Characterization and expression of a *Pinus radiata* putative ortholog to the *Arabidopsis SHORT-ROOT* gene

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Summary We characterized a *Pinus radiata* D. Don putative ortholog to the *Arabidopsis thaliana* (L.) Heynh. *SHORT-ROOT* gene (*AtSHR*) and analyzed its expression in different organs during vegetative development and in response to exogenous auxin during adventitious rooting. The predicted protein sequence contained domains characteristic of the GRAS protein family and showed a strong similarity to the SHORT-ROOT (SHR) proteins. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and in situ hybridization showed that the gene is predominantly expressed in roots, root primordia and in the cambial region of hypocotyl cuttings. Increased mRNA levels were observed, independently of the presence or absence of exogenous auxin, in the cambial region and rooting competent cells of hypocotyl cuttings within the first 24 h of adventitious rooting, before the activation of cell divisions and the organization of the adventitious root meristem. The expression pattern in organs and during adventitious rooting was similar to that of a *Pinus radiata SCARECROW-LIKE* (*PrSCL1)* gene, except that *PrSCL1* is induced in response to exogenous auxin. Results suggest that the *Pinus radiata SHORT-ROOT* (*PrSHR*) gene has a role in root meristem formation and maintenance and in the cambial region of hypocotyl cuttings.

Keywords: adventitious rooting, GRAS genes, pine, root meristem, vegetative propagation.

Introduction

In *Arabidopsis* and other plant species, such as pea, rice andmaize, the establishment of an embryonic root meristem involves members of the GRAS family of putative transcription factors which includes SCARECROW (SCR), SCARECROW-LIKE (SCL) and SHORT-ROOT (SHR) proteins (Di Laurenzio et al. 1996, Sabatini et al. 1999, 2003, Sassa et al. 2001, Helariutta et al. 2000, Wysocka-Diller et al. 2000, Kamiya et al. 2003, Heidstra et al. 2004, Lim et al. 2005, Levesque et al. 2006). These proteins are also involved in the radial patterning of roots, hypocotyls and aerial organs (Scheres et al. 1995, Fukaki et al. 1998, Helariutta et al. 2000, Wysocka-Diller et al. 2000). In addition, SCR and SHR play important roles in the regeneration of the root tip after laser ablation or excision in *Arabidopsis* and maize (Lim et al. 2000, Xu et al. 2006). In forest species, clones with homology to the *Arabidopsis GRAS* genes have been described. Stasolla et al. (2003) and Cairney et al. (2006) reported the induction of a *SCARECROW* gene during somatic embryogenesis in *Picea glauca* (Moench) Voss and *Pinus taeda* L., respectively. Laajanen et al. (2007) isolated a *Pinus sylvestris* L. *SCARECROW (PsySCR)* gene that is central to radial patterning of roots. Recently, our research group described a *Pinus radiate* D. Don *SCARECROW-LIKE (PrSCL1)* gene and a *Castanea sativa* Mill. *SCARECROW-LIKE (CsSCL1)* gene that are predominantly expressed in roots, are induced in the presence of exogenous auxin in rooting competent cuttings, and may play a role in the early stages of adventitious root induction (Sánchez et al. 2007).

Among GRAS proteins, *Arabidopsis* SHR (AtSHR) is involved in the specification of root stem cell identity and in radial patterning of roots in *Arabidopsis* (Benfey et al. 1993, Scheres et al. 1995, Helariutta et al. 2000, Nakajima et al. 2001, Sena et al. 2004, Cui et al. 2007). *AtSHR* mRNAis found in the stele, but the AtSHR protein moves from the stele to a layer of adjacent cells where it regulates root development by a transcriptional regulatory pathway that involves other *GRAS* genes, such as *SCR* and *SCL* (Helariutta et al. 2000, Nakajima et al. 2001, Levesque et al. 2006). Levesque et al. (2006) suggested that AtSHR is a critical component in the pathway regulating specification of the root meristem and differentiation of the root not only through a transcription regulatory network but also through hormonal and other signaling pathways. *SHR* genes have been described in rice (*Oryza sativa* L. *SHR, OsSHR*; Kamiya et al. 2003, Cui et al. 2007), poplar (*Populus tremula* L. *SHR*, *PttSHR*; Schrader et al. 2004) and *Medicago truncatula* Gaertn. (*MtSHR*; Imin et al. 2007). In rice, *OsSHR* genes seem to be involved in root development in a similar manner to that described in *Arabidopsis* (Cui et al. 2007). In addition, specific members of the *OsSHR* gene may be involved in the formation of leaf stomata (Kamiya et al. 2003).In poplar, *PtSHR* is expressed in the cambial zone of shoots (Schrader et al. 2004), and in *Medicago truncatula, MtSHR* expression has been studied during adventitious rooting of callus explants (Imin et al. 2007).

Molecular mechanisms regulating adventitious root formation are unknown. Adventitious rooting plays an important role in the vegetative propagation of forest species. Although auxins are required to induce adventitious roots in many of these species (Grönroos and Von Arnold 1985, 1987, 1988, Sánchez and Vieitez 1991, Greenwood and Weir 1995, Díaz-Sala et al. 1996, Goldfarb et al. 1998, Ballester et al. 1999), and genes related to adventitious root induction in forest species have been described (Hutchison et al. 1999, Lindroth et al. 2001*a*, 2001*b*, Gil et al. 2003, Goldfarb et al. 2003, Brinker et al.

2004, Busov et al. 2004), little is known about the mechanisms underlying root meristem formation in stem cuttings during adventitious root formation. In hypocotyls cuttings from young *Pinus taeda* seedlings, which consist mainly of primary tissues in which the cambium is not yet fully differentiated, the only cells competent to form roots are a small group of vascular parenchyma cells and cambial derivates located centrifugal to the resin canal. These cells differ from other parenchyma cells and cambial derivates in their ability to organize embryonic root meristems in response to exogenous auxin and the polar auxin transport, which is required only in the first 48 h of induction (Díaz-Sala et al. 1996). A similar pattern of adventitious root induction has been observed in hypocotyl cuttings of young seedlings of other pine species, such as *Pinus radiata* (Smith and Thorpe 1975), *Pinus contorta* Dougl. ex Lond. (Grönroos and Von Arnold 1987) and *Pinus strobus* L. (Goldfarb et al. 1998).

We report the isolation of a *Pinus radiata* putative ortholog to the *Arabidopsis SHR* gene, *Pinus radiata SHORT-ROOT* (*PrSHR*) that is predominantly expressed in roots, root primordia and the cambial region of hypocotyl cuttings. Increased *PrSHR* mRN levels were observed, independently of the presence or absence of exogenous auxin, in the cambial region and in rooting competent cells within the first 24 h of adventitious rooting, before the activation of cell divisions and the organization of the adventitious root meristem. A possible role of *PrSHR* and other *GRAS* genes during the early stages of adventitious rooting is discussed.

Materials and methods

Plant material and root induction

Pine (*Pinus radiata*) seeds were provided by Oihanberri (Vitoria, Spain). Seeds were germinated and seedlings were grown as previously described (Sánchez et al. 2007). Cuttings for dose-response experiments and for adventitious root induction were prepared according to Sánchez et al. (2007). Briefly, hypocotyl cuttings from 21-day-old seedlings, including the intact epicotyl, were prepared by severing the hypocotyls at its base and trimming it to a length of 2.5 cm from the cotyledons. Root induction was achieved by exposing the cuttings to 10 μM indole-3-butyric-acid (IBA) continuously. For dose-response experiments, cuttings from 21-day-old seedlings were exposed to 1, 10, or 20 μM IBA for 24 h. Cuttings without IBA treatment were used as controls. The IBA (IBA-K, Sigma) was dissolved in distilled water. Conditions for root induction were as described for seedling growth (Sánchez et al. 2007).

RNA extraction, quantification and cDNA synthesis

Thirty 1-cm basal segments of the hypocotyl cuttings were pooled for each treatment and measurement time as specified for each experiment, immediately frozen in liquid nitrogen and stored at –70 °C until total RNA isolation and quantification as described by Sánchez et al. (2007). Total RNA was also extracted from different organs of plant seedlings as

specified in each experiment. Synthesis of cDNA was performed with 1 µg of total RNA. For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), reverse transcription reactions were performed with 200 ng random primers and Super-Script™II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For 3′-rapid amplification of cDNA ends (RACE), cDNA was synthesized using a modified oligodT (3′-RACE System, Invitrogene) and SuperScript™II reverse transcriptase according to the manufacturer's instructions. For 5′-RACE, cDNA was synthesized using gene-specific primers designed based on the sequence obtained in our laboratory and Thermoscript reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

5 - and 3 –RACE

A full-length cDNA clone was constructed in a two-step procedure. We carried out 3′- and 5′- cDNA amplifications with *PrSHR*-specific primers designed based on a sequence of an initial fragment of *Pinus radiata* obtained in our laboratory. The initial fragment of *PrSHR* was isolated by PCR with *Pinus radiata* cDNA and primers designed based on the sequence of a *Pinus taeda* EST obtained from the TIGR database (http://www.tigr.org).We performed 3′- and 5′-RACE with the 3′- or 5′-RACE System (Invitrogen). Two independent RNA extractions were used as templates for reverse transcription (see above). Double stranded cDNA was synthesized by PCR amplification using Platinum Taq DNA polymerase (Invitrogen) in the presence of AAP or AUAP primers (Invitrogen) and forward and reverse *PrSHR*-specific primers (PrSHRFor: 5′-CCACGTTCTACAGCATGAACCC-3′ and PrSHRRev: 5′-CATGAAACCAGATAGTCGATCGC-3′). Cycling conditions were: initial denaturation at 94 °C for 5 min, then 94 °C for 30 s; 60 °C for 30 s; and 72°C for 2 min for 40 cycles. After a final extension at 72 °C for 10 min, the reactions were cooled to 4 °C. The reaction products were purified from agarose gels using the Quiaquick Gel Extraction kit (Qiagen, Germany), cloned using the TA Cloning kit (Invitrogen), and sequenced with an ABI-PRISM® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Clones from each experiment and species were sequenced at least twice. Nucleotide sequences were subjected to a BLASTx search (http:// www.ncbi.nlm.nih.gov) and compared (BLASTn) with loblolly pine and poplar ESTs available from the TIGR database (http://www.tigr.org).

Genomic DNA isolation and Southern blot

We isolatedDNAas described by Doyle and Doyle (1990). For Southern blot analysis, 40 µg of genomic DNA was digested with *Eco*RI and *Eco*RV (New England Bioloabs, Boston, MA) and the double digestions *Eco*RI+, *Hind*III, *Eco*RI + *Eco*RV and *Hind*III + *Eco*RV, separated on 0.8% agarose gel in TAE (40mMTris-acetate, 1mMEDTA pH 8) and blotted with 1M ammonium acetate onto a positively charged Nytran nylon membrane (Schleicher & Schuell, Germany) according to Sambrook et al. (1989). Hybridization was performed with a digoxigenin-labeled (Roche Diagnostics, Basel Switzerland) *PrSHR* probe under high stringency conditions following the procedure described by the manufacturer. CDP-Start (Roche) was used for chemiluminescent detection.

Phylogenetic analysis

The conserved carboxyl-terminal region of PrSHR and other GRAS proteins (Bolle 2004, Lim et al. 2005) was used for phylogenetic analysis. The polypeptides were aligned with ClustalW and subsequently analyzed with programs from the PHYLIP package (Phylogeny Interference Package, Version 3.6a3, Department of Genetics, University of Washington, Seattle). Bootstrap analysis was performed with SEQBOOT and generated 1000 replicates that were used to obtain a set of distance matrices with PROTDIST using the Dayhoff PAMmatrix algorithm. A set of unrooted phylogenetic trees was generated by the neighbor-joining method using NEIGHBOR, and a consensus tree was obtained with CONSENSE. A putative SCARECROW-LIKE protein encoded by a *Physcomitrella patens* (Hedw.) Bruch & Schimp. EST (Nishiyama et al. 2003) was used as the outgroup. The phylogenetic tree was drawn using Phylodendron Version 0.8d (D.G. Gilbert, Indiana University, Bloomington).

Quantitative RT-PCR (qRT-PCR)

Extraction of RNA, quantification and cDNA synthesis were done as previously described. Primer design, efficiency analyses, and polymerase chain reactions were carried out as described by Sánchez et al. (2007), with an 18S rRNA gene (*Ri18S*) as a control. *PrSHR*-specific primers (PrSHRtrFor1: 5′-GAACCAGTGCAAGGAGCATTG-3′ and PrSHRtrRev1: 5′-AAATCCTGCCTCCTTGAGCCT-3′) were designedbased on the pine RACE sequences obtained in our laboratory. Expression ratios were obtained from the equation $2^{-\Delta\Delta CT}$ (Applied Biosystems, Technical Bulletin #2, P/N4303859B). Results are expressed as mean values (± standard error) from at least two biological replicates.

In situ hybridization

Hypocotyl cuttings from 21-day-old seedlings were harvested after 0 and 24 h of IBA treatment. Untreated cuttings were used as controls. The basal 10 mm of each cutting was embedded and frozen in Jung Tissue Freezing medium (Leyca Microsystems Nussloch GMBH, Heildelberg, Germany) in dry ice. The basal 5 mm of each sample was cut in 10-µm transverse sections and collected on 3-aminopropyl-triethoxisilan glass slides. Cryostat sections were dried at 40 °C and fixed in 3:1 (v/v) ethanol:glacial acetic acid for 10 min followed by 5 min in 70% ethanol. To generate *PrSHR*- and *PrSCL1*-specific probes, a 350-bp fragment corresponding to the 3′-untranslated region of *PrSHR* or *PrSCL1* (lacking the poly (A) tail) was cloned into the PCR® II vector (Invitrogen) and amplified. The PCR fragment, flanked by the SP6 and T7 promoters, was used as template for synthesis of both sense and antisense DIG-labeled probes, with T7 or SP6 polymerase, respectively, according to the manufacturer's instructions (DIG RNA labelling kit SP6/T7, Roche Biochemicals). The probes were partially hydrolyzed to a mean length of 200 nucleotides by alkali treatment. The in situ hybridization was performed as described by Sánchez et al.

(1995). Sections were pretreated with 1 μg ml–1 proteinase K for 30 min at 37 °C before being incubated overnight at 43 °C with the RNA probes in a hybridization solution containing 40% deionized formamide. After washing four times in 2×SSC (1×SSC 150 mM sodium chloride, 15 mM sodium citrate) at 37 °C, slides were treated with 5 μgml–1 RNase A at 37 °C for 30 min, and washed twice with 0.1× SSC at 37 °C. The hybridization signal was detected with the DIG Nucleic Acid Detection kit (Roche Biochemicals) for 12 h in the dark following the manufacturer's instructions. Sections were dehydrated through an ethanol series (v/v) (50 and 70% for 30 s each, and 99% for 1 min twice), air dried and then mounted in Eukitt (O. Kindler, GmbH, Freiburg, Germany). Photographs were taken with an Olympus digital camera attached to Nikon microscope operating in bright-field illumination.

Statistical analysis

Comparison between two groups was made by Student's *t* test, and comparison among multiple groups was made by analysisof variance (ANOVA). The nucleotide sequence of *PrSHR* was deposited in the GenBank database under Accession no. EU044786.

Results

Isolation of a pine putative ortholog to the Arabidopsis SHORT-ROOT gene

To isolate a pine ortholog to the *Arabidopsis SHR* gene, *Pinus taeda* expressed sequence tags (ESTs) with homology to *Arabidopsis SHR* were identified in the loblolly pine TIGR database (http://www.tigr.org). By using 5′- and 3′-RACE, a full-length cDNA of 2 kb was identified. The cDNA contained a predicted protein sequence of 502 amino acids (Figure 1) designated PrSHR (*Pinus radiata* SHORT-ROOT protein). Comparison of the amino acid sequence with previously described proteins showed that it contained domains characteristic of SHR proteins, which belong to the GRAS protein family, including the presence within the carboxyl-terminal region of the highly conserved motifs, and the pairs of conserved residues, such as WX7G and WX10W, close to the SAW motif. In the amino-terminal domain, acidic residue-rich regions were found in addition to stretches of serines (Figure 1).

A phylogenetic analysis was performed to determine evolutionary relationships between PrSHR and other members of the GRAS family (Figure 2). For comparisons, representatives of different GRAS protein subfamilies (Bolle 2004, Lim et al. 2005) were selected, and the predicted sequence of a putative GRAS protein encoded by a *Physcomitrella patens* EST (Nishiyama et al. 2003) was used as the outgroup. Results indicated that PrSHR does not originate from a separate branch in the phylogenetic tree of known GRAS proteins: it formed a single clade with the previously described GRAS proteins included in the *Arabidopsis thaliana* SHR (AtSHR) branch, to which it showed the highest similarity (Figure 2).

Southern blot analysis revealed that a *PrSHR* probe hybridized to a single band in all restriction digests except for the *Eco*RI + *Eco*RV digestion under high-stringency conditions. This result is in agreement with the sequence restriction map (data not shown), and indicates that there is a single *PrSHR* gene in the *Pinus radiata* genome (Figure 3).

Expression of PrSHR in organs during vegetative development

We analyzed the expression pattern of *PrSHR* in different organs during vegetative development by qRT-PCR of RNA isolated from roots, hypocotyls, shoot apex nodal segments (including apical meristem, young needles, and shoot segment) and cotyledons from 35-day-old seedlings. Results are expressed relative to the expression in cotyledons, for which the lowest values of *PrSHR* expression were detected. Among the organs examined *PrSHR* mRNA accumulated in highest amounts in roots ($P < 0.01$) (Figure 4). *PrSHR* transcript levels higher than those measured in cotyledons were also detected in hypocotyls and shoot apex nodal segments, which comprised mostly apical meristem and young needles but also included a shoot segment (Figure 4). In situ hybridization showed that *PrSHR* was expressed in root primordial (Figures 5A and 5B) as well as in the vascular tissues, mainly the cambial region, of hypocotyl cuttings (Figure 5C). Because Sánchez et al. (2007) previously found that *PrSCL1*, a *Pinus radiata SCARECROW-LIKE* gene, showed a similar expression pattern in organs during vegetative development, we performed in situ *hybridization* using a *PrSCL1* probe to compare the expression pattern of both genes in these organs. Results showed that, similar to *PrSHR*, *PrSCL1* was expressed in root primordia (Figures 5E and 5F) and in the cambial region of hypocotyl cuttings (Figure 5G). No signal was observed when tissues were hybridized with either the *PrSHR* or the *PrSCL1* probe in a sense orientation (Figures 5D and 5H).

Temporal and spatial expression of PrSHR in auxin-treated rooting competent cuttings during adventitious rooting

Adventitious root formation in pine is dependent on the application of exogenous auxin (Díaz-Sala et al. 1996). Because *PrSCL1* and *PrSHR* were predominantly expressed in roots, root primordia and the cambial region of hypocotyl cuttings, and Sánchez et al. (2007) reported that *PrSCL1* is induced in the presence of exogenous auxin in cuttings during adventitious rooting, the expression pattern of *PrSHR* during adventitious root formation in rooting-competent cuttings that readily root within 15 days of culture (100% rooting, 12 induced roots per cutting), and its response to the application of exogenous auxin were also examined. Expression patterns were determined at the following times: (1) during the initial 48 h of the root induction process, a time when cell reorganization takes place, but before the resumption of cell division in the procambial region; (2) after 5 days of culture to allow time for the onset of rapid cell divisions followed by the reorientation of cellular division planes that originate the root meristem; and (3) after 15 days of culture to allow time for root primordium organization. Untreated cuttings were used as controls. The RNA was extracted from IBA-treated and untreated cuttings, and results expressed relative to values at time 0.

PrSHR mRNA was significantly ($P < 0.01$) increased within the first 24 h, both in the absence and presence of exogenous auxin (Figure 6A). No significant differences

between IBA treated and untreated cuttings were detected, except at Day 15 ($P < 0.01$). An increase in *PrSHR* transcripts was detected as early as 8 h and the increase continued for 24 h, after which there was a slight decline in *PrSHR* mRNA levels. After 5 days, the level of *PrSHR* mRNA did not change in the IBA-treated cuttings, which developed root primordia, whereas *PrSHR* transcript abundance decreased in the untreated cuttings. To confirm that the *PrSHR* pattern of expression was independent of the presence of exogenous auxin during the earliest stages of adventitious rooting, qRT-PCR was performed with RNA isolated from hypocotyl cuttings treated with different IBA concentrations for 24 h. Results showed no significant differences between untreated and IBA-treated cuttings after 24 h, independently of the IBA concentration tested, indicating that the *PrSHR* pattern of expression over the 24-h period was independent of the presence of exogenous auxin (Figure 6B). In situ hybridization showed that *PrSHR* was expressed in the vascular system in both the control and IBA-treated cuttings, specifically in the procambial region and the cells located centrifugal to the resin canal, which are the rooting-competent cells (Figures 7A and 7B). No signal was observed when tissues were hybridized with the *PrSHR* probe in a sense orientation (Figure 7E). In situ hybridization with the *PrSCL1* probe showed that this gene was expressed in the vascular system, specifically in the procambial region and the cells located centrifugal to the resin canal, but only in the IBA-treated cuttings (Figures 7C and 7D). No signal was observed when tissues were hybridized with the *PrSCL1* probe in a sense orientation (Figure 7F).

Discussion

PrSHR is a putative ortholog to the Arabidopsis SHORTROOT gene

We isolated and characterized a *Pinus radiata* putative ortholog to the *Arabidopsis SHORT-ROOT* gene. The deduced amino acid sequence of PrSHR (Figure 1) indicated that it belonged to the GRAS family of putative transcription factors that also includes SCR and SCL proteins (Bolle 2004, Lim et al. 2005), and it showed a high conservation at the carboxylterminal region with the characteristic features of *Arabidopsis* SHR (Helariutta et al. 2000), and other members of the GRAS protein family (Di Laurenzio et al. 1996, Pysh et al. 1999, Bolle 2004). Although the GRAS proteins share highly conserved domains in the carboxy terminus, the amino-terminal region is divergent among members of this family. The presence of stretches of serine in the amino terminal region of the PrSHR protein is similar to that found in AtSHR and OsSHR (Helariutta et al. 2000, Kamiya et al. 2003), as well as in other GRAS proteins of forest tree species, such as PrSCL1, CsSCL1 and PsySCR (Laajanen et al. 2007, Sánchez et al. 2007), and in distantly related plant species, such as AtSCR, *Zea mays* L. SCR (ZmSCR), *Pisum sativum* L. SCR (PsSCR) and *Lilium longiflorum* Thunb. SCL (LlSCL) (Di Laurenzio et al. 1996, Lim et al. 2000, Sassa et al. 2001, Morohashi et al. 2003). However, the homopolymeric stretches of proline, glutamine, histidine, threonine, alanine or glycine described in AtSHR (Helariutta et al. 2000), OsSHR (Kamiya et al. 2003), and other GRAS proteins, such as AtSCR (Di Laurenzio et al. 1996), ZmSCR (Lim et al. 2000) and PsSCR (Sassa et al. 2001) were not found in PrSHR. The amino-terminal region of PrSHR is rich in acidic residues, with which

it shares homology with the amino terminus of LlSCL, PrSCL1 and CsSCL1 (Morohashi et al. 2003, Sánchez et al. 2007). Specific acidic and neutral regions of the amino terminus of LlSCL protein have shown strong activity as transcriptional regulators (Morohashi et al. 2003). As suggested by Sánchez at al. (2007), the similar amino termini of PrSHR, PrSCL1, CsSCL1 and LlSCL could indicate a similar mechanism of transcriptional activation by the amino terminus that is evolutionarily conserved within the GRAS proteins. The specific leucin erich repeats found in the amino terminal domain of PsySCR (Laajanen et al. 2007) are not found in PrSHR, PrSCL1 or other GRAS proteins. Comparative analysis of PrSHR, AtSHR (Helariutta et al. 2000), OsSHR (Kamiya et al. 2003) and MtSHR (Imin et al. 2007) sequences revealed that PrSHR is highly similar to these proteins, grouping in the same clade (Figure 2). The conservation of the protein sequence (Figure 1), the evolutionary relationship to the SHR proteins, which are involved in root meristem specification (Figure 2), the conservation of gene structure (Figure 3) in an ancient organism such as pine, and its expression in roots and root primordia (Figures 4, 5A and 5B) indicate that *PrSHR* could be a putative ortholog to *AtSHR*, and suggest a conserved function of the gene in the regulation of root induction and development. Based on the evolutionary position of conifer tree species, these results together with those described for *Picea glauca* (Stasolla et al. 2003), *Pinus taeda* (Cairney et al. 2006), *Pinus radiata* and *Castanea sativa* (Sánchez et al. 2007) and *Pinus sylvestris* (Laajanen et al. 2007) confirm that GRAS proteins are an ancient family of proteins, as deduced by Sánchez et al. (2007) and by Nishiyama et al. (2003), who described the detection of ESTs with sequence similarity to GRAS proteins in bryophytes.

PrSHR is predominantly expressed in roots, root primordial and cambial region of hypocotyl cuttings

PrSHR expression in roots and root primordia (Figures 4, 5A and 5B) indicate that the gene likely has a function in root initiation or development, as described for *AtSHR* gene (Helariutta et al. 2000). Relatively high levels of *PrSHR* transcripts were also detected in other organs with proliferative meristems or active dividing cells, such as young hypocotyls and the shoot apex nodal segment (Figure 4), indicating a potential function of *PrSHR* in these organs in addition to its presumed role in roots and root primordia. In other plant species, *SHR* expression or function has been described in cell types other than root cells, usually associated with developmental processes involving asymmetric cell divisions (Scheres et al. 1995, Fukaki et al. 1998). In *Arabidopsis*, radial patterning of the hypocotyl is affected in *shr* mutants (Scheres et al. 1995). In rice, *OsSHR1* is expressed in stomatal precursor cells (Kamiya et al. 2003). *AtSHR* also participates in the maturation of ground root tissue independently of other signals (Paquette and Benfey 2005). In hypocotyls cuttings, which show a primary or a very initial secondary structure, *PrSHR* was detected in vascular tissues (Figure 5C), suggesting that it plays a role in the development of this tissue, perhaps regulating asymmetric cell division in the cambial region of hypocotyl cuttings or participating in the development and maturation of the vascular system. Schrader et al. (2004) reported that two close homologs of *AtSHR* from poplar, *PtSHR1* and *PtSHR2*, are expressed in the cambial zone and show increased expression toward the phloem.A role in stele development has also been described in *Arabidopsis* roots where the

AtSHR pathway modulates the expression of essential regulators of vascular development (Levesque et al.2006).

PrSHR transcript abundance in rooting competent tissues

Adventitious root formation is a postembryonic organogenetic process in which root meristems are induced where roots do not normally occur. In 21-day-old hypocotyls, *PrSHR* basal expression is predominantly confined to the cambial region, which is the tissue involved in the rooting response. In response to root-inductive conditions, an increase in *PrSHR* mRNA levels was detected within the first 24 h (Figure 6A) in the whole ring of the procambial region, that includes the rooting competent cells (Figures 7A and 7B), before the activation of cell divisions was observed (Díaz-Sala et al. 1996). Changes in gene expression within the first 24–48 h have been described in *Pinus radiata* and other pine species (Hutchison et al. 1999, Brinker et al. 2004, Busov et al. 2004, Sánchez et al. 2007). In other plant systems, *SHR* is expressed before root meristem formation (Helariutta et al. 2000).

Increased *PrSHR* transcript levels during the earliest stages of adventitious root formation is not a generalized response in hypocotyl cuttings, it is a highly localized response in specific vascular tissues that are involved in the formation of adventitious roots (Figures 7A and 7B). *PrSHR* transcript accumulation overlaps with that observed for *PrSCL1*, a pine gene encoding another GRAS protein (Sánchez et al. 2007); however, *PrSCL1* mRNA is increased only in the presence of exogenous auxin (Figures 7A–D). Therefore, an exogenous auxin-independent competence for increased *PrSHR* mRNA levels, and perhaps, for the induction of a *PrSHR*-mediated signalling pathway, and an exogenous auxin-dependent competence for increased *PrSCL1* mRNA levels seem to be features of the cambial region and of rooting competent cells before the activationof cell divisions that occur in the root primordium. Similarly, *Medicago truncatula SHR* gene (*MtSHR*) expression does not increase in response to exogenous auxin during adventitious rooting of callus explants (Imin et al. 2007). Although *PrSHR* accumulation was independent of the presence of exogenous auxin, its association with tissues involved in root meristem formation and its cellular localization suggest that *PrSHR* is involved in rooting, and that factors other than exogenous auxin may trigger its accumulation. There are many factors involved in the rooting response in addition to auxin, such as the developmental stage of the cutting and wounding. No changes in *PrSHR* mRNA abundance were observed in non-competent older hypocotyls during the early stages of adventitious rooting, and in non-competent epicotyls, a transient peak was not reached until 48 h of culture (authors' unpublished data). Although no conclusions can be drawn from these correlations between the pattern of *PrSHR* expression and competence for rooting, they indicate that *PrSHR* has a closer association with rooting than genes not directly involved in root meristem organization, but which are induced by other factors such as wounding. *PAL* gene expression, which has been analyzed in hypocotyls and epicotyl cuttings of young *Pinus taeda* seedlings during adventitious rooting (Díaz-Sala et al. 1997, Greenwood et al. 1997), is similar in competent and non-competent cuttings but a transient peak is reached sooner in epicotyls (3–6 h) than in hypocotyls (6–12 h). Our data and the results of the cited studies indicate that the increased *PrSHR* expression during the earliest stages of adventitious root formation is not simply a function of unspecific wounding.

The level of *PrSHR* mRNA in IBA-treated cuttings after 5 days (Figure 6A) could be related to the presence of developing root meristems (Figures 5A and 5B), which did not form in the untreated cuttings. The finding that once the primordium is organized, *PrSHR* and *PrSCL1* expression is confined to the root primordium and not to the vascular parenchyma between poles (Figures 5A, 5B, 5E and 5F) indicates roles for *PrSHR* and *PrSCL1* in adventitious root primordium organization and maintenance. The mechanisms by which *PrSHR* or *PrSCL1* is involved in adventitious root formation or maintenance are unknown. In *Arabidopsis*, AtSHR function in root development is mediated through its influence on the auxin signaling pathway, affecting the expression of genes involved in the auxin response, the activation of a network of transcription factors including AtSCR or AtSCL, or through its interaction with other signaling pathways such as receptor-like kinase pathways (Levesque et al. 2006). Therefore, possible interconnected pathways, including transcriptional regulatory networks of GRAS proteins, such as PrSHR or PrSCL1, and auxin signalling pathways, could be associated with adventitious root formation in *Pinus radiata*. Alternatively, the procambial region may serve as a source of PrSHR-mediated positional information required to orient auxin responses. The role of vascular tissue as a source of positional information has been described in the context of auxin signaling in the *Arabidopsis* root (Sabatini et al. 1999). In addition, the SHR-mediated radial patterning of root tissue (Helariutta et al. 2000) provides evidence for a role of vascular tissues, where *SHR* mRNA occurs (Nakajima et al. 2001), in plant organogenesis.

Acknowledgments

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Figures

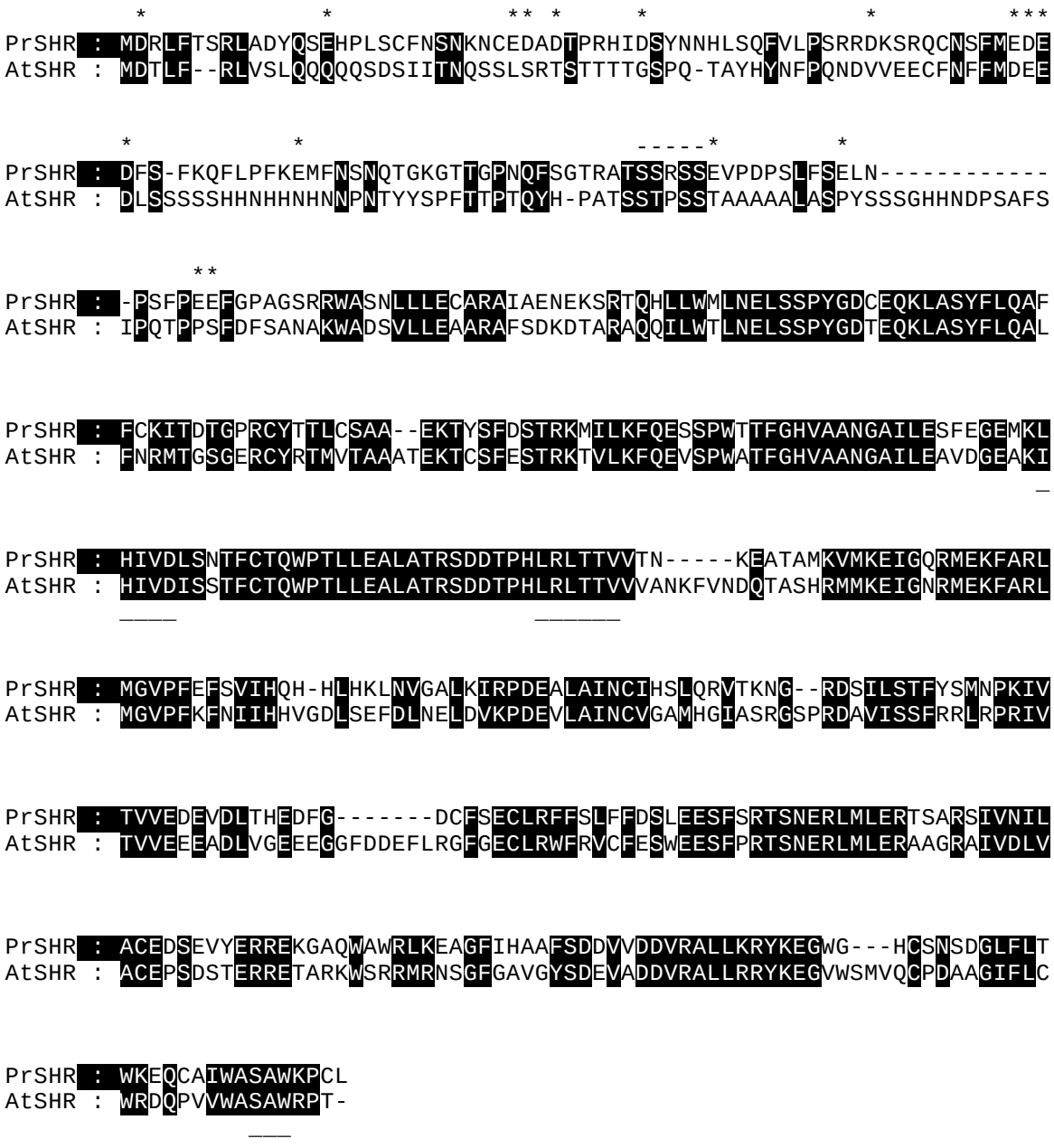

Figure 1. Alignment of deduced amino acid sequence of *Pinus radiata* PrSHR (EU044786) with *Arabidopsis thaliana* AtSHR (U62798). Conserved amino acids are displayed in black boxes. Specific conserved domains in the carboxyl-terminal region are underlined. Acidic amino acidsin the amino-terminal region and specific pairs of conserved residues in the carboxyl-terminal region are indicated with an asterisk. Conserved stretches of serine are double underlined.

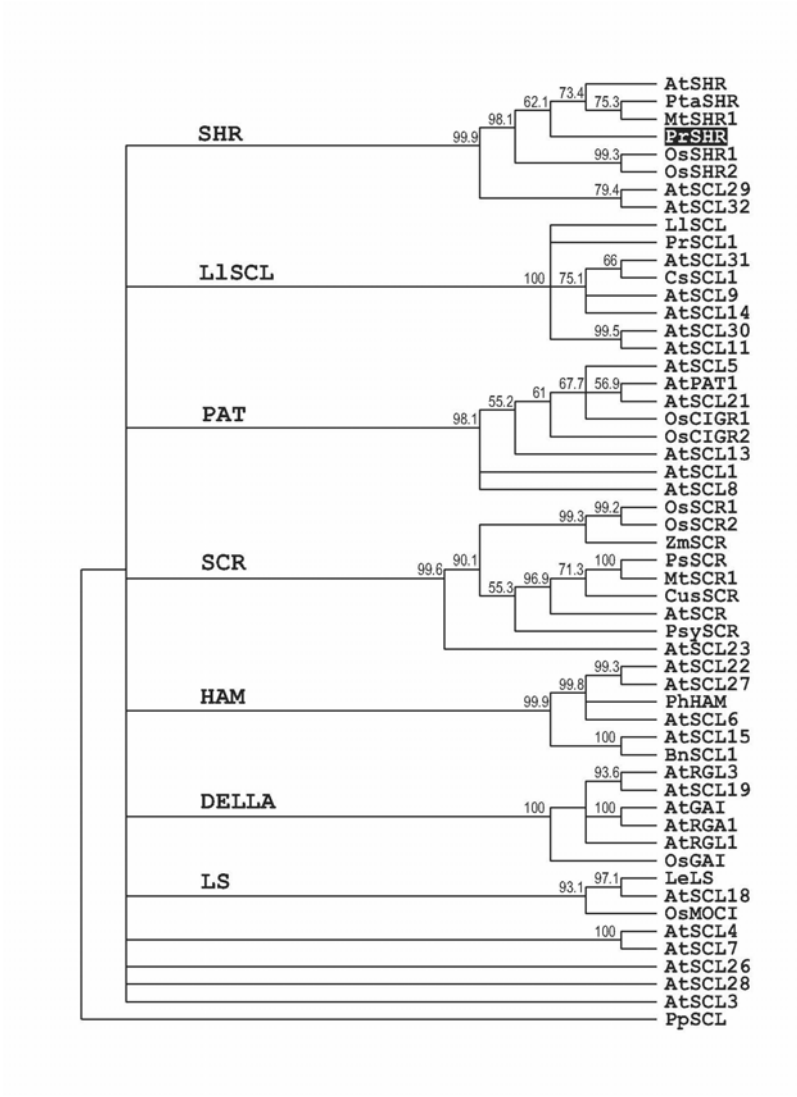

Figure 2. Phylogenetic tree of GRAS proteins from differentplant species (Accession nos. in parenthesis): *Arabidopsis thaliana* AtSCL1 (AF210731), AtGAI (Y15193), AtSCL3 (NM_103925), AtSCL4 (NM_126075), AtSCL5 (NM_103942), AtSCL6 (NM_116232), AtSCL7 (NM_114925), AtSCL8 (NM_1246), AtSCL9 (NM_129321), AtRGA1 (Y15194), AtSCL11 (NM_125336), AtSCL13 (AF419570), AtSCL14 (NM_100627), AtSCL15 (NM_119825), AtRGL1 (AY048749), AtSCR(U62798); *Petunia hybrida* PhHAM (AF481952); AtSHR (AF233752), AtSCL21 (AF210732), AtSCL22 (NM_115927), AtSCL23 (NM_123557), AtPAT1 (AF153443), AtRGL3 (AL391150), AtSCL26 (NM_116894), AtSCL27 (NM_130079), AtSCL28 (NM_104988), AtSCL29 (NM_112237), AtSCL30 (NM_114527), AtSCL31 (NM_100626), AtSCL32 (NM_114855); *Populus tremula* × *alba* PtaSHR (unpublished); *Oryza sativa* OsSHR1 (XM_468819), OsSHR2 (NP_911918), OsGAI (NM_001057567), OsCIGR1 (AY062209), OsCIGR2 (AY062210); *Castanea sativa* CsSCL1 (DQ683579); *Lilium longiflorum* LlSCL (AB106274); *Pinus radiata* PrSCL1 (DQ683567); *Brasica napus* BnSCL1 (AY664405); OsSCR1 (NP_001065617), OsSCR2 (NP_001066027); *Zea mays* ZmSCR (AF263457); *Cucumis sativus* CusSCR (AJ870306); *Pisum sativum* PsSCR (AB048713); *Lycopersicom esculentum* LeLS (AF098674); AtSCL18 (NM_104434), AtSCL19 (AC009895), OsMOCI (AY242058), PrSHR (EU044786); *Pinus sylvestris* PsySCR (ABH85406); *Medicago truncatula* MtSHR1 (1698.m00019) and MtSCR1 (EST592054). *Physcomitrella patens* PpSCL (BJ976460) was included as the outgroup. Bootstrap values of 1000 replicates are indicated. GRAS cladesare indicated. PrSHR is highlighted.

Figure 3. Genomic Southern blot analysis with a *PrSHR* probe. The

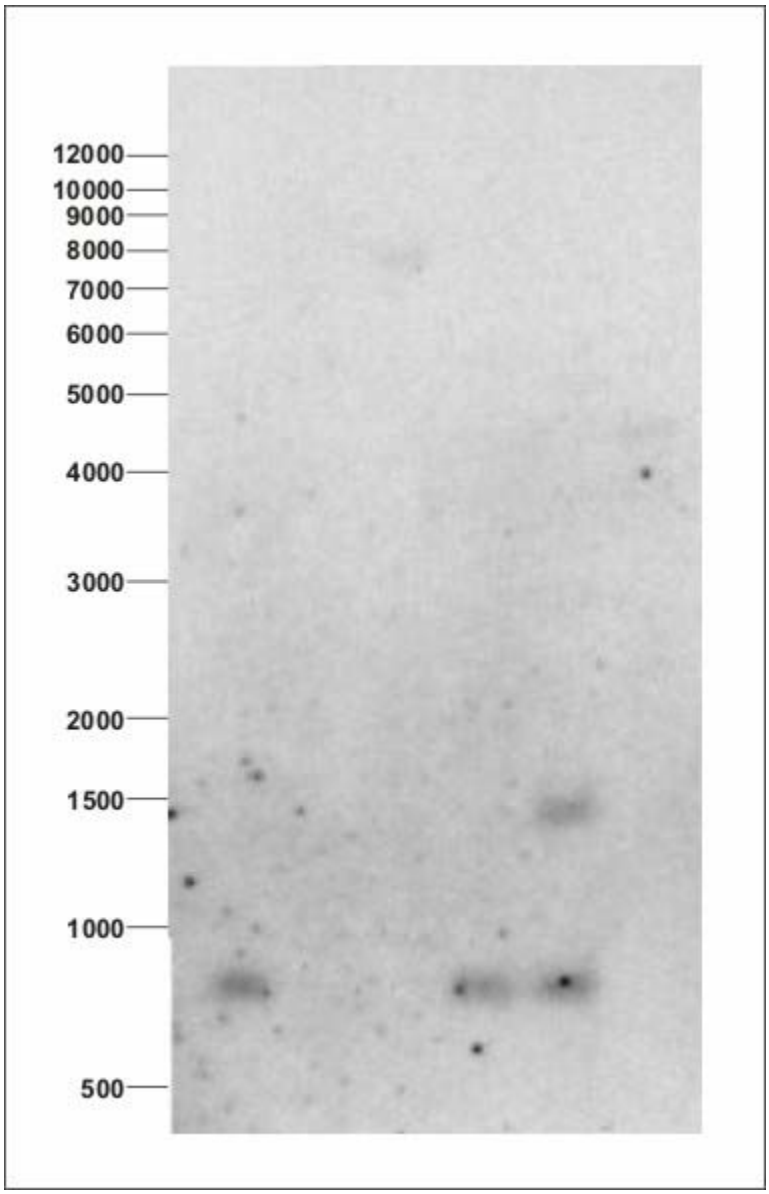

Figure 3. Genomic Southern blot analysis with a *PrSHR* probe. The DNA was digested with, from left to right, *Eco*RI, *Eco*RV and the double digestions *Eco*RI + *Hind*III, *Eco*RI + *Eco*RV and *Eco*RV + *Hind*III. Hybridization was performed under high stringency conditions.Molecular mass markers (bp) are indicated.

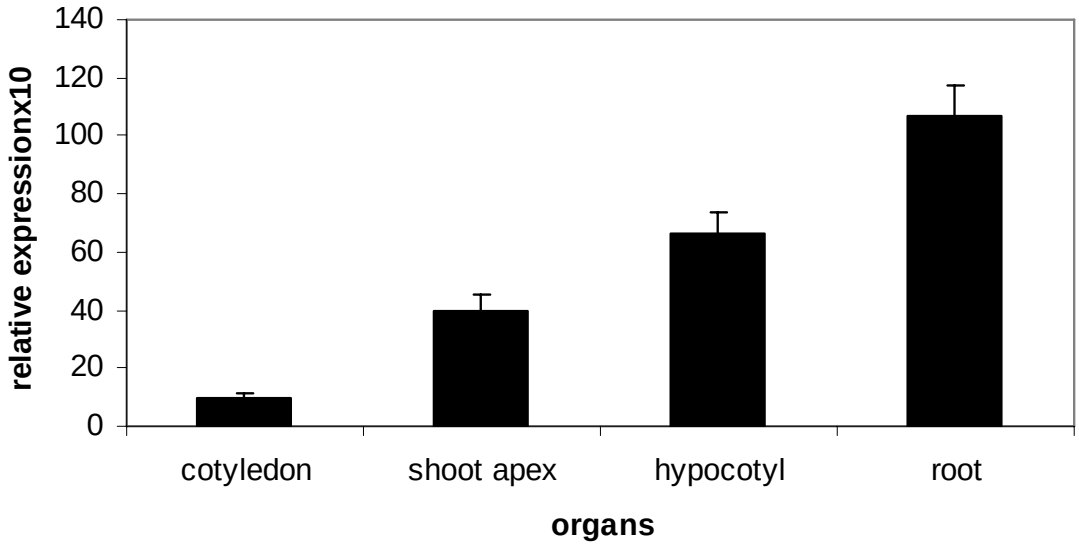

Figure 4. Expression of *PrSHR* in organs of *Pinus radiata* seedlings. Quantitative RT-PCR was performed with RNAs from roots, hypocotyls, shoot apex nodal segments or cotyledons. One µg RNA was reverse transcribed, and 12.5 ng of cDNA was amplified with 400 nM of *PrSHR*-specific primers. Pine *Ri18S* was used as a control. Results are expressed relative to the expression in cotyledons ± SE from three biological replicates.

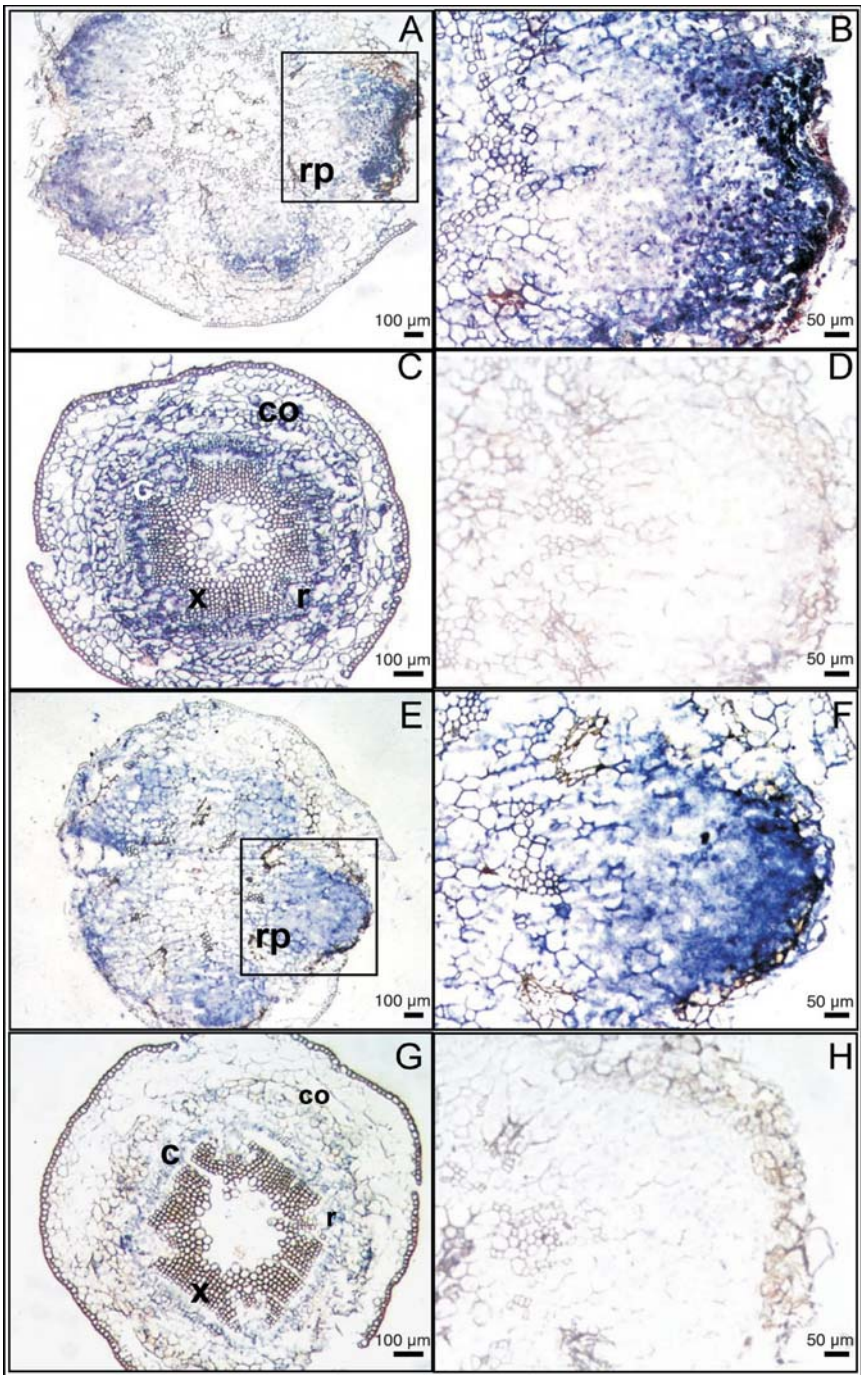

Figure 5. In situ localization of *PrSHR* (A, B, C, D) or *PrSCL1* (E, F, G, H) mRNA on sections of *Pinus radiata* seedlings. (A) Root primordia induced in hypocotyls from 21-day-old seedlings (inset A, B and D). (C) Hypocotyls from 21-day-old seedlings. (E) Root primordia induced in hypocotyls from 21-day-old seedlings (inset E, F and H). (G) Hypocotyls from 21-day-old seedlings. The sections were hybridized with RNA probes obtained by in vitro transcription of *PrSHR* in either the antisense (A, B, C) or sense (D) orientation, or with RNA probes obtained by in vitro transcription of *PrSCL1* in either the antisense (E, F, G) or sense (H) orientation. Note the absence of hybridization in the controls. Abbreviations: c, cambial region; co, cortex; r, resin canal; rp, root primordium; and x, xylem.

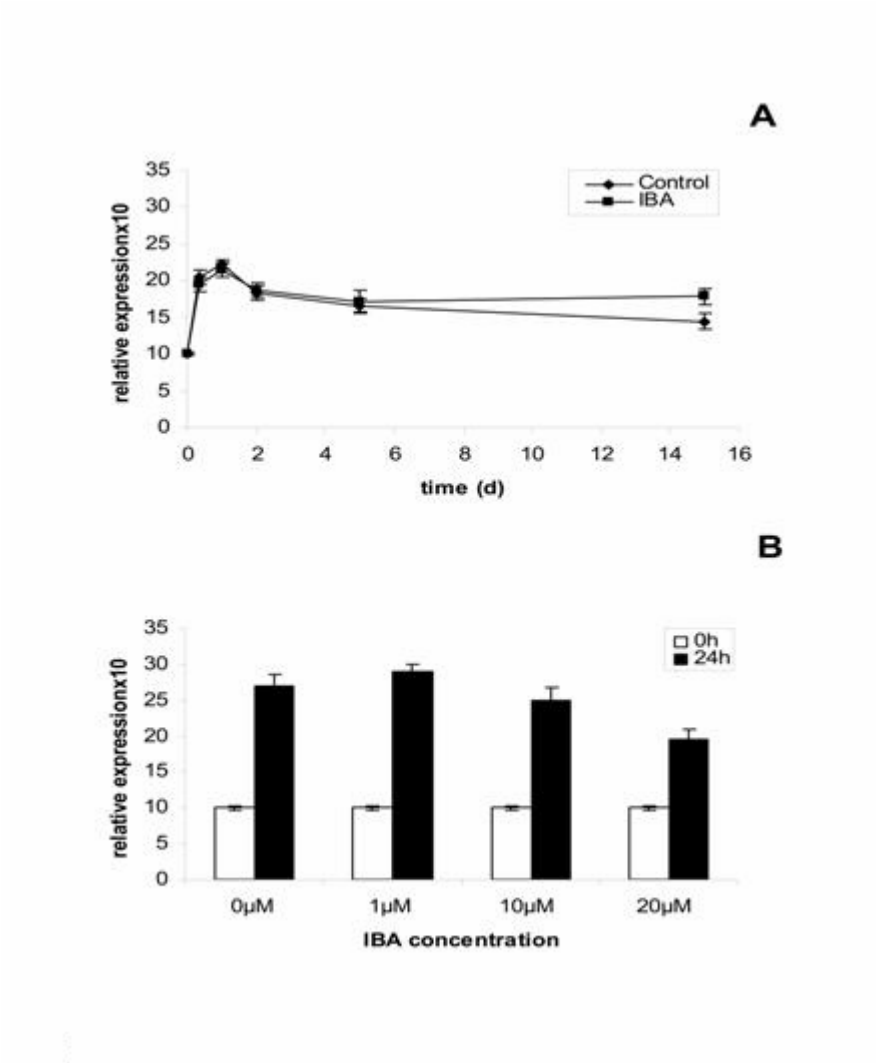

Figure 6. Quantitative RT-PCR analysis of RNA from hypocotyls of 21-day-old seedlings of *Pinus radiata*. One µg RNA was reverse transcribed, and 12.5 ng of cDNA was amplified with 400 nM of *PrSHR* specific primers. Pine *Ri18S* was used as a control. (A) Expression of *PrSHR* during adventitious root formation. Total RNA was extracted from the base of hypocotyl cuttings treated with or without 10 µM IBA at the indicated times. Results are expressed relative to the expression at time 0 ± SE from at least three biological replicates. (B)Effect of different auxin concentrations on the expression of *PrSHR*. Results are expressed relative to the expression at time 0 ± SE from two biological replicates.

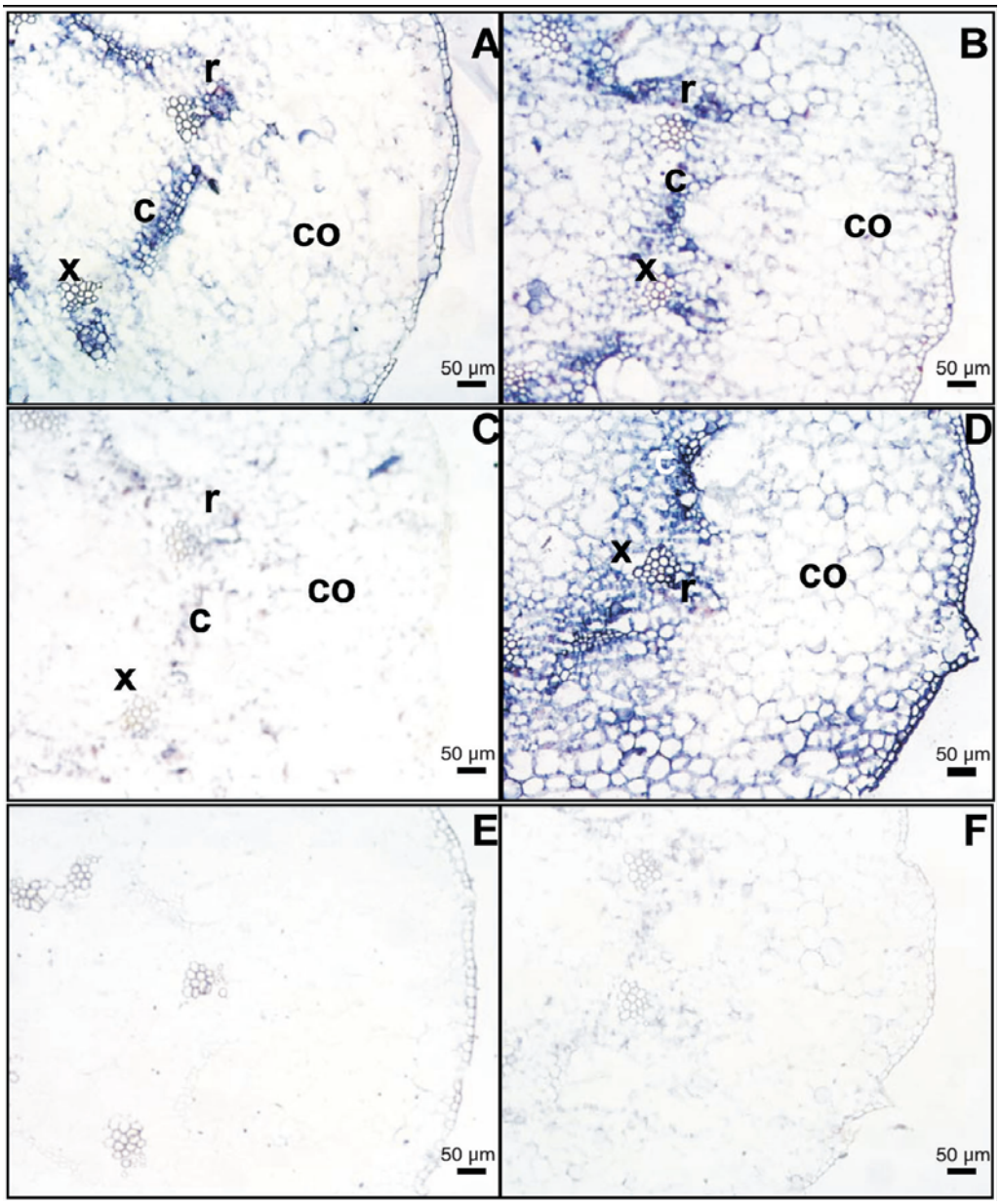

Figure 7. In situ localization of *PrSHR* mRNA on sections of hypocotyls from 21-day-old *Pinus radiata* seedlings in the absence (A) or presence (B, E) of 10 µM IBA for 24 h. In situ localization of *PrSCL1* mRNA on sections of hypocotyls from 21-day-old seedlings in the absence (C) or presence (D, F) of 10 µM IBA for 24 h. Sections were hybridized with RNA probes obtained by in vitro transcription of *PrSHR* in either the antisense (A, B) or sense (E) orientation, or with RNA probes obtained by in vitro transcription of *PrSCL1* in either the antisense (C, D) or sense (F) orientation. Note the absence of hybridization in the controls. Abbreviations: c, cambial region; co, cortex; r, resincanal; and x, xylem.