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ORIGINAL ARTICLE

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Molecular cloning of a pathogen/wound-inducible PR10 promoter from *Pinus monticola* and characterization in transgenic *Arabidopsis* plants

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Abstract In *Pinus monticola* (Dougl. ex D. Don), the class ten pathogenesis-related (PR10) proteins comprise a family of multiple members differentially expressed upon pathogen infection and other environmental stresses. One of them, *PmPR10-1.13*, is studied here by investigating its transcriptional regulation in transgenic *Arabidopsis* plants. For functional analyses of the *PmPR10-1.13* promoter, a 1,316-bp promoter fragment and three 5′ deletions were translationally fused to the ß-glucuronidase (GUS) reporter gene. The 1,316-bp promoter-driven GUS activity first appeared in hypocotyls and cotyledons in 2- to 3-day-old seedlings. As transgenic plants grew, GUS activity was detected strongly in apical meristems, next in stems and leaves. No GUS activity was detected in roots and in reproductive tissues of flower organs. In adult plants, the *PmPR10-1.13* promoter-directed GUS expression was upregulated following pathogen infection and by wounding treatment, which generally mimic the endogenous expression pattern in western white pine. Promoter analysis of 5′ deletions demonstrated that two regions between −1,316 and −930, and between −309 and −100 were responsible for the wound responsiveness. By structural and functional comparisons with *PmPR10-1.14* promoter, putative wound-responsive elements were potentially identified in the *PmPR10-1.13* promoter. In conclusion, *PmPR10-1.13* showed properties of a defence-responsive gene, being transcriptionally upregulated upon biotic and abiotic stresses.

Keywords Pathogen/wounding inducible · Pathogenesis-related · Promoter · PR10 protein · Transgenic plant · Western white pine

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Abbreviations 4MU: 4-Methylumbelliferone · RT-PCR: Reverse transcriptase-polymerase chain reaction · PR: Pathogenesis-related · X-Glu: 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Introduction

Genes encoding the class ten pathogenesis-related (PR10) proteins have been cloned and characterized in many angiosperm species and a few gymnosperm species. Although their nucleotide and protein sequences diverge considerably, several features are highly conserved in PR10 proteins, including their small size of 15–18 kDa, acidic pI, cytosolic localization, and similar 3-D structure (van Loon et al. 1994; Markovic-Housley et al. 2003). PR10 gene expression has been found to be activated by pathogen infection as part of plant defence response (Fristensky et al. 1988; Somssich et al. 1988; Matton and Brisson 1989; Schmelzer et al. 1989; Pinto and Ricardo 1995; Breda et al. 1996; Ekramoddoullah et al. 1998; McGee et al. 2001). A few purified PR10 proteins have been demonstrated to have in vitro RNase activity (Bufe et al. 1996; Bantignies et al. 2000; Wu et al. 2003). Recently, a novel PR10 member (Ocatin) has been identified from *Oxalis tuberosa* possessing antibacterial and antifungal activities (Flores et al. 2002). A role for PR10 proteins in plant defence against pathogens has been proposed (van Loon et al. 1994).

Apart from pathogen-inducible expression, PR10 genes are also expressed in an organ- or tissue-specific manner during development in healthy plants (Apold et al. 1981; Breiteneder et al. 1989; Crowell et al. 1992; Mylona et al. 1994; Warner et al. 1994; Constabel and Brisson 1995; Breiteneder et al. 1995; Vanek-Krebitz et al. 1995; Liu and Ekramodoullah 2003). Several works have shown that PR10 proteins have the ability to bind cytokinin (Fujimoto et al. 1998; Mogensen et al. 2002), brassinosteroids (Markovic-Housley et al.

2003), fatty acids, and flavonoids (Mogensen et al. 2002), suggesting that PR10 proteins are also involved in plant growth and development. Brassinolide, one of the most important brassinosteroids in the hormonal regulation of plant growth and development, was found to induce disease resistance in plants (Nakashita et al. 2003). Therefore, PR10 proteins may take part in the steroid hormone-mediated disease resistance in plant defence response. Although a wide range of studies have been performed on PR10 proteins in the last decade, little is known about their potential in vivo functions.

PR10 promoters have been cloned and functionally analysed in a few angiosperm species (Warner et al. 1993; Korfhage et al. 1994; Walter et al. 1996; Pühringer et al. 2000; Desveaux et al. 2000). Some PR10 gene promoters have been characterized for the presence of *cis* elements and their *trans*-acting protein factors, including the parsley (*Petroselinum crispum*) *PR1* genes (Meier et al. 1991; Rushton et al. 1996; Eulgem et al. 1999) and *pr2* gene (Korfhage et al. 1994) and potato (*Solanum tuberosum*) *PR-10a* gene (Desveaux et al. 2000). We have previously shown that in western white pine (*Pinus monticola*), multiple members of the PR10 gene family are differentially expressed upon pathogen infection, and other environmental stimuli (Liu et al. 2003), and one (*PmPR10-1.14*) is regulated in a root-specific manner in transgenic tobacco (Liu and Ekramoddoullah 2003). These results indicate that the promoters of western white pine PR10 genes might contain specific *cis*-regulatory elements to account for their differential expression in response to abiotic or biotic stresses. To test this hypothesis, we carried out characterization of the *P. monticola PmPR10-1.13* gene. Here, we report the identification of putative *cis*-regulatory elements and the functional analysis of the *PmPR10-1.13* promoter in transgenic *Arabidopsis* plants. We demonstrate for the first time that a conifer PR10 gene promoter directs wound/pathogen-inducible expression of the ß-glucuronidase (GUS) reporter gene by both negative and positive regulatory mechanisms.

Materials and methods

Plant materials and treatments

Western white pine samples of needles, stems, vegetative shoots, and roots were collected from greenhouse seedlings under ambient conditions in a green house. Developing male and female cones were collected in May 2000 from mature trees on the grounds of the Pacific Forestry Centre, Victoria, BC, Canada. *Arabidopsis thaliana* (Col-o, seeds from Dr. S. Shah, Alberta Research council, Canada) and T2 tobacco plants of transgenic lines harbouring *PmPR10-1.14* promoter GUS fusions (Liu and Ekramoddoullah 2003) were grown in growth chambers with a photoperiod of 16 h per day at 22°C. Fungal infection by *Cronartium ribicola* and wounding of western white pine seedlings were performed as described previously (Liu et al. 2003). *Pseudomonas syringae* (Psm4326) (from Dr. Xin Li, University of British Colombia, Canada) was used to infect transgenic *Arabidopsis* plants. The bacteria were streaked on an LB agar plate and incubated at 28°C for 2 days to grow single clones. Bacterial cultures from single colonies were grown for 8–12 h, and then the bacterial cells were collected and resuspended in 10 mM $MgCl_2$ to the optical density ($OD_{600} = 0.1$). Leaves were infiltrated with bacteria on their abaxial side using a 1-ml syringe and harvested at the indicated times post-inoculation (Katagiri et al. 2001).

T2 transgenic *Arabidopsis* plants were wounded by crushing lower rosette leaves with flat-tip forceps, and they were harvested 24 h after treatment. Wounding treatments on T2 transgenic tobacco plants were conducted on the fully developed leaves as described above for *Arabidopsis*.

Genomic DNA cloning of *PmPR10-1.13* gene

Genomic DNA was isolated from current-year needles of western white pine using a Plant DNeasy Extraction Kit (Qiagen, Mississauga, ON, Canada). We used a two-step PCR strategy to clone genomic DNA of the *PmPR10-1.13* gene as described previously (Liu and Ekramoddoullah 2003). An inverse polymerase chain reaction (IPCR) was performed first to obtain the flanking sequences of the coding region with two gene-specific primers: 5′-CCT TGC CTC CAC TTG AAC CAC CTC TTC CG-3′ (GSP1) and 5′-C CTC TCC AAT CCC AAC TTA TAC TG-3′ (3SE1). A pair of primers was then designed based on the flanking sequences obtained above to perform long-distance PCR to clone the whole *PmPR10-1.13* gene, including the promoter region, the coding region, and the 3′-untranslated region (UTR) downstream from the stop codon. The primers used in the genomic long-distance PCR were forward primer 5′-ATC GCA AAG CTT AGG AGG ATA GCA-3′ (XH5) and reverse primer 5′-GTA AGC GGA AAA TCC CAT TTA TCG-3′ (XH3).

Genomic DNA was digested with appropriate restriction enzymes and then circularized using T4 DNA ligase. The IPCR was performed using re-circularized genomic DNA as a template with an Advantage Genomic PCR Kit (Clontech Laboratories, Palo Alto, CA, USA). Thermal cycling conditions consisted of an initial 3-min denaturation at 94°C, 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 fragments were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA), and plasmid constructions and manipulation were carried out using standard methods (Sambrook et al. 1989).

DNA data analysis

Deoxyribonucleic acid sequences were determined for both strands on an ABI310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using a Thermo-cycle sequence kit (Amersham, Baie d'Urfe, Quebec, Canada) with T7, SP6, and other internal primers as needed, according to the manufacturer's instructions. DNA sequence data were compiled and analysed using BLAST (Altschul et al. 1997), DNASTAR software (DNASTAR Inc., Madison, WI, USA) and ExPASy Proteomics tools (Swiss Institute of Bioinformatics, http://us.expasy.org/tools/). The transcription start site in the promoter sequence was predicted using a promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html) (Reese 2001). The analysis of potential *cis*-regulatory elements in the promoter sequence was performed with the PlantCARE program (http://intra.psb.ugent.be:8080/PlantCARE/) (Lescot et al. 2002). The western white pine *PmPR10-1.13* genomic DNA sequence has been deposited in the GenBank database under accession number AY697416.

RNA isolation and analysis

Total RNA isolation and semiquantitative RT-PCR analyses were carried out as described by Liu and Ekramoddoullah (2003). To investigate *PmPR10-1.13* gene expression in western white pine, total RNA was treated with RQ 1 RNase-Free DNase (Promega) and purified using a Plant RNeasy Extraction Kit (Qiagen). Two micrograms of total RNA was reverse-transcribed using an Omniscript Reverse Transcriptase Kit (Qiagen) in a total volume of 20 µl. Semiquantitative RT-PCR was carried out with 1 µl of cDNA in 25 µl of PCR mix using a Taq PCR Master Mix (Qiagen) with gene-specific primers. *PmPR10-1.13* gene-specific primers were 5′-ATT AAT ATT GAA GAA ATA ACT ATT G-3′ (XA11-5) and 5′-GAT ACA GCC CAT AAA GAC GA-3′ (XA11-3).

The PCR product spans a genomic intron, which allows the visualization of potential genomic contamination. To monitor the amount of cDNA derived from plant tissues, QuantamRNA 18S rRNA Internal Standard Kit (Ambion, Austin, TX, USA) was used in control experiments with primers/competimers in the ratio of 3:7. The RT-PCR condition was determined experimentally with an incubation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and primer extension at 72°C for 90 s, with a final extension at 72°C for 7 min.

Construction of plasmids for promoter analyses

To construct the *PmPR10-1.13* promoter GUS fusion, PCR was performed for different progressive deletions from the 5′-end of the *PmPR10-1.13* promoter. The PCR-amplified promoter fragments were cloned into appropriate restriction enzyme sites of pBI101 (Jefferson 1987). All binary constructs contained the *β*-glucuronidase reporter gene in frame with a *PmPR10-1.13* promoter sequence from the start codon upstream to nucleotide positions −1316 (P-1316), −930 (P-930), −309 (P-309), and −100 (P-100) as numbered from the predicted transcription start site. The promoter region in each construct was verified by DNA sequence analysis.

Arabidopsis transformation

Following the introduction of plasmid DNA of each binary construct into *Agrobacterium tumefaciens* (LBA 4404), *Agrobacterium*-mediated transformation of *Arabidopsis* was performed following the floral dip transformation procedure (Clough and Bent 1998). The constructs CaMV 35S promoter::GUS (pBI 121) and promoterless::GUS (pBI 101) were used as positive and negative controls, respectively. Transgenic seeds were selected on medium containing 0.5 Murashige and Skoog basal salt mixture supplemented with vitamins (Sigma-Aldrich, Oakville, ON, Canada), 60 µg mL^{-1} kanamycin, and 50 µg mL^{-1} gentamycin. Selected plants were transferred to soil, numbered, and tested with PCR to confirm the presence of the constructs. Seeds were collected from each assigned line and grown for experiments.

Fluorometric and histochemical GUS assays

Plant samples from at least ten transgenic lines were collected for each construct to examine GUS activity. Fluorometric GUS assays of crude plant extracts were performed as described by Jefferson (1987). GUS activity was determined with a DyNAQuant 200 fluorimeter (Hoefer, CA, USA), and protein concentration of crude extracts was determined as described previously (Ekramoddoullah and Davidson 1995). Histochemical localization of ß-glucuronidase activity was performed essentially as described by Jefferson (1987) with 5-bromo-4-chloro-3-indolyl-ß-glucuronide (X-Gluc, Clontech) as a substrate. Plant materials were vacuum infiltrated for a few seconds 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. Stained samples were cleared of chlorophyll in 70% ethanol prior to visual analysis under a dissecting microscope (WILD, M32, Heerbrugg, Switzerland).

A one-way ANOVA for independent samples was used to assess significance in mean GUS activity in *Arabidopsis* leaves between the *PmPR10-1.13* promoter constructs. ANOVA was performed using a program of one-way analysis of variance for independent or correlated samples at the VassarStats web site (http://faculty.vassar.edu/lowry/VassarStats.html) for statistical computation. Tukey's multiple range comparison test

was carried out to determine the absolute difference between two sample means required for significance at $\alpha = 0.05$.

Results

Isolation and molecular characterization of the *PmPR10-1.13* genomic clone

The genomic DNA clone of the *PmPR10-1.13* gene contains a fragment about 2.3 kb, including two exons, one intron, and ORF flanking sequences (Fig. 1a). The intron is A/T-rich and localized between the 1st and 2nd bases of the codon for amino acid G_{61}; this intron position is conserved in the PR10 gene family. The 5′-exon/intron and 3′-intron/exon boundaries are the consensus GT/AG donor/acceptor. The nucleotide sequence of the genomic clones is identical to that of the cDNA clone we reported previously (Liu et al. 2003), except for two base differences in the 3′-UTR.

The transcriptional start site was predicted and designated as +1 on the *PmPR10-1.13* promoter sequence (Fig. 1b). The 1,316-bp sequence upstream of the puta-

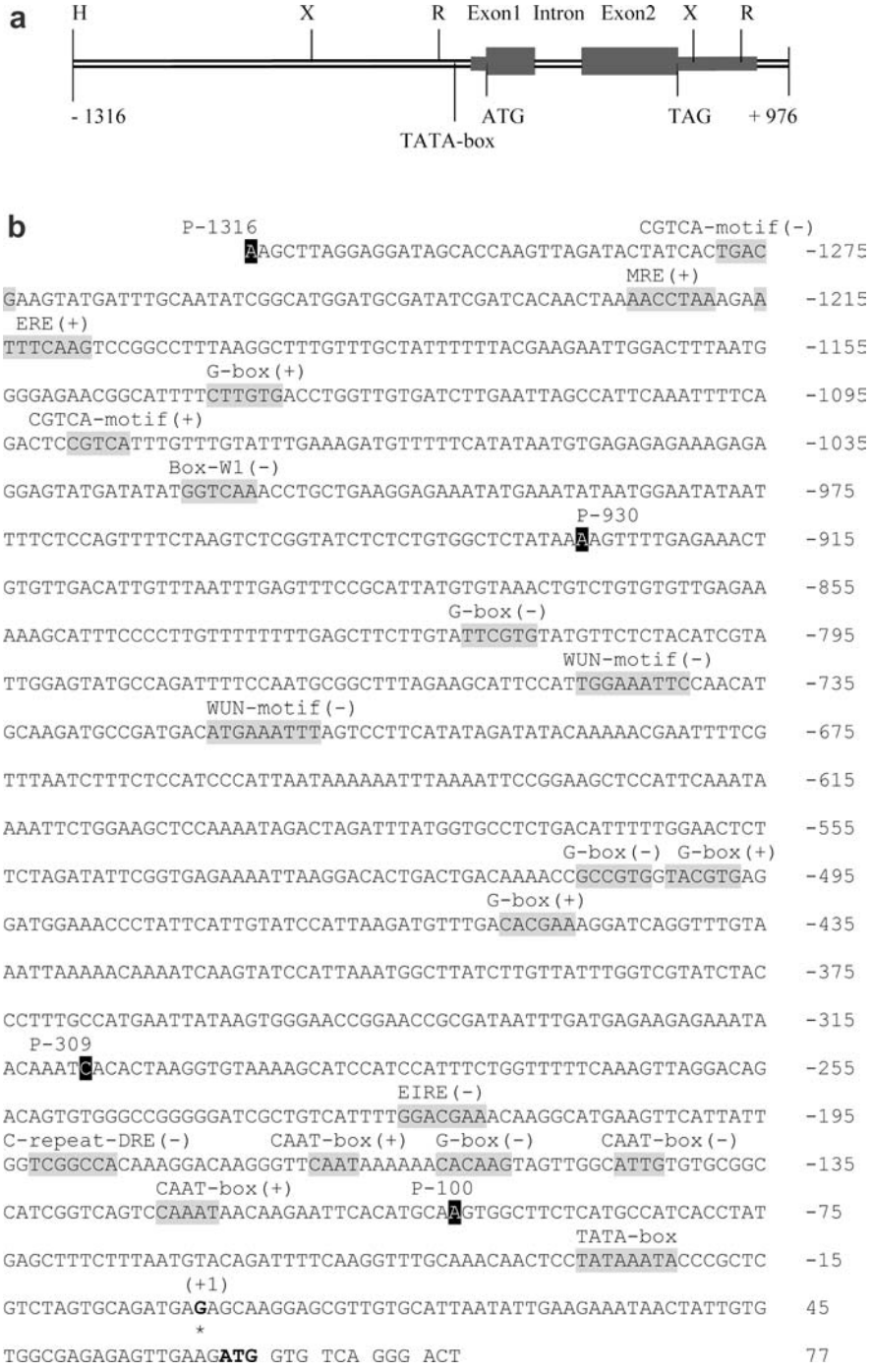

Fig. 1 Structure of the *PmPR10-1.13* gene. **a** Schematic representation of the *PmPR10-1.13* genomic clone. The *black rectangles* indicate two exons and the 5′- and 3′-untranslated regions. The region between two exons is intron. The start and stop codons of the open reading frame and the TATA-box position in the promoter region are shown. Restriction enzyme sites are labelled as follows: *H Hin*dIII, *R Eco*RI, and *X Xba*I. **b** Nucleotide sequence of the 5′-flanking fragment of western white pine *PmPR10-1.13* gene. Nucleotides are numbered relative to the putative transcription start site (+1) marked with an *asterisk*. The predicted regulatory *cis* elements are shaded in *grey*, and their orientations are shown in parenthesis. The positions of four 5′ deletions for the translational fusion of GUS reporter gene are indicated in *black*. The start codon ATG is in *bold* and the coding sequence downstream of ATG is presented in *triples*

tive transcriptional start site was determined. When 100% core similarity and 90% matrix similarity were set for the searching of putative *cis*-acting regulatory elements, several elements were found in this promoter. A putative TATA box was found at −30, whereas three CAAT boxes were identified at positions −123, −147, and −171. A typical box-W1 (TTGACC), the binding site of WRKY transcription factor for the fungal elicitor response (Rushton et al. 1996), was found at −1,021. A sequence of TTCGTCC, similar to another elicitor responsive element (EIRE) (Shah and Klessig 1996), was found at −224. Two CGTCA/TGACG-motifs were identified at −1,273 and −1,089, the same as the *cis*-acting regulatory element involved in the MeJA responsiveness (Rouster et al. 1997). An ERE-like motif (ATTTCAAG) at −1,216 shared high homology with an ethylene-responsive enhancer element (Itzhaki et al. 1994).

One C-repeat-DRE (TGGCCGAC, at −193) was identical to that in *Arabidopsis cor15a* promoter, believed to be involved in both drought- and cold-responsive gene expression (Baker et al. 1994). An MRE sequence (AACCTAA), the binding site of transcription factor MYB for light responsiveness in *Petroselinum crispum* (Feldbrügge et al. 1997), was found at −1,226. G-boxes or G-box-like motifs (Rouster et al. 1997) were observed throughout the promoter sequence at six positions (−1,138, −819, −510, −503, −456, and −161). Two WUN-like-motifs, responsible for wound-responsiveness (Pastuglia et al. 1997), were observed at −750 and −719. Most of the above motifs are involved in induced gene expression following environmental stresses in angiosperms, suggesting that *PmPR10-1.13* may be regulated in plant defence response.

GUS expression pattern of the *PmPR10-1.13* promoter

Because of high identities (>88%) of the *PmPR10-1.13* nucleotide sequence to other members among the subfamily I of *PmPR10* genes (Liu and Ekramoddoullah 2004), the expression of *PmPR10-1.13* in western white pine was investigated by RT- PCR analysis using gene-specific primers corresponding to the sequences in the 5′- and 3′-UTR. RT-PCR showed the high *PmPR10-1.13* transcript levels in vegetative shoots and stems, next in needles, and a weak signal in roots relative to the expression of the 18S-rRNA gene. No transcript was detected in the developing female and male cones (Fig. 2). A similar mRNA expression pattern was observed by Northern blot analysis with a *PmPR10-1.13* cDNA probe (data not shown).

ß-Glucuronidase expression in transgenic *Arabidopsis* with the longest promoter fragment (P-1316) generally mimicked the *PmPR10-1.13* expression pattern in western white pine as shown in RT-PCR analysis (Fig. 2). Histochemical staining showed that GUS activity appeared first in the hypocotyl and cotyledons of 2-day-old seedlings, but no signal was detected in the roots at this stage (Fig. 3a–c). As seedlings grew, the apical meristem showed the highest levels of GUS expression, with somewhat lower levels in stems and leaves (Fig. 3d). Occasionally, irregular staining spots were found in the roots of adult plants, which possibly resulted from unidentified stress factor(s). In a young inflorescence, weak GUS activity was detected on petal edges (Fig. 3e), and strong GUS activity only in the stamen filaments and the stigmas that were maintained throughout seed development (Fig. 3f–h). No GUS expression was found in the developing reproductive tissues of the anthers, pollens, ovaries, and seeds at any stage.

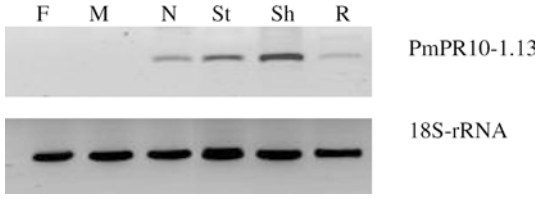

Fig. 2 RT-PCR analysis to detect *PmPR10-1.13* transcript in different organs of western white pine. Total RNA was isolated from developing *F* female cones, *M* male cones, *N* needles, *St* stems, *Sh* vegetative shoots and *R* roots. *Upper panel* Semiquantitative RT-PCR was performed with *PmPR10-1.13* gene-specific primers. *Lower panel* A control PCR using 18 S rRNA primers was performed to confirm RNA and cDNA quality in RT-PCR analysis

PmPR10-1.13 gene expression in response to pathogen infection and wounding stress

RT-PCR with gene-specific primers demonstrated that *PmPR10-1.13* mRNA was significantly induced by wounding relative to the expression of 18S rRNA gene (Fig. 4a). To confirm any wound-responsive regions in the *PmPR10-1.13* promoter, the response of transgenic *Arabidopsis* leaves to wounding was examined. It was found that the *PmPR10-1.13* promoter-regulated GUS activity was induced around wounding sites (Fig. 3i). *PmPR10-1.13* transcript level increased in a similar manner in western white pine needles post-inoculation with *C. ribicola* as in healthy needles post-mechanical wounding (Fig. 4a). Histochemical detection of GUS activity was performed with leaves of transgenic *Arabidopsis* locally injected with the pathogen *P. syringae*. The GUS activity was activated in the infiltrated area 24 h post *P. syringae* infection (Fig. 4b). It was noticed that the injection of 10 mM $MgCl_2$ (control) also induced GUS activity at a much lower level (Fig.4b).

Quantitative analysis of constitutive GUS expression levels in the leaves of different transgenics

To define the *cis*-acting regulatory regions responsible for the expression patterns above, a series of 5′ end truncations of *PmPR10.13* promoter were made at positions shown in Fig. 1b. Fluorescence assay of GUS

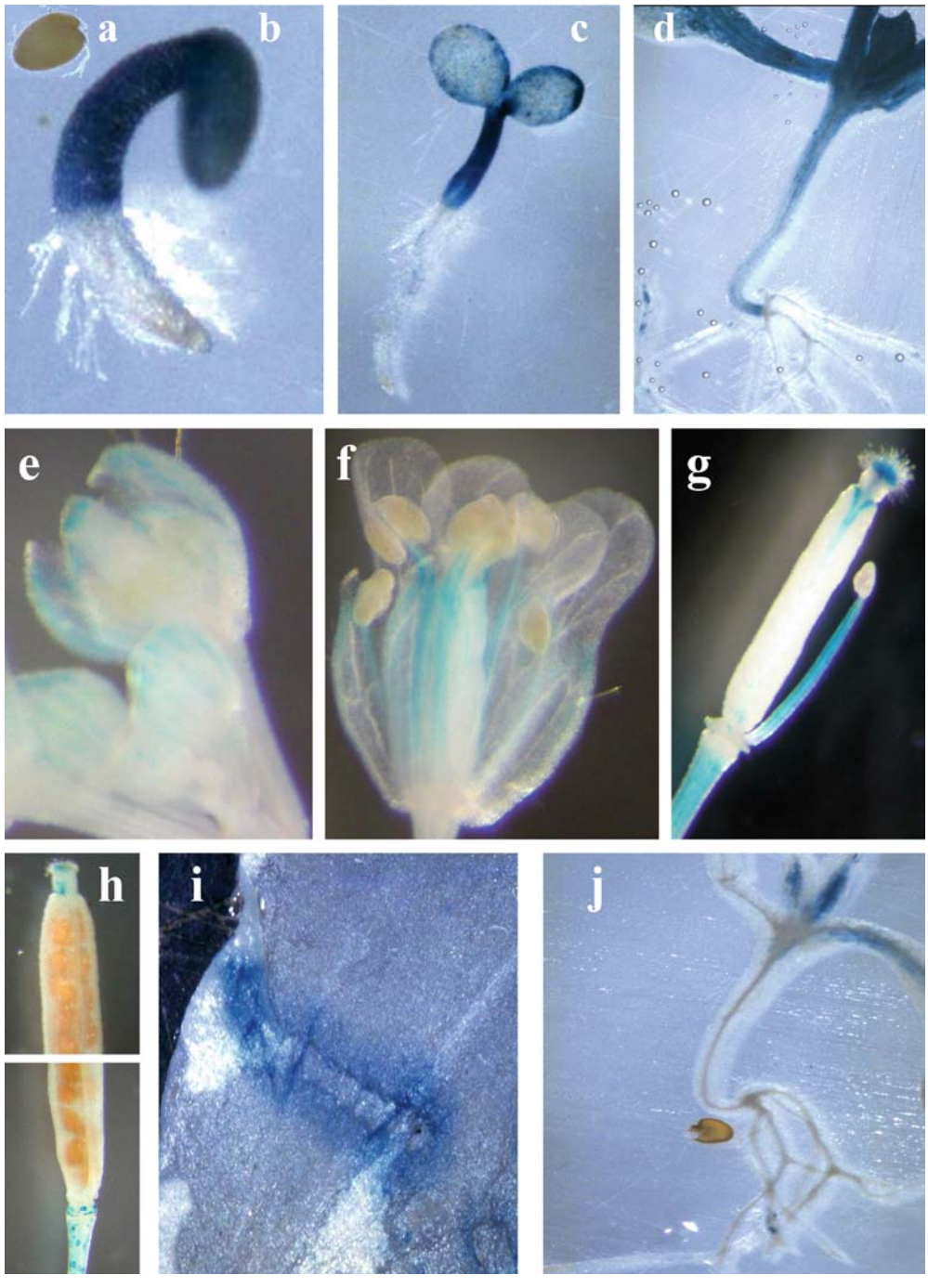

Fig. 3 GUS staining patterns of the organs of transgenic *Arabidopsis* plants carrying *PmPR10-1.13* promoter GUS fusions of P-1316 (**a**–**i**) and P-303 (**j**). **a** A soaked seed just before germination without any GUS staining. **b** A 2-day-old and **c** 5-day-old seedling showing GUS activity in hypocotyl and cotyledons. **d** A 2-week-old transgenic plant showing strong GUS activity mainly in the apical meristem, and some in stem and leaves. **e** A representative inflorescence at a developmental stage just before flower opening, showing weak GUS activity in petals. **f** A representative flower showing strong GUS activity in filaments of stamen. **g** GUS activity in a developing silique at an early stage and **h** at a mature stage (in two parts because of its length), showing GUS activity in the stigma. **i** GUS histochemical test of a leaf 24 h post-wounding treatment. **j** A 2-week-old seedling of P-309 transgenics, showing GUS activity in apical meristem

activity in leaves was conducted for the investigation of any possible quantitative differences in the level of transcriptional activity of the different promoter constructs (Fig. 5). The highest level of average GUS activity was observed in the P-1316 construct. The expression level dropped significantly approximately fivefold in the transgenic lines harbouring construct P-930 (<0.01), indicating that the promoter sequence between $-1{,}316$ and -930 bp contains positive regulatory elements. When the promoter sequence was progressively deleted to -309, the GUS expression levels increased significantly as compared to transgenics of P-930 (<0.01). The low level of GUS expression was seen in the P-100 construct, about ten times lower than the average from the construct P-1316. There were significant differences in GUS activity among all constructs (<0.01). Histochemical staining of GUS activity showed that the P-309 transformants had a similar expression pattern as that of the P-1316 construct, but at much lower levels (Fig. 3j). These results indicate that the *PmPR10-1.13* promoter has quantitative sequences for both activation and repression. Positive regulatory

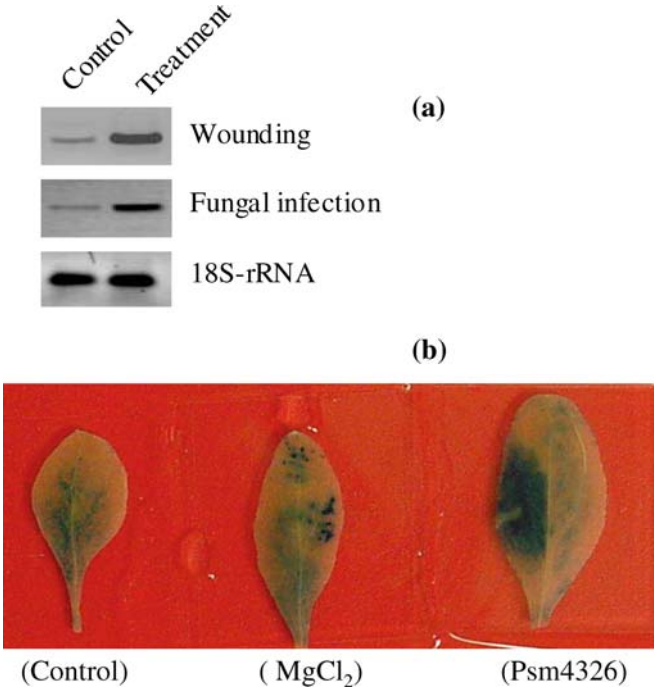

Fig. 4 Expression induction of *PmPR10-1.13* in response to pathogen infection and wounding. **a** RT-PCR analysis of western white pine needles inoculated with *Cronartium ribicola* or mechanically wounded. As a control, PCR using 18S rRNA primers was performed to confirm RNA and cDNA quality in RT-PCR analysis. **b** Histochemical localization of GUS gene expression in the leaves following bacterial infiltration of transgenic *Arabidopsis* plants. Leaves from transgenic *Arabidopsis* plant with *PmPR10-1.13*::GUS fusion (P-1316). Samples were harvested at 24 h post-inoculation and stained to detect GUS activity. *Control* An untreated leaf, *$MgCl_2$* a leaf infiltrated with 10 mM MgCl2, *Psm4326* a leaf infiltrated with *P. syringae* (Psm4326)

elements are located in two regions from −1,316 to −930 and from −309 to −100, and negative regulatory elements are present between −930 and −309.

Different promoter regions confer inducible expression in response to wounding

As shown in Fig. 6a, GUS activity in P-1316 transformants increased 26–130 times in different transgenic lines 24 h post-wounding treatment. 5′-deletion of this sequence to −930 (construct P-930) completely abolished the wound responsiveness. However, a further deletion to −309 (construct P-309) recovered wound-induction to 7–10 times higher levels. GUS activity in transformants with the construct P-100 exhibited no significant modulation in response to wounding. These results indicate that the 346 bp between −1,316 and −930 and the 209 bp between −309 and −100 contain *cis*-acting elements that are essential for wound responsiveness. Two WUN-like motifs at −750 and −719, identified from a search of transcription factor binding sites (Fig. 1), were unable to confer wound inducibility in the *PmPR10-1.13* promoter.

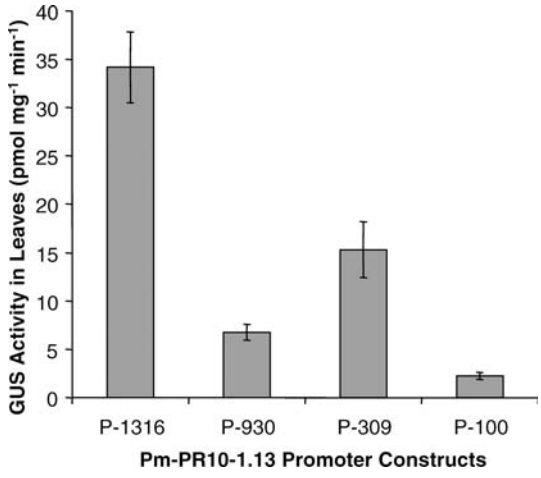

Fig. 5 Influence of 5′ deletions of the *PmPR10-1.13* promoter on GUS expression in transgenic *Arabidopsis* leaves. Each *bar* represents average GUS activity levels for different constructs. *Error bars* indicate the standard errors for transgenic lines tested with the same construct. A one-way ANOVA for independent samples was used to assess significance in mean GUS activity in *Arabidopsis* leaves of transgenic lines ($n = 16$) between the *PmPR10-1.13* promoter constructs. The experiment was repeated twice for each transgenic line. There was a significant difference in GUS activity between all constructs ($P < 0.01$)

ß-Glucuronidase activity in transgenic tobacco plants containing 5′ deletions of the *PmPR10-1.14* promoter was examined to see if they could regulate wound-inducible GUS expression. GUS activity driven by sequences of this promoter remained significantly constant regardless of the 5′ deletion (Fig. 6b). The comparison of nucleotide sequences of *PmPR10-1.13* and *PmPR10-1.14* promoters revealed that there was no significant similarity in the region upstream of position −309, while the regions from ORF start codon to −309 shared 85% identity (Fig. 7). These two promoters have a few common *cis*-acting regulatory elements in the region −100/−309, including C-repeat-DRE, CAAT- motif. However, a GGACGAA and a CACAAG sequence, similar to EIRE and G-box respectively, were only present in the *PmPR10-1.13* promoter.

Discussion

The *PmPR10-1.13* gene in transgenic *Arabidopsis* was expressed in aerial parts of plants. In contrast, the *PmPR10-1.14* promoter directed a root-specific GUS expression in transgenic tobacco (Liu and Ekramoddoullah 2003). Because of the high similarities of nucleotide sequences among closely related *PmPR10-1* genes, RT-PCR with gene-specific primers was employed to distinguish differential PR10 gene expression in western white pine. *PmPR10-1.13* transcript was detected at a high level in vegetative shoots and stems, and then in needles. A weak signal was still observed in roots (Fig. 2), possibly from a constant physiological stress from soil environment as speculated previously (Mylona

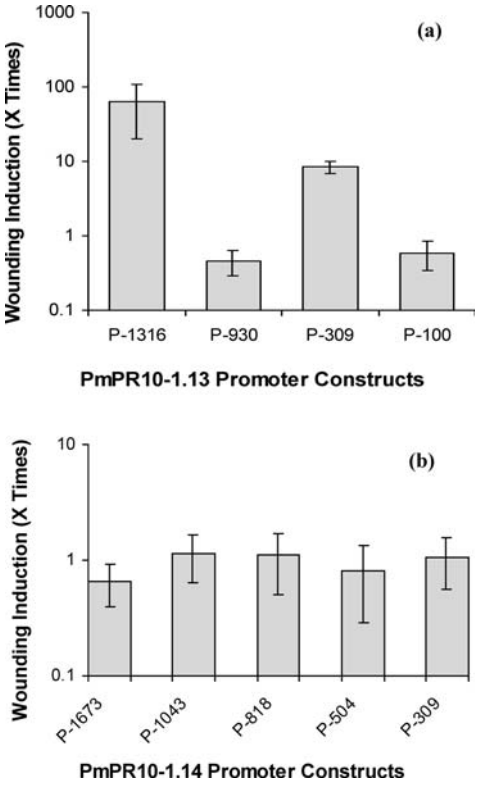

Fig. 6 Induction of GUS activity of several 5′ deletions of two *PmPR10-1* promoters in transgenic leaves. Each *bar* represents the average induction time for each construct. The induction time was the ratio of GUS activity of 24 h post-treatment to that of 0 h post-treatment (control). *Error bars* represent the standard deviation of transgenic lines ($n = 16$) of the same construct. The experiment was repeated twice for each transgenic line. **a** GUS expression directed by *PmPR10-1.13* promoter fusions in transgenic *Arabidopsis*. **b** GUS expression directed by *PmPR10-1.14* promoter fusions in transgenic tobacco

et al. 1994). The western white pine PR10 gene family consists of three subfamilies with at least 19 gene sequences (Liu and Ekramoddoullah 2004) and 12 PR10 protein isoforms detected immunologically in 2-D western blot analysis (Liu et al. 2003). The differential expression of highly similar PR10 genes in different plant organs and tissues provides a potential for plants to produce protein isoforms that are most selected evolutionarily in response to environmental stresses. Our previous study has demonstrated that this does happen, where PmPR10 protein accumulation patterns are almost identical under wounding stress and pathogen infection, but different under cold-hardiness (Liu et al. 2003). Based on these investigations, a PR10 gene expressed in response to wounding, such as *PmPR10-1.13*, should contain promoter region(s) responsible for pathogen/wound-responsiveness.

Promoter deletion from −1316 to −930 resulted in a drop in both constitutive expression levels in leaves (Fig. 5) and abolishment of wound responsiveness (Fig. 6a). Analysis of the sequence between −1,316 and −930 revealed a few motifs identical or similar to those well characterized in inducible promoters in response to a variety of environmental stresses. Among these motifs, it is worthy to point out that this region contains two typical TGACG/CGTCA-motifs at −1,273 and −1,089, one well-characterized box-W1 at −1,021, and one ERE-like motif (ATTTCAAG) at −1,216 (Fig. 1b). The GACG/CGTCA motif is a binding site for the *Arabidopsis* bZIP *trans*-activating factor TGA1a (Benfey and Chua 1989; Schindler et al. 1992) and has been identified as MeJA-responsive elements in the barley wound/MeJA-responsive *LoxA* promoter (Rouster et al. 1997). Our previous study showed that MeJA enhanced the wound-induced accumulation of PmPR10 proteins in western white pine needles (Liu et al. 2003). The wound-induced gene expression is mediated by the signal molecule MeJA (Creelman and Mullet 1995; Doares et al. 1995; Peña-Cortés et al. 1995) and ethylene (O'Donnell et al. 1996). Therefore, these motifs in the *PmPR10-1.13* promoter may be involved in wound responsiveness.

The second region in the *PmPR10-1.13* promoter responsible for wound-inducible GUS expression was localized between −309 and −100. An EIRE-like motifs (−224) and a G-box-like motif (−161) were found in this region of the *PmPR10-1.13* promoter, but they were absent in the *PR10-1.14* promoter that showed no wound responsiveness. As the binding site of a transcriptional factor in the tobacco *PR-2d* promoter, the EIRE motif (TTCGACC) was believed to be responsible for elicitor responsiveness (Shah and Klessig 1996). The involvement of G-box in wound-responsive expression was reported in the horseradish *prx C2* gene (Kawaoka et al. 1994). Comparison of *PmPR10-1.13* and *PmPR10-1.14* promoters between −309 and −100 also revealed an interesting sequence at −210 in the *PmPR10-1.13* promoter. This sequence (5′-ATGAAGTTCAT-3′) contains two inverted repeats of ATGAA and is able to form a perfect palindrome. Although palindromic regulatory elements have been identified in various species, any role that putative *cis*-acting regulatory elements and a palindromic element play in wound-responsive expression of the *PmPR10-1.13* gene will require further investigation.

The constitutive expression of *PmPR10-1.13* in aerial parts of transgenic *Arabidopsis* seedlings, even when the promoter was deleted to −309 (Fig. 3), suggests light-regulated *cis* elements in this promoter. Widely investigated *cis* elements related to light regulation are the G-box and G-box-like motif (Giuliano et al. 1988; Menkens et al. 1995). The *PmPR10-1.13* promoter contains three G-boxes (−1,138, −503, and −161) that are identical to those in *Antirrhinum majus* (Arguello-Astorga and Herrera-Estrella 1996) and *Hordeum vulgare* (Rouster et al. 1997). Three G-box-like motifs (−819, −510, −456) shared significant similarities with those in *Zea mays* (Arguello-Astorga and Herrera-Estrella 1996; Manjunath and Sachs 1997). The G-box binding factors (GBFs) belong to a family of basic leucine zipper transcription factors. Another motif involved in light responsiveness is MRE (AACCTAA) at −1,226, which is identical to that for the binding of PcMYB to mediate light-dependant activation of the chalcone synthase

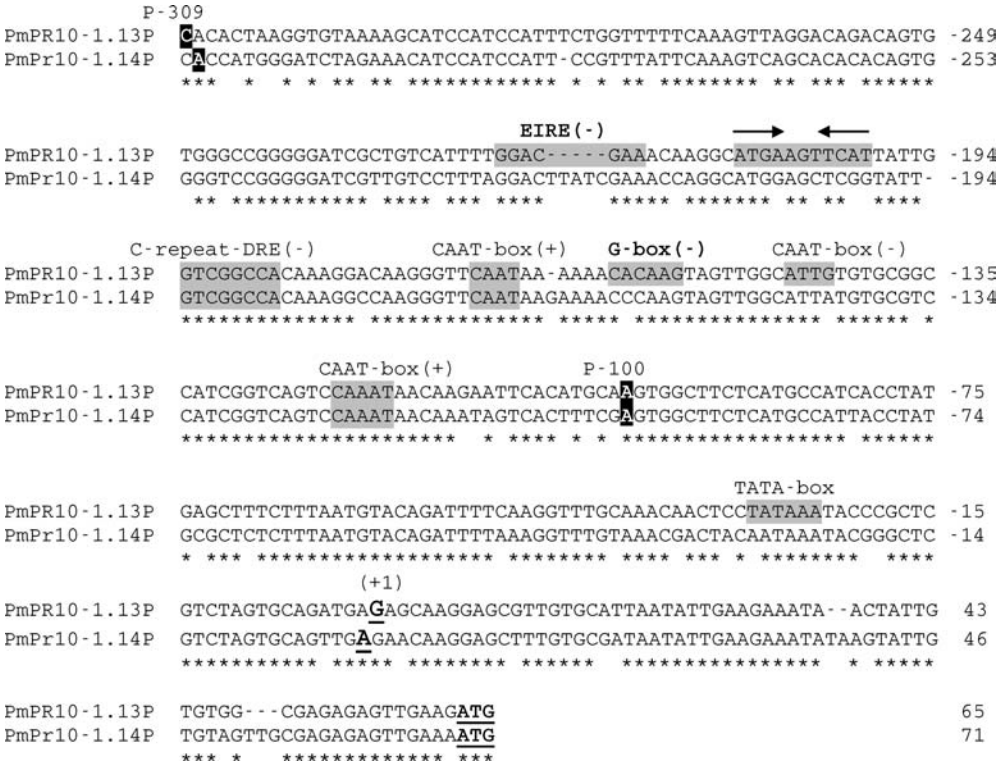

Fig. 7 Promoter nucleotide sequence comparison of *PmPR10-1.13* and *PmPR10-1.14* genes. *Grey boxes* mark the potential *cis*-acting regulatory elements. The putative transcription start sites are in *bold* and *underlined*. A palindromic sequence is indicated by *small arrows*. The deletion positions for GUS fusions are in *black*

minimal promoter in *Petroselinum crispum* (Feldbrügge et al. 1997). These elements possibly play a role in the expression pattern of *PmPR10-1.13*. However, the presence and function of GBFs and MYBs in a conifer species remain to be explored.

In the current study, we carried out a detailed deletion analysis of the *PmPR10-1.13* promoter, one of the environmental stress-responsive *PmPR10* genes. Our results highlighted several regions containing positive and negative regulatory elements in the *PmPR10-1.13* promoter. In *Asparagus* PR10 family, the *AoPR1* gene promoter displayed a wound-responsive expression pattern (Warner et al. 1993). The bean (*Phaseolus vulgaris* L.) PR10 gene (*Ypr10c*) promoter directed an organ-specific, dark-dependent and salicylic acid-inducible expression (Walter et al. 1996). The GUS activity regulated by the *Ypr10*a* promoter from apple (*Malus domestica*) was induced in young leaves by multiple stress factors, including pathogen attack and fungal elicitor (Pühringer et al. 2000). Detailed study on parsley PR10 promoter of *PR1* gene showed that the sequence from −240 to −130 of *PR1* promoter was essential for fungal elicitation (Meier et al. 1991). A further work revealed that the interaction of WRKY proteins and W-boxes was responsible for elicitor-responsive expression of parsley *PR1* gene (Rushton et al. 1996). In another parsley PR10 promoter of *pr2* gene, a 11-bp motif was necessary for elicitor-mediated expression (Korfhage et al. 1994). Both an elicitor response element and a silencing element have also been identified in the promoter regions of the potato *PR10a* gene responsible for its activation and repression respectively (Desveaux et al. 2000; Boyle and Brisson 2001). These findings suggest that there is a complex network modulating PR10 gene expression. The localization of short sequences in the *PmPR10-1.13* promoter involved in wound responsiveness will facilitate further study such as a gel shift assay to identify the transcriptional factors interacting with these regulatory sequences.

Differential expression of *PmPR10* genes is under developmental, organ-specific, and environmental regulation. The investigation of *cis*-acting regulatory elements that regulate the expression of PR10 genes may shed light on their complex and diverse patterns in plant growth and development and in plant defence response. It also provides clues for the further identification of transcriptional factors that modulate *PmPR10* gene expression.

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