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Root-specific expression of a western white pine PR10 gene is mediated by different promoter regions in transgenic tobacco⋆

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Abstract

We report here the isolation and characterization of a novel PR10 gene, *PmPR10-1.14*, from western white pine (*Pinus monticola* Dougl. ex. D. Don). The *PmPR10-1.14* gene encodes a polypeptide exhibiting high similarity with other members of the PR10 family and corresponds to one of six isoforms immunodetected in the roots of western white pine. Northern blot and western immunoblot analyses showed that expression of the PR10 gene family, including *PmPR10-1.14*, was detected in vegetative tissues constitutively, but not in developing reproductive organs. RT-PCR with gene-specific primers showed that the transcript of *PmPR10-1.14* gene was found only in lateral roots and needles during growth. To study PR10 gene regulation at the cellular level, *PmPR10-1.14* promoter was fused to the β-glucuronidase (*GUS*) report gene, and analyzed for transient and stable gene expression. The transient expression assays in agroinfiltrated tobacco leaves indicated that the core promoter of *PmPR10-1.14* gene resided in the sequence from -101 to $+69$ relative to the first nucleotide of PR10 cDNA. Furthermore, the promoter region from -311 to -101 acted as an enhancer, and the region from -506 to -311 as a silencer. Fluorometric GUS assays of transgenic tobacco plants demonstrated that the longest promoter of 1675 bp directed GUS expression constitutively at high levels in the roots of mature plants, but expression levels were too low to be detectable in other organs in histochemical assays. Histochemical localization analysis showed that *PmPR10-1.14* promoter directed a tissue-specific expression exclusively during the initiation and development of the lateral roots. The distal 5′ deletion of the promoter to -311 did not decrease the expression level significantly in the roots, suggesting that the *cis*-regulatory elements necessary for a high level of gene expression reside in the proximal fragment from -311 to $+69$. As one striking feature, *PmPR10-1.14* promoter contains two copies of direct repeated sequences as long as 281 bp at its distal 5′ region. Deletion of one copy (-1326 to -1045) or both copies (-1675 to -1045) of the repeated sequences increased gene expression significantly in leaves and stems, which was regulated developmentally. Further deletion to -820 erased the increased gene expression in leaves and stems. These experiments revealed that the root-specific expression of *PmPR10-1.14* gene is mediated by different promoter regions with both negative and positive regulatory mechanisms in transgenic tobacco plants.

Abbreviations: DIG, digoxygenin; GUS, β-glucuronidase; PR, pathogenesis-related; X-Gluc, 5-bromo-4- chloro-3-indolyl-β-glucuronide

Introduction

Pathogenesis-related (PR) proteins are well known proteins induced by pathogens and related abiotic stresses. Based on the sequence homology, serological relationships and biological activity, the PR proteins are grouped into eleven families (van Loon *et al.*, 1994). A particularly interesting family of PR proteins is the PR10 class. PR10 proteins are small, primarily acidic intracellular proteins of 15–18 kDa (Linthorst,

1991). PR10 proteins have been identified in a broad range of flowering plants. Many biotic and abiotic stresses have been shown to activate PR10 protein expression transcriptionally, suggesting their important roles during plant defense response (Matton and Brisson, 1989; Somssich *et al.*, 1988; Walter *et al.*, 1990; Warner *et al.*, 1992; Walter *et al.*, 1996). The structure of the birch pollen PR10 protein Betv1 determined by X-ray and NMR indicated the presence of a P-loop structure with the amino acid sequence GXGGXG that resembled a motif found in many nucleotide-binding proteins (Gajhede *et al.*, 1996). Recently some PR10 proteins with ribonuclease activity have been detected in birch pollens (Bufe *et al.*, 1996; Swoboda *et al.*, 1996) and lupine roots (Bantignies *et al.*, 2000), and PR10 protein with cytokinin binding ability has been detected in mung bean (Fujimoto *et al.*, 1998) and moss (Gonneau *et al.*, 2001). Despite their apparent importance in plant defense responses, the exact biological functions of the PR10 protein family are still unknown.

In addition to the proposed functions in plant defense response, PR10 proteins are also involved in plant development. Some PR10 proteins display constitutive expression patterns that are unrelated to the pathogenic response, and they are frequently subject to developmental control in healthy plants. As allergens, high levels of PR10 proteins are found in birch pollens (Breiteneder *et al.*, 1989), apple (Vanek- Krebitz *et al.*, 1995), and celery (Breiteneder *et al.*, 1995). It has been shown that PR10 proteins are developmentally regulated in different plant tissues and organs. In angiosperms PR10 proteins have been shown to be expressed constitutively in the reproductive organs of different flower parts (Breiteneder *et al.*, 1989; Warner *et al.*, 1993,1994; Constabel and Brisson, 1995; Swoboda *et al.*, 1996; Huang *et al.*, 1997) and seeds (Barratt and Clark, 1991; Warner *et al.*, 1994) and in the vegetative organs of roots (Crowell *et al.*, 1992; Mylona *et al.*, 1994; Walter *et al.*, 1996; Sikorski *et al.*, 1999), stems (Warner *et al.*, 1994) and leaves (Crowell *et al.*, 1992).

Our interest in conifer PR10 proteins and genes began with the identification of a PR10 protein (Pin l I) in sugar pine (*Pinus lambertiana* Dougl.) during the study of the host-pathogen interaction of the white pine/blister rust pathosystem (Ekramoddoullah *et al.*, 1995). Using an antibody against Pin l I, its homologous PR10 protein (Pin m III) in western white pine (*Pinus monticola*, Dougl. Ex D. Don) was found to be correlated with frost-hardiness of foliage (Ekramoddoullah and Taylor 1996). Subsequently one cDNA encoding for a PR10 protein was cloned and characterized (Yu *et al.*, 2000). Our previous study indicated that PR10 gene family contain multiple members in the genome of western white pine (Yu *et al.*, 2000), and the expression pattern of PR10 genes in healthy and stressed plants differed among conifer species and even among isoforms of the same species of western white pine. The complexity of the multigene family of PR10 proteins and their potential roles under normal and abnormal physiological conditions inspired us to investigate the gene regulation for the spatial and temporal expression pattern of PR10 genes in a conifer species. Here, we report the isolation, genomic organization and molecular characterization of a novel member of the PR10 gene family (*PmPR10-1.14*) in western white pine. We show that the promoter of the *PmPR10-1.14* gene is responsible for root-specific gene regulation in transgenic tobacco.

Material and methods

Plant material

Seedlings of western white pine were grown under natural conditions in the greenhouse, or in a growth chamber with a 16 h day (24 °C) and an 8 h night (20 °C) under mild illumination (2000 lux). Plant tissues were collected from the seedlings under natural winter conditions or growing in a growth chamber. The developing male cones and female cones were collected from mature trees in May 2000 at the Pacific Forestry Centre, Victoria, British Columbia, Canada. Tobacco plants (*Nicotiana tabacum* cv. ws38) were grown in growth chambers for agroinfiltration and gene transformation.

PR10 gene cloning and PCR amplification

Genomic DNA was isolated from current year needles of western white pine with a Plant DNeasy Extraction Kit (Qiagene, Mississauga, Ontario, Canada). We used a two-step PCR strategy to clone the genomic DNA of PR10 genes from western white pine. First, the inverse polymerase chain reaction (IPCR) was performed to get the flanking sequences of the coding region with reverse primer GSP1 and forward primer 3SE1. The primers GSP1 (5′-CCTTGCCTCCACTTGAACCAC CTCTTCCG-3′) and 3SE1 (5′-CCTCTCCAATCCCA ACTTATACTG-3′) hybridize to the sense and antisense strands of the PR10 cDNA of *Pin m III* (Yu

et al., 2000), respectively. Second, a pair of primers were designed based on the flanking sequences obtained above to perform long-distance PCR to clone the whole PR10 gene, including the promoter region, the coding region and the downstream region after the stop codon. The primers used in long-distance PCR for the genomic DNA cloning of full-length PR10 gene were forward primer B43-5′ (5′-AAAAGCTTCTCGAGATGACTCTTTTCCTGTGA CAC-3′) and reverse primer BG3 (5′-CATCGGATAG TATATGGATAGTGG-3′). Genomic DNA (1.5 μg) was digested with appropriate restriction enzymes (*Bam*HI or *Eco*RI), and then circularized with T4 DNA ligase. The IPCR was performed using 100 ng of re-circularized genomic DNA as a template in a final volume of 50 μl. PCR was carried out with an Advantage Genomic PCR Kit (Clontech Laboratories, Palo Alto, CA) using a Perkin-Elmer Thermocycler. Thermal cycling conditions consisted of an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, and primer annealing and extension at 68 °C for 5 min, with a final 10 min extension at 72 °C. PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI), and plasmid constructions and manipulation were carried out by standard methods (Sambrook *et al.*, 1989).

DNA sequences were determined on both strands with an ABI310 DNA sequencer (Applied Biosystems) using a Thermo-cycle sequence kit (Amersham) with a T7, SP6 primer and other internal primers as needed. DNA sequence data were assembled and analyzed with BLAST, ClastalW and ORF finder network services at the National Center for Biotechnology Information (NCBI). The calculation of molecular mass and isoelectric point of PR10 proteins, and the alignment analysis of the PR10 protein family were performed with the programs at http://www.expasy.ch/. A promoter prediction program, at http://www.fruitfly.org/seq_tools/promoter.html, was used for the database search to predict the transcription start site in the promoter sequence of the *PmPR10-1.14* gene. The analysis of potential *cis*-regulatory elements in the promoter sequence of *PmPR10-1.14* gene was performed with a program at http://www.itba.mi.cnr.it/cgi-bin/tradat/tradat_launcher (Quandt *et al.*, 1995).

RNA isolation and analysis

Total RNA was isolated according to the method of Liu *et al.* (1998) with some modifications. There was some genomic DNA in the isolated total RNA, which was removed by LiCl precipitation. For northern blot analysis, total RNA (10 μg) was electrophoresed in a denaturing agarose gel and blotted onto nylon membranes (Hybond-N+, Amersham). A probe from *PmPR10-1.14* coding region labeled with digoxygenin (DIG) using a random primer labeling kit (Boehringer Mannheim) was used to hybridize to the PR10 gene. Hybridizations and washing were carried out according to standard methods (Sambrook *et al.*, 1989). For semiquantitative RT-PCR, total RNA was treated with RQ 1 RNase-Free DNase (Promega) and purified with a Plant RNeasy Extraction Kit (Qiagen). A 2 μg portion of total RNA was reverse-transcribed with the Omniscript Reverse Transcriptase Kit (Qiagen) in a total volume of 20 μl. PCR was carried with 1 μl of cDNA in 25 μl of PCR mix with *Taq* PCR Master Mixture (Qiagen). Based on DNA sequence comparison among PR10 genes from western white pine (GenBank accession numbers AY064193–AY064206), two gene-specific primers of *PmPR10-1.14* spanning the genomic intron were designed, which would allow us to visualize potential contamination of genomic DNA, GB435 (5′-CAACGAAAGACGGCCACG-3′) and GB433 (5′-GGAACCAATTTCACCTCAAG-3′). To monitor the amount of cDNA derived from plant tissues, the Quanta mRNA 18S rRNA Internal Standard Kit (Ambion, Austin, TX, USA) was used in control experiments. RT-PCR condition was determined experimentally as that of incubation at 94 °C for 1 min, followed by 30 cycles (59 °C, 30 s; 72 °C, 90 s; 94 °C, 30 s), and a final extension at 72 °C for 7 min.

Protein extraction and western immunoblot analyses

Total proteins were extracted from tissues of western white pine (Ekramoddoullah and Hunt 1993). The protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), or two-dimensional (2-D) PAGE, and transferred onto a nitrocellulose membrane. For immunodetection, the blots were incubated with polyclonal antibodies against a PR10 protein (Pin l I) from sugar pine as described by Ekramoddoullah *et al.* (1995). Scanning, detection and quantification of the blots were performed with PDI Quantity One software (Version 3.0, PDI, Huntington Station, NY).

Constructs for promoter analyses

To construct binary vectors, PCR was used to introduce appropriate restriction enzyme sites at both 5′ and 3′ ends of the promoter sequence of the *PmPR10-1.14* gene with the forward primer B43-5 and a reverse primer PBS (5′-AAGGATCCGTCGACCATTTTCAA CTCTCTCGCAAC-3′). Primer B43-5 was incorporated with synthetic *Hin*dIII/*Xho*I restriction enzyme sites at its 5′ end. *Bam*HI/*Sal*I restriction enzyme sites were introduced just behind the start codon (ATG) of the coding region in primer PBS. The promoter fragment was cloned into pGEM-T Easy Vector (Promega). A series of 5′ deletions were made with double restriction enzyme digestion of *Xho*I combined with *Bam*HI, *Eco*RI, *Sac*II, or *Nco*I to obtain the promoter deletion at −1045 (BX), −820 (RI), −506 (Sc II), and −311 (Nc), respectively. Single enzyme digestion of *Bam*HI was used to delete one copy of a 281 bp repeated sequence to get promoter deletion BB-Δ-281. The deletion of *PmPR10-1.14* gene to −101 (ST) was made by PCR with primer PBS and an additional forward primer B-101 (5′-AAAAGCTTAGTGGCTTCTCATGCCAT-3′). All deletions were verified by DNA sequencing analysis. *Hin*dIII/*Sal*I-digested promoter fragments were inserted into the polylinker *Hin*dIII/*Sal*I sites of pBI101 vector (Jefferson, 1987; Clontech Laboratories), resulting in chimeric constructs FL (−1675), BB (−Δ−281), BX (−1045), RI (−820), Sc II (−506), Nc (−311), and ST (−101) (Figure 1).

Agroinfiltration and stable transformation of tobacco plants

Plasmid DNA of binary constructs was introduced into *Agrobacterium tumefaciens* (LBA 4404) with a freeze-thaw method. Transient gene expression by agroinfiltration was performed according to the procedure described by Yang *et al.* (2000). The constructs of CaMV 35S promoter::GUS (pBI 121) and promoterless::GUS (pBI 101) were used as positive and negative controls, respectively. Four independent experiments were performed for the quantitative evaluation of transient GUS gene expression driven by *PR10-1.14* promoter fragments. For stable gene expression, tobacco plants were co-cultivated with *A. tumefaciens* by the standard leaf-disk infection method (Horsch *et al.*, 1985). Transformants were selected on Murashige and Skoog's medium supplemented with 200 μg/ml kanamycin and 500 μg/ml carbenicillin. Regenerated plants were analyzed for

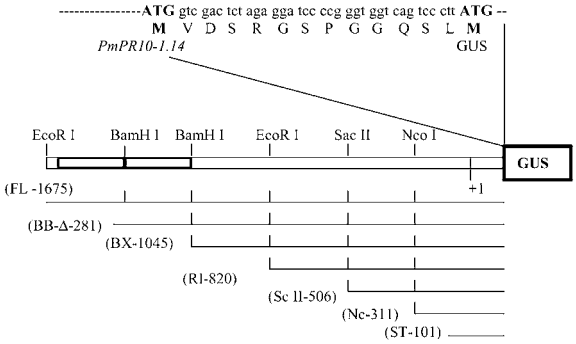

Figure 1. Schematic representation of the different *PmPR10-1.14::GUS* fusions. The promoter fragments (FL to ST) were inserted into pBI101 to construct plant gene expression vectors. The length of the promoter deletion, and the positions of restriction enzyme sites used for vector construction are indicated schematically. Position (+1) corresponds to the first nucleotide of PR10 cDNA. The DNA and amino acid sequences are shown to describe the fusion of the *uidA* gene to the *PmPR10-1.14* promoter. Codons in lower-case letters are derived from the sequence of pBI101.

the integration of the promoter-*uidA* fusion genes into the plant genome by PCR. The forward primer (BI-For) was synthesized on the sequence upstream from the polylinker region of vector pBI-101 (5′-ACACAGGAAACAGCTATGACCATG-3′). The reverse primer was PBS that was previously used for binary vector construction, or primer BI-Rev (5′-GGTTTCTACAGGACGTAACATAAG-3′). Kanamycin-resistant transformants of these lines were grown in soil in a controlled environmental chamber under the same conditions described previously. Primary transgenic plants and progeny were referred to as T_1 and T_2 plants, respectively.

To determine GUS activity in T_1 plants, tissue samples were collected from each transgenic line about 2 months after plantlets were transplanted into soil from Murashige-Skoog selection medium. Roots were washed carefully to remove any soil, and samples were taken from various parts of the root bale. Leaf samples were collected from young ones just after their full expansion about the third one from the top, and stem samples were collected at about the same position. Flower organs were collected about one day before flower opening. All T_1 plants grew under the same conditions, and the samples were collected at the same developmental stage. For experiments with T_2 seedlings, seeds from the T_1 generation were sterilized, germinated, and selected on Murashige-Skoog medium supplemented with 10 g/l sucrose and 400 μg/ml kanamycin, and grown in a sterile envi-

ronment under the conditions described above. At the times indicated in the figure legends, seedlings or the roots, stems and leaves of the seedlings were sampled and assayed for GUS activity.

Fluorometric and histochemical GUS assays

Fluorometric GUS assays of crude plant extracts were performed as described by Jefferson (1987). Tissue samples were collected in 1.5 ml Eppendorf tubes and ground in 450 μl GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% v/v Triton X-100, 0.1% Sarkosal, 10 mM 2-mercaptoethanol). After centrifugation at 12 000 $\times$ *g* for 10 min at 4 °C, the supernatant of crude extract (50 μl) was mixed with 1 mM 4-methylumbelliferyl glucuronide (4-MUG) in 0.45 ml extraction buffer. Aliquots of 50 μl were removed at time 0 h and 1 h of incubation at 37 °C and mixed with 2 ml of stop solution (0.2 M Na_2CO_3) to terminate the reaction. GUS activity was determined with a DyNAQuant 200 fluorometer (Hoefer, CA), and protein concentration of crude extracts was determined as described previously (Ekramoddoullah and Davidson, 1995).

Histochemical localization of β-glucuronidase activity was performed essentially as described by Jefferson (1987) with 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-Gluc, Clontech) as a substrate. Transgenic samples were vacuum-infiltrated for a few minutes with 1 mM X-Glu in 50 mM sodium phosphate pH 7.0, 0.02% Triton X-100 and 0.5 mM each of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$, and incubated at 37 °C for 3–16 h. After embedding in 7% agarose solution, sections (ca. 100 μm) from fixed tissues were cut with a vibrotome (Campden Instruments, London, UK). Stained tissue was cleared of chlorophyll in 70% ethanol prior to visual analysis under a microscope.

Statistical analysis of GUS activity data

Because the gene expression in the T_1 plants usually does not follow a normal distribution (Nap *et al.*, 1993), a non-parametric Mann-Whitney test was used for the distribution-free statistical analysis on the data (Neave and Worthington, 1988).

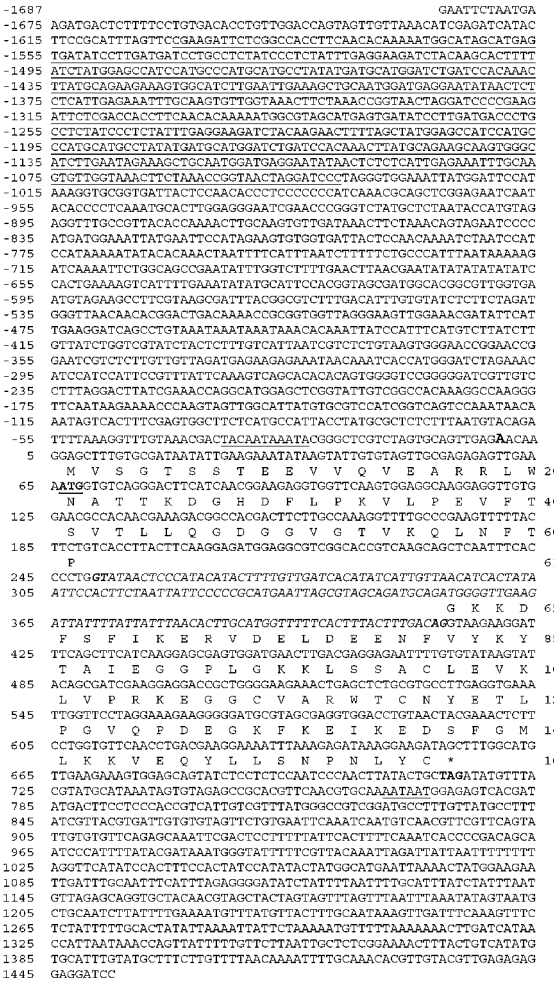

Figure 2. Nucleotide sequence of the *PmPR10-1.14* gene and flanking regions. Nucleotides are numbered on the left side, with the position of the first nucleotide of cDNA designated as +1. Amino acids are numbered on the right side. The translation start codon at position +69 is in bold. The direct repeats from −1600 to −1039, a putative TATA box at −34, and a polyadenylation signal at +767 are underlined. The intron is in italics and its border GT/AG in bold. The nucleotide sequence data reported will appear in the GenBank Nuceotide Sequence Database under the accession number AY064206.

Results

*Organization of the PR10 gene (*PmPR10-1.14*) and promoter*

A 3140 bp DNA fragment of a PR10 gene (designated it as *PmPR10-1.14*), including exonic, intronic, and flanking sequences, was isolated and determined (Figure 2). To facilitate the sequence numbering, the first nucleotide of *PmPR10* cDNA has been designated as +1. The coding region of *PmPR10-1.14* is 486 bp

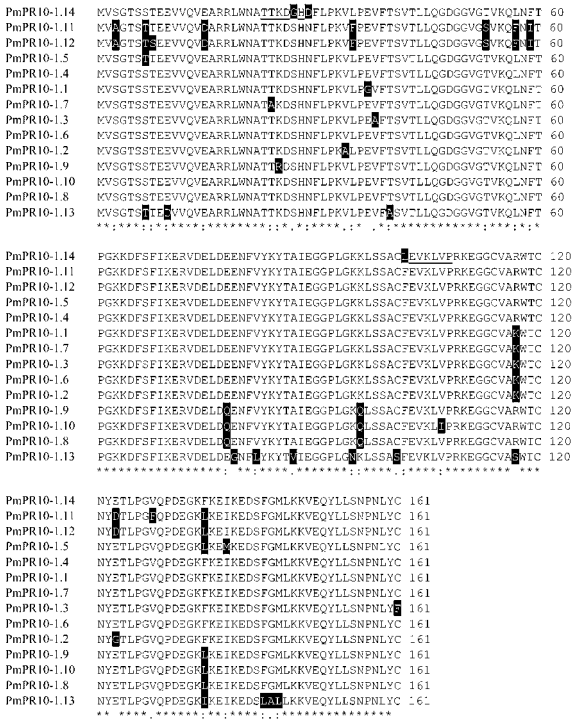

Figure 3. Comparison of the deduced amino acid sequences of PR10 genes from western white pine (accession numbers AY064193–AY064206). The alignment was made with the ClustalW program. Numbers of amino acids are indicated on the right. The underlined regions are the sequences from which the primers were designed for RT-PCR amplification of *PmPR10-1.14* gene. The different amino acid residues are shaded in black.

long and interrupted by an intron spanning 164 bp at amino acid position 62. This intron position is highly conserved among PR10 protein genes from both angiosperms and gymnosperms (Ekramoddoullah *et al.*, 2000). The 5′ exon/intron and 3′ intron/exon boundaries conformed to the known GT/AG donor/acceptor site rule. The intron is A/T-rich (66%), which is an essential characteristic for splicing. One poly(A) signal (AATAAT) is found at position +767, 51 bp downstream of the stop codon (TAG).

The coding region of the cloned PR10 gene predicted a putative polypeptide of 161 amino acids with a molecular mass of 17.96 kDa and an isoelectric point of 5.34. Alignment analysis of the deduced amino acid sequences showed that it shared 90–98% similarity with PR10 cDNAs cloned from needles of western white pine (Yu *et al.*, 2000; Liu *et al.*, manuscript submitted), and belonged to a super gene family of PR10 proteins (Figure 3). The putative polypeptide encoded by *PmPR10-1.14* has high identities (61–78%) with those isolated from other conifer species (Ekramoddoullah *et al.*, 2000; Matheus *et al.*, 2000; Dubos and Plomion, 2001), and less than 40% with those from angiosperms. Among them, *PmPR10-1.14* gene shares 31% similarity with parsley *PR1* at protein level (Somssich *et al.*, 1988), a typical member of the PR10 family (van Loon *et al.*, 1994).

A database search with the promoter sequence of the *PmPR10-1.14* gene identified a putative transcription start site, an adenine, just 2 bp upstream of the first nucleotide of PR10 cDNA in western white pine. Inspection of upstream sequences revealed a TACAATAAATA motif at −34 as a potential TATA box and a putative CAAT box located at −173.

A search for potential *cis*-regulatory elements in the promoter region of the *PmPR10-1.14* gene revealed a number of *cis*-regulatory elements that have been shown to be the binding sites of transcription factor proteins in angiosperms (Table 1). Nineteen AAAG boxes were found throughout the whole promoter region, eleven of them in an inverse orientation. The AAAG element is recognized by a class of plant-specific transcription factor-Dof proteins (Yanagisawa, 2000). Nine gibberellin (GA)-responsive elements, i.e., TAACAAA-like boxes, were detected, and five of them were in an inverse orientation. The

Table 1. The putative *cis*-regulatory elements in the *PmPR10-1.14* promoter.

Trans factor	Core sequence[a]	Sequence[b]	Position	Reference
Dof/PBF	nnnwAAAGnnn	tttTAAAGgtt (+)	−55 to −45	Yanagisawa and Schmidt (1999)
		catTAAAGaga (−)	−72 to −62	
		ctcGAAAGtga (−)	−110 to −100	
		ccaCAAAGgcc (+)	−191 to −181	
		tccTAAAGgac (−)	−238 to −228	
		attCAAAGtca (+)	−278 to −268	
		tgaCAAAGagt (−)	−398 to −388	
		tgtCAAAGacg (−)	−566 to −556	
		ctgAAAAGtca (+)	−653 to −643	
		ttcAAAAGacc (−)	−689 to −679	
		ataAAAAGatc (+)	−723 to −713	
		agaAAAAGatt (−)	−743 to −733	
		ccaTAAAGgtg (+)	−1019 to −1009	
		ataGAAAGctg (+)	−1128 to −1118	Vicente-Carbajosa *et al.* (1997)
		gctAAAAGttc (−)	−1223 to −1213	
		attGAAAGctg (+)	−1349 to −1399	
		gaaGAAAGtgg (+)	−1427 to −1417	
		gatAAAAGtgc (−)	−1403 to −1393	
		aggAAAAGagt (−)	−1770 to −1760	
GAMYB	YAACsrmm	CAACgatc (−)	−245 to −238	Gubler *et al.* (1999)
		CAACttcc (−)	−497 to −490	
		TAACcacc (−)	−505 to −499	
		CAACaaaa (+)	−793 to −886	
		TAACggca (−)	−890 to −883	
		CAACacc (+)	−996 to −989	
		TAACtctc (+)	−1101 to −1094	
		TAACtctc (+)	−1382 to −1375	
		CAACaggt (−)	−1654 to −1647	
SBF1	kwrTnGTTAAwwwn	gatggGTTAAgttc (+)	−538 to −525	Lawton *et al.* (1991)
		tagttGTTAAacat (+)	−1640 to −1627	
bZAP910	GGRTGCTGACGT	GTGTGCTGACTT (−)	−273 to −262	Martinez-Garcia *et al.* (1998)

[a]N=A+T+C+G, S=C+G, M=A+C, Y=C+T, W=A+T, K=T+G, R=A+G, M=A+C.
[b](−), DNA sequence of antisense strand; (+), DNA sequence of sense strand.

TAACAAA- like box is a conserved GA response element in the promoters of GA-regulated genes expressed in cereal aleurone cells (Gubler *et al.*, 1999).

Present at −1640 and −538 were two sequences homologous to the silencer consensus sequence GGTTAA(A/T)(A/T)(A/T), which was first observed in the bean chalcone synthase promoter, and involved in organ-specific expression in plant development (Lawton *et al.*, 1991). Another element, GTGTGCTGACTT, at −273, matched in 10 of 12 positions the consensus sequence GGRTGCTGACGT, which is the binding site of the transcription factor bZIP910 that regulates gene expression in a flower-preferential manner in *Antirrhinum* (Martinez-Garcia *et al.*, 1998). One of the most striking features of *PmPR10-1.14* promoter is the presence of two highly conserved direct repeated sequences of 281 bp located in the 5′-distal region which share 97% similarity with each other.

Transient gene expression of the PmPR10-1.14 *promoter::GUS fusion in agroinfiltrated tobacco leaves*

To investigate the functional properties of *PmPR10-1.14* promoter, a series of 5′ deletions of the promoter fused to the *uidA* gene were constructed (Figure 1), and introduced into tobacco leaves by agroinfiltration for the analysis of transient gene expression. As shown in Figure 4, all of the *PmPR10-1.14* promoter::GUS fusions actively directed GUS gene expression, in-

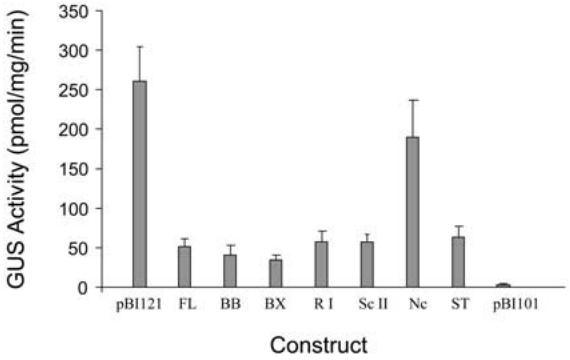

Figure 4. Fluorometric GUS assay in agroinfiltrated tobacco leaves carrying different *PmPR10-1.14*::GUS fusions. The GUS activities are expressed as pmol 4-MU per mg protein per minute. The bars represent the mean ± SD of the measurements from four independent experiments.

cluding construct ST that contained the shortest promoter fragment (−101 to +69). In the negative control, GUS activity was not detectable in young tobacco leaves agroinfiltrated with the promoterless::GUS construct (pBI 101). The promoter sequence of *PmPR10-1.14* from −101 to +69, the start site of coding region, was sufficient to direct active transcription. No significant difference in GUS activity levels was observed among all constructs of *PmPR10-1.14* promoter::GUS fusions except the construct Nc with the promoter sequence from −311 to +69. The expression level of construct Nc was three to four times higher than those of the other constructs, almost the same level as that of CaMV 35S promoter (Figure 4).

Root-specific expression of PmPR10-1.14*::GUS transgene in mature transgenic tobacco plants*

To investigate the spatial and developmental regulation of *PmPR10-1.14*, the constructs of *PmPR10-1.14* promoter::GUS fusions were also transformed into tobacco plants for the analysis of stable gene expression in transgenic plants. Transgenic tobacco plants with CaMV 35S promoter::GUS fusion of pBI 121, as a positive control, exhibited strong GUS activity in all organs and in T_2 seedlings (data not shown). For the transformants of construct FL with the longest promoter fragment from −1675 to +69, ten independent transgenic lines were selected randomly for fluorometric assay for GUS activity to monitor promoter activity in different organs in mature plants. The roots of the transformants showed different intensities of GUS activity, the highest being six-fold more than the lowest (Table 2). This variability of GUS expression is probably due to the position effect, i.e., the effect on transcriptional activity of the region where the fusion gene was inserted. The GUS activity level was different enormously among the ten primary transformants, but all showed a similar pattern of high GUS activity in roots. Only low levels of GUS activity were measured in the extracts of other organs including leaves, stems, and flower organs, including sepals, petals, stamens and pistils (Table 2). On the average, GUS activities in the roots were hundred times higher than that in the leaves and stems. Correspondingly, histochemical assays showed that GUS staining was very strong in the roots, but it was not detectable in the other tissues.

PmPR10-1.14*::GUS fusion is specifically regulated during the development of lateral root*

Representative transgenic lines harboring construct FL of the *PmPR10-1.14* gene were allowed to self- pollinate and set seeds. Starting at seed germination, T_2 plantlets were histochemically stained with X-Gluc to assess GUS activity. GUS activity was first observed in 6- to 8-day old seedlings when the lateral root began to develop, demonstrating that the root-specific expression pattern of *PmPR10-1.14* was controlled developmentally in transgenic tobacco seedlings (Figure 5). At this developmental stage, *in situ* GUS staining was only detected in the upper part of the primary root connected to the hypocotyl where lateral roots were initiating (Figure 5A). Here, GUS activity was very strong in the primordium of lateral roots, weak in the cortex cells of primary roots (Figure 5B), and absent elsewhere. After the lateral root forced its way through the surrounding cortical tissue of the primary root, strong GUS activity was observed in the root cap and meristem (Figure 5C and D). As the lateral root grew, GUS activity was observed in the epidermis cell and root hairs (Figure 5E). Longitudinal sections of lateral roots revealed a high level of GUS expression in cortex cells, but none in the root vascular cylinder (Figure 5F). As plants grew, no GUS staining was observed in leaves, stems or flower organs, including petal, sepal, stamen and pistil (Figure 5G). Transgenic line F16 was an exception with some GUS activity detected in its pistils (Table 2). A longitudinal section of the ovary of F16 T_2 plants revealed weak staining in the ovules (Figure 5H). GUS activity was not detectable in the pollen grains from the mature anthers (Figure 5I), or in embryos at different development stages (Figure 5J) or in seeds even in transgenic line F16. These results

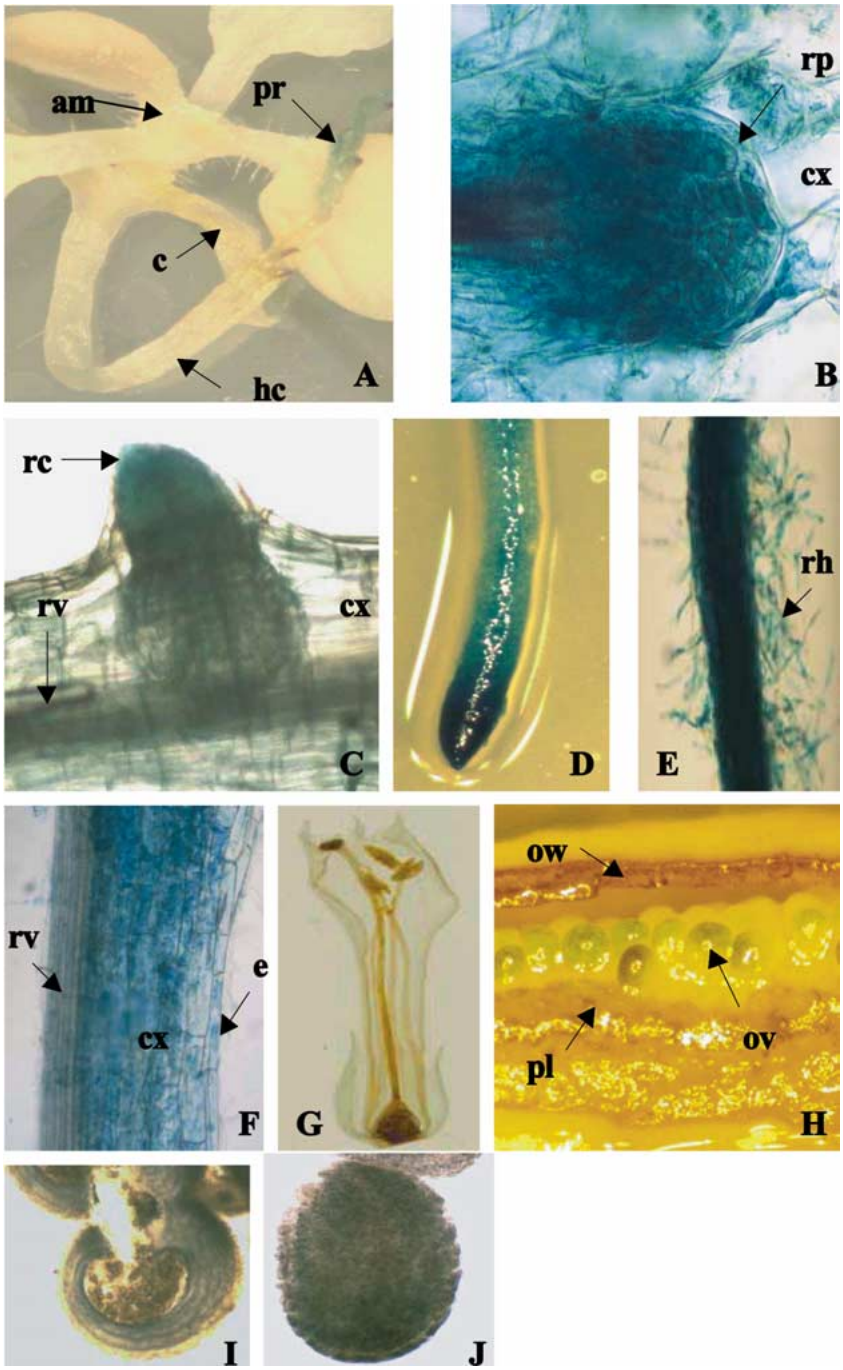

Figure 5. In situ histochemical localization of GUS activity in transgenic tobacco plants containing *PmPR10-1.14:: GUS* fusion under control of the promoter sequence from −1675 to +69 in construct FL. A. Ten-day old T_2 seedling with GUS staining only in the primary root (pr). B. Origin of the second lateral root primordium in the pericycle of the primary root. C. The young lateral root tip growing out through the surrounding cortical tissue of the primary root with strongest GUS staining at root cap and meristem. D. A lateral root showing the root cap and the root meristem part with strongest GUS activities. E. The zone of root hair from lateral roots. F. Longitudinal section of lateral root showing strong GUS staining in cortex tissue and no signal in the vascular cylinder. G. A flower from a T_2 plant; half of it was removed to show stamen and pistil. H. Longitudinal section of ovary showing a weak GUS staining in ovule (ov) in transgenic line F16. I. Cross section of anther to show no signal in pollen grains. J. The developing embryo without signal. am, apical meristem; c, cotyledons; cx, cortex; e, epidermis; hc, hypocotyl; pr, primary root; rc, root cap; rh, root hair; rp, root primordium; rv, root vascular cylinder; ow, ovary wall; pl, placenta; ov, ovule.

Table 2. Fluorometric quantification of GUS activity in the tissues of different transgenic T_1 plants harboring *PmPR10-1.14*::GUS fusion vector FL. GUS activities in protein extracts were determined twice and expressed in pmol of 4-methylumbelliferyl-β-D-glucuronide per mg protein per minute.

Plant	Plant tissue							GUS activity ratio	
line	leaf	stem	root	sepal	petal	stamen	pistil	root/leaf	root/stem
FL2	31.35	33.88	3413.33	16.29	30.06	29.86	9.95	108.15	100.74
FL4	40.90	14.22	2993.83	13.53	26.21	46.28	31.50	73.19	210.53
FL6	26.57	24.67	2621.21	16.54	14.81	10.13	9.52	98.65	106.25
FL3	19.86	25.89	7611.65	17.99	19.20	20.44	11.95	383.26	293.99
FL9	23.22	51.46	3666.67	18.12	10.95	17.31	9.46	157.91	71.25
FL8	11.29	20.66	1294.63	28.57	16.60	4.19	4.44	114.67	62.66
FL10	9.92	58.20	2115.56	13.01	10.98	28.63	11.88	213.26	36.34
FL5	26.57	17.22	2482.53	17.46	9.79	42.62	20.00	93.43	144.16
FL11	18.94	112.64	2725.93	17.40	27.84	15.26	17.96	143.92	24.20
FL16	51.93	113.56	1983.84	31.57	39.51	88.19	242.04	38.20	17.42
Mean	26.06	47.24	3090.92	19.35	20.59	30.34	14.59	142.46	106.75
±SE	9.40	29.38	1191.05	5.87	8.24	12.05	6.42	65.69	65.68

demonstrated that the *PmPR10-1.14* gene in transgenic tobacco seedlings was characteristically under the control of an exquisite tissue-specific expression program during the development of lateral roots.

The spatial expression of the endogeneous PR10 gene in western white pine

To determine whether the behavior of the endogenous white pine PR10 gene mimics that of the transgene, northern blot, RT-PCR and western immunoblot analyses were performed to monitor the expression profile of the PR10 genes, including *PmPR10-1.14*, in western white pine. Northern blot analyses showed that PR10 transcripts were most abundant in the roots, less abundant in stems and vegetative shoots, and at the lowest level in needles collected under dormant condition in January 2000 (Figure 6A and B). However the distribution of PR10 mRNAs in tissues was changed when the seedlings were growing in a growth chamber. PR10 mRNAs were most abundant in apical shoots and stems, less abundant in roots, and at the lowest level in needles (Figure 6A and B). RT-PCR with gene-specific primers of *PmPR10-1.14* showed the presence of its mRNA only in lateral roots and needles of western white seedlings under growth conditions (Figure 6C).

Western immunoblot analysis on SDS-PAGE blots showed that PR10 proteins were most abundant in roots, less abundant in stems and apical shoots, and at the lowest level in needles in the winter samples. By contrast, in seedlings from the growth chamber the PR10 proteins were most abundant in stems and apical shoots, less abundant in roots, and at lowest level in the needles (Figure 7). Neither PR10 transcripts nor proteins were detectable in the immature male cones and female cones (Figures 6 and 7), which was similar to the expression pattern observed in transgenes. Western immunoblot analyses on 2-D blots showed that six PR10 isoforms were detected in the roots of seedlings from the growth chamber (Figure 8). These six isoforms were distinguished according to their isoelectric points: two isoforms with a pI of 5.9–6.0, three with a pI of 5.5–5.6, and one with a pI of ca. 5.3. According to the calculated pI and molecular mass, the isoform encoded by *PmPR10-1.14* may correspond to one with a pI of ca. 5.3.

The effect of 281 bp repeated sequences on the gene expression in the leaves and stems of transgenic tobacco

To reveal the promoter regions of *PmPR10-1.14* that mediate the root-specific expression, other constructs of *PmPR10-1.14* promoter 5′ deletions (Figure 1) were transformed into tobacco to analyze stable gene expression. Different independent transgenic lines from each construct were selected to assess GUS activity in leaves and roots of mature plants. As shown in Figure 9, all six constructs drove GUS gene expression at high levels in the roots and at low levels in leaves. As the promoter was deleted from the 5′-distal region

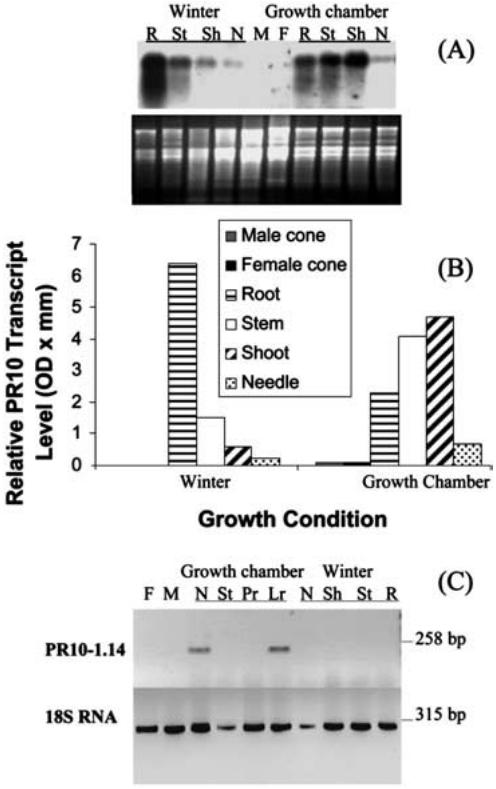

Figure 6. PR10 gene expression in western white pine. A. Northern blot analysis; 10 μg of total RNA in each lane was hybridized with DNA probe of *PmPR10-1.14*. To control for equal loading, the gel was stained with ethidium bromide (bottom). B. PR10 transcript levels shown above were quantified with PDI software. C. Semi-quantitative RT-PCR analysis of RNA transcripts extracted from various tissues. The RT-PCR products for *PmR10-1.14* (top) and control 18S rRNA (bottom) were separated on a 1.2% agarose gel. The sizes of amplified bands are indicated at the right. St, stems; Sh, apical shoots; N, needles; R, whole root system; Pr, primary roots; Lr, lateral roors; M, immature male cones; F, female cones.

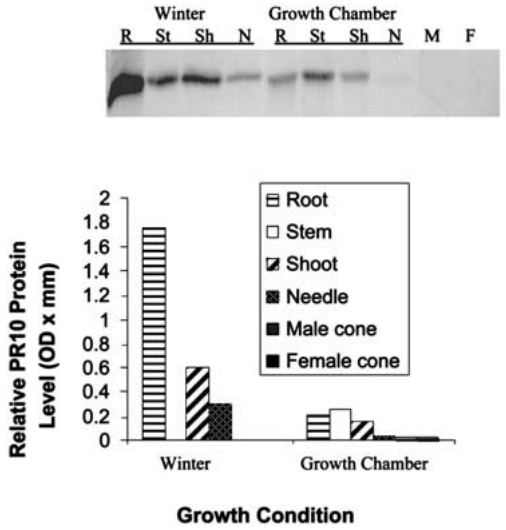

Figure 7. Western immunoblot analysis of PR10 protein in different organs of western white pine. Tissue samples were the same as described in northern blot analysis. A 10 μg portion of total protein was loaded in each lane and separated by electrophoresis on SDS-polyacrylamide gels, blotted onto PVDF membranes, and analyzed with an antiserum against the N-terminal region of a PR10 protein (Pin l I) from sugar pine. Bottom: PR10 protein levels in the upper panel quantified with PDI software.

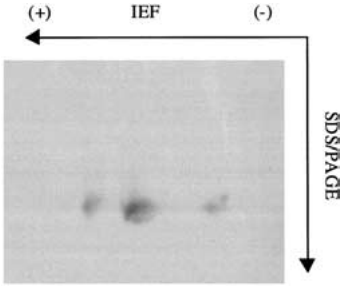

Figure 8. PR10 protein profile from roots of western white pine using immunodetection of 2-D blots. For 2-D blots, the first dimension was IEF (pH 3–10), and the second dimension was SDS-PAGE. A 100 μg portion of total protein was loaded on the IEF gel. The pI and position of each PR10 spot was determined by the related migration rate with the references of standard proteins and recombinant PR10 protein of *Pin m III* from *Escherichia coli*.

to -311, GUS activity showed a decreased trend in the roots, but statistical analysis showed no significant difference in gene activity in roots among these six constructs. Furthermore, the average ratio of GUS activity in roots to those in leaves established by the -1675 promoter sequence (FL) was as high as 142; as the 5′ deletions continued to -311, the average ratio was still at 32 (Figure 10).

One striking feature of the *PmPR10-1.14* promoter is that it possesses two copies of repeated sequences as long as 281 bp from -1600 to -1039. To study the *cis*-regulation functions of these repeated sequences on gene expression, two constructs were made. In construct BB, one copy of the repeated sequence from -1326 to -1045 was deleted; and in construct BX, both copies of the repeated sequences were deleted from -1675 to -1045. Non-parametric statistical analysis of the transgenic T_1 plants showed that the deletion of one copy or both copies of repeated sequences resulted in a significant increase of expression levels in leaves ($P = 0.014$ between construct FL and BX; $P = 0.031$ between construct BX and RI) (Figure 9B). GUS activity in stems with constructs BB and BX also increased significantly (data not shown). However, in the flower tissues and in developing

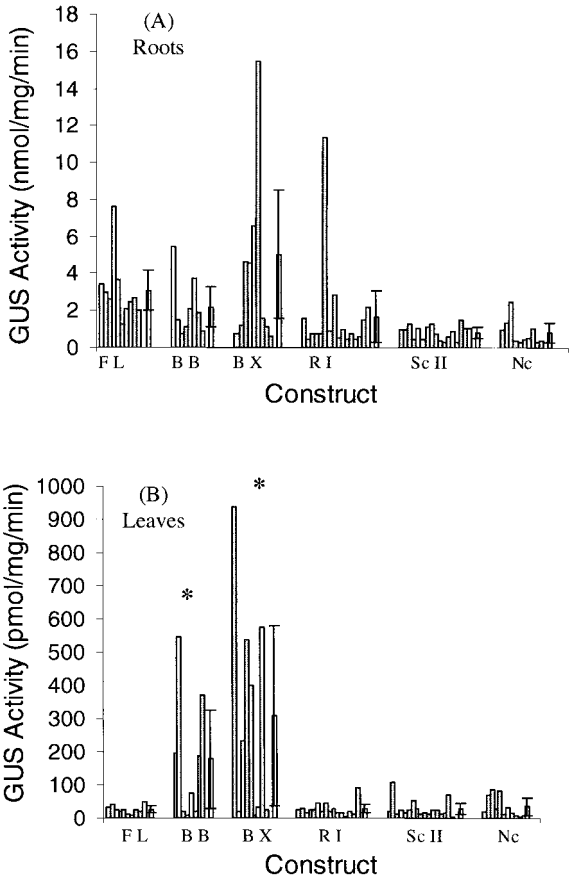

Figure 9. Effect of 5′ deletion on GUS expression in leaves and roots of *N. tabacum* transformed with various *PmPR10-1.14::GUS* fusions. Each gray bar represents the value of a transgenic line, a white bar represent the mean value ± deviation of the mean. The increased GUS activities in plants harboring constructs BB and BX are significant ($P = 0.031$ and $P = 0.014$, respectively), indicated with *. Statistical analyses show no significant differences of GUS expression in roots among different constructs.

seeds, GUS activity was low with these two constructs, as it was with construct FL (data not shown). Further 5′ deletion to −820 in construct RI recovered a gene expression pattern similar to that of construct FL (Figure 9).

The developmental regulation of gene expression directed by the *PmPR10-1.14* promoter fragment from −1045 to +69 in construct BX was further investigated using *in-situ* GUS staining on T_2 plantlets. Histochemical localization showed an expression pattern in roots similar to that of construct FL. At the same developmental stages (6 to 8 days old), GUS activity was detected in the lateral roots of seedlings, and also in the apical shoot and developing leaves (Figure 11A). GUS activity was highest in vascular bundles and in the glandular trichome of the developing leaves of T_2 seedlings (Figure 11B). As plants grew, GUS expression in leaves decreased greatly (Figure 11C). In mature plants strong GUS staining was observed in all types of cells of the leaves before the differentiation of the mesophyll (Figure 11F). After differentiation of the mesophyll, only weak GUS activity was detected in the palisade mesophyll and spongy mesophyll, and none was detected in the epidermis except in the glandular trichome (Figure 11G). Histochemically detectable GUS activity was high in the young stem close to the apex meristem of the mature plants (Figure 11E, left), and GUS staining was observed in most of the tissues except the xylem and the cells around the external phloem (Figure 11H and I). On the other hand, GUS staining was detected only in the epidermis cells of the part of the stem distal from the apex and the petiole (Figure 11J and D).

Discussion

A western white pine PR10 gene and gene expression

In this work, we present the isolation and characterization of a genomic DNA clone of a PR10 gene (*PmPR10-1.14*), which corresponded to one of six PR10 isoforms immunodetected in healthy roots of western white pine. In conifers, PR10 proteins have been shown to be up-regulated after infection of fungal diseases (Ekramoddoullah *et al.*, 1998, 2000). Furthermore, histochemical localization demonstrated that the PR10 protein was bound to the fungal cell walls in the infected needles of western white pine (Ekamoddoullah, data unpublished). A subset of the PR10 family is inducible upon wounding in the needles of western white pine, and their protein level is enhanced by the global defense signal molecule methyl jasmonate and by phosphatase inhibitor (Liu *et al.*, manuscript submitted). These studies imply a role for PR10 proteins in the conifer defense response.

Multiple isoforms of PR10 protein are expressed constitutively in all vegetative organs, especially in roots of western white pine (Figures 6 and 7). Expression of PR10 protein is highest in roots in winter and correlates significantly with frost hardiness, implying a role in cold acclimation (Ekramodoullah *et al.*, 1995). Noteworthy in this regard is the change in distribution of both PR10 transcripts and proteins among organs under growth conditions (Figures 6 and 7), suggesting a complicated mechanism regulating the accumulation of PR10 proteins in winter.

The constitutive or inducible expression of PR10 genes in roots have been reported in a few angiosperm species (Crowell *et al.*, 1992; Mylona *et al.*, 1994; Moons *et al.*, 1997; Utriainen *et al.*, 1998; Sikorski *et al.*, 1999; Bantignies *et al.*, 2000). RT-PCR with gene-specific primers showed that the *PmPR10-1.14* gene was only expressed in lateral roots and needles under growth condition, different from most of other PR10 genes in western white pine seedlings (Figure 6), but its promoter establishes an exquisite root-specific expression program in transgenic tobacco (Figure 5). This discrepancy may be due to different regulatory mechanisms in tobacco and white pine, or the binding of RT-PCR primers to some other unidentified PR10 genes in western white pine, or some enhancers present in the other gene regions rather than the 1675 bp promoter sequence of *PmPR10-1.14*. Although the expression of a set of PR10 genes are inducible by wounding in the needles of western white pine (Liu *et al.*, manuscript submitted), the expression of *PmPR10-1.14* fusion gene was not affected by wounding treatment in transgenic tobacco (data not shown). Apart from RNase activity (Bufe *et al.*, 1996; Swoboda *et al.*, 1996; Bantignies *et al.*, 2000), cytokinin-specific binding activity has been detected in some PR10 proteins (Fujimoto *et al.*, 1998; Gonneau *et al.*, 2001). As plant growth hormone cytokinins control differentiation and proliferation of plant cells. Two tobacco PR proteins with cytokinin-binding properties were proposed an important role in hormone signal transduction of cytokinin related to morphogenesis (Kobayashi *et al.*, 2001). Our observations suggest the expression of *PmPR10-1.14* may undertake important functions not only in defense response to some constant physiological stresses as proposed by Mylona *et al.* (1994), but also in the development of lateral roots.

Regulation of root-specific expression of PmPR10-1.14 *by multiple promoter regions*

Tissue-specific expression is presumably due to the interaction of a set of regulatory proteins that bind to a special combinatory set of *cis*-regulatory elements of the promoter (Singh, 1998). Therefore, the expression pattern of a designed gene is determined by *trans* factors, the transcription proteins available from the background cells, and *cis* factors, the *cis*-regulatory elements in the DNA sequences of gene regions. Some *cis*-regulatory elements are found in the intron (Mascarnhas *et al.*, 1990), the coding region and the downstream region (Sessa *et al.*, 1995), or in the distal region as far as 10 kb from start codon (Wasylyk, 1988), but most of them are located in the proximal region about 500 bp from the transcription start site. In this study the 1675 bp promoter region of *PmPR10-1.14* gene is shown to direct gene expression in a root-specific pattern during lateral root initiation and development in transgenic plants (Table 2 and Figure 5). Characterization of *PmPR10-1.14* allowed us to identify potential *cis*-regulatory elements in the promoter regions for the regulation of its root-specific expression.

A surprising feature of *PmPR10-1.14* promoter structure is that it contains many Dof *cis* elements and TAACAAA-like boxes (Table 1). Dof *cis* elements are involved in a variety of signal-responsive and/or tissue-specific gene expressions in plants (Baumann *et al.*, 1999; Yanagisawa and Schmidt, 1999). TAACAAA-like boxes are the binding sites of a class of transcription factor GAMYB, which is responsible for the tissue-specific expression of GA-regulated genes in cereal aleurone cells (Gubler *et al.*, 1999). This type of distribution of *cis*-regulatory elements and promoter organization of the *PmPR10-1.14* gene predicts tissue-specific expression.

The analysis of transient gene expression in agroinfiltrated tobacco leaves showed that the core promoter of *PmPR10-1.14* might be the sequence from -101 to $+69$. The gene sequence from -101 to $+69$ contains one putative TATA box-like element at -34, two Dof *cis* elements, plus the 5′-untranslated region, which may serve as the *cis*-regulatory elements for basic gene expression. 5′ deletions of the *PmPR10-1.14* gene from -1675 to -506 were found to have no obvious effects on transient gene expression. But a further deletion to -311 was shown to increase gene expression three to four times higher (Figure 4), suggesting the enhancer element(s) reside in the region from -311 to -101, and silencer element(s) reside in the sequence from -506 to -311.

Furthermore, the level of GUS activity measured in the roots of transgenic plants harboring the -311 bp promoter was not significantly different from that of transgenic plants containing the -1675 bp promoter of *PmPR10-1.14*. 5′ deletion of the promoter to -311 still drove gene expression at high levels in roots (Figure 9). Thus, positive *cis*-regulatory elements that regulate the constitutive expression of *PmPR10-1.14* in roots of transgenic tobacco may reside between -311 and $+69$. In the meantime, when the ratios of GUS activity in roots to that in leaves are ana-

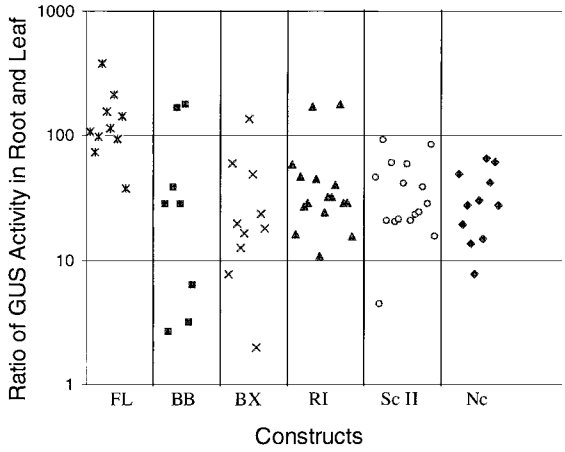

Figure 10. The relative GUS activity in roots and leaves of tobacco plants transformed with chimeric reporter gene constructs using different promoter fragments. The ratio between root GUS activity and leaf GUS activity was determined for individual T_1 transgenic lines with different promoter deletions.

lyzed, the ratio established by the −311 promoter was about 25% of that observed for the −1675 promoter (Figure 10). Therefore, some positive quantitative elements responsible for root-specific expression may be located between −1675 and −311.

From −506 to −311 there are two TAACAA-like boxes and one Dof *cis* element (Table 1). The *cis* elements associated with the TAACAA-like boxes appear to be analogous to enhancer elements in angiosperms (Gubler *et al.*, 1999). In maize, different Dof proteins bind to different AAAG elements with positive and/or negative effects on promoters in different contexts (Yanagisawa, 2000). Further study is needed to explore the regulatory sequence in this region.

The promoter region from −311 to −101 is rich in various *cis* elements, including one CAAT box, four Dof *cis* elements, one CAACAAA-like box, and one *cis* element for the binding of bZIP910 (Table 1). It is noteworthy that the potential binding sites for bZIP910 (−273) and one Dof protein (−278) are close to each other in the promoter of the *PmPR10-1.14* gene, suggesting that there might be interaction of different transcription factors. The interactions of Dof and bZIP proteins have been observed in the promoters of maize α-zein gene (Vicente-Carbajosa *et al.*, 1997) and the *Arabidopsis* glutathione *S*-transferase (GST) gene (Zhang *et al.*, 1995). Therefore, the presence and interaction of these putative *cis* elements may be a significant factor in the gene expression at high levels in the transient expression assays and in the roots of transgenic plants.

An unusual feature of the *PmPR10-1.14* promoter is the presence of two copies of repeated sequences at its 5′-distal region (Figure 1). Surprisingly, removal of one copy or both copies of these repeated sequence results in significantly increased expression levels in the leaves (Figure 9) and stems (Figure 11), but not in other tissues. When the progressive 5′ deletion went from −1045 to −820, the increased GUS gene expression in leaves and stems was abolished (Figure 9). These results revealed that for the gene expression in leaf and stem tissues the repeated sequence of 281 bp functioned as a *cis*-regulatory element with a negative effect and the sequence from −1045 to −820 as a *cis*-regulatory element with a positive effect. Therefore, both enhancer and silencer elements may function in the leaves and stems. There are two Dof *cis* elements and one CAATAAA element in the repeated sequence; however, these elements or unidentified elements seem unrelated to the increased expression in leaves and stems because there was no observed difference between deletions of one copy or two copies of the repeated sequence. The repeated sequences are dispersed throughout the genomes of higher plants, and some of them have been identified in the promoter regions of plant genes (Inaba *et al.*, 1999), suggesting their functions in the regulation of gene expression (Bureau and Wessler, 1994; Strömvik *et al.*, 1999). The long repeated sequence found in the *PmPR10-1.14* promoter has the ability to form a stable secondary structure, i.e., a hairpin structure, which might provide a binding site for a regulatory protein. However, to understand the mechanism by which this repeated sequence acts as a silencer, further study is required.

Roots are very important in plant development, but so far our understanding of the regulation of gene expression in roots is very limited. Short elements that contribute to root-preferred expression have been identified. These elements are involved predominantly in root-specific expression as enhancers, such as the *ocs* elements (Ellis *et al.*, 1987), *ocs*-like *mas* elements (Feltkamp *et al.*, 1995) and *as-1* elements (Lam *et al.*, 1989; Benfey *et al.*, 1990). We have isolated and characterized a western white pine PR10 gene and reported the first plant PR10 promoter to drive GUS gene expression in a root-specific manner in transgenic plants. Our experimental results showed that the root-specific expression of a gymnosperm promoter is mediated by different promoter regions in an angiosperm plant. Further work is needed to identify *cis* elements that are responsible for root-specific gene ex-

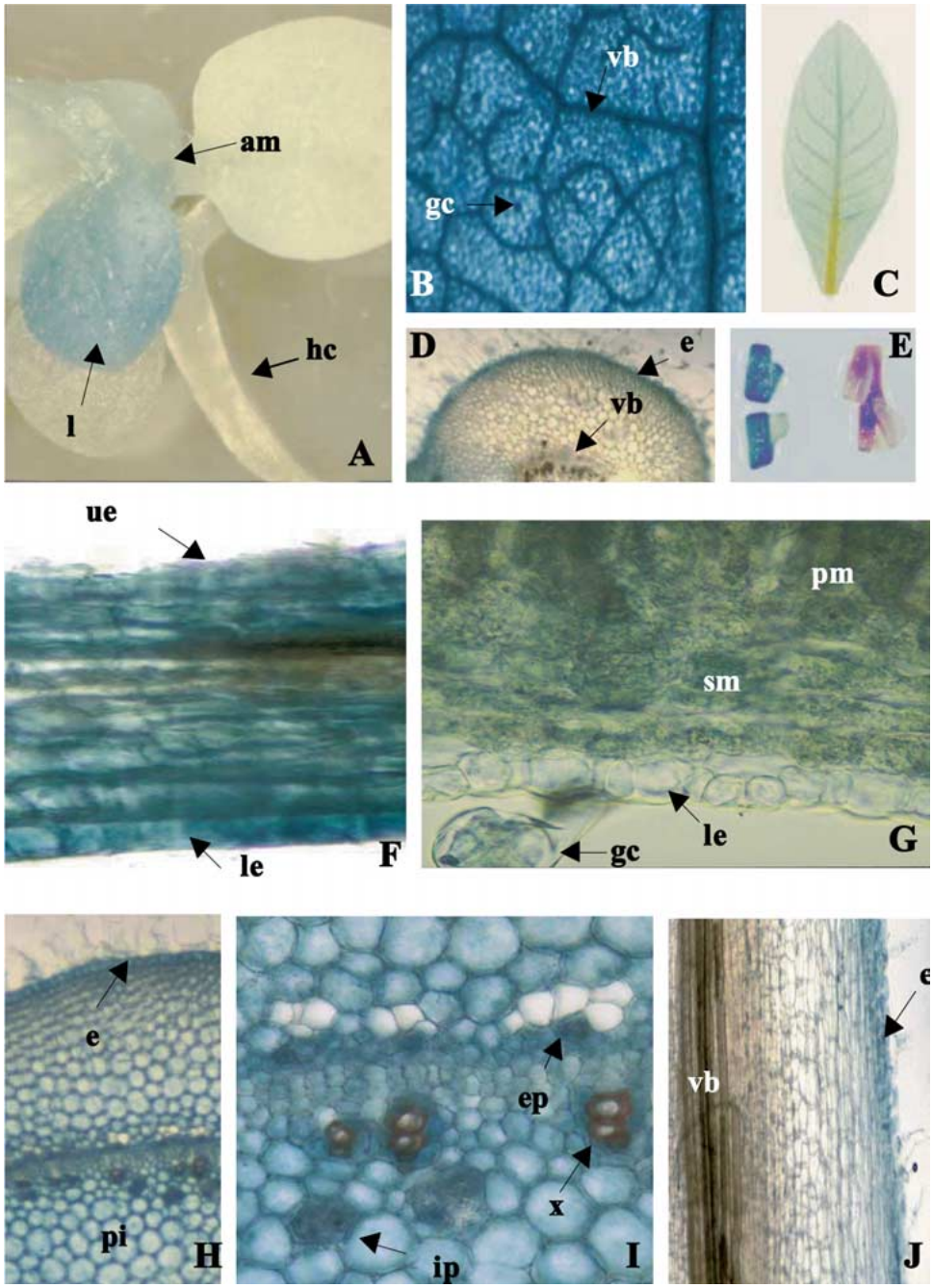

Figure 11. Histochemical localization of GUS activity in transgenic tobacco plants containing *PmPR10-1.14:: GUS* fusions under the control of the promoter sequence from +69 to −1045 in construct BX. A. Ten-day old seedling showing GUS activity in young leaves and apical meristem. B. Partial region of the young leaf from A to show strongest GUS staining in glandular trichome and vascular bundle. C. Young leaf from mature plants. D. Cross-section of petiole from young leaves to show GUS activity only in epidermis from young plants. E. Stems from mature plant, the left from plant with construct BX, the right from plant with construct FL. F: cross-section of young leaf before differentiation of mesophyll from the mature plant. G. Cross-section of young leaf after differentiation of mesophyll from the mature plant to show weak GUS signal in mesophyll cells and glandular cells and no signal in epidermis cells. H. Cross-section of young stem close to apical meristem. I. Magnified partial region from H to show no signal in xylem and cells around external phloem. J. Longitudinal section of stem far away from the apical meristem to show GUS staining mainly in epidermis. am, apical meristem; e, epidermis; le, lower epidermis; ue, upper epidermis; gc, glandular cells; hc, hypocotyl; l, leaf; vb, vascular bundle; pi, pith; ep, external phloem; ip, internal phloem; x, xylem; pm, palisade mesophyll; sm, spongy mesophyll.

pression in the *PmPR10-1.14* promoter. Root-specific promoters are of special interest because of the physiological functions in roots, and the susceptibility of roots to attack by many pathogens and pests. Such tissue-specific promoters as the *PmPR10-1.14* promoter offer a wide range of potential applications to promote expression of endogenous or foreign proteins in roots to enhance plant resistance to pathogens and pests, and to increase tolerance to heat, salt or drought. Other important potential applications are to improve the nutritive value of edible root plants, and to produce recombinant proteins aimed at molecular farming and phytoremediation.

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