Characterization, expression and evolution of two novel subfamilies of *Pinus monticola* cDNAs encoding pathogenesis-related (PR)-10 proteins

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**Summary** Proteins of the pathogenesis-related (PR)-10 family are induced in many plants by phytopathogens and environmental stresses. A multi-gene family of PR10 proteins has previously been found in the genome of western white pine (*Pinus monticola* Dougl. ex D. Don). We isolated two novel subfamilies of PR10 cDNAs (*PmPR10-2* and *PmPR10-3*) from *P. monticola* that are distinct from other PR10 genes (*PmPR10-1.1–1.14*) reported from the same species. The *PmPR10* proteins are grouped in three subfamilies based on similarity in amino acid sequences. The sequence identities of *PmPR10* proteins are much higher among members within a subfamily than among members of different subfamilies (86–99% versus 59–68%). Induction of both *PmPR10-2* and *PmPR10-3* mRNAs was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in needles in response to wounding treatment. *PmPR10-3* was also expressed in needles during cold acclimation in winter. Transcript levels of both *PmPR10-2* and *PmPR10-3* were less than the detectable levels of constitutive expression in roots, stems and vegetative shoots, whereas *PmPR10-1.10* mRNA of subfamily I was expressed at various levels. Phylogenetic analysis showed that *PmPR10* and PR10 proteins from other conifers are grouped within one clade that is distinct from that of angiosperm PR10 proteins. In the conifer monophyletic group, PR10 sequences diversify into three distinct clusters. Among these three clusters, some PR10 proteins from single conifer species showed greater divergence distances than sequences from other conifer species, suggesting that, within the conifers, the multi-gene family underwent great diversification during evolution. Based on ratios of nonsynonymous to synonymous nucleotide substitutions (Ka/Ks), we speculate that positive selection resulted in the divergence of *PmPR10* subfamilies I and III. Possible mechanisms and significance of PR10 gene evolution are discussed.

**Keywords:** adaptive gene evolution, environmental stress, gene divergence, phylogenetic analysis, RT-PCR, wound-induced expression.

**Introduction**

Among pathogenesis-related (PR) proteins, members of the PR10 protein family are small, acidic, intracellular proteins with molecular masses ranging from 15 to 18 kDa (van Loon et al. 1994). PR10 proteins or their genes have been identified in many seed plants, including a few conifer species (Ekramoddoullah and Hunt 2002). Based on similarities in amino acid sequences, the PR10 family comprises three distinct groups: intracellular pathogenesis-related proteins (IPR) (van Loon et al. 1994), cytokinin-specific binding proteins (CSBP) (Fujimoto et al. 1998) and major latex proteins (MLP) (Os-mark et al. 1998).


Despite the ubiquitous presence of PR10 proteins in higher plants, little is known about their biochemical activity. One conifer PR10 protein has been found to accumulate in the pathogen cell wall in infected plant tissue (Ekramoddoullah and Hunt 2002). An explanation linking PR10 proteins to plant defense response against pathogen invasion is based on the
finding that a few PR10 proteins exhibit in vitro ribonuclease activity (But et al. 1996, Bangnies et al. 2000, Wu et al. 2003), which may either help protect plants during programmed cell death around infection sites, or act directly on the pathogens. The physiological role of ligand-specific binding activity has been demonstrated for PR10 proteins in several species. Ligands bound by PR10 proteins include cytokinin (Fujiimoto et al. 1998, Gonneau et al. 2001, Mogensen et al. 2002), brassinosteroids (Markovic-Housley et al. 2003), fatty acids and flavonoids (Mogensen et al. 2002).

Crystal structures have been determined for some members of the PR10 protein family, including a major birch pollen allergen, Bet v 1 (Gajhede et al. 1996), and its low allergenic isoform, Bet v 11 (Markovic-Housley et al. 2003), a major cherry allergen, Pru av 1 (Neudecker et al. 2001) and classic PR10 proteins from yellow lupine, LIPR10-1A and LIPR10-1B (Biesiadka et al. 2002). Three-dimensional (3-D) structures of these PR10 proteins are similar: each consists of a large, hydrophobic, Y-shaped cavity has been found in 3-D structures of PR10 proteins. It forms between residues lining the interior face of the β-sheet and the three α-helices, and functions as the ligand-binding site (Biesiadka et al. 2002). However, structural data of the conserved P-loop motif (GxGGxGxxK) of the PR10 3-D structure (Biesiadka et al. 2002) does not support the suggestion that the P-loop is a binding site for nucleotides involved in RNase activity. The lack of support makes the RNase hypothesis of PR10 protein function controversial. On the other hand, large, hydrophobic, Y-shaped cavity has been found in 3-D structures of PR10 proteins. It forms between residues lining the interior face of the β-sheet and the three α-helices, and functions as the ligand-binding site (Biesiadka et al. 2002, Markovic-Housley et al. 2003). The PR10 transport of physiologically active ligands may be an important step in plant defense, as well as in plant growth and development.

A multiple gene family of PR10 proteins has been identified in some angiosperms. In gymnosperms, multiple copies of PmPR10 genes have been found in the western white pine (Pinus monticola Dougl. ex D. Don) genome (Yu et al. 2000, Liu et al. 2003). However, little is known about PR10 gene evolution in the plant kingdom. In the present study, we cloned two novel subfamilies of PR10 genes in western white pine and characterized their expression. A phylogenetic study of conifer PR10 genes suggests a succession of adaptively driven amino acid substitutions may have played a key role in the diversification of the PR10 gene family.

Materials and methods

Plant materials and experimental treatments

Pinus monticola (Dougl. ex D. Don) seeds of Seed lot 3278 were sown and grown at the Pacific Forestry Centre, Victoria, BC, Canada. For wounding treatments, fully developed current-year needles were collected in July and August of 2000 and 2001. The needles were crushed at 3- to 4-mm intervals with fine forceps before being transferred onto 3-mm Whatman paper dampened with sterile water in petri dishes, and incubated at room temperature (22 °C) in an 18-h photoperiod. For the low-temperature treatment, seedlings were grown in a controlled environment chamber at 24 °C in a 16-h photoperiod and then transferred to a controlled environment chamber set at 5 °C with a 16-h photoperiod. Tissue samples were collected at different times for total RNA extraction. Each treatment was repeated three times. At each harvest, samples were pooled from three individual seedlings, frozen in liquid nitrogen and stored at –80 °C until analyzed.

PR10 cDNA cloning

Total RNA was isolated as described previously (Liu et al. 2003). First-strand cDNA was synthesized using a SMART cDNA library construction kit according to the manufacturer’s instructions (Clontech Laboratories, Palo Alto, CA). According to the PR10 cDNA sequences from western white pine (Liu and Ekramoddoullah 2003), one reverse primer GSP-1, 5'-GCC GAC GCC TCC ATCTCC TTG AAG TAA G-3', was designed at the highly conserved P-loop region. We performed reverse transcriptase-polymerase chain reaction (RT-PCR) with first-strand cDNA as a template and primer GSP-1 and Clontech PCR 5' primer from the SMART cDNA library construction kit (Clontech Laboratories). The PCR conditions consisted of an initial denaturation step at 94 °C for 30 s, followed by 30 cycles of 15 s at 94 °C and 3 min at 65 °C for primer annealing and extension, with a final 10-min extension at 72 °C. The amplified DNA fragments with expected sizes were purified and cloned into pGEM-T easy vector (Promega, Madison, WI) for nucleotide sequence analysis. From the amplified DNA fragments, two partial PR10 sequences were identified as novel. Their 5'-UTR sequences were used to design gene-specific forward primers that were combined with oligo d(T)30 primer to clone their full-length cDNAs respectively. The PmPR10-2 gene-specific forward primer was 5'-GAG TGC ACA ATA GCA CAG CTT C-3', and the PmPR10-3 gene-specific forward primer was 5'-CAC ACC ACA AGC AGC TTA CGT-3'.
Repeated detection of RT-PCR analysis of PmPR10 expression

PdbViewer software was used (Guex and Peitsch 1997). To manipulate protein structure and model the PmPR10-3 protein, the Deep View Swiss-ProtViewer software was used (Guex and Peitsch 1997).

RT-PCR analysis of PmPR10 expression

Repeated detection of PmPR10-2 or PmPR10-3 gene expression by Northern blot analysis using their 3′-UTR as probes showed no significant hybridization signals compared with PmPR10-1 gene expression. To circumvent this difficulty, RT-PCR was performed to quantify gene expression of three PmPR10 subfamilies in western white pine. Total RNA was treated with RQ 1 RNase-free DNase (Promega) and purified using a Plant RNeasy Extraction Kit (Qiagen). Reverse transcription was performed with 2 µg total RNA as the template in a final volume of 20 µl at 42 °C for 1 h using an Omniscript Reverse Transcriptase Kit (Qiagen). To determine expression of western white pine PR10 genes, semi-quantitative RT-PCR was performed with gene-specific primers. Coupled with the gene-specific forward primers mentioned above, two reverse primers (5′-AGC CCA AGC CTA TTC TTT ATT GAG-3′ and 5′-TTC GTT CCA TAA AAC ATA GTT TGG-3′) were designed for PmPR10-2 and PmPR10-3 genes respectively. A forward primer (5′-ATG GTG TCA GGG ATC TCA TC-3′) and a reverse primer (5′-CTA GCA GTA TAA GTT GGG AT-3′) were used for PmPR10-1.10 gene. The cDNA amplisons of all of these primer pairs can be distinguished from those resulting from genomic contamination based on size differences. Semi-quantitative RT-PCR was carried out with 1 µl of cDNA in 25 µl of PCR mix using Taq PCR Master Mixture (Qiagen). A negative control (DNA-free) was performed with each RT-PCR. The negative control results are not shown, because there was no amplification. The RT-PCR amplifications were performed three times for each RNA sample. In control experiments to verify the quality of cDNAs isolated from the different tissue samples, cDNA of 18S rRNA was amplified with a Quanta mRNA 18S rRNA internal standard kit (Ambion, Austin, TX). The PCR conditions consisted of an initial denaturation step at 94 °C for 30 s, followed by 30 cycles of 15 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C, with a final 10 min extension at 72 °C.

Results

Isolation of two distinct subfamilies of PR10 cDNAs (PmPR10-2 and PmPR10-3) in P. monticola

To characterize induced PR10 gene expression in plant defense response, RT-PCR was performed to amplify the 5′-end partial sequences of PR10 cDNAs from wounded needles with GSP-1 primer and Clontech 5′-primer. The RT-PCR differential display showed that a PR10 cDNA fragment of about 200 bp was specifically amplified from needles 1 day after wounding treatment, but was undetectable in the untreated needles (Day 0 control) (data not shown). Because primer GSP-1 was designed at the conserved P-loop region of conifer PR10 proteins, the RT-PCR differential display led to amplification of diverse members of the same gene family. After the amplified fragments had been cloned, 54 recombinants of partial PR10 clones were identified by DNA sequence analysis. Two of them encoded novel PR10 proteins (designated PmPR10-2 and PmPR10-3), suggesting that their expression levels may be lower compared with those of other PmPR10 genes (PmPR10-1s).

We performed PCR with gene-specific forward primers and oligo d(T)30 to clone full-length coding regions. Two cDNA clones, PmPR10-2.1 and PmPR10-2.2 (GeneBank accession nos. AY596271 and AY596272, respectively), share almost identical nucleotide sequences except for five base changes and length difference in the 3′-UTRs that may have resulted from DNA polymorphisms. The deduced PmPR10-2 protein has a predicted molecular mass of 18.2 kDa and an isoelectric point (pI) of 5.86. PmPR10-3.1, PmPR10-3.2 and PmPR10-3.3 cDNA sequences (GenBank accession nos. AY596273 to AY596275) had 94 to 99% homology. Their encoded proteins possess calculated molecular masses of 18.1, 18.2 and 18.0 kDa, and pIs of 5.45, 5.15 and 5.03, respectively.

Compared with other conifer PR10 proteins, PmPR10-2 had the highest identity with white spruce Picg1 (89%), whereas identity with PmPR10-1s and PmPR10-3s ranged from only 59 to 65%. PmPR10-3.2 shared the highest homologies among themselves (86 to ~97%), followed by 72–77% homology to Pinus contorta (55%) and Pinus ponderosa (51%) and 67–75% homology to P. pinaster (59 to 65%). PmPR10-2.1 had 94 to 99% homology. Their encoded proteins possess calculated molecular masses of 18.1, 18.2 and 18.0 kDa, and pIs of 5.45, 5.15 and 5.03, respectively.

Phylogenetic analysis of PR10 family

To explore evolutionary relationships between IPR-type PR10 genes, a phylogenetic tree was constructed based on amino acid sequence alignment analysis of 87 PR10 proteins from both angiosperm and gymnosperm species. No IPR-type PR10...
protein has been identified in the Arabidopsis genome, so an
Arabidopsis MLP with similarity to IPRs was included as an
outgroup. As seen in Figure 2, a phylogenetic tree constructed
from aligned sequences contains two major branches or
clades: PR10 proteins from conifers form one main clade, and
those from both monocots and dicots form the other. This re-
sult suggests that PR10 proteins from conifers and angio-
sperms have the same origin, and that divergence of the PR10
genes may have occurred as the last common ancestor of seed
plants separated into conifers and angiosperms.

In the conifer clade, PR10 proteins are subgrouped into
three clusters that may have evolved from different ancestor
genes (Figure 2). The pairwise distance analysis showed
amino acid variance between clusters was significantly greater
than that within the same cluster. Cluster I comprised
PmPR10-1.10, Douglas-fir PsemI, and white spruce Picg2 and

Figure 1. Alignment of deduced amino acid sequences of PR10 genes from conifers. Amino acid residues identical to
PmPR10-1.10 are indicated by asterisks. Grey boxes indicate strictly conserved residues. Gaps were introduced to optimize alignment (indicated by dashes). Black boxes indicate P-loop motifs (G47–T53), E103, E150 and Y152, which are believed to be responsible for ribonuclease activity. Amino acid sequences are deduced from cDNA or genomic DNA sequences from P. monticola (GenBank accession nos. AY064120–AY064206, and AY596271–AY596275), white spruce Picg2, PsemI, and maritime pine PpPR10 (GenBank accession no. CAC83079).
TWO SUBFAMILIES OF PR10 PROTEINS IN PINUS MONTICOLA

Figure 2. Phylogenetic tree based on the alignment of the deduced amino acid sequences of PR10 proteins from western white pine, other conifers and angiosperms. The tree was constructed by the neighbor-joining method provided in MEGA2. Arabidopsis thaliana (L.) Heynh. major latex protein (MLP)-related protein (At-MLP, NM_128163) is included as an outgroup. Sequence abbreviations of PR10 proteins are given with GenBank accession nos. in parentheses: western white pine PmPR10-1.1–1.14 (AY064193–AY064206), PmPR10-2.1 (AY596271) and PmPR10-3.1–3.3 (AY596273–AY596275); white spruce Picg1, -2, -4 (AAF12810–AAF12812); P. pinaster PpPR10 (CAC83079); Douglas-fir Pseml (AAF60972); Arabis hypogaea L. Ara-h8 (AY320880); Asparagus officinalis L. AoPRP (X62103), AocPR2 to AocPR7 (X69562, X69563, AJ132610 to AJ132613); Betula pendula Roth Betv1 (Z72429), Betv1-sc1, -sc3 (X77599, X77601), Bppy10a–b (AJ289770, AJ289771); Capsicum annuum L. CaPR-10 (AF244121); Corylus avellana L. Cor-a02–a04 (AF332973–AF332975); Daucus carota L. Dau-c (AF456481); Gossypium arboreum L. GaPR10 (AF416652); Gossypium barbadense L. GbPR10 (AY588276), GbPR10-5, -12 (AY560553, AY560552); Glycine max (L.) Merrill. GmPR10 (AF529303); Gossypium hirsutum L. GhPR10-5, -12, -16 (AF305064, AF305066, AF30567); Hordeum vulgare L. HvPR-10 (AY220734); Lilium longiflorum Thunb. LloPR10 (AAD17336), LloPR10-1 (AF021848) and LloPR10-7 (AF021853); Lupinus luteus L. LloPR10.1a–c (AF002277, AF002278, AF180941), LloPR10.1a.1a, e, f (AF170091, AF170092, AY288355, AY303549); Lupinus albus L. LaPR10 (AJ000108); Malus x domestica Borkh. Ypr10*a–d (AY026908–AY026911) and Ypr10*Md.b (AY186248); Medicago sativa L. MsPR10.1 (AJ311104); Medicago truncatula Gaertn. MtPR10-1 (Y08641); Nicotiana tabacum L. NtPR10a (AY055111); Oryza sativa L. OsPR10a (AF395880), OsPR10b (AF274850), OsPR10b (AF274851) and OsPR10 (AB127580); Oxalis tuberosa Molina Ot-Oca (AF333436); Pachyrhizus erosus (L.) Urb. SPE16 (AY433943); Petroselinum crispum (Mill.) Nyman ex A. W. Hill Pepr1 (X96868), Pepr1-1 (U48862), Pepr1-3 (X12573) and Pepr2 (X85698); Pisum sativum L. PsDRR49a (U31669) and PsRH2 (S74512); Solanum tuberosum L. pSTH2 (M25155) and pSTH21 (M25156); Sorghum bicolor (L.) Moench. ShPR-10 (U60764); Vitis vinifera L. VvPR10.1 (AJ291705) and VvPR10.2 (AJ291704).
Picg4. PmPR10-1s have shorter $\rho$-distances among themselves (0.00813 to ~0.11382), as well as to Picg2 (0.20325 to ~0.24390), Picg4 (0.19151 to ~0.22764) and Psem (0.21138 to ~0.24390), than to Picg1 (0.31707 to ~0.42276) and PpPR10 (0.34146 to ~0.38211). PmPR10-2.1 is grouped with Picg1 in Cluster II, which shows closer $\rho$-distance (0.10569) than to other PR10s from the same species or other conifers (0.31707 to ~0.42276). Cluster III contains PmPR10-3s and PpPR10. The $\rho$-distances among three PmPR10-3s ranged from 0.03252 to 0.11382, and are clearly separated from other PmPR10s (0.30081 to ~0.35772). However, PpPR10 showed closer $\rho$-distances (0.17073 to ~0.21138) to PmPR10-3s than to any PR10 from other conifers (0.30081 to ~0.38211). Therefore, the three PmPR10 clusters in the phylogenetic tree (Figure 2) correspond to their three protein subfamilies. The analysis suggests that these three PR10 ancestor genes arose from earlier DNA duplication and mutation may have been present in the common ancestor of conifers about 300 to 350 million years ago.

Expression of PmPR10-2 and PmPR10-3 transcripts in P. monticola

To determine the spatial expression of PmPR10 genes in P. monticola, RT-PCR analysis was carried out with total RNA extracted from roots, stems, needles and vegetative shoots collected under different growing conditions. The mRNA of the PmPR10-1.10 gene was detected in different organs under favorable growing conditions (growth chamber at 24 °C) and under dormant conditions in winter. Its induced expression was also observed in needles in response to wounding and in stems after exposure to low temperatures (5 °C) for 24 h (Figure 3). Northern blot analysis showed a similar pattern to the RT-PCR data when the 3'-UTR region of PmPR10-1.10 was used as a DNA probe for hybridization (data not shown). In contrast, neither PmPR10-2 nor PmPR10-3 was constitutively expressed in plant vegetative organs. The PmPR10-2 transcript was induced in needles by mechanical wounding, not by other environmental stresses such as low temperature and natural cold-acclimation during winter (Figure 3). PmPR10-3 mRNA accumulated in needles in response to both mechanical wounding and natural cold-acclimation during winter (Figure 3).

Ratios of nonsynonymous to synonymous substitutions in PmPR10 proteins

Nonsynonymous to synonymous substitution ratios (Ka/Ks) were analyzed for all pairwise comparisons of PmPR10 genes within the PmPR10-1 and PmPR10-3 subfamilies. In 94 pairwise nucleotide sequence comparisons of PmPR10 coding regions, ten had Ka/Ks ratios larger than 1.0 (Figure 4). For all pairwise comparisons of the three PmPR10-3s, Ka/Ks ratios increased to 2.74, 3.26 and $\infty$. In the absence of selective pressure on codon usage, Ka/Ks ratios were predicted to be 1.0. A Ka/Ks ratio significantly larger than 1.0 indicates that a gene or gene fragment has undergone adaptive selection during evolution (Messier and Stewart 1997, Zhang et al. 1997, Yang and Nielsen 1998). The analysis of Ka/Ks ratios in PR10 proteins (Figure 4) indicate that some PmPR10 genes, especially those in subfamily III, experienced strong, steady selective pressure.

To obtain insight on how adaptive selection may have affected conifer PR10 proteins, we modeled the 3-D structure of PmPR10-3 protein. A Y-shaped hydrophobic pocket similar to that of Bet v 1 was detected, and is formed by three $\alpha$-helices and one seven-$\beta$-sheet. Mapping the detected amino acid residue substitutions onto the 3-D structure of PmPR10-3 revealed that substitutions occurred in the binding site of this Y-shaped pocket. All four substitutions are nonsynonymous between PmPR10-3.2 and PmPR10-3.3. The first two of these nonsynonymous changes (Y74 to D74 and L76 to I76) occurred in the $\beta$-sheet. Mapping the detected amino acid residue substitutions onto the 3-D structure of PmPR10-3 revealed that substitutions occurred in the binding site of this Y-shaped pocket. All four substitutions are nonsynonymous between PmPR10-3.1 and PmPR10-3.2, 13 of which were detected at 0 (Ck) and 24 h post-treatment (W). Controls corresponding to total RNA templates (negative controls) are not shown because they consistently produced no bands.
tected in the seven-β-sheet. A neutral rate test for the PmPR10 subfamily III demonstrated that Ka was significantly greater than Ks. These analyses suggest that the putative ligand-binding site of PmPR10 proteins may have undergone rapid adaptive evolution.

Discussion

We identified PR10 genes up-regulated during plant defense response to wounding. The GSP-1 primer designed for differential RT-PCR, which was targeted at the conserved P-loop region, is 28 bases long and has a high G+C content at its 5'-terminal region. We set the primer annealing and extension steps at a lower temperature than the standard PCR conditions (65 °C instead of 68 °C) over an extended time (3 min instead of 30 s) to enable us to clone two novel subfamilies of PR10 cDNAs that had low homology with the GSP-1 primer in the critical 3'-terminal region.

Alignment analysis of deduced amino acid sequences, including those identified in this and other studies with other conifers and also angiosperms, showed that the P-loop motif and other amino acids essential for ribonuclease activity are present in the polypeptides of western white pine. Eighteen functional PR10 genes were isolated in western white pine. All sequences share high homology with PR10 proteins isolated from other species (Figure 1). Their predicted 3-D structure and other structural characteristics are similar to those of other PR10 proteins that have been shown to have enzyme functions or ligand-binding activities. However, biochemical functions such as putative ribonuclease activity or ligand-binding ability of these PmPR10 proteins remain to be explored.

A phylogenetic gene tree, which was constructed by alignment analysis of PR10 protein sequences isolated from various species (Figure 2), suggests that plant PR10 genes originated from a common ancestor. In this phylogenetic tree, PR10 genes from seed plants form two major clades corresponding to the angiosperm and conifer groups. The conifer monophyletic group subdivides into three distinct clusters that include PR10 genes from several conifer species. For example, cluster III contains PR10 members from both western white pine and white spruce, and different PR10 proteins from a single species, such as western white pine, may be divided among more than one cluster. This pattern suggests that duplication of an ancestral PR10 gene may have occurred prior to the divergence of Pinus and Picea. This gene duplication may have resulted in the progenitor of PmPR10 subfamily I on the one hand and the progenitor of subfamilies II and III on the other. Therefore, the most recent common ancestor of Pinus, Picea and Pseudotsuga would contain distinct subfamilies of PR10 genes, and extant descendants of those PR10 genes remain in the genome of western white pine today. During early evolution of flowering plants, an ancestral phylogenetic burst might have occurred, resulting in PR10 gene duplication or PR10 gene alleles.

More than 100 PR10 or PR10-related sequences have been identified from various flowering plants, but the physiological functions of PR10 proteins have been poorly explored. The 3-D structures of birch Bet v 1 (Gajhede et al. 1996) and yellow lupine (Lupinus luteus L.). PR10 protein (Biesiadka et al. 2002) show that E96, E148 and Y150 (as positioned in Bet v 1) are close together, and are predicted to form the active site of ribonuclease. Associated with ribonuclease-like activity, the P-loop (GxGGxGxxK) motif is involved in ATP or GTP binding. However, RNase activity was detected in only a few PR10 proteins. Recent data indicate that not all PR10 proteins possess this catalytic property. For example, LlPR10.1B has some RNase activity, but its homologous protein from the same species, LlPR10.1A, has shown none at all (Biesiadka et al. 2002). The recombinant protein of PmPR10-1.10 also lacks RNase activity (Liu and Ekramoddoullah, unpublished observations). Our work indicates that PmPR10 proteins comprise a highly diverse family, with complex expression patterns (Figure 3, Liu et al. 2003). Therefore, it is reasonable to postulate that some PmPR10 proteins have evolved the capacity for catalytic activity in response to diversifying pressure.

Apart from RNase activity (Bufe et al. 1996, Swoboda et al. 1996, Bantignies et al. 2000), cytokinin-specific binding activity has been detected in PR10 proteins from Vigna radiata (L.) R. Wilcz. (Fujimoto et al. 1998) and Physcomitrella patens (Hedw.) Bruch & Schimp. (Gonnau et al. 2001). Cytokinins are plant growth hormones that control differentiation and proliferation of plant cells. Recently, it has been shown that PR10 protein Bet v 1 has the capacity to bind a range of physiologically important ligands, including fatty acids, flavonoids and cytokinins (Mogensen et al. 2002). The Arabidopsis receptor kinase (BR11) has an extracellular leucine-rich repeat and a transmembrane domain that are involved in the binding of brassinosteroids (He et al. 2000). PR10 proteins may act in the plant cell as a carrier to move ligands to their soluble receptors, both increasing ligand concentration and providing the means for specific interaction (Markovic-Housley et al. 2003). This suggests a transport or storage function for PR10 protein that may be involved in signaling of phytohormones (Mogensen et al. 2002). A 30-Å-deep, Y-shaped, hydrophobic pocket in the crystal structure of birch pollen allergen Bet v 11 is responsible for specific non-covalent interaction with apolar ligands (Markovic-Housley et al. 2003). We found a putative ligand-binding site in the 3-D structure of PmPR10-3 protein that is similar to those of Bet v 11 and Pru av 1 (data not shown). After mapping onto the 3-D structure of PmPR10-3, we identified some amino acid residues subject to adaptive replacements in the putative binding site of this Y-shaped pocket. These analyses suggest that the putative ligand-binding site of PmPR10-3 proteins might have been under positive selective pressure during evolution, allowing a homologous PR10 protein to obtain a new function with little change in 3-D structure.

During the evolution of this multiple-gene family, different members may have been co-opted to serve additional functions. In the multiple family of winter rye PR3-protein chitinases, two cold-induced members possess antifreeze activity, whereas other pathogen-induced members have enzymatic activity only (Yeh et al. 2000). An antifreeze protein in carrot
shares sequence similarity with polygalacturonase-inhibitor proteins (Worrall et al. 1998). In western white pine, at least 12 PmPR10 proteins are differentially expressed in response to different environmental stresses (Liu et al. 2003). It is believed that the role of ligand binding and transport in PR10 proteins may be crucial to the plant-defense response to pathological situations, as well as being important for growth and development (Markovic-Housley et al. 2003). Studies on ligand-binding affinity and specificity and genetic mutants at the putative binding site of PR10 proteins should yield interesting information about the mechanism(s) underlying the evolution and functional adaptation of PR10 proteins in plants.

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