm$ SE) of three independent experiments.

was generally parallel to that for multiple wounding, but the induction was delayed and protein level lower at all times (Fig. 3B). Although a low level of PmPR10 mRNA was detected in unwounded needles 4 and 8 day after systemic wounding (Fig. 2B), the PmPR10 protein level was probably too low to be detected in the same needle samples even 8 days after treatment (Fig. 3C). Taken together, these results demonstrate that PmPR10 protein expression is activated in response to mechanical damage. The wound-induced PmPR10 protein level is related to the extent of wounding on each needle and displayed a dose-dependent effect. Generally, the induction pattern of the PmPR10 proteins observed on western immunoblot analyses is in agreement with the accumulation pattern of PmPR10 transcripts following wounding. These comparison data from plants that received different treatments suggest that the wound-

inducible PmPR10 protein accumulation may be regulated at the transcriptional level.

Differential regulation of multiple PmPR 10 proteins in response to wounding

Because a set of PmPR10 cDNAs was cloned from wounded needles (Table 1), we speculated that multiple PmPR10 proteins were also synthesized upon wounding. The single immunostained band observed in western immunoblot analysis of SDS-PAGE (Fig. 3) is likely to be composed multiple polypeptides of similar molecular masses but possessing different isoelectric points (pI). To determine this, western immunoblot analyses were performed following protein electrophoresis on IEF gels or 2-D gels. As shown in Fig. 4A, six bands were detected on the IEF blots after wound induction. On 2-D blots a similar pattern of PmPR10 proteins was detected except that the first and sixth bands on the IEF blot were separated into two and three spots, respectively (Fig. 4B and E). According to their pI values, the wound-inducible PmPR10 proteins can be classified into three groups, each containing three PmPR10 spots (Fig. 4B and E). Group I with pI about 5.9–6.0 includes PmPR10 spots a, b and c. Group II with pI about 5.5–5.6 includes PmPR10 spots d, e and f. Group III with pI about 5.2–5.3 includes PmPR10 spots g, h and i. Image density analysis showed that the PmPR10 proteins in group II were the dominant ones, accounting for about 68% of the total wound-inducible PmPR10 proteins. Group I and III accounted only for 19 and 13%, respectively. Most PmPR10 cDNAs identified in this study encoded proteins corresponding to the PmPR10 protein spots in group II. *PmPR10-1.1* and *PmPR10-1.2* encoded PmPR10 proteins corresponding spots in group I and *PmPR10-1.13* encoded a protein from group III (Table 1). This was partially confirmed by the recombinant Pin m III protein (*PmPR10-1.10*) expressed in *E. coli*, whose pI and MM values on IEF and 2-D blots fell in the same range as that observed in group II (data not shown). Because of the high polymorphism of PmPR10 gene family in western white pine, it is difficult from our current data to determine exactly which PmPR10 cDNA encodes which PmPR10 protein. The pIs and molecular masses of some predicted PmPR10 proteins are very close to each other (Table 1); therefore, some PmPR10 protein spots identified on 2-D blots, especially those in group II, may be mixtures of PR10 proteins translated from different mRNAs. In summary, the results of cDNA cloning and protein immunodetection demonstrate that multiple PmPR10 proteins are expressed differentially in response to mechanical damage.

Differential regulation of wound-inducible PmPR10 proteins in response to wounding, cold hardening and disease-infection

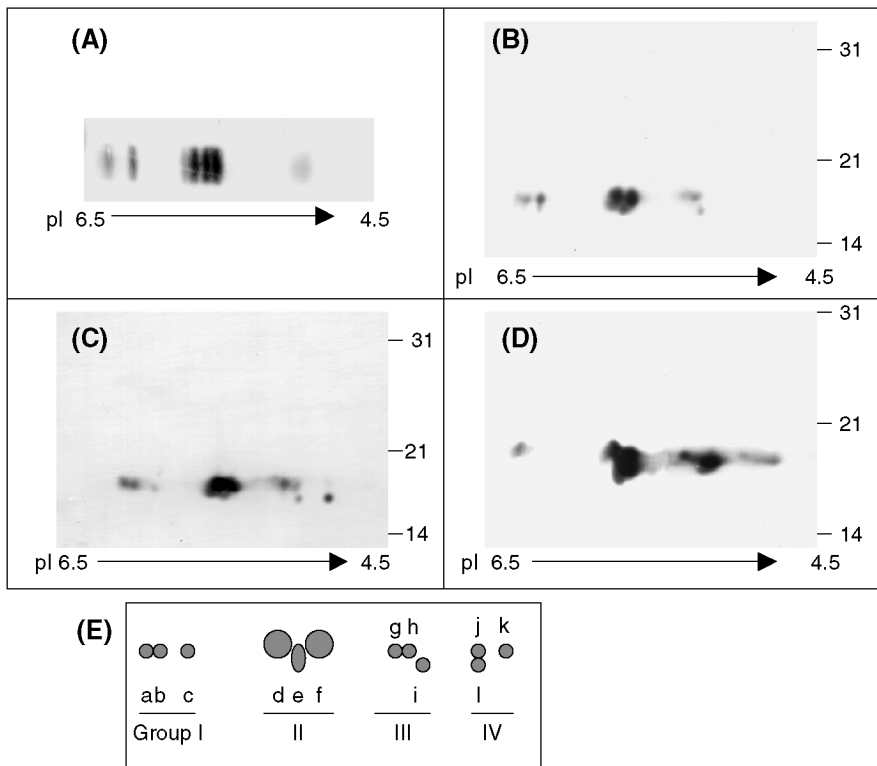Low temperature is one key factor in the cold-hardening process. Therefore, we analysed the accumulation of

Fig. 4. Comparison of PmPR10 protein profiles induced by wounding, cold-hardening and fungal infection of western white pine by *C. ribicola* using immunodetection after IEF or 2-D PAGE. Only the area corresponding to pH gradient from 4.5 to 6.5, where PmPR10 proteins are detected, is shown. Proteins were extracted from needles 4 days after wounding treatment with multiple sites, and then detected after IEF (A) and 2-D PAGE (B). (C) PmPR10 proteins from summer needles after *C. ribicola* infection; (D) PmPR10 proteins from healthy needles collected in winter. Fifty micrograms of total soluble proteins was loaded for each IEF gel. Protein transfer and immunodetection were as described in Fig. 3. The pI and position of each PR10 spot was determined relative the migration of protein standards and recombinant Pin m III protein from *E. coli*. (E) A schematic representation of PmPR10 protein profiles summarized from (B), (C) and (D).

PmPR10 protein after low temperature treatment (5°C) under controlled experimental conditions. Western immunodetection of SDS-PAGE blots showed that low temperature (5°C) did induce PmPR10 protein expression; however, the PmPR10 protein levels in the tissues exposed to cold (5°C) in controlled conditions were lower than in tissues collected in winter, infected tissues, or wounded tissues (Fig. 5). These experiments indicate that the temperature change could account for, at least partially, the accumulation of PmPR10 proteins in western white pine in the autumn and winter.

To investigate the expression of multiple PmPR10 proteins under different environmental stresses, we further compared the profiles of PmPR10 proteins extracted from needle tissues after wounding, blister rust (*C. ribicola*) infection and winter cold-hardening. These analyses showed that PmPR10 protein profiles were different for different environmental stresses (Fig. 4B, C and D). The PmPR10 protein expression profile in response to cold hardening during winter was more diverse than that evoked by wounding. In addition to the three PmPR10 groups induced by wounding, a group IV with pI about 5.0–5.1 was detected, which include two PmPR10 spots j and k (Fig. 4D). PmPR10 proteins in group II and III were up-regulated, but only one PmPR10 protein spot (a, b or c) in group I was detected in healthy needles after winter cold hardening. In the fungus-infected needles from the summer the PmPR10 protein profile was almost the same as that in wounded needles, only one new PmPR10 protein (spot l) was detected in the pI range of group IV. PmPR10 protein spots a, g and l were up-regulated, whereas spot c was down-regulated upon infection by *C. ribicola* (Fig. 4B and C). The PmPR10 proteins in group II were always the dominant ones in the above three cases.
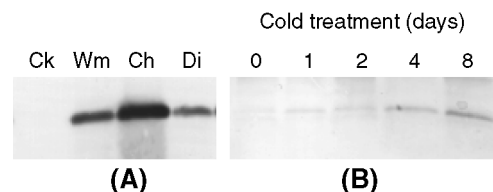

Fig. 5. Western immunoblot analysis of PmPR10 protein accumulation in western white pine needles following different environmental stresses. Protein transfer and immunodetection were as in Fig. 3. (A) PmPR10 protein induced by wounding, cold hardening in winter and fungal infection. Twenty micrograms of total proteins was loaded in each lane. Ck, healthy needles without treatment collected in August of 2000 as control. Wm, healthy needles 4 days after wounding treatment with multiple sites collected in August of 2000. Ch, healthy needles after cold hardening collected in February of 2000. Di, diseased needles infected by *C. ribicola* collected in August of 2000. (B) Accumulation of PR10 proteins upon cold treatment at 5°C in western white pine needles. Fifty micrograms of total proteins was loaded in each lane. Total proteins were extracted from needle samples at different times following cold treatment. These experiments were repeated at least twice with similar results.

Western immunoblot analyses of 2-D blots established the complex differential expression pattern for PmPR10 proteins in western white pine needles, demonstrating that different members of the PmPR10 gene family are differentially regulated upon different environmental stresses.

Effects of MeJA, SA, ABA and OA on the accumulation of wound-inducible PmPR10 proteins

PmPR10 proteins are induced upon wounding, fungal infection and cold hardening, which suggests that they may play an important role in the plant defence response. However, no direct evidence is available on the signalling pathways or components regulating their expression in western white pine. To study the characteristics of wound-activation of PmPR10 protein synthesis, the responses to global defence-related signal compounds and OA were investigated. As shown in Fig. 6, western immunoblot analysis shows the time courses of accumulation of PmPR10 proteins in multiple-wounded needles
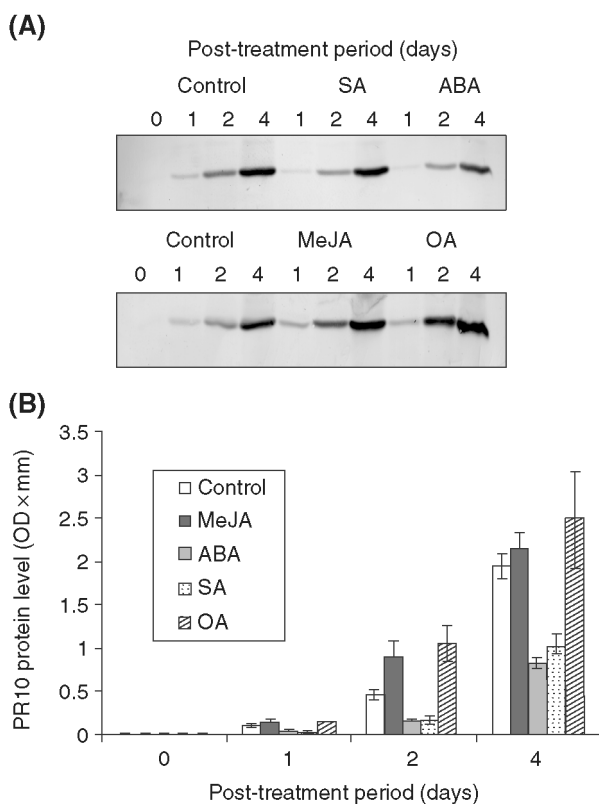
(A)

(B)

Fig. 6. Effect of treatments with SA, ABA, MeJA and OA on the wound-inducible accumulation of PmPR10 proteins. (A) PmPR10 protein was determined by western immunoblot analysis of SDS-PAGE. Protein transfer and immunodetection were as in Fig. 3. Wounded needles were treated for the indicated periods without (control) or with $100 \mu M$ SA, $100 \mu M$ ABA, $1 \mu M$ MeJA, or $1 \mu M$ OA. Proteins were extracted and $20 \mu g$ were loaded in each lane. (B) Relative PR10 protein levels were quantified using the QUANTITY ONE software. Each point represents the means ($\pm$ SE) of three independent experiments.

treated with or without chemicals (MeJA, SA, ABA and OA). MeJA at $1 \mu M$ stimulated the accumulation of PmPR10 protein within 2 days of treatment. The enhancement of the wound-induced accumulation of PmPR10 protein by MeJA continued for 4 days, the longest time period analysed (Fig. 6), suggesting that JA or MeJA may act as a signal chemical in western white pine. Compared with the controls (mock treatment, wounding only), the treatment with $1 \mu M$ OA also resulted in increased accumulation of the PmPR10 proteins. Okadaic acid is a membrane-permeable inhibitor of serine/threonine phosphatase type 1 and type 2 A (Cohen et al. 1990). The increased PmPR10 protein level by OA suggests that hyper-phosphorylation of one or more target proteins may be involved in the signal transduction pathway of the defence response to wounding in western white pine as that observed for potato PR10a (Després et al. 1995).

By contrast, ABA and SA, both at $100 \mu M$, partially suppressed the accumulation of PmPR10 protein after wounding (Fig. 6). All of these chemical treatments affected the level of PmPR10 proteins, but did not change the expression pattern of PmPR10 proteins detected on IEF blots (data not shown). These results suggest the involvement of signal components of plant defence in western white pine PmPR10 gene expression. All of the wound-inducible PmPR10 proteins are also induced by fungal infection, suggesting that cross-talk between the pathogen signalling pathway and the wound response pathway may be present. But the similarity of the PmPR10 protein profiles between wounded needles and winter needles is much more lower (Fig. 4B and D). These observations imply that different signal transduction pathways might be involved in the activation of different PmPR10 genes in response to different environmental stresses.

Discussion

Our interest in the role of PmPR10 proteins in the plant defence response led to the investigation of their expression upon wounding stress. Many biotic and abiotic stresses can activate PR10 gene expression in plants. PR10 proteins have been shown to be induced by elicitors (Walter et al. 1990, 1996), pathogens (Swoboda et al. 1995, Ekramoddoullah et al. 1998), copper stress (Utriainen et al. 1998), salt stress (Moons et al. 1997), cold acclimation (Ekramoddoullah et al. 1995) and drought stress (Dubos and Plomion 2001). We showed that PmPR10 transcripts accumulated after wounding treatments (Fig. 2). Similar observations have been reported for an asparagus PR10 gene (AoPR1) (Warner et al. 1992, 1993) and for the PR10a gene in potato (*S. tuberosum*) (Constabel and Brisson 1992, Després et al. 1995).

Comparison of PmPR10 expression showed the different PmPR10 protein profiles in the needle tissues upon wounding, cold-hardening and fungal infection (Fig. 4). The impact of different environmental stresses on plants

is not well understood. Different stress factors may have both positive and negative effects on plant defence-related gene expression. Some PmPR10 proteins were expressed to higher or lower levels in response to fungal infection and winter environmental stimuli relative to the levels expressed after wounding (Fig. 4). PmPR10 protein was induced to the highest level by winter cold-hardening (Fig. 5A), but induced to much lower levels by a controlled low temperature of 5°C (Fig. 5B). Similar to the synergistical induction of some other low temperature-responsive proteins in angiosperms, our results suggest that the accumulation of PmPR10 proteins in winter might be caused by the combined effects of many environmental factors, such as temperature and photoperiod.

Some angiosperm PR10 proteins have been reported to have ribonucleolytic activity (Moiseyev et al. 1994, 1997, Bufe et al. 1996, Swoboda et al. 1996, Bantignies et al. 2000). The structure of birch pollen Betv1 determined by X-ray and NMR indicate that its P-loop structure resembles a motif found in many nucleotide-binding proteins (Gajhede et al. 1996). The P-loop sequence is conserved in all PmPR10 proteins as well as in the PR10 proteins from other species. Sugar pine PR10 protein (Pin l I) (Ekramoddoullah et al. 1995) was accumulated in needles and 10 PmPR10 proteins were induced by infection with blister rust fungus (Fig. 4). Immunocytochemical localization showed that the PmPR10 proteins were bound to the cell wall of blister rust fungus both intracellularly and extracellularly in the western white pine needles (Ekramoddoullah, unpublished data). This observation implie that the PR10 proteins are synthesized and released from the host cells damaged by the fungus penetration, suggesting their defence role(s) to pathogen attack. Although there is as yet no proof for a ribonuclease function for PmPR10 protein, such proteins with possible ribonuclease activity expressed in the plant defence response may play an important role in injured plant cells, or in pathogen cells after their penetration into plant tissues.

Multiple members of the PR10 gene family are differentially expressed under different environmental stresses (Fig. 4) or at different stages during plant growth and reproduction (Breiteneder et al. 1989, Crowell et al. 1992, Warner et al. 1993, Swoboda et al. 1995, 1996, Walter et al. 1996, Huang et al. 1997). The multigenic characteristic of *PmPR10*, as seen in many other genes in plants, indicates that gene duplication and subsequent diversification has occurred during the evolution of the PR10 gene family. This may explain the induction of a large number of PmPR10 proteins under different environmental conditions (Fig. 4). It is therefore not reasonable to assign a unique function to all PR10 proteins throughout the plant kingdom. The functional analysis of different PR10 proteins is very important for us to understand their physiological roles in plants. During evolution of a multiple gene family, different members may have been co-opted to perform an additional function. In winter rye PR3 family two cold-responsive PR3 protein chitinases have been identified with ice-binding activity (Yeh et al.

2000), but other members of this family are pathogen-induced with only enzymatic activity. Previously we observed that expression levels of PmPR10 proteins were correlated with frost-hardiness of foliage in western white pine (Ekramoddoullah et al. 1995). However, the physiological roles of PR10 proteins in cold acclimation are still unclear. The *Pin m III* (*PmPR10-1.10*) gene has been transformed into *Arabidopsis* to investigate the effect of PmPR10 gene expression on frost-tolerance in transgenic plants.

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