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ORIGINAL ARTICLE

Concepción Ávila · Josefa Pérez-Rodríguez
Francisco M. Cánovas

Molecular characterization of a receptor-like protein kinase gene from pine (*Pinus sylvestris* L.)

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Abstract We are developing molecular approaches to study the growth and development of woody plants. As part of our research efforts, we report the molecular cloning and characterization of *PsRLK* here, a cDNA from the conifer Scots pine (*Pinus sylvestris L.*) encoding a polypeptide similar to the receptor protein kinases described in angiosperms. A full-length clone was isolated from a cDNA library constructed with poly (A)+ enriched RNA prepared from germinating pine seeds. Characterization of the isolated sequence revealed that it contains multiple leucine-rich repeats in the N-terminal region and a characteristic Ser/Thr protein kinase domain in the C-terminal region. N- and C-terminal conserved domains are separated by a putative membrane spanning sequence. PsRLK protein is encoded by a single gene in the pine genome. A comparison of the pine sequence with the LRR-RLKs from *Arabidopsis* revealed that *PsRLK* is phylogenetically related to the LRR XI subfamily members. RT-PCR analyses of transcript abundance in pine tissues suggest that the gene expression pattern of *PsRLK* reflects the plant body formation programme, with increased levels during development of pine seedlings. The precise localization of *PsRLK* transcripts revealed that gene expression was restricted to specialized phloem cells suggesting a possible function of the putative receptor-like protein kinase in this particular vascular element.

Keywords Pine seedlings · *PsRLK* · Leucine-rich repeat · Tree development · In situ hybridization

Abbreviations EST: Expressed sequence tag · LRR: Leucine-rich repeat · RLK: Receptor-like kinase

C. Ávila · J. Pérez-Rodríguez · F. M. Cánovas (✉)
Departamento de Biología Molecular y Bioquímica,
Instituto Andaluz de Biotecnología,Unidad Asociada UMA-CSIC,
Campus Universitario de Teatinos, Universidad de Málaga,
29071 Málaga, Spain
E-mail: canovas@uma.es
Tel.: +34-95-2131942

Introduction

Receptor protein kinases play important roles in cellular signalling processes in living organisms. A cell surface receptor commonly contains three functional domains: an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. Studies of animal receptor protein kinases have suggested a model for receptor kinase mediated cellular signalling processes: a ligand binds to the extracellular domain and causes the receptor dimerization, which triggers the subsequent activation of the intracellular kinase domain. The activated kinases then phosphorylate substrate proteins within the cell, resulting in transduction of the molecular signals (Hunter 1995). In the last few years, a number of gene expression products with this characteristic architecture have been identified in plants. These gene products are referred to as receptor-like kinases (RLKs). The sequence homology and structural similarity of these RLKs with animal receptor kinases have suggested a similar biological mechanism of action for RLKs in plants. The major group of plant RLKs is the leucine-rich repeat (LRR) RLKs, which contain imperfect repeats of a 24-amino acid leucine-rich motif in the extracellular domain. Plant LRR RLKs have been found to play functions in mediating a variety of cellular processes, including morphogenesis (Torii et al. 1996), embryogenesis (Schmidt et al. 1997), meristematic growth (Clark et al. 1997; Suzaki et al. 2004) and pollen self-incompatibility (Muschietti et al. 1998). Some RLKs regulate responses to environmental signals, such as light (Deeken and Kaldenhoff 1997), hormones (van der Knaap et al. 1996; Hong et al. 1997; Li and Chory 1997; Li et al. 2002) and pathogen attack (Song et al. 1995). Database searches for LRR RLKs showed that many of them are present in the *Arabidopsis thaliana* genome (Shiu and Blecker 2001, 2003)where they are distributed on all five of the *A. thaliana* chromosomes. Recent bioinformatic analyses have even found a more complex superfamily of LRR RLKs in the rice genome

(Shiu et al. 2004). Although important advances have been made in the characterization of this group of plant proteins, the biological functions of LRR RLKs are mostly unknown. As part of our research efforts addressed to identify genes involved in growth and development of woody plants, a full-length cDNA encoding an LRR-RLK from pine seedlings has been cloned, sequenced and its structure compared to LRR-RLKs previously characterized in angiosperms. As an approach to get insights on the function of this gene its expression pattern has been analysed by RT-PCR during the initial stages of tree development. The spatial distribution of gene expression in pine seedlings has also been precisely determined by in situ hybridization. The results of this work are presented here, and it represents to the best of our knowledge the first report describing the molecular characterization of an RLK in gymnosperms. This contribution is an initial step to determine whether or not genes and mechanisms involved in cell signalling are conserved between gymnosperms and angiosperms.

Materials and methods

Plant material

The Scots pine seeds (*P. sylvestris L.)* used in all experiments were from Servicio de Material Genético, INFOCA (Spain). Seeds were imbibed in deionized water for 12 h under continuous aeration, and then germinated and grown as described previously (Cánovas et al. 1991).

cDNA library construction and screening for *PsRLK* clones

Total RNA was isolated from cotyledons of Scots pine seedlings (2.5 cm in length) using the phenol/SDS method (Ausubel et al. 1987; Claros and Cánovas 1998). For cDNA library construction, Poly (A)+ RNA was prepared from total RNA using two sequential rounds of affinity chromatography on oligo (dT) columns. Synthesis of cDNA was achieved using the Stratagene synthesis kit. cDNA fragments were cloned into λZAP vector and library plated following the instructions of the manufacturer. Plaque screening hybridizations were performed in a solution containing 6×SSC, 5×Denhardt's solution, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA at 65°C. Filters were washed twice in 2×SSC, 0.1% SDS for 30 min at 65°C followed by 0.2×SSC, 0.1% SDS for 30 min at 72°C. An EST clone previously isolated in our laboratory from developing seedlings was used as a probe. Several putative clones were isolated and characterized. A positive clone containing the longest insert (3.9 kb) was selected for DNA sequencing.

DNA sequencing and structural analysis

Restriction fragments derived from the 3.9 kb cDNA clone were subcloned into pBluescript SK to serve as templates for DNA sequencing by standard procedures using an ABI automated sequencer. The primary sequencing data were analysed by databases searches with the BLAST programme (Altschul et al. 1990). Amino acid sequences of all the LRRRLKs used in the study were aligned using the Clustal X programme (Higgins et al. 1996).

RT-PCR analysis

Total RNA from different developmental stages including three embrionary and two plantular samples stages was obtained as described before (Ausubel et al. 1987; Claros and Cánovas 1998). The poly (A)+ samples were obtained as described elsewhere (Avila and Cánovas 2001). The PCR reaction was performed using two specific primers that amplified 280 bp of the *PsRLK* 3′ untranslated region. The nucleotide sequences were as follows: 5′-CATTCTTCGGTTTCCAA-3′ and 5′- AGCGGTCCAGTCCGAGC-3′. The StrataScript RT-PCR system from Stratagene was used following instructions of the manufacturer. As control, a fragment of 300 bp of the constitutive *eiF4a*-2 gene was amplified by using the following primers: 5′-GGCTTCAAGGATCAGAT-3′ and 5′-CTGGTCCATGTCTCCATG-3′. Samples were separated in agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labelled probes. Quantification was performed using the Bio-imaging analyzer BAS-1500 (Fuji Photofilm). Expression levels were normalized against the control.

Southern analysis

Genomic DNA was isolated from pine seedlings, 2.5 cm of cotyledon length, by using standard protocols (Dellaporta et al. 1983; Ausubel et al. 1987). DNA was enzymatically digested overnight, fractionated on 0.8% agarose gel and transferred to a nylon membrane. The membrane was hybridized using a *PsRLK* probe containing the amino-terminus coding region of the cDNA.

In situ hybridization

Pine tissues were fixed and paraffin-embedded as described by Cantón et al. (1999). Gene-specific antisense riboprobes were synthesized from *PsRLK* (this work; EMBL accession number AJ250467) and *PsGS1b* (Avila-Sáez et al. 2000; EMBL accession number AJ005119) cDNAs. Preparation of digoxigenin (DIG)-labelled riboprobe was performed according to the method of Langdale (1993). As a control, a single-stranded DIG-labelled sense riboprobe was synthesized

from the linearized template. In situ hybridisations on apex, cotyledon, hypocotyl and root tissue sections were carried out as previously described (Cantón et al. 1999).

Results and discussion

A cDNA library prepared from RNA isolated from pine cotyledons was used for the screening of RLK clones. A cDNA clone of 3,880 bp was shown to contain an open-reading frame of 3,435 bp encoding for a protein of 1,145 amino acids. The *Ps*RLK sequence will appear in the EMBL database under the accession number AJ250467. Sequence analysis revealed high identity with LRR-RLKs previously characterized in angiosperms (Fig. 1). The predicted polypeptide contains multiple LRRs in the N-terminal region, a putative membrane spanning sequence and a characteristic domain of Ser/Thr protein kinases in the C-terminal region (Fig. 1a). A conserved LRR region of 568 amino acids was identified, which contained 23 imperfect tandem repeats of a 24-amino acid leucine-rich motif plus an additional truncated LRR. LRRs function as protein–ligand (often protein–protein) interaction domains (Kobe and Deisenhofer 1995; Kobe and Kajava 2001). The predicted major extracellular domain of the PsRLK polypeptide contained the LRR region flanked by pairs of conservatively spaced cysteines. The LRRs of PsRLK have the consensus sequence LxxLxLxxNxLxGxIPaEaGxL/Cxx. A consensus residue is defined as that appeared >50%

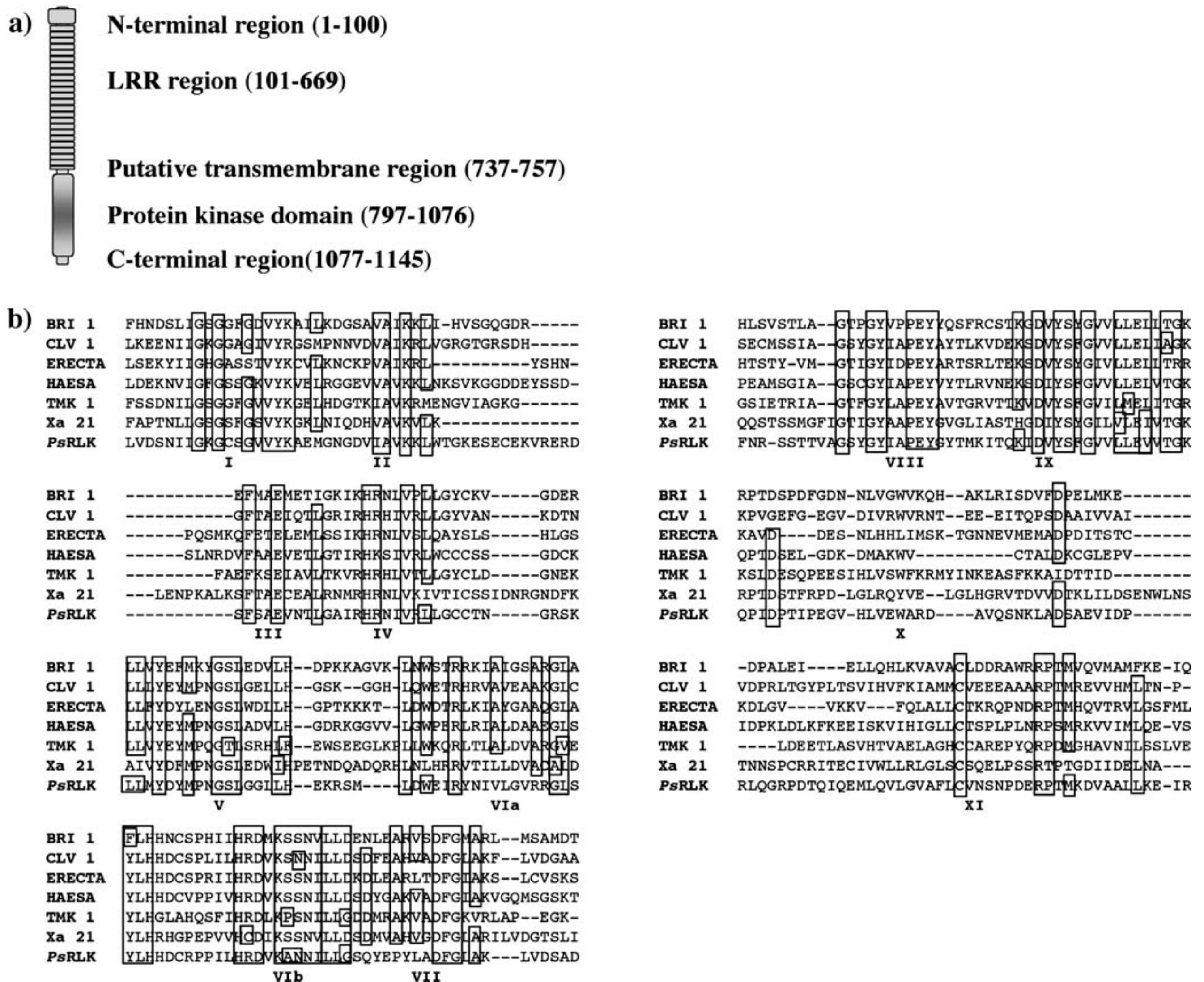

Fig. 1 Structural analysis of PsRLK. **a** Diagram showing putative protein domains in PsRLK. LRR represents LRR domain. *Numbers* indicate the amino acid extent of the domains in the primary structure. **b** Sequence alignment of the kinase domains of PsRLK and several LRR receptor kinases in plants. The following sequences are included (*accession numbers in brackets*): BRI1 (AF017056), CLV1 (U96879), ERECTA (U47029), HAESA (M84660), TMK1 (Y07748), Xa21 (U37133) and *Ps*RLK (AJ250467). The conserved protein kinase domains are indicated I to XI (Hanks and Quinn 1991). Residues that are conserved among at least five of the compared sequences are *boxed*

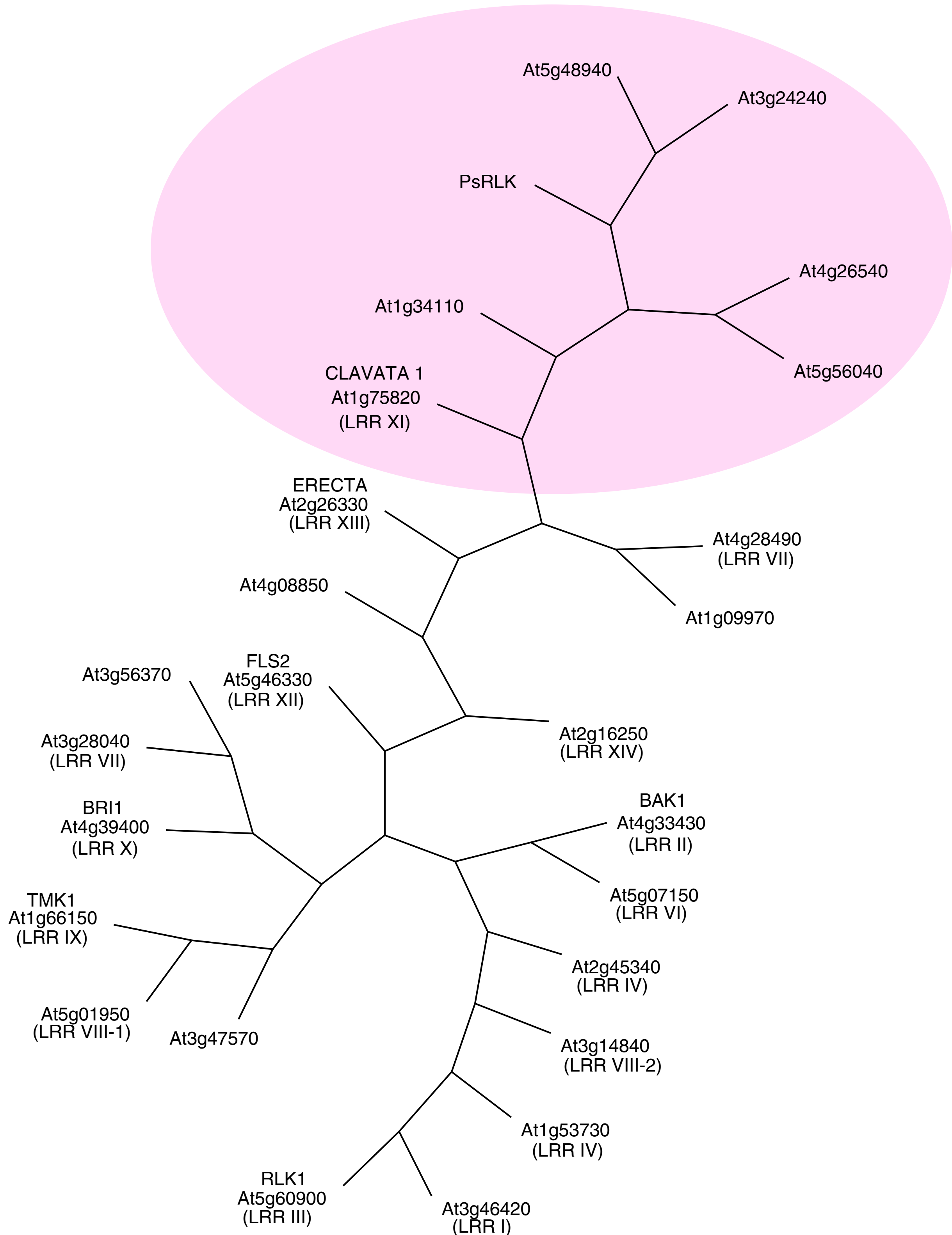

Fig. 2 Phylogenetic tree of the *Pinus sylvestris* and *Arabidopsis thaliana* LRR-RLKs. The unrooted tree was constructed with the Clustal X programme (Higgins et al. 1996). Acession numbers: PsRLK (AJ250467), At5g4890 (NM 124271), At3g24240 (NM 113329), At4g26540 (NM 118787), At5g56040 (NM 124986), At1g34110 (NM 103134), At1g75820 (NM 106232), At4g28490 (NM 118991) At1g09970 (NM 100871), At4g08850 (NP 192625), At2g16250 (NM 127181), At5g46330 (NM 124003), At3g56370 (NM 115495), At3g28040 (NM 113722), At4g39400 (NM 120100), At1g66150 (L 00670), At5g01950 (NM 120273), At3g47570 (NM 114625), At4g33430 (NM 119497), At5g07150 (NM 120797), At2g45340 (NM 130097), At3g14840 (NM 112345), At1g53730 (NM 104251), At3g46420 (NM 114509), At5g60900 (M 84658)

frequency at each position. The position that exhibits no clear consensus was represented as ‘x’. Any aliphatic residue (L, I, V, A, F and M) was represented as ‘a’ (data not shown). The catalytic kinase domain in the carboxy terminus was used to perform sequence alignment with other plant receptor kinases including: BRI1 the putative brassinosteroid receptor (AF017056) (Li and Chory 1997), the developmental regulators CLAVATA1 (CLV1) (U96879) (Clark et al. 1997) and ERECTA (U47029) (Torii et al. 1996), a race-specific disease-resistance gene of rice Xa21 (U37133) (Song et al. 1995), a putative LRR-RLK from rice TMK (Y07748) (van der Knaap et al. 1996) and HAESA from *Arabidopsis* (M84660) (Walker 1993). The alignment of the kinase domains in the carboxy terminus of the plants RLKs is shown in Fig. 1b. Protein kinase domain of PsRLK has all 11 conserved subdomains of eukaryotic protein kinases and all invariant amino acid residues in their proper positions (Hanks and Quinn 1991). PsRLK appears to fall into the serine/threonine class of protein kinases, since it contains diagnostic sequences of this family (subdomains VIb and VIII in Fig. 1b) (Hanks and Quinn 1991).

The genomic organization of *PsRLK* in *P. sylvestris* was investigated using its cDNA as a molecular probe. Genomic DNA was digested with the restriction enzymes *Hind*III and *Bam*HI, which do not cut the probe and *Eco*RI, which recognize a single site in the cDNA sequence. Two hybridizing bands were visible in the *Eco*RI lane of the Southern blot, whereas only a single major band was detectable in the *Hind*III and *Bam*HI lanes (not shown). These data strongly suggests that *PsRLK* is present as a single copy gene in the pine genome.

Although large-scale EST sequencing projects have been developed for pine (Allona et al. 1998; Cantón et al. 2003), the availability of full-length cDNA from conifers is still very limited and consequently this report represents a valuable contribution in the field of conifer molecular biology. ESTs databases for loblolly and maritime pines have been established, and the information is accessible on the Internet (http://www.pinetree.ccgb.umn.edu; http://www.cbi.labri.fr/outils/SPAM/index.php). A search in the databases identified a total of 38 ESTs with sequence similarity to *PsRLK*.

Shiu and Blecker (2001) have categorized more of 600 RLKs in the *Arabidopsis* genome into subfamilies based on both the identity of extracellular domains and the phylogenetic relationship between the kinase domains of subfamily members. According to these criteria the LRR-containing RLKs have been grouped into 14 distinct subfamilies. To examine the phylogenetic relationship of the *PsRLK* sequence with LRR-RLKs from *Arabidopsis*, an unrooted phylogenetic tree was constructed with 25 LRR-RLKs including a representative member of each LRR subfamily. As shown in Fig. 2, *PsRLK* is closely related to At5g48940 and At3g24240 genes encoding putative RLKs of unknown function in *Arabidopsis*. In the same cluster are included At4g26540, At5g56040, At1g34110A and CLAVATA1which are classified into the LRR XI subfamily. The clustering of *PsRLK* with CLAVATA1 suggests it may be involved in the control of tree growth and development. The close situation of ERECTA in the phylogenetic tree is also supporting the earlier assumption.

Since we do not know about how and where receptors of any type function in woody plants, we decided to examine whether the pattern of *PsRLK* expression could be related to CLAVATA1, the most similar gene of a known function in the phylogenetic analysis (Fig. 2). Thus, the cellular distribution of *PsRLk* transcripts was determined in the shoot apex of 2 weeks old pine seedlings by in situ hybridization (Fig. 3). It is well known that CLAVATA1 is specifically expressed in a broad group of cells in the central region of the shoot meristem (Clark et al. 1997; Clark 1997). However, as shown in Fig. 3a, *PsRLK* gene expression was quite unrelated and found largely associated to elongated cells along the

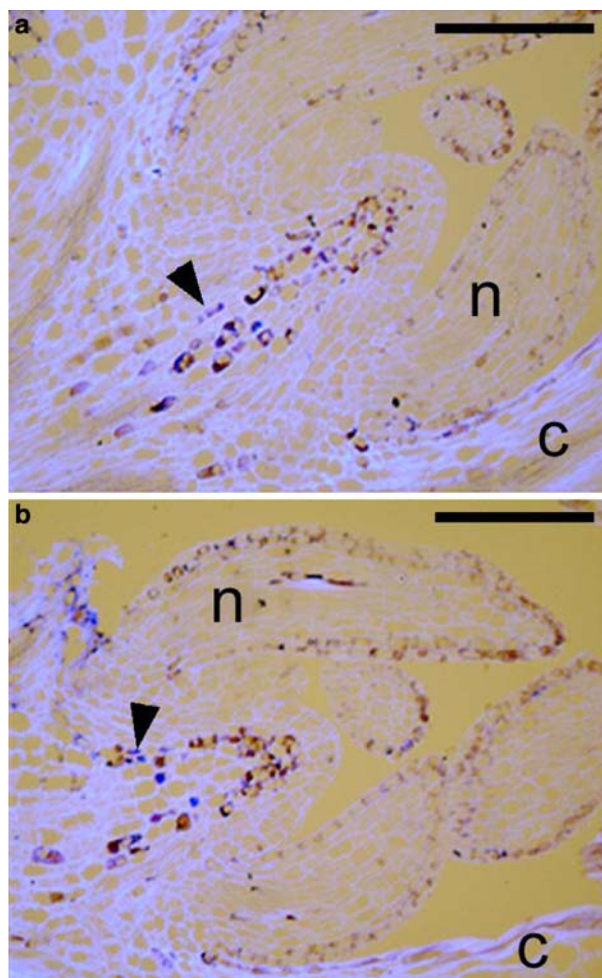

Fig. 3 Determination of the *PsRLK* expression pattern in pine apex. RNA in situ hybridizations using antisense *PsRLK* (**a**) and *PsGS1b* (**b**) riboprobes were performed as described in Materials and methods on longitudinal sections of pine apex; *n* needle; *c* cotyledon. Gene expression signals were observed in *purple colour* (*arrowheads*). *Scale bars* represent 1 mm

young stem which are characteristics of the emerging vascular bundles. No expression signals were seen in the first layers of cells of the apex central region. The particular expression of *PsRLK* in these cellular types was confirmed by the localization of *PsGS1b* transcripts in the same cross sections of the shoot apex (Fig. 3b). *PsGS1b* is a gene of nitrogen metabolism exclusively expressed in the vascular cells (Avila et al. 2001; Suárez et al 2002; Gómez Maldonado et al. 2004) and it has been recently proposed as a suitable molecular marker for early vascular differentiation in pine (Pérez-Rodríguez et al. 2006).

In order to get further insights into the function of the gene the relative transcript abundance was determined by RT-PCR in pine seedlings at different stages of development including three embrionary and two plantular stages (Fig. 4). PCR reaction was performed using two specific primers that amplified 280 bp of the 3′ untranslated region of poly (A)+ samples obtained from each developmental stage as described elsewhere (Ávila and Cánovas 2000). As a control, a fragment of 300 bp of the constitutive *eiF4a-2* gene was amplified in the same developmental stages analysed for *PsRLK* gene expression. *PsRLK* mRNAs accumulated progressively in all five stages considered, from the mature dry embryo to developing seedlings (Fig. 4). However, the abundance of *PsRLK* transcripts was particularly high in the two plantular stages where the levels were about two to nine times (P1 and P2, respectively) higher than those observed in the dry embryo (DE). No significant differences were observed in the relative abundance of *eiF4a-2* transcripts (results not shown).

The precise localization pattern for PsRLK transcripts within specific plant tissues at specific stages of development could provide additional information on its functional role. For this reason the distribution of *PsRLK* gene expression in different cell types was also

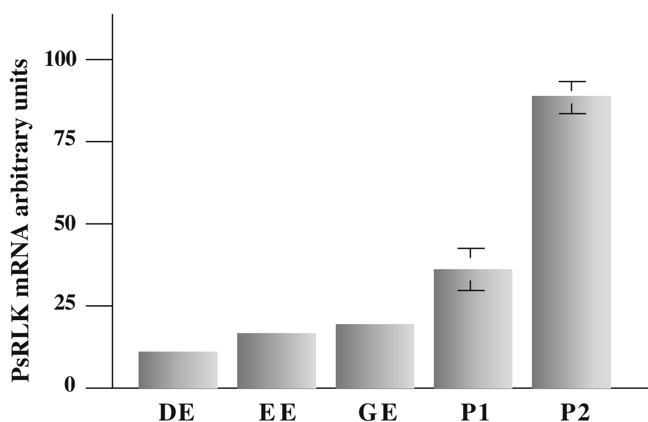

Fig. 4 Analysis of *PsRLK* mRNAs during the initial stages of pine development. Relative transcript levels of *Ps*RLK were determined by RT-PCR using specific oligonucleotides. Five developmental stages were analysed: *DE* dry embryo; *EE* embedded embryo; *GE* germinating embryo; *P1* first plantular stage corresponding to seedlings of 0.5 cm cotyledon length; *P2* second plantular stage corresponding to seedlings of 2.5 cm cotyledon length

determined in roots, hypocotyls and cotyledons of seedlings by in situ hybridization (Fig. 5). Relatively high levels of *PsRLK* transcript were detected in sections of hypocotyls and cotyledons, while in roots gene expression was hardly detected. However, *PsRLK* mRNAs were always found associated to phloem elements of the seedling. Interestingly, a significant number of pine ESTs showing identity with *PsRLK* are expressed in roots and vascular elements (data not shown) what is consistent with the observed gene expression pattern.

Plant development requires the integration of various signalling pathways that recognize and respond to spatial and temporal information. Although *PsRLK* expression is specific of vascular cells (Fig. 5), its expression levels increased during the initial stages of seedling development (Fig. 4). Thus, the above data suggest that the observed gene expression pattern might reflect the progressive differentiation of the vascular system in developing pine. In fact, in pine embryo where no mature vascular elements are present, the transcript starts to be visible only when procambial cells differentiate into xylem and phloem elements during germination (data not shown).

Furthermore, the predicted PsRLK protein has all the canonical features for a potential role in intercellular signalling: an extracellular putative ligand-binding domain, a transmembrane domain and an intracellular kinase domain, possibly involved in a phosphorylation cascade. If PsRLK acts as a receptor, the signal molecule and the components of the signalling pathway have to be determined. Nevertheless, whatever the ligand is, it seems to be present and available along the seedling, given the specificity of *PsRLK* expression, since only differentiated cells of the phloem could respond to it. Furthermore, the PsRLK protein contains a large LRR region, which is believed to be a specific ligand-binding site for small peptide(s) or glycoprotein(s) (Kobe and Deisenhofer 1994), or pathogenic elicitor(s) (Baker et al. 1997). Based on the studies of other plant LRR receptor kinases the ligand for PsRLK receptor is likely a small polypeptide. In addition to the pathogenesis elicitors, the most characterized peptide ligand is the small-secreted peptide CLV3 that is thought to bind CLV1 in cooperation with another protein CLV2 (Clark et al. 1997; Jeong et al. 1999; Trotochaud et al. 2000; Fiers et al. 2005). Database searches show that the LRR RLKs represent a large group of RLKs in higher plants. However, the understanding of the functions of each LRR-RLK awaits the availability of further studies.

In summary, we present here for the first time the molecular structure of a conifer LRR-RLK as well as expression studies showing the temporal and spatial distribution of specific transcripts at early stages of pine development. This new knowledge provides insights into the function of this particular gene in conifers and, at the light of the findings reported here, it is temptative to speculate that PsRLK could be a plant receptor kinase involved in vascular development. However, additional

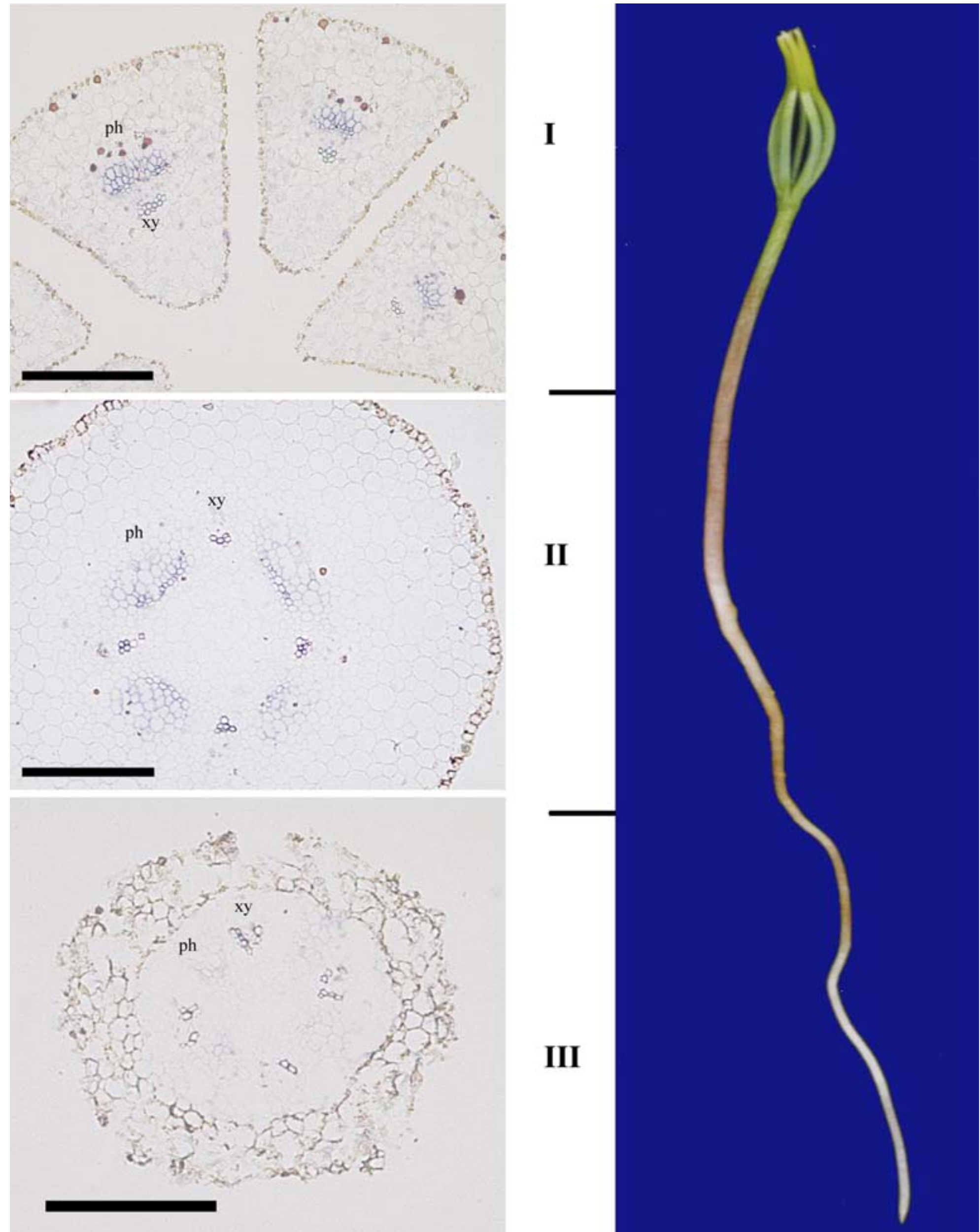

Fig. 5 In situ localization of *PsRLK* transcripts in phloem cells of pine seedlings. Transversal cross sections through the seedling were used for localization of *PsRLK* mRNA by in situ hybridization with an antisense RNA probe. In all cases the specificity of the signals was established by in situ hybridization of control sections with sense probes. (*I*) cotyledons; (*II*) hypocotyl; (*III*) root; (*ph*) phloem, (*xy*) xylem. *Scale bars* represent 200 μm

research work is necessary to understand the function of *PsRLK* in pine trees and to dissect the putative transduction pathway where it could be involved.

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