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SHORT COMMUNICATION

Molecular cloning and characterization of a lipid transfer protein gene (*PsLTP1*) from *Pinus sylvestris* (L.)

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Abstract Plant nonspecific lipid transfer proteins (nsLTPs) are widely distributed through plant kingdom and are characterized by the presence of a central hydrophobic cavity, suitable for binding various hydrophobic molecules. Despite extensive research on nsLTP in different plant species, mostly angiosperm, and the great diversity of physiological processes in which they seem to be involved, their exact functions still remain unclear. Also, very limited experimental data are available on nsLTP in gymnosperm. In this study, we report for the first time on the molecular cloning of nsLTP, from *Pinus sylvestris* L. (*PsLTP1*, GenBank accession JN980402.1) and the expression pattern of *PsLTP1* during ontogenesis and in response to environmental stress conditions. Total RNA from roots of 7-day old pine seedlings was used to isolate the cDNA clone, corresponding to Scots pine lipid transfer protein. The open reading frame of *PsLTP1* consists of 372 bp encoding a protein of 123 amino acids. Amino acid sequence alignment revealed that mature *Ps*LTP1 shares high level of similarity with nsLTP from other conifers and with well-studied nsLTPs from angiosperms. The *Ps*LTP1 contains a 27-amino-acid N-terminal signal sequence and presents all the features of a plant nsLTP. Amino acid comparison analysis and 3D structure prediction showed that *Ps*LTP1 is a type 1 nsLTP. The results of the expression analysis of Scots pine *PsLTP1* gene revealed that its transcripts accumulate in actively growing tissues. Furthermore, transcription of *PsLTP1* was upregulated in response to cold and salt treatments, and downregulated during acidic, osmotic and water stresses.

Keywords Scots pine · Nonspecific lipid transfer protein (nsLTP) · Molecular cloning · Expression · Abiotic stresses

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Introduction

Conifers are one of the oldest seed plants that have survived into the present day and are thus called living fossils (Hensiruk 2002). Scots pine (*Pinus sylvestris* L.) is an important tree species in northern Eurasia. In Ukraine, Scots pine covers 23% of the total forest area, and in Polissya, that area increases to 64.5%, making this tree species very important for Ukrainian forestry (Hensiruk 2002). Scots pine is economically valuable for its soft timber and derivative products that are widely used for pharmaceutical purposes. Besides the great economic benefit, boreal forests with Scots pine provide high biodiversity and oxygen. Conifers, like other plant species, are constantly exposed to a great variety of environmental

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conditions at every stage of their life cycle, including biotic (such as pests and pathogenic microorganisms) and abiotic stresses (drought, salt, alkali, flood, high and low temperature, oxidative stress and heavy metal toxicity). All these factors modulate plant vitality, negatively affecting plant growth and development and thereby decreasing productivity. To decrease the negative effects, conifers developed a set of defense mechanisms that include structural barriers, antimicrobial chemicals, hypersensitive response and pathogenesis-related (PR) protein genes and their products with strong antibacterial and antifungal activities (Adomas et al. 2008; Veluthakkal and Dasgupta 2010). Studies from various laboratories have revealed that some PR-genes are expressed constitutively while the expression of others is triggered only under certain conditions, such as infection or exposure to abiotic stresses. In recent years, considerable interest has developed in using specific genes and nature-friendly products to reduce damaging effects of different environmental stimuli and to enhance stress tolerance in plants. Genes encoding pathogenesis-related (PR) proteins are attractive candidates for that role (Goyala and Mattoo 2014; Sels et al. 2008). The best-known products of PR-genes in plants are chitinases, peroxidases, defensins, thaumatins, thionins and lipid transfer proteins (Goyala and Mattoo 2014).

Plant nonspecific lipid transfer proteins (nsLTPs) belong to the multigene PR-14 family with antimicrobial and antifungal activity in vitro (García-Olmedo et al. 1995). Research on nsLTPs has focused on their structure, subcellular localization, classification, expression patterns and evolution. The first nsLTP was identified nearly 40 years ago, and since then, they have been isolated from more than 50 plant species, and that number is still growing (José-Estanyol et al. 2004; Kader 1996). NsLTPs are low molecular weight basic proteins, ranging in size from 6.5 to 10.5 kDa, and typically are synthesized from a precursor that has a signal sequence, varying from 21 to 27 amino acids. Their tertiary structure is characterized by a conservative eight-cysteine motif (8 CM), where cysteine residues form four disulfide bonds, necessary to stabilize the hydrophobic cavity. In vitro studies showed that the cavity binds a diverse range of lipid molecules, including fatty acids, fatty acyl-CoA, phospholipids, glycolipids, hydroxylated fatty acids and prostaglandin B2 (Carvalho and Gomes 2007; Douliez et al. 2000, 2001; Tassin-Moindrot et al. 2000). The compact structure of nsLTPs is stabilized by the disulfide bonds, conferring stability to heat and resistance to proteolysis (Scheurer et al. 2004).

The two main types of nsLTP in flowering plants, termed nsLTP1 and nsLTP2, differ in molecular size (LTP1 consists of 90 amino acids; LTP2 has 70 amino acids.) and by disulfide bond arrangement (In the LTP1 type, cysteine 1 forms a disulfide bond with cysteine 6, and cysteine 5 bonds with cysteine 8; in the LTP2 type, cysteine 1 is paired with cysteine 5, while cysteine 6 is linked with cysteine 8) (Kalla et al. 1994). In addition, both LTPs have different internal cavities for binding hydrophobic ligands: LTP1 is characterized by a long tunnel-like cavity, suitable for binding fatty acids, while LTP2 has two adjacent hydrophobic cavities allowing to accommodate sterols, which is not possible for LTP1 (Cheng et al. 2004; Samuel et al. 2002). Beside LTP1 and 2, more isoforms have been discovered in recent years (Boutrot et al. 2008; Wang et al. 2012). Some are restricted to a single plant species, while others are apparently present in all plants, except for algae (Edstam et al. 2011). The absence of any nsLTP in algae indicates that nsLTPs arose during the conquest of the land by plants, thus, making them key elements in adaptation to harsh environment. Indeed, they are abundant in plants and constitute about 4% of the total soluble proteins (Kader 1996).

Plant lipid transfer proteins display complex expression patterns. They are present in different tissues and are upregulated at different stages of development. NsLTPs have been found in seeds, leaves, stems, roots, flowers and fruits (Pyee et al. 1994; Thoma et al. 1994; Yubero-Serrano et al. 2003). Members of this family are responsive to abiotic stimuli such as drought, cold, salt stress, and their expression is also induced by bacterial and fungal pathogens (Carvalho et al. 2006; Edstam et al. 2014; Guo et al. 2013; Jung et al. 2003).

Despite recent progress, the function of nsLTPs in plants is not well understood. Initially, nsLTPs were thought to be involved in lipid trafficking, due to their ability to bind and transfer lipids in vitro (Kader 1996). Later, LTPs were found to be secreted, but their function in the extracellular matrix was not defined (Sterk et al. 1991; Thoma et al. 1994). Studies of *Arabidopsis* to knockdown, knockout or overexpress members of the *nsLTP* family have uncovered the involvement of nsLTP in key physiological processes such as long-distance signaling, defense against pathogens and pests, pollen tube adhesion, cuticle formation, suberin biosynthesis, embryogenesis, seed germination, fruit ripening and allergen action (DeBono et al. 2009; Edstam et al. 2013; Eklund 2003; Maldonado et al. 2002; Park et al. 2007). Recent studies further support the role of nsLTPs in lipid trafficking based on their dynamic distribution (Ambrose et al. 2013; Edstam et al. 2013; Pagnussat et al. 2012).

To date, the research has mainly focused on nsLTP functions in angiosperms rather than gymnosperms. Members of nsLTP family identified in *Ginkgo biloba* were shown to function as proteinase inhibitors. In Norway spruce, nsLTPs are involved in somatic embryogenesis (Sabala et al. 2000; Sawano et al. 2008). Notably, nsLTPs in pines have not been studied.

In this paper, we describe for the first time the identification of a cDNA encoding Scots pine nsLTP (*PsLTP1*) and analyze the predicted amino acid sequence and the three-dimensional (3D) structure of the *Ps*LTP1 protein. Furthermore, the *PsLTP1* expression pattern was studied in different organs from Scots pine during ontogenesis and under different abiotic stresses.

Materials and methods

Plant material and treatment

Seeds of Scots pine from Lviv Forestry Breeding Seed Center (Lviv region, Ukraine) were washed with distilled water three times and placed on sterile filter paper soaked with distilled water on a Petri dish. They were then left to germinate in a growth chamber in a 16 h-photoperiod at 24 °C. Seedlings were collected at 3 and 7 days after sowing and organs excised separated (roots, hypocotyls and cotyledons). Needles, roots, buds, bark, female and pollen cones were also separately collected from 15-year-old Scots pine from the Ukrainian National Forestry University (UNFU) Dendrobotanical garden. Seeds were harvested from 2-year-old female cones. Endosperms and embryos were collected from mature seeds. All samples were immediately frozen in liquid nitrogen and used for RNA extraction.

For stress treatments in a growth chamber, twenty 7-day-old seedlings were used per experiment at same conditions as mentioned above. For acidulation and NaCl treatments, seedlings were transferred onto sterile filter paper in a Petri dish, soaked with 10 mM HCl or 250 mM NaCl, respectively. For osmotic and oxidative stresses, seedlings were placed onto sterile filter paper that had been moistened with 100 mM mannitol or 10 mM hydrogen peroxide, respectively. Solutions were prepared in a final volume of 5 mL of distilled water and sterilized using a 0.2 µm filter. For these treatments, seedlings were later kept at 24 °C, for 24 h. For cold treatment, plates with seedlings were placed at 4 °C for 24 h. For heat-shock stress, plates with seedlings were incubated at 37 °C for 24 h. For water stress, 7-day-old seedlings were submerged in distilled water on plates, kept at 24 °C and collected after 24 h. Non treated seedlings were grown at 24 °C as the control. After treatments, samples were stored at − 80 °C until total RNA isolation. For each treatment, three replicates were used and treated independently.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from freshly frozen seedlings using lithium chloride precipitation with minor modifications (Chang et al. 1993). The quality of total RNA samples was verified using 1% agarose gel electrophoresis and the quantity measured using the WPA UV 1101 Biotech Photometer UV1101 (Biochrom, Holliston, MA, USA) at OD_{260} with ratio OD_{260}/OD_{280} determination. cDNA was synthesized from 1 µg of total RNA using reverse transcriptase RevertAid Premium (Fermentas, Vilnius, Lithuania), according to the manufacturer protocol.

Cloning Scots pine lipid transfer protein gene (*PsLTP1* gene)

To clone the cDNA of lipid transfer protein-1 gene from *P. sylvestris*, we used a PCR-based approach. Primers for Scots pine *LTP* cloning were designed using the known *LTP* cDNA sequence of *Pinus resinosa* Aiton (accession number AF223405.1, NCBI database). Both primers contained sites for restriction enzymes: NcoI in the forward primer and EcoRI in the reverse primer (listed in Table 1 as F-*Ps*LTPc and R-*Ps*LTPc, respectively; restriction sites are underlined). cDNA from roots of 7-day-old seedlings was used as a template. PCR was run under the following conditions (95 °C for 5 min; 30 cycles of 95 °C for 30 s, 54 °C for 1 min, 72 °C for 1 min; final extension at 72 °C for 5 min). PCR products were separated by gel electrophoresis and excised from the gel. For obtaining the *pET23d-Ps*LTP1 expression plasmid, PCR products were digested with both NcoI and EcoRI and subcloned into the bacterial cloning/expression vector pET23d (+) (Novagen, Merck KGaA, Darmstadt, Germany) digested with the same enzymes. The resulting construct was used to transform *E. coli* strain DH5α, and recombinant clones were grown on selective Luria–Bertani (LB) medium containing ampicillin 100 µg/mL. The presence of a DNA insert carrying the complete coding region for *PsLTP1* was verified by PCR analysis. Inserts from positive colonies were purified and sequenced using the automatic DNA sequencer ABI 73 TM (SEQGEN, Torrance, CA, USA).

Sequence analysis and three-dimensional (3D) structure prediction of *Ps*LTP

The amplified and cloned sequence of the *PsLTP1* cDNA was analyzed using the Expasy Translate tool (Gasteiger et al. 2003). The presence of the signal peptide was verified using the online program SignalP, version 3.0 (Bendtsen et al. 2004). BLAST was used for searches of sequence databases (https://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments (detailed later) were created using Clustal Omega, version 1.2.1 (Sievers et al. 2011). Default parameters were used. The phylogenetic tree was constructed through comparison with sequences from the

Table 1 Primers used in this study

Primer	Sequence (5′-3′)	Use
F-*PsLTP*c	TTATTCCATGGCTGTGAAGAAGATG	Cloning primer, forward
R-*PsLTP*c	CACCTGAATTCTCAGTGAACCTTGGAACAG	Cloning primer, reverse
PsLTP-F	ATGGCTGTGAAGAAGATG	Semiquantitative RT-PCR, forward
PsLTP-R	TCAGTGAACCTTGGAACAG	Semiquantitative RT-PCR, reverse
RPL44-F	CAAAGCTTGCAAAAAGCACA	Standard control primer, forward
RPL44-R	TTCCCTTCCCCTTCTTGTC	Standard control primer, reverse

BLAST databases using the Clustal W method in LaserGene software (DNASTAR, Madison, WI, USA). LTP sequences used for multiple sequence alignment and phylogenetic tree construction were from Monterey pine (*Pinus radiata* D. Don), GO448405; loblolly pine (*Pinus taeda* L.), U10432; shore pine (*Pinus contorta* Douglas ex Loudon), GT250957; red pine (*Pinus resinosa* Aiton), AF223405; Japanese black pine (*Pinus thunbergii* Parl.), FY841227; Jack pine (*Pinus banksiana* Lamb.), GW765141; Japanese red pine (*Pinus densiflora* Siebold and Zucc.), FG617230; maritime pine (*Pinus pinaster* Aiton), CT576220; Scots pine (*Pinus sylvestris* L.), JN980402; white spruce [*Picea glauca* (Moench) Voss], EX413050; Norway spruce [*Picea abies* (L.) H. Karst], GT886696; black spruce [*Picea mariana* (Mill.) Britton, Sterns and Poggenb.], JZ079655; Sitka spruce [*Picea sitchensis* (Bong.) Carrière], GW719531; ginkgo (*G. biloba* L.), DQ836633; barley (*Hordeum vulgare* L.), M15207; *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.], AF159798; tree tobacco (*Nicotiana glauca* Graham), AY621631; maize (*Zea mays* L.), Uniprot P19656; strawberry [*Fragaria* ×*ananassa* (Duchesne ex Weston) Duchesne ex Rozier], AJ315844. The 3D structure of *Ps*LTP1 was predicted by submitting the primary sequence to I-TASSER and Phyre2 servers (Kelley et al. 2015; Yang et al. 2015). The signal peptide of *Ps*LTP1 was removed from the sequence submitted to I-TASSER and Phyre2 since it is absent in the mature protein. Both 3D structures were determined using the automated modeling mode. The protein structures were visualized using ChimeraX version 0.1 (Pettersen et al. 2004).

Gene expression analysis by semiquantitative multiplex RT-PCR

After first-strand cDNA synthesis, the cDNA samples were used as a template for semiquantitative multiplex RT-PCR (mxPCR) using a Proteus thermocycler (Helena Bioscience, Gateshead, UK) (95 °C for 5 min; 35 cycles at 95 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s; final extension at 72 °C for 7 min). The housekeeping gene 60S ribosomal protein L44 (RPL44; GenBank EL342388) was used as a standard control for the mxPCR. The primer sequences used in the mxPCR are listed in Table 1. All mxPCR products were separated by electrophoresis in 1.5% (w/v) agarose gel.

Statistical analyses

The data were analyzed statistically using ANOVA. Results are represented as the mean ± SE. Densitometrical analysis of the electrophoregrams was performed using GelProAnalyzer 4.0 (MediaCybernetics, Rockville, MD, USA). The values for expression level of *PsLTP1* were calculated relative to 60S *rpl44*. The experiments were run in triplicate.

Results

Molecular cloning and sequence analysis of *PsLTP* cDNA

To obtain the full-length cDNA encoding Scots pine lipid transfer protein (*PsLTP1)*, RT-PCR was performed using specific primers and mRNA extracted from seedling roots (GenBank JN980402.1). The *PsLTP1* ORF is predicted to encode a polypeptide of 123 amino acid residues containing a signal peptide of 27 amino acids at the N-terminus (Fig. 1). After the signal sequence was removed, the predicted molecular mass of the mature form of *Ps*LTP1 is 9.83 kDa with a theoretical pI of 8.12.

By multiple sequence alignment, *Ps*LTP1 was shown to share identities with other known nsLTPs. Comparison analyses with other nsLTPs from angiosperms and gymnosperms showed amino acid identity ranging from 39% for *Arabidopsis* to 100% for three *Pinus* species in the database: Jack pine, Maritime pine and Japanese red pine (Fig. 2a). LTPs from other pine species also showed high identity with Scots pine *Ps*LTP1 (99% with black pine nsLTP, 98% with red pine and shore pine nsLTPs, 97 and 96% with loblolly and Monterey pine, respectively). An identity of 86% was found for *Ps*LTP1 from Sitka and 91% for nsLTPs from white spruce, respectively, from genus *Picea*. Despite high similarity between nsLTPs from gymnosperms, Gb-nsLTP1 from ginkgo showed only 37%

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atggctgtgaagaagatggtggagggtgtgtttgtggttgggcttgtggttacaatgatg
 M  A  V  K  K  M  V  E  G  V  F  V  V  G  L  V  V  T  M  M
attgtgcggggtgctgtgagtgtggaaggtgccatatcatgcaaccaggtggtgagtgcc
 I  V  R  G  A  V  S  V  E  G  A  I  S  C  N  Q  V  V  S  A
atgactccatgcgctacataccttctaggaaatgcagcaacccctgctgcggcttgttgt
 M  T  P  C  A  T  Y  L  L  G  N  A  A  T  P  A  A  A  C  C
ccttccattcgtgggttggactcacaagtgaaagccacccctgataggcaggctgtttgc
 P  S  I  R  G  L  D  S  Q  V  K  A  T  P  D  R  Q  A  V  C
aactgcctcaagacacaagcacagtcatatggggtcaagttgggaaaggccgccaatctg
 N  C  L  K  T  Q  A  Q  S  Y  G  V  K  L  G  K  A  A  N  L
ccgggtctgtgcaaggtcaccgatctcaacgtgcctatctctcctaatgttgactgttcc
 P  G  L  C  K  V  T  D  L  N  V  P  I  S  P  N  V  D  C  S
aaggttcactga
 K  V  H  -
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Fig. 1 Nucleotide and deduced amino acid sequences of *Ps*LTP1. The predicted signal peptide is boxed. The position of tryptic peptides, identified by mass spectrometry is shaded in gray

sequence identity with Scots pine *Ps*LTP1, while barley nsLTP and 41% identity. Furthermore, 51% identity was found with tree tobacco and 47% identity with strawberry *Fa*LTP. nsLTP from maize, which also was used for 3D modeling, had 45% identity with Scots pine *Ps*LTP1. To further investigate the relationship between *Ps*LTP1 and nsLTPs, we constructed a phylogenetic tree (Fig. 2b), which showed that *Ps*LTP1 formed one monophyletic group with other pine members and had a closer relationship with nsLTPs from the gymnosperms than with angiosperms, except for Gb-nsLTP1 from ginkgo.

Scots pine *Ps*LTP1 was further analyzed using 3D structure modeling based on templates of the lipid transfer protein from *Zea mays* L. (PDB codes 1fk5 and 1ahf), where both PDB codes correspond to the same protein sequence (Uniprot P19656), but the structure of 1ahf was experimentally determined by NMR spectroscopy and the structure of 1fk5 was established by X-ray crystallography. For building the 3D model using the I-TASSER server, 1ahf was used as a template, while coordinates from 1fk5 were applied using the Phyre2 server. In both cases *Ps*LTP1 had 45% amino acid sequence identity with *Z. mays*. The predicted model of *Ps*LTP1 using I-TASSER consisted of 54% α-helices (Fig. 3a). Phyre2 predicted a model with 100% confidence and 96% coverage. From 96 amino acids, which codify the *Ps*LTP1, 54% consisted of α-helices, and 38% of the query was found to be disordered (Fig. 3b). The 3D structural model of *Ps*LTP1 shows that the protein consists of four α-helices, where the secondary structures are stabilized by four disulfide bridges and an unstructured C-terminal tail. The four pairings are formed between eight cysteines: C7–C55 (cysteine pair 1–6), C17–C32 (cysteine pair 2–3), C33–C77 (cysteine pair 4–7) and C53–C92 (cysteine pair 5–8).

Expression profile of *PsLTP1* gene

In the expression analysis of *PsLTP1* in different tissues during ontogenesis using mxPCR, *PsLTP1* transcripts were expressed in all organs of the seedlings and an adult tree. The relative transcript levels in young seedlings were higher than in the adult tree. In seedlings, the highest level of *PsLTP1* transcripts accumulated in apical parts of the seedlings and gradually decreased in lower parts (Fig. 4).

In organs of an adult tree, the quantity of *PsLTP1* transcripts was relatively low compared to the level in seedlings. As shown in Fig. 4, *PsLTP1* transcripts are present in all investigated organs, and were relatively high in female and pollen cones and buds. *PsLTP1* transcripts were not detected in the endosperm, but levels were high in embryos collected from dry seeds, indicating that *PsLTP1* gene expression is tissue- and organ-specific and time-dependent.

The analysis of *PsLTP1* expression in response to several abiotic stresses (Fig. 5) showed that cold and salinity led to an upregulation of *PsLTP1* expression. Treatment with hydrogen peroxide and high temperature did not substantially affect expression in pine seedlings, while HCl and mannitol induced a downregulation that was more pronounced than that caused by water stress (32% when compared to the control).

Discussion

In this study, we cloned a cDNA carrying the entire coding sequence for an nsLTP (*PsLTP1* gene) from *P. sylvestris*. The deduced amino acid sequence of *PsLTP1* shares most of the characteristics present in other plant nsLTPs,

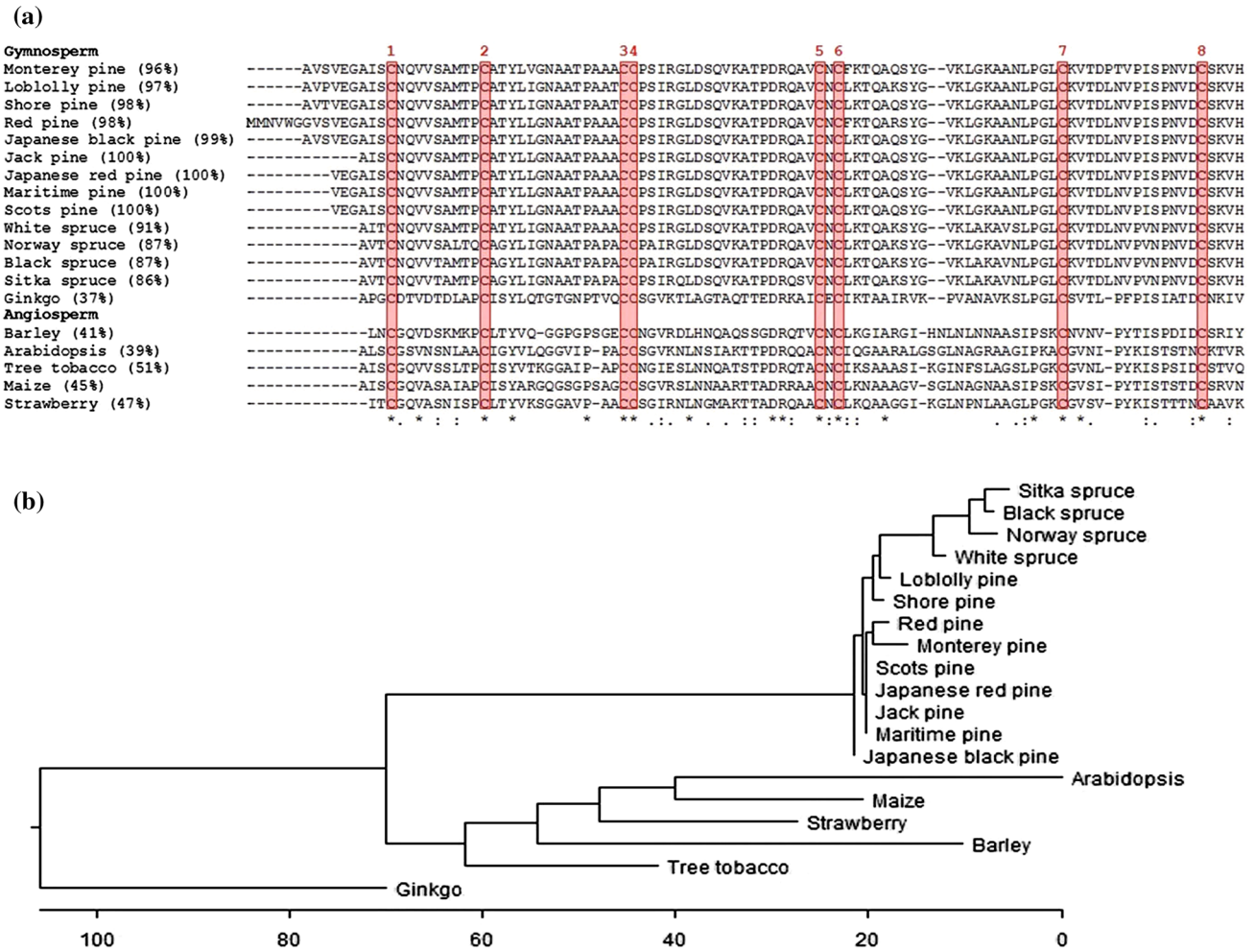

Fig. 2 Sequence analysis of *Ps*LTP1 with 18 plant nsLTPs. **a** Sequence alignment of mature *Ps*LTP1 from seedling roots with mature nsLTPs from gymnosperms and angiosperms. All N-terminal signal sequences were removed from the alignment using SignalP. The eight strictly conserved cysteine residues are shaded in red. Asterisks below the sequences indicate identical amino acids; colon indicates strong amino acid similarity, and a period indicates weak similarity. Percentage of identity between *Ps*LTP and each amino acid sequence used in the alignment is reported for each sequence. LTPs sequences were: Monterey pine, loblolly pine, shore pine, red pine, Japanese black pine, Jack pine, Japanese red pine, maritime pine, Scots pine, white spruce, Norway spruce, black spruce, Sitka spruce, ginkgo, barley, *Arabidopsis*, tree tobacco, maize, strawberry. **b** Phylogenetic tree of *Ps*LTP1 and other plant nsLTPs constructed by Clustal W program using neighbor-joining method. The length of each pair of branches displays the distance between sequence pairs, and units at the bottom of the tree indicate the number of substitution events

including the presence of eight cysteines in defined positions, crucial for correct disulfide bridge formation. *Ps*LTP1 is a low molecular weight protein with basic pI. Signal P predicted an N-terminal signal peptide of 27 amino acids that engages the protein into the secretory pathway (Carvalho and Gomes 2007). The predicted mature *Ps*LTP1 is a type 1 nsLTP with a calculated molecular mass of approximately 10 kDa and a positive net charge. Using I-TASSER and Phyre2 programs generated the hypothetical 3D structure of *Ps*LTP1, consisting of 4 α-helices and a C-terminal tail. The disulfide bond arrangements C7–C55 (cysteine pair 1–6) and C53–C92 (cysteine pair 5–8) corroborate that *Ps*LTP1 belongs to type 1 in the nsLTP family. The predicted structure with internal cavity allows the protein to accommodate hydrophobic compounds such as fatty acids, but further in vitro and in vivo studies are required to validate this prediction. Multiple sequence alignment with nsLTP from other plant species revealed that *Ps*LTP1 shares a high degree of identity with nsLTPs from other conifers, and they clustered together in the phylogenetic tree. Thus, the mature *Ps*LTP1 protein shares 100% identity of its amino acid sequence with three other members of the same genus: Jack pine, maritime pine and Japanese red pine. Notably, all have different geographical locations, very distant from each other. Scots pine is native to northern Eurasia, maritime pine is native

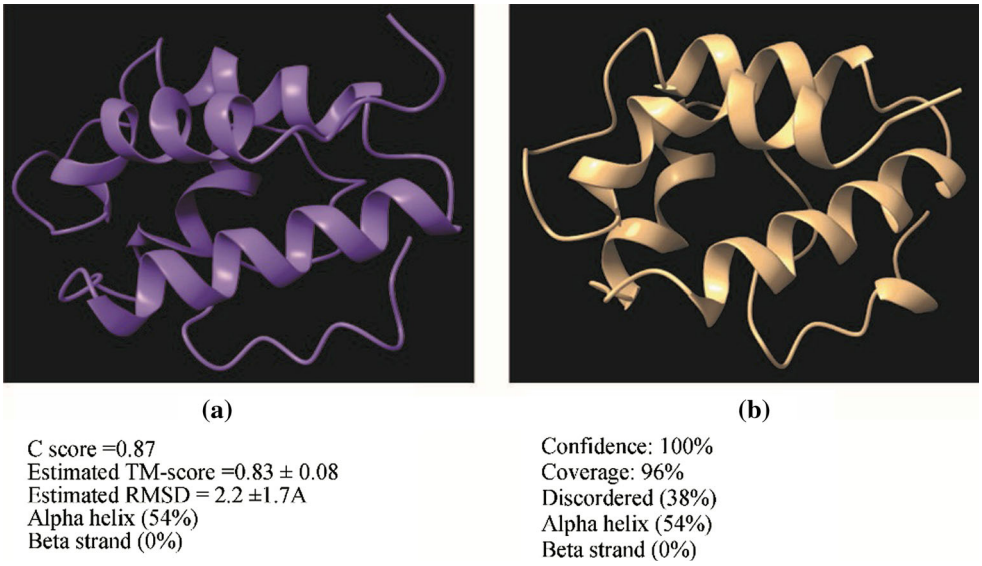

Fig. 3 Three-dimensional model predicted for *Ps*LTP1. The hypothetical 3D structural modeling was performed based on a template of lipid transfer protein from *Zea mays* (PDB codes 1fk5 and 1ahf). To build the 3D model, the I-TASSER program used 1ahf (**a**) as a template, while the Phyre2 used 1fk5 (**b**). In both cases, *Ps*LTP had 45% amino acid sequence identity with *Z. mays*. The 3D structural model of *Ps*LTP shows that the protein consists of four α-helices, where the secondary structures are stabilized by four disulfide bridges and unstructured C-terminal tail. The four pairings are formed between eight cysteines: C7–C55, C17–C32, C33–C77 and C53–C92

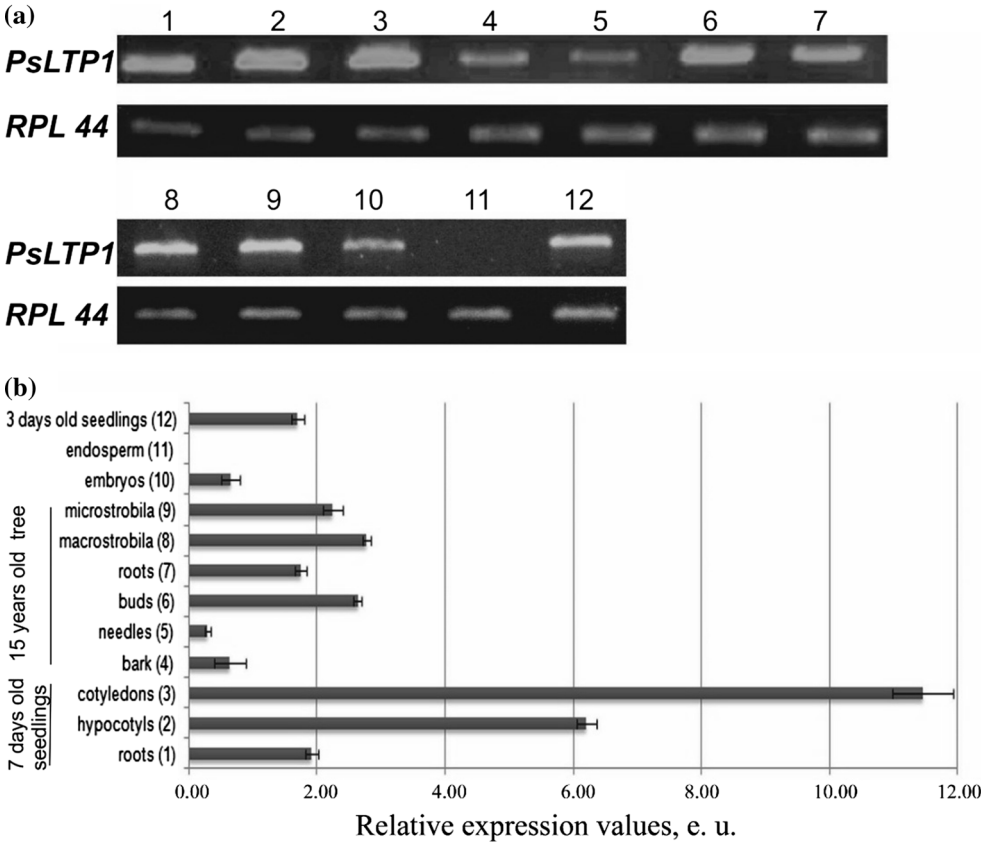

Fig. 4 Expression analysis of *PsLTP1* gene in different organs of Scots pine. Top panels: **a** electrophoresis of mxPCR products using RNA from roots (1), hypocotyls (2), cotyledons (3) of 7-day-old seedlings; bark (4), needles (5), buds (6), roots (7), female cones (8), pollen cones (9) of 15-years-old tree; embryos (10), endosperms (11) and 3-day-old seedlings (12). Bottom panel, **b** transcript levels calculated relative to housekeeping gene *rpl44* from Scots pine as an internal control

to the Mediterranean region, Jack pine is an eastern North American pine, and Japanese red pine is native to the northeastern part of China and the Korean peninsula, Japan and the far eastern part of Russia. From these data, we can conclude that *Ps*LTP1-like sequences are highly conserved inside this genus.

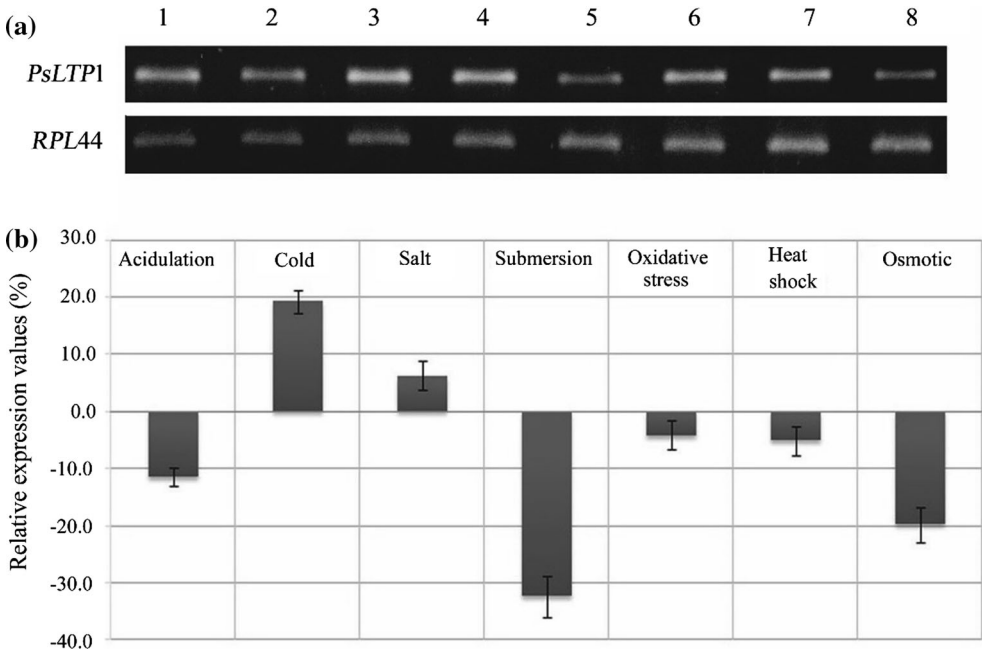

Fig. 5 Expression of Scots pine *PsLTP1* under various environmental stresses. **a** Electrophoresis of mxPCR products using RNA extracted from seedlings treated with water as the control (1), exposed to HCl (2), cold (3), or salt (4), submersed in water (5), exposed to H_2O_2 (6), heat shock (7) or osmotic stress (8). **b** Expression level of *PsLTP1* relative to *rpl44* from Scots pine as the internal control. Expression of the control, grown under typical conditions, was set to zero. Values below zero indicate downregulation above zero indicate upregulation of *PsLTP1*

PsLTP1 showed was expressed at the highest level in young tissues, including roots, hypocotyls and cotyledons of pine seedlings and in buds, female and pollen cones of the adult tree. Moreover, *PsLTP1* transcripts were abundant in embryos and 3-day-old seedlings but were absent in the endosperm. A thorough search of the EST database revealed that nucleotide sequences of similar *nsLTPs* have been isolated mostly from young seedlings. Similar results were also obtained in studies of other species in which expression of *nsLTPs* tends to be higher in aerial plant organs and in actively growing tissues. In *Betula plathyphilla* Sukaczev, expression of *BplLTP1* was much higher in young organs than in adults (Guan et al. 2013). In *Arabidopsis*, nsLTPs are expressed in vascular tissues and epidermal cells during development and in broccoli in the cuticular waxy layer (Pyee et al. 1994; Thoma et al. 1994). The expression of ns*LTP* genes is necessary for normal embryo development in both *Arabidopsis* and Norway spruce (Sabala et al. 2000; Vroemen et al. 1996). Interestingly, nsLTPs decrease during plant aging and in fully expanded tissues in angiosperms (Pyee et al. 1994; Thoma et al. 1994).

Some abiotic stresses caused downregulation of the *PsLTP1* gene, but low temperature and high salinity led to the upregulation of *PsLTP1* in Scots pine seedlings. A thorough search of EST libraries of *P. sylvestris* revealed that several other sequences identical to *PsLTP1* have been isolated from young pine plants after treatment with ozone. Similar expression patterns were described for other plant species for which several stress-induced *LTP* genes have been isolated and characterized. For example, *TSW12* in tomato was upregulated after NaCl treatment (Torres-Schumann et al. 1992; Trevino and O'Connell 1998). Two pepper genes (*CALTPI* and *CALTPIII*) were upregulated during drought, in a high salt concentration, at low temperature and after wounding (Jung et al. 2003). In strawberry, *FaLTP* is upregulated during fruit development, and *FaLTP* transcripts increase after ABA, SA and wounding treatments but decrease slightly after cold treatment; they do not accumulate after salt treatment (Yubero-Serrano et al. 2003). Three *LTP* transcripts (*PpLTPG3*, *PpLTPG8* and *PpLTPG9*) in the moss *Physcomitrella patens* (Hedw.) Bruch and Schimp. accumulate to high levels during cold treatment, and the expression of four *LTP* genes (*PpLTPG2*, *PpLTPG3, PpLTPG6*, *PpLTPG9*) associated with the synthesis and/or deposition of cutin and cuticular wax is upregulated during dehydration (Edstam et al. 2014).

Because members of the *nsLTP* gene family vary widely in their expression patterns during development, in different tissues and in response to environmental stresses, clarifying the exact physiological role of each member of this gene family is a complex task. Taking into account that *PsLTP1* transcripts were detected mostly from actively growing young tissues, the homology among members from different pine species is very high, and their expression profiles also seems to be similar, it is not unreasonable to think that the identified *Ps*LTP1 could be involved in pine development and response to abiotic stress.

Previously, we isolated a nsLTP (p1 protein) from Scots pine seedlings and confirmed its identity by mass spectrometry (Kovaleva et al. 2009). Two peptides were obtained by tryptic digestion RP-HPLC of this endogenous p1 protein (VTDLNVPISPNVDCSK and AATPAAACC*PS*IR), then

identified using MS. A search of different databases revealed that both peptides are similar to the nonspecific lipid-transfer protein precursor (nsLTP) from *P. resinosa* (GenBank AF22340) (Kovaleva et al. 2009). Further studies allowed us to isolate a cDNA clone, encoding *P. sylvestris* LTP, which we termed *PsLTP1*. Sequence analysis of the coding region and the predicted translated protein revealed that *Ps*LTP1 contains the same two peptide sequences obtained by tryptic digestion of the endogenous nsLTP (p1 protein) from Scots pine seedlings. Taken together, our studies suggest that *Ps*LTP1 could have a similar structure and/or role in defense as the p1 protein previously described. However, the biological function of *Ps*LTP1 needs to be further investigated.

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