Characterization of Pin m III cDNA in western white pine

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Summary  Maximum accumulation of Pin m III protein in western white pine (Pinus monticola Dougl. ex D. Don) needles occurred during the winter months. To characterize Pin m III, an expression cDNA library from poly(A)+ mRNA of needles was immunoscreened and the full length cDNA was cloned. An open reading frame of 486 bases encodes a protein of 161 amino acid residues with a molecular mass of 18 kD and a predicted isoelectric point of 5.5. The deduced amino acid sequence had some similarities (37%) with an intracellular PR protein from garden asparagus (Asparagus officinalis L.) and the major pollen allergen from white birch (Betula verrucosa J. F. Ehrh.), which are members of the ribonuclease-like PR-10 family. Phylogenetic analysis provided circumstantial evidence that Pin m III may be grouped with intracellular PRs from asparagus and potato (Solanum tuberosum L.), while the allergens formed another subgroup. Northern analysis showed that the Pin m III gene was preferentially expressed during cold acclimation with the highest expression in the fall and winter months, preceding the peak of Pin m III protein accumulation. Tissue specificity expression analysis indicated that the gene was strongly expressed in roots and twigs. Higher amounts of the homologous protein (Pin l I) and its transcript accumulated in sugar pine (Pinus lambertiana Dougl.) needles infected with blister rust compared with healthy needles.

Keywords: intracellular PR protein, PR-10 family, seasonal regulation, sugar pine, white pine blister rust.

Introduction

Western white pine (Pinus monticola Dougl. ex D. Don) is a commercially important, fast-growing species widely distributed in the Pacific Northwest, Idaho and Montana. Although this species has been devastated by the white pine blister rust, it has recently been reintroduced as a commercial species because of its high wood quality and resistance to laminated root rot (Phellinus weirii Murr.).

White pine blister rust is caused by the fungus Cronartium ribicola J.C. Fisch. ex Raben, which has five spore stages on two unrelated hosts, western white pine and Ribes spp. (currants, gooseberries). Basidiospores are produced on Ribes in the fall and are dispersed by wind to western white pine foliage, where they germinate and infect the needles through the stomata, producing an infection spot. Hyphae grow down the needles into the bark, causing perennial cankers that eventually kill the tree by the formation of canker girdles around the main stem. Although some large older trees can survive the infection, younger trees almost always die.

Several types of plant proteins are induced in response to both abiotic and biotic stresses including vegetative storage proteins, heat shock proteins and pathogenesis-related proteins (Hightower 1991, Linthorst 1991, Mason and Mullet 1990, Ekramoddoullah et al. 1995, Sabehat et al. 1996). Pathogenesis-related proteins (PRs) are a heterogeneous group of host-encoded proteins that are induced following exposure to pathogens such as viruses, bacteria and fungi or certain chemicals (van Loon et al. 1994). On initiation of plant systemic acquired resistance, PRs are induced and account for part of the multicomponent resistance system (Ward et al. 1991). Van Loon et al. (1994) classified PRs into 11 families. Among them, PR-10 is defined as a family of intracellular defense-related proteins with a ribonuclease-like structure, typified by parsley (Petroselinum crispum (Mill.) Nyman ex A.W. Hill) “PR1” (van Loon et al. 1994). The PR-10 is a ubiquitous family of conserved proteins found as intracellularly localized, acidic, low molecular mass (16–19 kDa) proteins (Walter et al. 1990). Members of PR-10 include STH-2 in potato (Solanum tuberosum L.) (Matton and Brisson 1989), garden asparagus (Asparagus officinalis L.) PR1 (Warner et al. 1992), birch (Betula sp.) major pollen allergen BET V I (Breiteneder et al. 1989), and bean (Phaseolus vulgaris L.) PR1 and PR2 (Walter et al. 1990). Some members of PR-10 have ribonuclease activities (Moiseyev et al. 1994, Swoboda et al. 1995, Bufer et al. 1996). Members of the PR-10 family are transcriptionally upregulated following pathogen infection, treatment with elicitors, wounding or in response to various stresses (Walter et al. 1990, Paakkonen et al. 1998).

Recently, a 19-kDa protein Pin I I was detected in sugar pine (Pinus lambertiana Doug.) foliage in increasing amounts in autumn (Ekramoddoullah et al. 1995). A homolog of this protein, named Pin m III (Ekramoddoullah et al. 1995), was identified in western white pine foliage. The N-terminal amino acid sequence of Pin m III was 89% similar to that of Pin I I...
(Ekramoddoullah and Taylor 1996). The accumulation of Pin m III in western white pine seedlings is seasonally regulated, increasing during the fall and reaching a maximum in winter, then declining to a minimum in the summer (Ekramoddoullah et al. 1995). The amount of Pin m III is also correlated with frost hardiness of western white pine (Ekramoddoullah et al. 1995). Because Pin m III accumulates in western white pine trees inoculated with blister rust fungus (Ekramoddoullah et al. 1998), we investigated its possible relationship to known pathogenesis-related proteins, by cloning and characterizing the gene encoding Pin m III. An expression cDNA library from poly(A)+ mRNA of winter needles was generated and the Pin m III cDNA was cloned by antibody screening. BLAST search and subsequent sequence comparisons indicate that Pin m III may be a member of the intracellular PR-10 protein family. We report the complete sequence of the cDNA encoding Pin m III from western white pine, its possible phylogenetic relationship to other angiosperm PR-10 proteins, analysis of its temporal and spatial expression, and its expression pattern in infected and healthy foliage of resistant and susceptible plants.

Materials and methods

Plant material collection and treatment

Total RNA was extracted from foliage samples of western white pine originating from Seedlots 2881, 2888 and 3159 as previously described (Ekramoddoullah et al. 1995). Current-year needle samples used for the expression cDNA library were collected in January 1996 from a western white pine tree growing at the University of Victoria (Victoria, British Columbia, Canada). For the analysis of tissue-specific expression, current-year and 1-year-old needles and their corresponding twigs and roots were harvested from 2-year-old white pine seedlings. Samples were collected in September 1996 from an open-sided greenhouse (shelter house) at the Pacific Forestry Centre (Seedlot 3144, Victoria, British Columbia, Canada), frozen in liquid nitrogen and lyophilized.

For the analysis of disease-specific expression, 4-year-old sugar pine seedlings that were inoculated with blister rust fungus in August 1995 were used. Samples were collected in September 1996 for the extraction of total RNA and protein. Sugar pine needles from resistant (R4) or susceptible (S4) seedlings were harvested in December 1996 for the extraction of total RNA and protein. The remaining spotless portions of the infected needles were also pooled and analyzed. Pycnia and aecia were visible on the susceptible tree (S4) in 1998, whereas no cankers were observed on the stem of the resistant tree (R4).

RNA extraction

To construct the cDNA library, total RNA was extracted from 10 g of current-year western white pine needles as previously described (Schultz et al. 1994). Needles were ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was transferred to cooled centrifugation tubes with extraction buffer and further homogenized in a polytron. Poly(A)+ mRNA was isolated by using the mRNA Purification Kit from Pharmacia Biotech according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). For northern blot analysis, total RNA was extracted by the method of Wang and Vodkin (1994). The RNA concentration was determined spectrophotometrically and confirmed by agarose gel electrophoresis.

Construction of expression cDNA library and antibody screening of the library

An expression cDNA library was constructed in λgt22A from poly(A)+ mRNA of western white pine needles, according to the manufacturer’s protocol (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). The library was screened with a polyclonal antibody against a synthetic N-terminal (17 amino acid residue) peptide of the sugar pine protein Pin I that cross reacted with Pin m III (Ekramoddoullah et al. 1995). In the first 17 amino acid residues, Pin m III and Pin I differed in a single amino acid at position 13 (Ekramoddoullah et al. 1995, Ekramoddoullah and Taylor 1996). Immunoscreening was conducted following Stratagene’s picoBlueTM immuno-screening instruction manual (Stratagene, La Jolla, CA) with some modifications; 5% skim milk powder (5% w/v in TBS) was used as a blocking agent and incubated overnight. Three initial positive plaques were identified and one was purified through three rounds of screening. The cDNA insert was subcloned into pBluescript KS+ (Stratagene).

Northern blot analysis

For northern blot analysis, 20 μg of total RNA per lane was separated on 1.2% agarose gels containing 2.2 M formaldehyde (Sambrook et al. 1989), photographed, and transferred to Zeta-Probe GT membranes according to the manufacturers instructions (BIO-RAD, Mississauga, Ontario, Canada). The Pin m III cDNA insert and the 18S rRNA gene insert were labeled with the random primers DNA labeling system with [α-32P]dCTP and hybridized according to the manufacturer’s instructions (Gibco BRL). The membranes were washed under high stringency according to the manufacturer’s instructions (BIO-RAD). Membranes were exposed to BioMax film (Kodak, Rochester, NY) for 3.5 hours for the 18S rRNA gene probe, and for 3–7 days for the Pin m III probe with intensifying screens at −80 °C. Northern blot analysis was repeated three times for the analysis of the seasonal expression and repeated twice for the analyses of tissue specificity and disease response. The autoradiograms of northern blots were scanned with a laser scanner (Model 110A, Molecular Dynamics, Sunnyvale, CA) interfaced with a workstation (SPARK 1, Sun Microsystems of Canada Inc., Vancouver, British Columbia,
Genomic DNA extraction and southern analysis

Genomic DNA was extracted from freeze-dried current-year foliage samples by the CTAB method (Wagner et al. 1987). Thirty μg of DNA was digested with restriction enzyme overnight at 37 °C, fractionated through 0.8% agarose gels and blotted onto Zeta-Probe GT membranes. The Pin m III cDNA insert (Eco RI fragment) was labeled, hybridized and washed as described for the northern blot analysis. Southern analysis was repeated three times.

DNA sequencing

Lambda DNA was purified by the QIAGEN Lambda Maxi Kit (QIAGEN GmbH, Hilden, Germany). The inserts from the λgt22A vector were purified by fractionating the digestion mixture by electrophoresis on 1% agarose and extracting the insert by QIAquick Gel Extraction Kit (QIAGEN GmbH). The inserts from the cDNA insert by QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). The inserts from the cDNA insert (Eco RI fragment) was labeled, hybridized and washed as described for the northern blot analysis. Southern analysis was repeated three times.

Western immunoblot

Extraction of proteins for tissue specificity and sugar pine needle infection analysis was carried out as described by Ekramoddoullah and Hunt (1993). Polyclonal antibody against the Pin l I N-terminal 17 amino acid residues was used in the western blot analysis of Pin m III as described previously (Ekramoddoullah et al. 1996).

Results

Cloning and characterization of Pin m III cDNA

Immunoscreening of the λgt22A western white pine needle cDNA expression library with the polyclonal antibody against synthetic N-terminal Pin I I peptide identified three positive clones in the first round. The second and third rounds of immunoscreening of one of the three clones confirmed a specific signal. After three rounds of consecutive screening, the sequence of a cDNA encoding the Pin m III protein was obtained. The Pin m III cDNA sequence was 806 bp in length with a 5′ untranslated region of 54 bp, a 486-bp open reading frame encoding the Pin m III protein, and a 266-bp 3′ untranslated region including the poly(A) tail (Figure 1). The deduced amino acid sequence of Pin m III had two more strongly acidic (negatively charged) residues (D, E) than strongly basic (positively charged) amino acids (K, R) (22 acidic versus 20 basic amino acids). It had 50 hydrophobic amino acids (A, I, L, F, W, V) and 44 polar amino acids (N, C, Q, S, T, Y). The predicted isolectric point is 5.5, which is consistent with the pI value of 5.4 observed in 2-dimensional gel electrophoresis analysis of Pin m III (Ekramoddoullah et al. 1996). At pH 7.0 the predicted charge is ~2.024. Hydrophobicity analysis by the Kyte-Doolittle method (Kyte and Doolittle 1982) predicted that the protein is hydrophilic (data not shown).

Figure 1. Complete nucleotide (nt) sequence of Pin m III and the predicted amino acid (aa) sequence. The aa sequence is shown in single letter code under the nt sequence. The open reading frame is capitalized and the 5′ and 3′ untranslated regions are in lower case. The start codon and its surrounding consensus regions are underlined. The 17 underlined and italicized amino acids were used in synthesizing the peptide for the polyclonal antibody [N, instead of V underlined. The 22 amino acid sequence derived from microsequencing is italicized and the 5′ untranslated region contained a putative polyadenylation signal (AATAAA) located at position 590 and the poly(A) tail was located 221 nucleotides downstream from the TAG translation termination codon. The deduced amino acid sequence of Pin m III had two more strongly acidic (negatively charged) residues (D, E) than strongly basic (positively charged) amino acids (K, R) (22 acidic versus 20 basic amino acids). It had 50 hydrophobic amino acids (A, I, L, F, W, V) and 44 polar amino acids (N, C, Q, S, T, Y). The predicted isolectric point is 5.5, which is consistent with the pI value of 5.4 observed in 2-dimensional gel electrophoresis analysis of Pin m III (Ekramoddoullah et al. 1996). At pH 7.0 the predicted charge is ~2.024. Hydrophobicity analysis by the Kyte-Doolittle method (Kyte and Doolittle 1982) predicted that the protein is hydrophilic (data not shown).

Pin m III is similar to AoPR1 and Bet v I and is a member of the PR-10 family

A BLAST search (Altschul et al. 1997) of the protein databases revealed similarities among the sequences of Pin m III and a group of intracellular pathogenesis-related (PR) proteins and major tree pollen allergens (Figures 2 and 3). Pin m III shares 22 to 36.7% amino acid similarity with the representative intracellular PR and allergen family members from angio-
sperm plants. Highest similarity is with PR1-ASPOF (AoPR1) from asparagus (36.7%). The intracellular PRs form a phylogenetic subgroup (except the celery (Apium graveolens L.) allergen), and the allergens form another subgroup (Figure 3).

A small multigene family may code for PR-10 in western white pine

Thirty µg of genomic DNA extracted from foliage samples was digested with the restriction enzymes Xba I, BamHI, Hind III or EcoRI and analyzed by Southern blot hybridization with the PR-10 sequence. On DNA blots the Xba I digestion gave at least seven bands (Figure 4, Lane 1), whereas Hind III and EcoRI digestion gave at least four bands (Figure 4, Lanes 3 and 4). BamHI digestion also had at least four distinct bands (on another repeated blot). Thus, we have preliminary evidence that the western white pine genome has a small family coding for PR-10-related sequences. Eastern white pine (Pinus strobus L.) genome had a similar banding pattern to Pinus strobus. Pine genome had a similar banding pattern to western white pine when digested with Hind III (Figure 4, Lane 5) had twice as much transcript (based on the densitometric analysis, data not shown). The expression of Pin m III in the current-year and previous year seedling sample, the amounts of transcript and protein in stem (stems) samples from the current-year and previous year lot 3144 were harvested in September 1996. Foliar and twig residues are indicated by an asterisk. Homologous amino acid differences are indicated by an asterisk.

Expression of the Pin m III transcripts and protein in healthy and diseased tissues

To study the expression of Pin m III in different tissues of western white pine seedlings, 2-year-old seedlings from Seedlot 3144 were harvested in September 1996. Foliar and twig (stems) samples from the current-year and previous year (1 year older) and the mixed root systems from the seedlings were collected and analyzed for amounts of transcript and protein. Because the samples were collected in September, the expression of Pin m III was low in the foliage (Figures 5 and 6). There was a large accumulation of Pin m III transcripts in the root system and the corresponding Pin m III protein concentration was higher in the root system than in the other tissues analyzed (Figure 7, Lane 5; Figure 8, Lane 5). The amounts of transcript and protein in the foliage and stem were low compared with the root system (Figures 7 and 8). From the same seedling sample, the amounts of transcript and protein in stem samples (Figure 7, Panel A, Lanes 3 and 4; Figure 8, Lanes 3 and 4) were higher than in foliage samples (Figure 7, Panel A, Lane 1; Figure 8, Lanes 1 and 2). Compared with the October foliage sample of Seedlot 2888 collected in 1992 (Figure 7, Panel A, Lane 6), which had the highest amount of transcript (Figure 6, Lane 3), the seedling root system (Figure 7, Panel A, Lane 5) had twice as much transcript (based on the densitometric analysis, data not shown). The expression of Pin m III was highest in roots, followed by stems and then foliage.

To investigate the role of Pin m III in disease resistance, the sugar pine–white pine blister rust pathosystem was used. In...
sugar pine, resistant trees carry the dominant R gene, as homozygous (RR) or heterozygous (Rr), which confers resistance to white pine blister rust (Kinloch et al. 1970). Healthy or in-

Figure 3. Phylogenetic tree of 30 representative members of the intra-

Figure 4. DNA blot analysis of genomic DNA from western white pine, related pine species and tobacco. Thirty µg of genomic DNA was used in each overnight digestion. Lane 1, western white pine DNA cut with *Xba* I; Lane 2, *BamH* I; Lane 3, *Hind* III; Lane 4, *EcoR* I; Lane 5, eastern white pine genomic DNA digested with *Hind* III; Lane 6, ponderosa pine genomic DNA digested with *Hind* III; Lane 7, tobacco genomic DNA digested with *Hind* III; Lane 8, western white pine genomic DNA cut with *Hind* III (same as Lane 3).
Infected needles from 4-year-old sugar pine seedlings were collected and used in northern or western analysis. In both susceptible and resistant sugar pine seedlings, the amounts of protein and transcript of Pin l I (homologue of Pin m III) were higher in infected needles than in healthy needles (Figure 7, Panel B; Figure 8, Lanes 6–11). The susceptible sugar pine showed higher amounts of Pin l I transcript and protein than the resistant sugar pine, indicating that the expression of Pin l I and the amounts of Pin l I transcript and protein were correlated with the severity of the fungal infection.

Discussion

During investigations of the host–pathogen interaction of the white pine blister rust pathosystem (Ekramoddoullah and Hunt 1993), a sugar pine protein, Pin l I, was detected in in-
creases amounts in the fall (Ekramoddoullah et al. 1995). Its homolog, Pin m III, was detected in western white pine using an anti-Pin l I antibody. Furthermore, the concentration of Pin m III, which reached its maximum in winter months, was significantly correlated with frost hardiness of western white pine foliage (Ekramoddoullah et al. 1995). To characterize the Pin m III protein, we constructed an expression cDNA library and isolated full-length cDNAs. Sequence analysis revealed some similarities between the deduced Pin m III polypeptide and the PR-10 family of ribonuclease-like PR proteins, including major tree pollen allergens such as BV1A-BETVE (Bet v I) from birch pollen (Breiteneder et al. 1989) and asparagus intracellular PR proteins PR1-ASPOF (Warner et al. 1992). The intracellular PRs grouped together, whereas the allergens formed a second group, suggesting a possible divergence of functions inherent to the groups as shown in the phylogenetic tree in Figure 3. Pin m III is most closely related to the PR1-ASPOF (Warner et al. 1992) from asparagus (36.7% similarity).

Intracellular PR proteins are known to be up-regulated in plants by pathogens or by treatment of cell cultures with microbial elicitors (Warner et al. 1992). In Medicago sativa L., accumulation of MsPR10-1 transcript increased in leaf blades during an incompatible interaction and the expression of this gene was induced by salicylic acid treatment of the leaves (Breda et al. 1996). Furthermore, during the incompatible interaction with P. syringae pv. pisi, several genes or allelic variants of PR-10 class were expressed. These findings are indicative of a close correlation between PR mRNA accumulation and the disease-resistance response of the plant and the restriction of fungal growth.

In the sugar pine model, infection analysis suggested that Pin l I is upregulated at the mRNA and protein levels in both resistant and susceptible sugar pine seedlings (Figures 7 and 8). Ekramoddoullah et al. (1998) also observed a high amount of Pin m III protein in infected spots and cankered tissues of a large number of infected mature and young western white pine trees. Although there is no evidence that Pin m III or its homolog provides protection against fungal infection, there is sequence similarity with known intracellular PR proteins, such as the PR protein from potato (Matton and Brisson 1989). Furthermore, the expression of many PR proteins is temporal and spatial, particularly in infection sites, suggesting a defensive role of Pin m III in white pine blister rust pathosystem. It is worth noting that chitinase, a well-known PR protein, was detected in large amounts in a susceptible rice cultivar (Anuratha et al. 1996) and in susceptible western white pine (Davidson and Ekramoddoullah 1997).

The timing of seasonal Pin m III accumulation parallels the accumulation of vegetative storage proteins, which store nitrogen over winter for mobilization for new growth in the spring (Wetzel et al. 1989, Wetzel and Greenwood 1991, Roberts et al. 1991). However, Pin m III lacks the properties of vegetative storage proteins, which are usually basic, 30–32 kDa in size and are rich in glutamine and asparagine. The hydophobicity of Pin m III is an important characteristic of cryoprotectants. Thus the accumulation of Pin m III in winter (Ekramoddoullah et al. 1995, 1996) may reflect its role as an antifreeze protein.

The finding that Pin m III, which normally accumulates in winter months, can also be induced by a fungal pathogen is similar to some pathogenesis-related proteins that are induced by both biotic and abiotic stress (Lin et al. 1996). There may be a common receptor that perceives signals by two different stresses. For example, in overwintering plants that have to survive frost, the ability to limit water potential could be important for survival. It has been suggested that regulation of water potential by host plants restricts microbial colonization (Pearce 1996). Overwintering grasses that have to survive freezing temperature and desiccation are known to have increased resistance to fungal diseases (Tronsmo 1984, 1985, Tronsmo et al. 1993). Based on the genotypic correlation between freezing tolerance and resistance to the snow molds Typhula ishikariensis Imai (Tish) and Fusarium nivale (Fr.)

Figure 8. Western analysis of Pin m III in tissues of healthy western white pine seedlings (Seedlot 3144) and its homolog in infected and healthy foliage of sugar pine in September 1996. Tissues were Lane 1, current-year foliage of western white pine; Lane 2, one-year-old foliage; Lane 3, current-year twig; Lane 4, 1-year-old twig; Lane 5, seedling root system; Lane 6, sugar pine Seedlot S4 (susceptible) infected needles infection spots; Lane 7, Seedlot S4 infected needles spotless regions; Lane 8, Seedlot S4 healthy needles; Lane 9, Seedlot R4 (resistant) infected needles infection spots; Lane 10, Seedlot R4 infected needles spotless regions; Lane 11, Seedlot R4 healthy needles.
Ces. (Tronsmo et al. 1993), Hon et al. (1995) suggested that the same genetic trait(s) may be involved in both disease and freezing tolerance.

We postulate that the Pin m III gene encodes a member of the PR-10 family of intracellular PR proteins. It is seasonally regulated and is upregulated by Cronartium ribicola.

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