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The pine *Pschi4* promoter directs wound-induced transcription

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Abstract

Mechanical wounding stimulates the accumulation of *Pschi4* transcripts (encoding a putative extracellular chitinase) in pine trees. To gain insight into the transcriptional regulatory region(s) in this gymnosperm defense gene, the 5′-flanking region of *Pschi4* was fused to the *uid*A reporter gene encoding β-glucuronidase (GUS), and the construct was functionally tested for transient expression in onion and pine cells. As little as 200 bp of the upstream sequence was sufficient to promote activated transcription and nearly maximum levels of transient expression in onion cells. Similar results were obtained in bombarded pine cells, although maximum expression appeared to require the entire 4.5 kb of upstream sequence. Having defined potential regulatory regions in the 5′ flanking region of *Pschi4* in transient assays, the pine promoter-*uid*A fusions were functionally tested in stably transformed tobacco plants. As little as 200 bp of the upstream sequence was sufficient to direct wound-inducible transcription. Furthermore, particle bombardment was sufficient to stimulate transgene expression in the transgenic tobacco, indicating that bombardment per se leads to wound responses in vivo. Taken as a whole, our data demonstrate that a gymnosperm promoter can recruit a wound-regulated signal transduction pathway in angiosperms. This suggests that the downstream components of wound signaling pathways are functionally conserved across broad taxonomic groups.

Keywords: Angiosperm; Bombardment; Gymnosperm; Promoter; Wounding

1. Introduction

Mechanical wounding is an environmental stress factor that influences all higher plants. Trees are particularly long-lived plants, so an individual tree can be subjected to persistent mechanical wounding throughout its life-cycle due to herbivory, parasitism, wind damage, and anthropogenic sources. Wounds can directly disrupt physiological function, and furthermore wounds are potential infection courts for opportunistic pathogens. Consequently, wound sensing mechanisms have evolved whose function in plants is to activate expression of genes involved in wound repair and plant defense. Wound sensing appears to be important in all higher plants, because wound responses are widespread among plants and wound regulation is common among genes.

We have focused our attention on a gene from pine trees (*Pschi4*) that apparently encodes an extracellular chitinase [1]. We were interested in using this gene to illuminate several aspects of

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pine gene regulation. First, we wanted to test the regulation of this pine gene in intact pine trees. Previous analyses were limited to the expression of this gene in cell cultures. Second, we wanted to determine the location of the *Pschi4* promoter. Relatively few functional tests of gymnosperm promoters have been performed, and one that has been rigorously dissected appeared to contain regulatory elements downstream of the coding sequence [2]. Consequently, the location of promoters in gymnosperm genes was not obvious when our investigations began. Third, we wanted to examine functional homology between promoters in gymnosperms and angiosperms. Results from various laboratories in which transgenes were introduced into gymnosperm cells have been highly variable in terms of transgene expression levels [3]. This raised questions as to whether aspects of transcriptional control may differ in angiosperms and gymnosperms.

Herein we report that *Pschi4* is mechanically wound-induced in stems of pine trees. We also show that the core promoter region of *Pschi4* is sufficient to recruit a wound signal transduction pathway in angiosperms, which supports the hypothesis that wound-induced transcriptional control mechanisms are conserved in distantly related plant taxa.

2. Materials and methods

2.1. Plant materials

Slash pine seeds (*Pinus elliottii* var. *elliottii*, open-pollinated seed collected from female 88-55 and kindly provided by Dr Tim White and Greg Powell of the Cooperative Forest Genetics Research Program, University of Florida) were treated with hydrogen peroxide (15%) for 5 min, rinsed three times in sterile distilled water, and sown on moist vermiculite. After germination, seedlings were transferred to 3.8-l pots or 4 × 22 cm tubes containing Metromix 250 and fertilized twice weekly with 1 g/l Peter's 20:7:7 with micronutrients. White pine seeds (*Pinus strobus*, open-pollinated seed collected from genotype # 23) were incubated in 10% commercial bleach (0.525% NaOCl) for 1 min, washed twice and then soaked in tapwater for 24 h. Seeds were blotted with paper towels until semi-dry, then wrapped in moist cheesecloth and stored in sealable plastic bags for 45 days at 4°C. Seeds were then sown in Rediearth soil-less mix supplemented with 17-6-13 Sierra Osmocote plus minor nutrients at a rate of 300 g/8.2 kg Rediearth and placed in a greenhouse under shade cloth until germination. Seedlings were potted individually in 4 × 20 cm pots. Seedlings were grown to a height of ca. 20 cm (slash pine) or 10 cm (white pine) and were mechanically wounded with pliers.

Wounding was imposed so that at the time of harvest, approximately one-half of the foliage was crushed and approximately one-half of the surface area of the stem was bruised so as to impose damage but not disrupt vascular transport processes. In the first experiment, two wounding treatments were imposed at 0 and 18 h on slash pine and white pine seedlings, and shoots from wounded and unwounded seedlings were excised and immediately frozen in liquid nitrogen at 21 h. In the time course experiment, wounding was imposed at 0 h and seedlings were harvested at 1, 6, 12 and 24 h.

Onion, maize and pine cells were used in particle bombardment experiments. White onions were purchased from local grocery stores. Maize suspension cell line PC-5 was kindly provided by Dr L.C. Hannah (University of Florida). Cell cultures of slash pine (*P. elliottii* var. *elliottii*, genotype 52–56) were initiated and maintained according to previously described methods [4]. Tobacco plants (*Nicotiana tabacum* var. Samsun) were used as an explant source for stable transformation. Plants were grown from seeds on agar-solidified MS medium (purchased from Sigma) and maintained in aseptic cultures.

2.2. RNA analysis

Total RNA was extracted from pine shoots using previously described methods [5] and subjected to RNA blot analysis as we previously reported [1]. The *Pschi4* probe was a 668-bp *Sac*I-*Bam*HI fragment that spans most of the coding sequence. RNA samples were quantified by spectrophotometry, and equal loading of RNA samples was confirmed by ethidium bromide staining of the gel and by hybridization with constitutively expressed cDNA probes [6].

2.3. Plasmid construction

The 5′-flanking region of *Pschi4* was amplified by PCR from the plasmid containing the genomic clone, by using a pair of primers (Forward: M13 universal primer F; Reverse: 5′-GCCAACG-CAAATTGTGGTGATGATCCC-3′ complementary to positions 735–761 of *Pschi4*, numbers corresponding to that in Fig. 2 of Ref. [1]). The PCR fragment was treated with T_4 DNA polymerase, then digested with *Bam*HI and cloned into *Bam*HI–*Eco*RV sites of pBlueScript. The resulting plasmid was digested with *Cla*I, filled by Klenow fragment of *Escherichia coli* DNA polymerase I, digested with *Xba*I, and subcloned into *Xba*I–*Sma*I sites of the binary vector pBI101.1 (Clontech) for use in creating stably transformed lines of tobacco, or subcloned into pGUS (the *Hin*dIII–*Eco*RI fragment from pBI101.1 subcloned in pUC19) for use in transient expression assays. The resulting binary vector construct was named pBI-4.5, and the pUC derivative was named pGUS-4.5.

Plasmid DNA of pGUS-4.5 was digested with *Pst*I and *Sal*I, then incubated with exonuclease III at 37°C for various periods of time (25, 50, 75, 100, 125 s). At each time point, reactions were stopped by adding an equal volume of 2 × Exo III stop buffer (0.3 N NaCl, 7.5 mM EDTA) and incubated at 70°C for 2 min. Fragments were made blunt by treatment with Klenow fragment, the DNAs were self-ligated and transformed into *E. coli*. This created a nested 5′ deletion series *Pschi4* putative promoter-*uid*A constructs in pUC19 with promoter sizes of 1.2, 0.8, 0.6, 0.5, 0.4 and 0.2 kb, which were designated by size (pGUS-1.2 for the 1.2-kb fragment, etc.). Individual transformants were screened by PCR to determine the length of the promoter fragments. These constructs, including the full-length construct pGUS-4.5, were tested by particle bombardment in transient expression assays. Some of these constructs (0.8, 0.4 and 0.2 kb) were subsequently subcloned into the *Hin*dIII site of pBI101.3 for stable transformation of tobacco, and were designated pBI-0.8, pBI-0.4 and pBI-0.2, respectively.

To normalize for transformation efficiency in the transient assay, a maize ubiquitin promoter-luciferase construct (kindly provided by Dr Don McCarty, University of Florida) was included in each bombardment mixture. Thus, expression data are reported as ratios of β-glucuronidase to luciferase (GUS/LUC) activities [7].

2.4. Particle bombardment

Particle bombardment was performed as described previously [8] using a DuPont PDS-100 particle gun. Those cells to be bombarded were prepared as follows. The inner epidermis of an outer layer of onion was peeled using a pair of fine forceps and placed on the center of MS-agar plates. Slash pine suspension cells were transfered to fresh medium at 7-day intervals. Three days after transfer, cells from several flasks were pooled to create an experimental population. Approximately 1 ml (settled cell volume) of cells was placed on a filter paper on MS-agar plate and dried for 3–10 min. The drying time varied from plate to plate and needed to be adjusted empirically to ensure that there was no liquid medium on top of the cells. Maize suspension cells were similarly prepared for bombardment. Each experiment represented three to ten replicate bombardments, and was repeated at least once with similar results.

2.5. Incubation and extraction of proteins

Following bombardment, culture dishes were incubated at room temperature in constant light for 24 h. Bombarded onion, pine or maize cells were ground with a mortar and pestle aided by addition of glass beads in 200–800 μl (depending on the mass of the tissue to be extracted) of GUS/LUC extraction buffer (0.1 M potassium phosphate (pH 7.8), 2 mM EDTA (pH 8.0), 2 mM DTT, 5% glycerol). The homogenates were centrifuged and supernatants were transferred to clean tubes and used for enzyme assays.

2.6. Quantification of transient expression

Quantitative measurement of GUS activity was performed as described [9], except that the substrate 4-methylumbelliferyl β-D-glucuronide MUG was dissolved in the extraction buffer described above. For the luciferase assay, an automated luminometer (AutoLumat model # LB953, Wallac, MA) and Promega's Luciferase Assay Kit were used, according to the manufacturer's instructions. The unit of measurement was the Relative Light Unit (RLU).

2.7. Analysis of promoter deletion constructs in stably transformed tobacco

Deletion constructs of the *Pschi4* promoter-GUS were introduced into tobacco using *Agrobacterium*-mediated transformation as described [10]. Primary transgenic plants were allowed to flower and set seed. A total of 21 transgenic lines from constructs pBI-4.5 (five lines), pBI-0.8 (seven lines), pBI-0.4 (five lines), and pBI-0.2 (four lines) were determined to be kanamycin resistant based on germination on MS-agar plates containing kanamycin (50 μg/ml), and the young leaves were used in mechanical wounding or bombardment experiments. For wounding treatments, a single leaf was cut in two along its longitudinal axis. One half was frozen in liquid nitrogen immediately, and the other half remained attached to the plant and was mechanically wounded with hemostats around the leaf margin. Wounding was performed at 0, 2 and 4 h, and the leaf was collected at 7 h. Protein extraction and GUS quantification were the same as described above. A minimum of three stably transformed lines containing each promoter construct were tested for wound inducibility in this fashion.

3. Results

3.1. Wound-induced expression of Pschi4 in pines

We previously reported that *Pschi4* was chitosan-induced in pine cells and in transgenic tobacco plants containing *Pschi4* under the control of its own 5′ and 3′ regulatory sequences [1]. Fig. 1 shows that *Pschi4* is also wound-induced in two different species of pines, *Pinus strobus* and *P. elliottii* var. *elliottii*. The magnitude of transcript accumulation was less for (twice-)wounded samples than for chitosan treated cell cultures, which were incubated with chitosan and therefore continuously 'elicited' for 6 h (Fig. 1A, lane 6).

A time course experiment indicated that after a single wounding event, *Pschi4* transcripts accumulated in stems within 6–12 h and after 24 h transcript accumulation had begun to subside (Fig. 1B). Organs that were detached from the plant and then mechanically wounded consistently showed little or no *Pschi4* transcript accumulation (data not shown). In contrast, wounding of tissues on intact plants such as that reported in this report, yielded consistent *Pschi4* transcript accumulation.

3.2. Transient expression of Pschi4 promoter-GUS fusions in pine suspension cells

To gain insight into the location of the regulatory sequences that may control wound-induced *Pschi4* transcription in pines, putative promoter-*uid*A constructs were made and then introduced into pine cells by particle bombardment. The purpose of the transient expression assays was to quickly identify regions sufficient to direct activated transcription. The results of this analysis are summarized in Fig. 2. The longest construct that was tested (4.5 kb) consistently directed higher levels of transgene expression relative to the promoterless control. However, progressive 5′ deletions of this putative promoter region that were intermediate in size (1.2, 0.8, 0.6, 0.5, 0.4 and 0.2 kb) directed levels of expression that were similar to one another, and only slightly higher than the promoterless control (Fig. 2, additional data from other experiments are not shown). These observations did not reveal any obvious *cis*-elements that directed GUS expression above endogenous levels in pine cells, with the possible exception of a putative enhancer region far upstream of the transcription start site (i.e. in the region between −4500 and −1200).

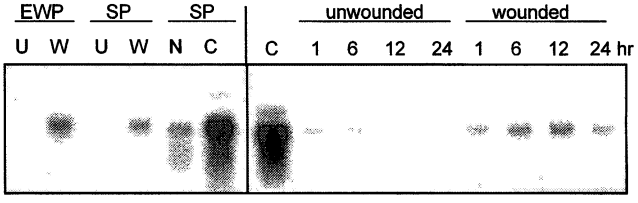

Fig. 1. *Pschi4* is wound-induced in pine seedlings. Northern blots contained total RNA (10 μg/lane) from unwounded (U) and wounded (W) seedlings, and from non-elicited (N) and chitosan-elicited (C) slash pine cultured cells [1] as controls. Left panel, the first and second lanes are RNA from shoot tissues of unwounded or wounded eastern white pine (EWP) seedlings, respectively; the third and fourth lanes are stems from unwounded or wounded slash pine (SP) seedlings, respectively; the fifth and sixth lanes are cultured cells after 0 and 6 h of chitosan treatment, respectively. Wounded seedlings were wounded at 0 and 18 h, and harvested at 21 h. Right panel, the first lane is RNA from cultured cells after 6 h of chitosan treatment, and the remaining lanes are from slash pine seedlings that were subjected to a single wound treatment and stem samples were collected from pools of three seedlings 1, 6, 12, and 24 h after wounding.

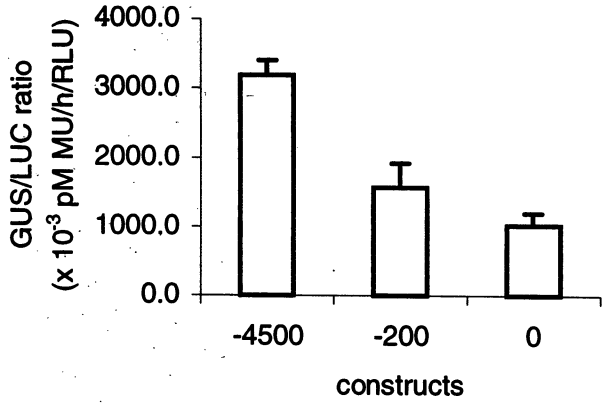

Fig. 2. Promoter activity in pine cells. Pine suspension cells were co-bombarded with 5 μg of indicated pGUS constructs and 5 μg of Ubi-LUC and cultured for 24 h. GUS and LUC activity were measured as described in the methods (Section 2). The '0' construct denotes the promoterless *uid*A construct used as a negative control. Each bar represents mean (± S.E.) of six replicates.

Incubation of cells with chitosan, either before or after bombardment, did not stimulate *Pschi4*-GUS expression. In general, the expression of *Pschi4–uidA* and *ubi*–LUC reporter genes was low in pine cells compared to the endogenous levels of GUS, which limited the utility of the bombardment approach to identify regulatory sequences. Alternative sources of plant material were used to meet the objectives of the study.

3.3. Transient expression in onion cells

Peeled epidermal cells from white onions provide a uniform, terminally differentiated source of cells with large nuclei, no pigmentation, and no background GUS or LUC activity [11]. Transient expression assays in onion cells revealed that all constructs directed activated transcription except the promoterless control. Results from a representative experiment are shown in Fig. 3. No consistent differences in activity were observed among the −200 to −4500 constructs within any particular bombardment experiment. The smallest promoter construct (−200 bp) contained sufficient regulatory elements to direct activated transcription.

Consistent with our previous results in pine, incubation of onion explants with chitosan before or after particle bombardment did not affect *Pschi4* transgene expression levels (data not shown).

3.4. Wound-induced expression in stably transformed tobacco plants

To confirm and extend the findings from the transient assays (which suggested that the core promoter was within 200 bp of the transcription start site) pGUS constructs were subcloned into the binary vector pBI101 and introduced into tobacco by *Agrobacterium*-mediated transformation. To minimize variation among assays within transgenic lines, the progenies of primary transgenic plants were used for mechanical wounding experiments. Fig. 4 shows the results from a typical experiment. Consistent with the transient expression data in onion suggesting the presence of *cis*-element(s) within the −200 region, only 200 bp of upstream sequence was sufficient to direct wound-induced expression in stably transformed tobacco plants. In all cases in which a plant line was confirmed to be kanamycin resistant, the wounded sample exhibited higher GUS activity than the unwounded control sample. Quantitative comparisons of GUS induction among different constructs were not made, because of the limited number of different lines that were analyzed. No GUS expression was detected in transgenic lines containing promoterless GUS constructs, or in normal untransformed plants.

3.5. Bombardment-induced expression in tobacco

Since particle bombardment involves mechanical injury to cells, we asked if the activity we observed in transient assays might be due in part to wounding associated with bombardment, which could then stimulate transcription of the P-GUS constructs. To determine if particle bombardment

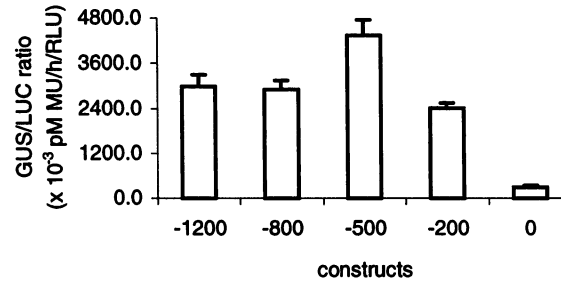

Fig. 3. Pine promoter activity in onion cells. Onion epidermis cells were bombarded with the constructs indicated. The '0' construct denotes the promoterless *uid*A construct used as a negative control. Data represent mean (± S.E.) of five to six replicates.

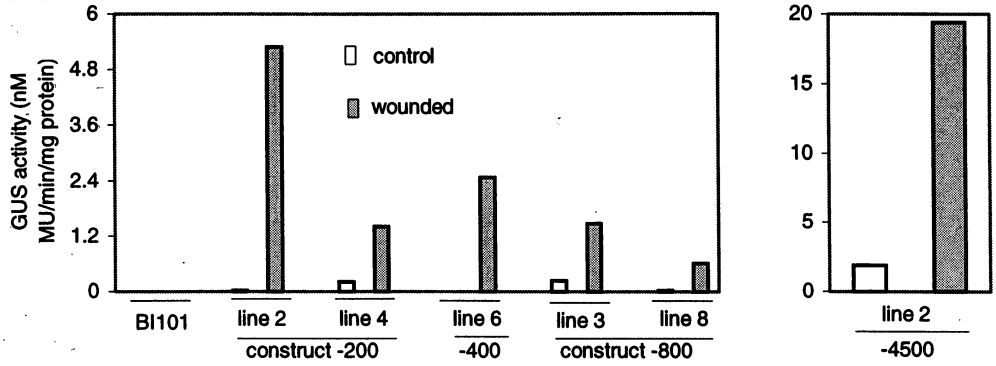

Fig. 4. Mechanical wound-induced transcription, driven by the 5′ region flanking *Pschi4*, in transgenic tobacco plants. Seeds from transgenic tobacco containing each construct were germinated on kanamycin-MS agar plates and then transferred to soil. A single fully expanded leaf from each line was selected and one-half of that leaf was harvested immediately (no treatment) and the other half remained on the plant for wound treatments as described in Methods (Section 2). Data from a representative experiment are presented.

per se could induce transcription, leaves from unwounded transgenic tobacco containing pBI-4.5 were bombarded with gold particles alone. Particle bombardment increased GUS levels three- to four-fold in transgenic tobacco (Fig. 5). Similar results were obtained when transgenic lines containing pBI-0.2 and pBI-0.4 constructs were bombarded with gold particles (data not shown), which confirmed that the −200 region of the promoter was sufficient to recruit a wound-induced signal transduction pathway.

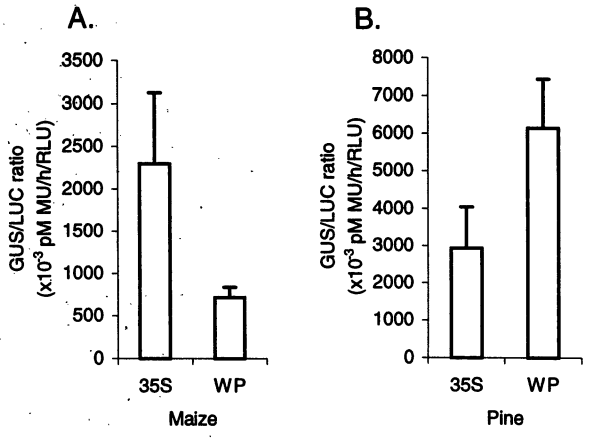

Fig. 5. Particle bombardment per se induces promoter activity. Four leaf disks (diameter = 1.5 cm) were cut from a single leaf of transgenic tobacco line 2 (containing pBI-4.5), or pBI101 vector only. Each leaf disk was then placed on the center of an MS-agar plate and subjected to no bombardment or no-DNA bombardment for one, two or three shots, respectively. The leaf disks were then incubated in 50 mM KCl for 22 h before total protein was extracted and assayed for GUS activity. Data represent means ± S.E. of two replicates.

3.6. Promoter comparison in maize and pine suspension cells

The commercially available CaMV 35S promoter (35S) has been demonstrated to direct high levels of activated transcription in many cell types of angiosperms. However, there are conflicting data regarding the efficiency of the 35S promoter in gymnosperms [3]. The availability of a promoter from pine allowed us to directly compare the levels of expression directed by 35S and the *Pschi4* promoter from white pine (WP). Constructs containing either 35S-GUS or pGUS-4.5 were bombarded into maize and pine suspension cells (Fig. 6). In maize cells, 35S was a stronger promoter than WP (Fig. 6A). In contrast, WP was a stronger pro-

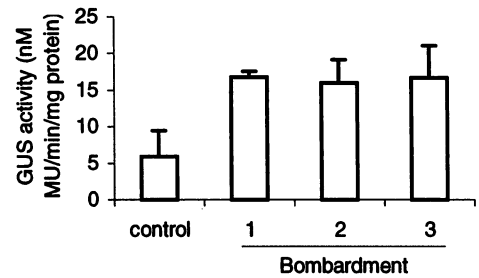

Fig. 6. Promoter comparison in maize and in pine cells. Maize and pine suspension cells were bombarded with pBI221 containing the CaMV 35S promoter (designated '35S') or pBI-4.5 (4.5 kb of the 5′ region flanking *Pschi4*; designated 'WP'). Ubi-LUC was used as an internal control. Data are expressed as the ratio of GUS activity to luciferase activity. All data represent means ± S.E. of two to five replicates.

moter than 35S in pine cells (Fig. 6B). It should be noted that the internal control, Ubi-LUC, also showed much lower activity in pine (average = 3.7×10^4 RLU) than in maize (average = 2.6×10^6 RLU), which in part explains why the ratio of GUS/LUC was higher in pine than in maize (note that the scales are different).

4. Discussion

We showed that *Pschi4* is induced by wounding in two different species of pine, *P. strobus* and *P. elliottii* var. *elliottii*. We then dissected the 5′ region flanking *Pschi4* and identified a region within 200 base pairs of the transcription start site that recruits a wound signal transduction pathway in transgenic tobacco plants. A 4.5-kb region 5′ to the *Pschi4* gene contains sufficient sequence to direct activated transcription in a gymnosperm (pine), and in angiosperms including monocots (onion and maize) and a dicot (tobacco).

4.1. Pschi4 is wound-inducible

Pschi4 transcripts accumulate in stems of wounded pine seedlings (Fig. 1). Some transcript accumulation is detected in stems, leaves and cell cultures prior to treatments, which could be due to constitutive expression of certain *Pschi4*-like genes in the genome. Southern blots suggested that the *Pschi4* probe hybridizes to four to six different genes in pines [1], so it would not be surprising if family members differed in their regulation.

Wound-induced expression was found in stems of pine (Fig. 1). Stems are infection courts for several important pine pathogens, and *Fusarium subglutinans* is one such pathogen that infects wounds on slash pine stems, leading to pitch canker disease [12]. Because pitch canker disease causes significant losses to forests in the southeastern US, there is a good deal of interest in understanding the mechanisms by which certain genotypes are resistant to the disease. Because *F. subglutinans* infects wounds on slash pine stems, and rapid chitinase induction is associated with resistance responses in other pathosystems [13], wound-inducible chitinases like *Pschi4* may be involved in conditioning the outcome of the host:pathogen interaction. Even if the Pschi4 protein is not involved in resistance responses, its induction may be a useful indicator of resistance in slash pine genotypes. Experiments are ongoing to assess the induction and potential role of *Pschi4* in pine:pathogen interactions.

4.2. The −200 region is sufficient to recruit wound signals

We assessed the transient expression of *Pschi4* promoter-*uid*A fusions in onion and pine cells, and measured stable expression in transgenic tobacco plants. This showed that the −200-bp upstream region contained sufficient promoter sequences to direct wound-induced transcription. It is reasonable to speculate that one or more wound-regulated proteins (transcriptional coactivators) may bind to a small portion of this 200-bp sequence and induce *Pschi4* gene transcription. A sequence was found between −140 and −132 (5′-TTACCTTCT-3′, identities underlined), that is similar in composition and relative location to a *cis* element implicated in wound and elicitor responsiveness of proteinase inhibitor genes in potato and tobacco plants (5′-GTACCTTGCC-3′; [14,15]). This same sequence found in the *Pschi4* promoter (5′-TTACCTTCT-3′, identities underlined) is also similar to the 10-bp TCA motif that was initially identified by binding of nuclear factors in tobacco nuclei, and was then detected by a sequence survey to be present in more than 30 pathogen-inducible promoters (TCA consensus = 5′-TCATCTTCTT-3′; [16]).

We used particle bombardment in the transient expression assays to identify promoter elements that direct activated transcription. We also bombarded transgenic tobacco leaves with metal particles containing no plasmid DNA. The results of the latter experiments confirmed the sufficiency of the −200 promoter region to recruit a wound signal, and further demonstrated that particle bombardment induced gene expression from a wound-inducible promoter. We infer from this result that the transient assays may not have detected upstream *cis* elements in the *Pschi4* promoter because their expression may have been masked by the large magnitude of the wound response.

While it is known that most transcriptional control elements reside within the 5′-upstream region of the coding sequence in higher plants, reports have also indicated that this may not

necessarily be true in pine trees. The regulatory sequences of a xylem-specific promoter do not lie exclusively within the 5′-upstream, but rather a 3′-flanking sequence appears to be important in silencing transcription in non-xylem cells [17]. In the present study, a wound response element was detected within 200 bp of the transcription start sequence, and in this respect appeared similar to most higher plant promoters. There are some putative differences between angiosperms and gymnosperms that were suggested by the transient expression data, and which deserve further study. There may be an enhancer region between −4500 and −1200 that was evident in pine cells, but not in onion cells. The presence of such an element can be tested by expressing deletion constructs in stably transformed pines. Production of these transgenic lines is in progress [18].

It is relevant to note that we did not observe chitosan-induced expression in transient assays with onion or pine, or in stably transformed tobacco plants (data not shown). In contrast, chitosan-induced *Pschi4* mRNA accumulation was observed in non-transgenic pine suspension cells, as well as in transgenic tobacco plants containing a full-length g*Pschi4* genomic clone [1]. We speculate that the putative chitosan-response *cis* elements could reside downstream of the *Pschi4* coding sequence.. It would appear that the chitosan and wound-response signal transduction pathways are distinct, which is in contrast to the model proposed by Doares et al. [19] that both chitosan and wounding stimulate gene transcription by inducing octadecanoid synthesis. However we cannot exclude the possibility that chitosan uptake was poor in our current experiments, or that onion and Samsun tobacco are relatively insensitive to chitosan. The presence and possible location of elicitor-response elements in pine genes will be further tested as we examine pathogen-induced defense responses in pines.

4.3. *Pschi4* promoter versus *CaMV 35S* promoter

Promoters from gymnosperm genes may offer some advantages to genetic engineering efforts in the commercially important conifers. In the present study, the *Pschi4* promoter activated higher levels of reporter gene expression than did the 35S promoter in transient assays with pine cells (Fig. 6). This implies that the *Pschi4* promoter could be useful to direct heterologous gene transcription in transgenic pines.

Acknowledgements

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References

[1] H. Wu, C.S. Echt, M.P. Popp, J.M. Davis, Molecular cloning, structure and expression of an elicitor-inducible chitinase gene from pine trees, Plant Mol. Biol. 33 (1997) 979–987.

[2] C.A. Loopstra, R.R. Sederoff, Xylem-specific gene expression in loblolly pine, Plant Mol. Biol. 27 (1995) 277–291.

[3] D.D. Ellis, Transformation of gymnosperms, in: S. Jain, P. Gupta, R. Newton (Eds.), Somatic Embryogenesis in Woody Plants, vol. 1, Kluwer, Dordrecht, 1994, pp. 227–251.

[4] M.S. Lesney, Growth responses and lignin production in cell suspensions of *Pinus elliottii* "elicited" by chitin, chitosan or mycelium of *Cronartium quercuum f. sp. fusiforme*, Plant Cell Tiss. Org. Cult. 19 (1989) 23–31.

[5] S. Chang, J. Puryear, J. Cairney, A simple and effective method for isolating RNA from pine trees, Plant Mol. Biol. Rep. 11 (1993) 113–116.

[6] M.E. Mason, J.M. Davis, Defense response in slash pine: chitosan treatment alters the abundance of specific mRNAs, Mol. Plant-Microbe Interact. 10 (1997) 135–137.

[7] U. Hoecker, I.K Vasil, D.R. McCarty, Integrated control of seed maturation and germination programs by activator and repressor functions of Viviparous-1 of maize, Genes Dev. 9 (1995) 2459–2469.

[8] M.G. Taylor, I.K. Vasil, Histology of, and physical factors affecting transient GUS expression in pearl millet (*Pennisetum glaucum* (L.) R. Br.) embryos following microprojectile bombardment, Plant Cell Rep. 10 (1991) 120–125.

[9] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.

[10] S.G. Rogers, R.B. Horsch, R.T. Fraley, Gene transfer in plants: production of transformed plants using Ti plasmid vectors, Methods Enzymol. 118 (1986) 627–640.
[11] L. Meisel, E. Lam, The conserved ELK-homeodomain of KNOTTED-1 contains two regions that signal nuclear localization, Plant Mol. Biol. 30 (1996) 1–14.
[12] L.D. Dwinell, E.G. Kuhlman, G.M. Blakeslee, Pitch canker of southern pines, in: P.E. Nelson, T.A. Toussoun, R.J. Cook (Eds.), Fusarium: Diseases, Biology, and Taxonomy, The Pennsylvania State University Press, University Park and London, 1981, pp. 188–194.
[13] D.B. Collinge, K.M. Kragh, J.D. Mikkelsen, K.K. Nielsen, U. Rasmussen, K. Vad, Plant chitinases, Plant J. 3 (1993) 31–40.
[14] C.J. Palm, M.A. Costa, G. An, C.A. Ryan, Wound-inducible nuclear protein binds DNA fragments that regulate a proteinase inhibitor II gene from potato, Proc. Natl. Acad. Sci. USA 87 (1990) 603–607.
[15] T. Balandin, C.J.M.V. van der Does, J.F. Bol, H.J.M. Linthorst, Structure and induction pattern of a novel proteinase inhibitor class II gene of tobacco, Plant Mol. Biol. 27 (1995) 1197–1204.
[16] A.P. Goldsbrough, H. Albrecht, R. Stratford, Salicylic acid-inducible binding of a tobacco nuclear protein to a 10 bp sequence which is highly conserved among stress-inducible genes, Plant J. 3 (1993) 563–571.
[17] C.A. Loopstra, E.-G. No, Elements required for xylem-specific expression are located downstream of a loblolly pine gene. In: T. White, D. Huber, G. Powell (Eds.), Proceedings, 24th Biennial Southern Forest Tree Improvement Conference, Orlando, FL, June 9–12, 1997, National Technical Information Services, US Department of Commerce, Springfield pp. 374–376.
[18] C.H. Michler, T.M. Hubacher, Development of an adventitious shoot regeneration procedure for transformation of eastern white pine. In Vitro Cell. Dev. Biol. 34 (1998) Part 11, 69A.
[19] S.H. Doares, T. Syrovets, E.W. Weiler, C.A. Ryan, Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway, Proc. Natl. Acad. Sci. USA 92 (1995) 4095–4098.