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ORIGINAL ARTICLE

Characterization of a *Pinus sylvestris* thaumatin-like protein gene and determination of antimicrobial activity of the in vitro expressed protein

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Abstract
Thaumatin-like proteins (TLPs) are pathogenesis-related proteins, which are involved in plant defense responses to pathogen infection. Expression of the *Pinus sylvestris* L. *TLP* gene is up-regulated by methyl jasmonate treatment and inoculation with *Heterobasidion annosum*. A full-length *Pinus taeda TLP* gene sequence was used to design PCR primers for amplification of the full-length *TLP* gene from *P. sylvestris*. A putative 705-bp open reading frame of *TLP* gene was cloned into *Escherichia coli* cells, and then subcloned into the overexpression vector pET100 using BL21 Star expression bacteria. Optimization of the expression of recombinant TLP was achieved by decreasing both expression temperature and IPTG concentration. The purified 24.6-kDa TLP shows antimicrobial activity against 12 fungal species.

Keywords *Pinus sylvestris* · Thaumatin-like protein · Methyl jasmonate · Protein expression · Antimicrobial activity

Introduction

Plants need to be able to respond and adapt to recurring abiotic and biotic stresses. Induced resistance is an increased expression of natural defense mechanisms in plants against repeated exposure to adverse factors. One of the most important inducible defense mechanisms in plants is the biosynthesis of pathogenesis-related (PR) proteins. Members of the PR-5 group are called thaumatin-like proteins (TLPs) because their amino acid sequences are homologous to thaumatin, a sweet-tasting protein from the West African shrub *Thaumatococcus danielli* (Velazhahan et al. 1999). Under normal conditions, TLPs are expressed at low levels in certain organs at specific developmental stages (Liu et al. 2010a). TLPs can play a role in cold tolerance, salinity, and drought resistance (Singh et al. 1989; Jung et al. 2005; Joosen et al. 2006; Deihimi et al. 2013). Many PR-5 proteins are induced in plants as a response to infection by pathogens, osmotic stress (Singh et al. 1987), treatment with abscisic acid (Singh et al. 1989), ethylene (Koiwa et al. 1994), salicylic acid, methyl jasmonate (Reymond and Farmer 1998), and wounding (Neale et al. 1990). In addition, overexpression of a TLP gene in tobacco increased tolerance to both fungal pathogens and abiotic stress conditions (Rajam et al. 2007). The plant growth regulator jasmonate and its methyl ester (MeJA) are present in plant tissues and are involved in both regulation of plant development and response to environmental stresses such as wounding and pathogen infection. Exogenous application of MeJA stimulates the synthesis of PR proteins including TLPs, chitinases, peroxidases, proteinase inhibitors, and several other proteins in conifers (Piggott et al. 2004; Pervieux et al. 2004; Liu et al. 2005). Some plant TLPs exhibit a strong antifungal activity while other TLPs have no or only weak antifungal activities (Wang and Ng 2002; Krebitz et al. 2003; Jayaraj et al. 2004; Vitali et al. 2006; Mohamed et al. 2011). The inhibitory effects of TLPs can differ depending on the fungal species tested (Vitali et al. 2006). The conserved hydrophobic domain of thaumatin may interact with components of fungal cell membranes, and lead to structural disruption and formation of trans-membrane pores, resulting in rapid release of cytoplasmatic contents (Woloshuk et al. 1991). Several TLPs have been reported to bind to ß-1,3-glucans, a common component of fungal cell walls. Interaction of TLPs

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with ß-1,3-glucan may act in concert with PR2 enzymes to disrupt fungal cell wall synthesis and/or prevent proper fungal wall assembly during hyphal extension (Osmond et al. 2001). The presence of TLP gene and protein families has been reported in a range of species, including *Pinus monticola*, where a total of 10 TLP genes with differential expression in various tissues and times have been reported (Liu et al. 2010a). However, this gene family has not been as well characterized in other *Pinus* species, with only one full-length *P. taeda* TLP gene sequence in the NCBI database (accession no. EF532603), which was used to characterize a full-length *P. sylvestris* TLP gene (JX461338) (this study). These genes are most similar to the *P. monticola* TLP genes *-L6*, *-L2*, and *-L5* (82, 82, and 80% sequence identity, respectively). The *-L2* gene was the most abundantly expressed gene in *P. monticola*, particularly in needles and roots. The *-L6* gene was preferentially expressed in roots, while the expression of *-L5* was low (Liu et al. 2010a).

Scots pine (*Pinus sylvestris*) is one of the most important forest tree species in Latvia, both ecologically and economically, due to its wide distribution, productivity, and high wood quality. Fungal pathogens can cause significant losses in forestry, and in particular, the forest tree root rot-causing pathogen *Heterobasidion annosum* has become widespread and is causing considerable economic damage (Van Damme et al. 2002; Gaitnieks et al. 2007). Needle cast (caused by *Lophodermium seditiosum*) can affect pine trees of all ages, but it is a significant disease in forest nurseries and young plantations because it can be fatal to young *Pinus sylvestris* seedlings. Development of needle cast is favored by warm and rainy conditions in autumn and mild winters. Currently, serious damage from needle cast is rare in forest plantations, but may increase due to changes in climatic conditions and a tendency to reduce seedling age and planting density in order to decrease forest regeneration costs (Jansons et al. 2008). Use of fungicides in forests is forbidden; therefore, research on novel, environmentally friendly means of disease control is necessary, for example, tree breeding for increased resistance and the induction of resistance mechanisms in seedlings. The *TLP* gene utilized in this study has been previously shown to exhibit gene copy number variation polymorphism in *P. sylvestris* (Šķipars et al. 2017), and transcriptome sequencing indicated that the expression of this gene increased after MeJA treatment (Kānberga-Siliņa et al. 2017) and inoculation with *H. annosum* (Šķipars, unpublished). Therefore, in this study, we describe the identification of a full-length *TLP* gene sequence from *P. sylvestris*. To confirm the antimicrobial activity of this gene, the encoded TLP was expressed in vitro, and antifungal activity of the expressed TLP against a range of fungi commonly found in Latvian forests was determined. Induction of expression of the *TLP* gene in *P. sylvestris* after inoculation with *Heterobasidion annosum* as well as application of MeJA was confirmed.

Materials and methods

Plant material, MeJA treatment, *Heterobasidion annosum* inoculation, and real-time PCR quantitation

P. sylvestris seedlings (1 year old) were obtained from the Latvian State Forests department "Seeds and Plants", Jaunkalsnava, Latvia, and placed in growth chambers at 17 to 22 °C and 10 h light and 14 h dark. Each tray (10 seedlings) was treated with 5 ml of either 10, 30, 50, or 100 mM MeJA dissolved in 0.1% Tween 80. Two control groups were utilized, seedlings treated with 5 ml 0.1% Tween 80, and seedlings treated with distilled water. The seedlings treated with MeJA were kept in plastic bags for 24 h after treatment to allow the volatilization of the excess MeJA before being placed in the same growth chamber as the controls. Needles were harvested prior to and 2 weeks after treatment (Heijari et al. 2005; Moreira et al. 2009).

For *Heterobasidion annosum* inoculation, 1-year-old ramets of three genotypes were used (seven ramets of each genotype, five were inoculated and two were wounded as controls). These genotypes were identified as having TLP gene copy number variation polymorphism (Šķipars et al. 2011). Expression of the *TLP* gene in inoculated samples was compared to the expression in wounded controls 7 days after treatment. Wounding of the control ramets was performed by removing bark from the stem with a scalpel and applying sterile malt extract agar (MEA) medium. Inoculation was performed in the same way but medium containing a pure culture of *H. annosum* was applied to the wound. The treated part of the ramets was wrapped in Parafilm, and stem samples were collected 7 days after treatment.

For *TLP* gene expression analyses, RNA was extracted from stem cross sections as described previously (Šķipars et al. 2014); 500 ng of RNA was reverse transcribed using the TaqMan Reverse Transcription kit (Thermo Fisher Scientific) following manufacturer's instructions.

Real-time PCR relative quantification was done using comparative C_T ($\Delta\Delta C_T$) analysis and Maxima Probe/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific) or Hot FIREPol EvaGreen qPCR Mix plus Rox kit (Solis BioDyne). The reaction with probe contained 15.0 μl of 2× Maxima Probe/ROX qPCR Master Mix, 20 ng cDNA, 900 nM forward and reverse primer (final concentration), and 250 nM probe (final concentration). DEPC (diethyl pyrocarbonate)-treated water was used to bring the reaction volume to 30 μl. PCR conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and hybridization and elongation 60 °C for 1 min. Reaction setup with EvaGreen qPCR Mix plus Rox kit (Solis BioDyne) per sample was as follows (reaction volume 20 μl): 4 μl of master mix (5×), 400 nM of each primer, and 32 ng of cDNA was used as a PCR template. The following PCR program was used: 95 °C for 15 min., 40 cycles of 95 °C for 15 s, 60 °C

for 20 s, 72 °C for 20 s. Thermal cycling was performed on Applied Biosystems StepOne Plus instrument. The *TLP* gene-specific primers and probes used for real-time PCR were as follows: F: 5′- CAGTGCCCACAGGCATACAG - 3′, R: 5′- CCACCAGGGCAGGTGAAG - 3′, probe: 5′- 6-FAM-TATGCCAAGGACGATGCCACCAGC -TAMRA - 3′. The *P. sylvestris GAPDH* gene (GenBank Acc. No. L07501) was used as the endogenous control: F: 5′- ACGGTTTTGGTCGA ATTGGA - 3′, R: 5′- CCCCACGAGCTCGATATCAT - 3′, probe: 5′- VIC- CTCGTCGCCCGTGTGGCTCTG -TAMRA - 3′. Real-time PCR analyses were performed in three technical replicates. Data were analyzed using StepOne software v2.2, ANOVA ($P < 0.05$).

Identification and analysis of the *Pinus sylvestris* TLP gene sequence

A full-length *Pinus taeda TLP* gene sequence (GenBank Acc. No. EF532603) was used to design primers for amplification of the full-length *TLP* gene from *P. sylvestris*. Genomic *P. sylvestris* DNA was isolated using a CTAB method (Murray and Thompson 1980). Approximately 400 ng genomic DNA was utilized for amplification of a 936-bp fragment of the *P. sylvestris TLP* gene with forward primer: 5′- GTGTTGAG TTATACTGTTTAACCTGCT - 3′ and reverse primer: 5′- CTATTGTTACTATATGAGTAGGAAGATAT - 3′. The PCR mixture contained 0.2 mM (each) dNTP, 2 mM $MgCl_2$, 1.5 U Taq polymerase, and 0.8 μM of each primer. PCR cycling conditions were 95 °C, 5 min; 40 cycles: denaturation 95 °C, 30 s, annealing 50 °C, 35 s, elongation72 °C, 1 min 30 s; final elongation 72 °C, 30 min. PCR products were analyzed by 1.75% agarose gel electrophoresis and purified using Sephadex G-50. The concentrations of purified PCR products were measured using a Qubit fluorometer (Invitrogen) with the Quant-iT dsDNA BR Assay Kit (Invitrogen). The purified PCR product was ligated into the vector pTZ57R/T using the InsTAclone PCR Cloning Kit (Thermo Fisher Scientific), which was used to transform *E. coli* DH5 α cells. Plasmid DNA was isolated using an alkaline lysis mini-prep protocol. Plasmids were sequenced with M13 primers using an ABI PRISM 3130xl Genetic Analyzer and analyzed using BLAST. Sequence and predicted protein features, such as an open reading frame, signal peptide, pI, and molecular mass were evaluated using protein analysis tools (http://expasy.org/tools) and the ORF finder available at NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/).

For intron verification, RNA isolation was done according to a modified protocol (Šķipars et al. 2014). RNA was isolated from two to three fresh needles using Genomic DNA Purification Kit (Thermo Fisher Scientific), originally intended for isolation of DNA, followed by several purifications with DNase I (Thermo Fisher Scientific). Removal of DNA was confirmed using a standard PCR protocol with intron-spanning primers and visualization of PCR products on agarose gels. RNA concentration was measured using a Qubit fluorometer (Invitrogen) with the Quant-iT RNA BR Assay Kit (Invitrogen). cDNA was synthesized using TaqMan reverse transcription reagents (Applied Biosystems) and oligo $d(T)_{16}$ primer, following the manufacturer's protocol. Reverse transcription was performed as follows: 10 min at 25 °C, 30 min at 48 °C, and 5 min 30 s at 95 °C.

The synthesized cDNA and genomic DNA were analyzed by PCR using the primers F: 5′- TGCGCAGGGTCCCTTTG - 3′ and R: 5′- CAGTCACCAGTGGTACAGCTACCT - 3. The PCR products were also sequenced.

Subcloning and in vitro expression of thaumatin-like proteins

Phire® Hot Start II DNA polymerase (Thermo Fisher Scientific) and cDNA were used to produce blunt-ended PCR products. PCR primers were designed for amplification of the *PsTLP* gene without the 5′ signal sequence, and cloned into the pET100/D-TOPO vector for expression of recombinant protein with the Xpress epitope and 6× His-tag. The PCR mixture contained 1× Phire®Plant PCR buffer (Thermo Fisher Scientific), 0.4 μl Phire® Hot Start II DNA Polymerase (Thermo Fisher Scientific), 0.5 μM each primer: forward primer 5′-**CACC**ATGACCGTCAAAAACCA GTG-3′ and reverse primer 5′- CTAACCACAGAAGACGA CGTTGTAA-3′ (start and stop codons are underlined, overhang sequence for pET TOPO vector is in bold). The conditions for PCR were 98 °C, 5 min; 35 cycles: denaturation 98 °C, 10 s, annealing 65 °C, 10 s, elongation 72 °C, 20 s; final elongation 72 °C, 20 min. PCR products were analyzed by 1.75% agarose gel electrophoresis and concentrations were measured using a Qubit fluorometer (Invitrogen) with the Quant-iT DNA BR Assay Kit (Invitrogen).

Approximately 2.5 ng of the 613-bp PCR product was cloned into the pET100/D-TOPO vector (Invitrogen). The pET TOPO construct was used to transform competent *E. coli* One Shot TOP10 cells (Invitrogen) using heat shock for 30 s at 42 °C.

Successfully transformed *E. coli* cells containing the TLP/pET100 vector were grown in S.O.C. medium at 37 °C for 1 h [2.0% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast-extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose] (Invitrogen) and selected by plating the transformed cells on Luria-Bertani broth (LB) agar plates [1.0% (*w*/*v*) tryptone, 0.5% (*w*/*v*) yeast-extract and 1.0% (*w*/*v*) NaCl at pH 7] containing ampicillin (100 μg/ml). Correct orientation of the insert was confirmed by sequencing using T7 primers: forward primer 5′-TAATACGACTCACTATAGGG-3′ and reverse primer 5′-TAGTTATTGCTCAGCGGTGG - 3′. Plasmid DNA was isolated using an alkaline lysis mini-prep protocol.

Plasmids containing the correct TLP/pET TOPO construct were transformed into *E. coli* BL21 Star (DE3) One Shot cells (Invitrogen) by heat shock for 30 s at 42 °C and grown in S.O.C. medium (Invitrogen) at 37 °C for 30 min and then overnight in LB media with ampicillin (10 mg/ml) at 37 °C with shaking. The overnight culture was added to LB medium and grown for 2 h at 37 °C. At this time point, IPTG (1 mM final concentration) was added to the media followed by incubation at 37 °C for 4 h. The cells were collected by centrifugation at 5000 rpm for 10 min and re-suspended in lysis buffer [50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 1 mM imidazole at pH 7.8] according to the Champion pET Directional TOPO Expression Kit (Invitrogen) protocol, followed by three rounds of freeze (liquid nitrogen)/thawing (42 °C). The TLP was collected by centrifugation at 13,000 rpm for 5 min to separate the soluble protein fraction (supernatant) from the insoluble fraction (pellets).

Optimization of recombinant TLP expression

Modifications were made to the TLP expression protocol to maximize protein production. Optimization of expression of the recombinant TLP was achieved by decreasing both the expression temperature and IPTG concentration and addition of glucose.

The induction process was optimized in the following ways: at 25 °C overnight using different IPTG concentrations (0, 0.05, 0.1, 0.5, and 1 mM); at 25 °C overnight with 1 mM IPTG and 1% glucose; and at 37 °C for 4 h adding 1 mM IPTG to the culture when OD600 reached approximately 1.4 to 1.6 and adding 1% glucose. Optical density was measured with a Lambda 25 UV/VIS spectrophotometer (Perkin Elmer). The cells were collected by centrifugation at 5000 rpm for 10 min. Soluble proteins were extracted as described earlier and analyzed by 12% tricine SDS-PAGE gel electrophoresis.

Protein analysis, quantitation, and purification

Chip-based analysis was performed with the Agilent 2100 Bioanalyzer system (Agilent Technologies). The Protein 80 kit was used for sizing and quantification of protein samples, according to the manufacturer's protocol.

The recombinant TLP was expressed with a 6× His-tag. After induction, the cells were heat-shocked and the recombinant protein was purified from the supernatant under native conditions using Ni-NTA IMAC (immobilized metal affinity chromatography) (QIAGEN). The optimization of purification was achieved by using different concentrations of imidazole in lysis buffer and washing buffer. After shaking the bacterial extract in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 1 mM or 10 mM or 40 mM imidazole, pH 8.0) with Ni-NTA resin, the lysate was applied to the column, and the resin was washed three times with 250 μl wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM or 30 mM or 40 mM imidazole, pH 8.0). The TLP was then eluted with 50 μl elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0) and the cell extract, the flow through, and the eluate were analyzed by 12% tricine SDS-PAGE and Agilent 2100 Bioanalyzer.

Enterokinase was added to the purified protein at a ratio of approximately 0.02 units per 1 mg of protein and mixed. Incubation was done at 25 °C for 16, 24, and 32 h. Protein concentrations were determined by reaction with Bradford reagent using bovine serum albumin as a standard (Bradford 1976).

Assessment of antimicrobial activity of the recombinant TLP

The antifungal activity of the expressed recombinant TLP was tested on fungal isolates from the collection of the Latvian State Forest Research Institute "Silava" Forest phytopathology and mycology laboratory, which represent fungal species found in Latvian forest ecosystems. The following isolates were tested: *Amylostereum areolatum*, *Sistotrema brinkmannii*, *Resinicium bicolor*, *Bjerkandera adusta*, *Climacocystis borealis*, *Gloeophyllum sepiarium*, *Stereum sanguinolentum*, two *Heterobasidion annosum* (Fr.) Bref. isolates (50 and V Ma15), two *Heterobasidion parviporum* (Fr.) Niemelä & Korhonen isolates (17 and 162), *Lophodermium seditiosum*, *Lophodermium conigenum*, and a saprotrophic soil fungus from the genus *Trichoderma*. To initiate fungal mycelial growth, an agar plug containing mycelia of fungi was placed at the center of a Petri dish containing 20 ml of 2% malt extract agar and incubated at 25 °C until the mycelia reached a diameter of 2 cm (1–10 days depending on the fungal species). Various amounts of the expressed TLP (20, 30, 50, 100, 200, 300 μg) with the His-tag were pipetted into wells that were punched in the agar plate surrounding the plugs 1 cm from the rim of the mycelial colony. Ni-NTA elution buffer was utilized as a control. Inhibition of mycelial growth was recorded after 48 h of incubation at 25 °C or when mycelium reached the edge of control wells. Data are presented as the mean of three independent experiments. Mycelial growth inhibition was scored as the following: − no inhibition of mycelial growth; + inhibition zone width 1–2 mm; ++ inhibition zone 2–3 mm; and +++ inhibition zone > 3 mm.

Results

PCR primers designed using the *P. taeda TLP* gene sequence amplified a PCR product of 936 bp from *P. sylvestris* genomic DNA (GenBank Acc. No. JX461338). The *Ps*TLP coding sequence was 705 bp, encoding a protein of 234 amino acids,

with a molecular weight of approximately 24.61 kDa, and pI 4.51 (an acidic protein), including 16 cysteine residues typical of L-type TLPs, and was highly similar to the TLP mRNA coding sequence from *Pinus taeda* (99%). Also, this sequence has significant sequence similarity with other thaumatin-like proteins, sharing 88% amino acid identity with *Picea glauca* TLP, *Picea sitchensis*—87%, and *Pinus monticola*—82%. The disulfide bridges formed by these cysteine residues have an important role in maintaining protein stability and correct folding and preserving high activity under extreme temperatures and pH conditions. In addition, the deduced protein has a thaumatin family signature peptide sequence G-x-[G/F]-x-C-x-T-[G/A]-D-C-x(1,2)-[G/Q]-x(2,3)-C at the 86-102 aa position (Liu et al. 2010b). The sequence of the first 30 aa at the N-terminal end is typical of signal peptides with a hydrophobic core. Comparison of the *Pinus sylvestris* and *Pinus taeda* TLP sequences indicated that both ORFs encode a protein of 234 aa with only 7 aa changes between the two predicted proteins (in non-conserved positions). Also, both sequences have the five conserved amino acid residues forming the acidic cleft conferring antifungal activity. This conserved acidic cleft is comprised of five amino acids (R, E, D, D, D) and is believed to be involved in binding to β-1,3-glucan in the fungal cell wall, resulting in a targeted interaction between the host TLP and the fungal cell. This would lead to permeabilization of the fungal cell membrane and disruption of the osmotic balance inside hyphal cells, resulting in cell rupture (Osmond et al. 2001).

To verify the *P. sylvestris* mRNA and gene sequence and position of the intron, RNA and DNA were extracted from methyl jasmonate (MeJA) treated plants, as no expression of the *P. sylvestris* TLP gene in untreated plants was detected by agarose gel electrophoresis. Electrophoresis of PCR amplicons confirmed the presence of an intron as the fragment amplified from cDNA amplicons were shorter in length compared with the respective genomic DNA amplicons (Fig. 1). Sequencing of genomic DNA and cDNA was used to confirm the size of the intron in the *TLP* gene (145 bp) and to verify the predicted intron position. The splicing site was verified by sequencing of cDNA. Nucleotide sequence analysis of the TLP genomic DNA and cDNA identified an open reading frame (ORF) of 850 bp, which had a 5′GT and 3′AG splice junction, characteristic of eukaryotic introns.

Up to a 10-fold increase in TLP gene expression ($P < 0.05$) was observed in needles of seedlings after treatment with various MeJA concentrations (Fig. 2). Treatment with increasing MeJA concentrations significantly affected seedling growth and treatment with MeJA concentrations above 10 mM resulted in chlorotic or dead needles. After 3 weeks, all of the seedlings treated with 30, 50, and 100 mM MeJA concentrations had died. Inoculation with *H. annosum* also resulted in an increase in expression of the *TLP* gene in all genotypes ($P < 0.05$). Expression was compared at early stage of infection; at this time, strong expression of genes involved in cell defense and protein synthesis can be observed (Li and Asiegbu 2004). An increase in relative expression 7 days post inoculation was seen in all three analyzed Scots pine genotypes, ranging from a 1.4-fold increase in the genotype Sm3-II-1 up to a 6.5-fold increase in the genotype Sm9-III-2 (Fig. 3). Differences in expression levels between genotypes were also observed, similarly as for the MeJA-treated seedlings. The results indicate that expression of the *P. sylvestris* TLP gene is increased in response to both inoculation by a specific fungal pathogen (*H. annosum*), as well as in response to a more generalized induction of defense responses (MeJA treatment). A more detailed analysis using additional time points and control genes will enable a more precise characterization of responses to various pathogens and identification of variation in breeding material and natural populations. To study the effect of the *Pinus sylvestris* TLP on fungal growth, the coding sequence without the signal peptide sequence was cloned into *Escherichia coli*, and then subcloned into the expression vector pET100 using BL21 Star bacteria. Comparing soluble proteins extracted from induced and non-induced cells by SDS-PAGE, non-induced cells showed darker protein bands than induced cells. In addition, the recombinant TLP of the expected size was not visible in the cell lysates and the optical density of *E. coli* cells after 4 h induction was lower than that of non-induced cells, indicating that expression of the recombinant TLP was toxic to the *E. coli* cells (data not shown). Optimization of the expression of the recombinant *Ps*TLP was done by modifying the conditions (decreasing IPTG concentration, decreasing the induction temperature and/or adding 1% glucose). Glucose was added to the bacterial culture medium to repress basal expression of T7 RNA polymerase and to stabilize the construct. Decreasing the level of recombinant protein expression also decreases the toxicity to the bacterial cells. Initially, cells were induced with 1 mM IPTG at 37 °C at a higher OD_{600} (1.6 vs 0.6) and grown for 4 h (in medium containing 1% glucose). Therefore, there were more *E. coli* cells synthesizing the recombinant *Ps*TLP. A moderate amount of TLP was detected in induced cells with the plasmid/vector containing the TLP construct by SDS-PAGE. Also, the presence of a fragment of the same size as the expected TLP in the non-induced cultures indicated the possibility of some background protein expression.

Mohamed et al. (2011) reported that the maximum amount of soluble NP24I protein (belonging to the TLP family) was obtained at 0.05 mM IPTG at 25 °C; therefore, in an additional experiment, cells were incubated at 25 °C overnight using various concentrations of IPTG (0.05, 0.1, 0.5, 1 mM, no IPTG) without glucose. By decreasing the incubation temperature, higher amounts of *Ps*TLP were synthesized, indicating that the *Ps*TLP is not as toxic to the *E. coli* cells at lower levels

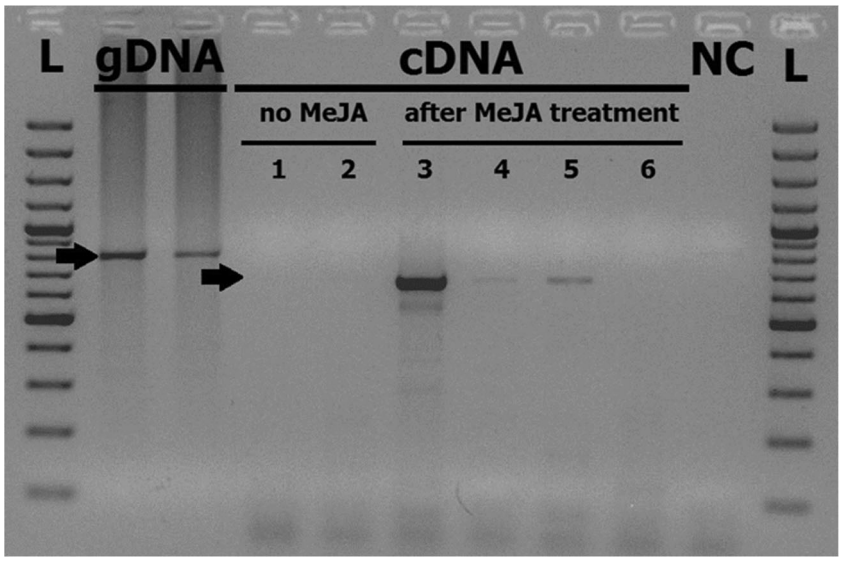


Fig. 1 Verification of intron in the *P. sylvestris TLP* gene. 1, 2—no MeJA treatment; 3—application of 5 mM MeJA; 4, 5—10 mM MeJa; 6—30 mM MeJA; NC—negative control of PCR, L—GeneRuler DNA ladder mix (Thermo Fisher Scientific). Arrows indicate *TLP* gene PCR products (amplicon length from genomic DNA is 818 bp and from cDNA—673 bp)

of expression. Different concentrations of IPTG had no significant effect on the amount of expressed recombinant *Ps*TLP protein.

Analyzing lysate samples by SDS-PAGE, nonspecific proteins of the same molecular weight as the expected *Ps*TLP or slightly larger were visualized (Fig. 4). However, protein size analysis was also performed with the 2100 Bioanalyzer and Protein 80 kit, which resulted in distinct protein bands compared to the SDS-PAGE analysis and no nonspecific proteins were detected. The observed doublets in the SDS-PAGE analysis were probably gel artifacts resulting from re-oxidization of the protein samples during the run or incomplete disulfide bond dissociation.

As a result of the optimization experiments, the most appropriate conditions for expression of the recombinant *Ps*TLP protein were determined to be induction with 1 mM IPTG at OD600 1.4–1.6 with 1% glucose and incubation at 25 °C overnight. The yield of recombinant protein was found to be 2.6 g/L.

To optimize protein purification under native conditions using Ni-NTA IMAC (QIAGEN), different concentrations of imidazole were investigated. Initially, the protein purification step was performed using the recommended concentration of imidazole (10 mM imidazole in the lysis buffer and 20 mM imidazole in the wash buffer) but SDS-PAGE analysis showed a high level of background proteins in almost all purification steps. Therefore, imidazole concentration was increased to 20 mM in the lysis buffer and 40 mM in the wash buffer to reduce nonspecific binding. Under these conditions, background proteins in eluates were reduced but the PsTLP was eluted in the flow through by the wash buffer, indicating that imidazole concentration in the lysis buffer was too high and TLP binding to the membrane was inhibited due to imidazole

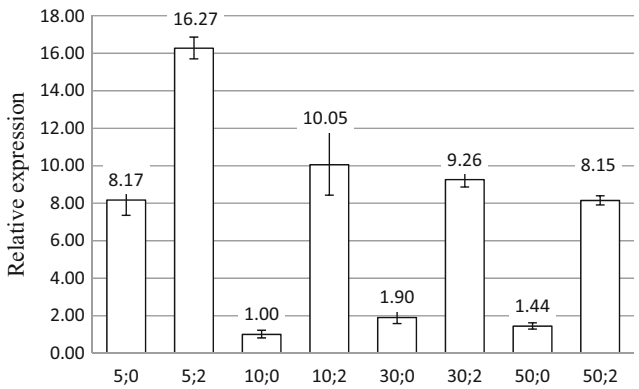


Fig. 2 Expression of the TLP gene in *P. sylvestris* seedlings. In the sample names, the first symbol indicates sample type (5; 10; 30; 50 = samples treated with indicated MeJA concentration) and symbol after comma indicates treatment stage (0—before treatment, 2—2 weeks after treatment). Real-time PCR analyses were performed in three technical replicates

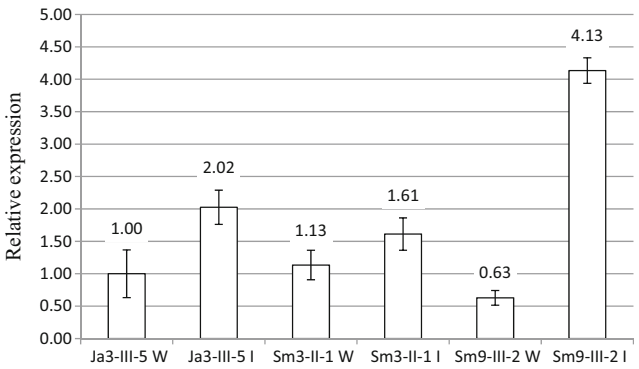


Fig. 3 Relative expression of the *TLP* gene in three Scots pine clones after inoculation with *H. annosum*. Expression was determined 7 d.p.i. W—wounded ramets, I—inoculated ramets. Ja3-III-5 W was used as the reference sample. Real-time PCR analyses were performed in three technical replicates

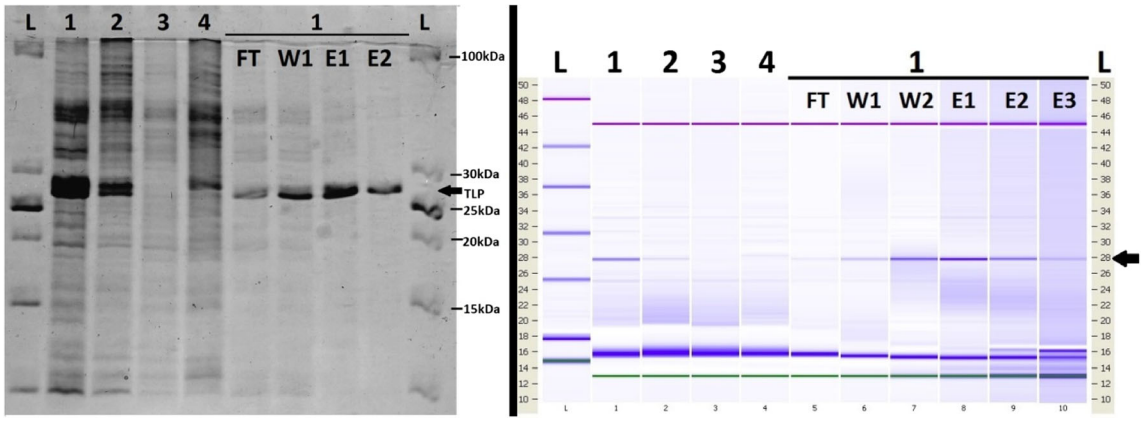


Fig. 4 Analysis of cell lysate aliquots in parallel using the 2100 Bioanalyzer and 12% tricine SDS-PAGE. 1—lysate from IPTG induced *E. coli* cells with TLP construct (with 1% glucose); 2—lysate from IPTG induced *E. coli* cells with TLP construct (without 1% glucose); 3—lysate from non-induced *E. coli* cells with TLP construct (with 1% glucose); 4—lysate from non-induced *E. coli* cells with TLP construct (without 1% glucose); L—protein ladder. Ni-NTA purification (sample No. 1): FT—flow through; W 1–2—wash; E 1–3—eluates. Arrow indicates TLP (expected size of TLP with the N-terminal His-tag was 27.61 kDa)

binding to the nickel ions and disruption of binding of histidine residues in the recombinant TLP. Subsequently, imidazole concentration was reduced to 1 mM in the lysis buffer and 40 mM in the wash buffer, which provided the best conditions for protein purification (Fig. 4). After optimization, there was still a significant amount of recombinant TLP detected in flow through and wash buffers.

In our experiments, enterokinase showed poor cleavage efficiency or no cleavage at all. Imidazole inhibits enterokinase activity so we attempted to remove it with Sephadex G-50 using Multiscreen Filter plates (Millipore). However, this did not successfully remove imidazole as enterokinase did not cleave the His-tag after purification (the molecular weight of the recombinant TLP did not change) (Fig. 5).

To test the inhibitory effect of the in vitro expressed *Ps*TLP on fungal growth, various amounts of the purified protein (20, 30, 50, 100, 200, 300 μg) were placed into wells on plates containing fungal cultures, and the inhibition zone surrounding the wells was measured (Table 1). After incubation for several days at 25 °C, inhibition zones were seen around the wells containing TLP. Differing degrees of inhibition of fungal growth were observed depending on the amount of protein applied (Fig. 6). No inhibition zones were observed around wells with Ni-NTA elution buffer. *S. brinkmannii*, *C. borealis*, and *R. bicolor* were most sensitive to TLP and were inhibited by 20 μg of TLP. *H. parviporum 17*, *G. sepiarium*, *A. areolatum*, *H. annosum VMa15*, *H. parviporum 162*, *S. sanguinolentum*, *B. adusta*, and *L. conigenum* were inhibited by 30 μg TLP. *H. annosum 50*, *Trichoderma* sp., and *L. seditiosum* were inhibited only by 100 μg of TLP. The longest inhibition time was detected in *R. bicolor*, *G. sepiarium*, *H. parviporum*, *H. annosum*, and *L. seditiosum*. There was less inhibitory activity of faster growing fungi such as *B. adusta* and *Trichoderma* sp.

Discussion

The *P. sylvestris* TLP gene characterized in this study is most similar to the *P. taeda* TLP gene (99% sequence similarity). With regard to other characterized and published *Pinus* TLP family genes, these two genes are most similar to the *P. monticola* TLP genes *-L6*, *-L2*, and *-L5* (82, 82, and 80% sequence identity, respectively). A partial TLP gene sequence from *Pseudotsuga menziesii* (accession no. AJ131731) has 86% sequence similarity. In addition, there are a number of unannotated expressed sequences from *Picea glauca* and *Picea sitchensis* with a sequence similarity of over 80%.

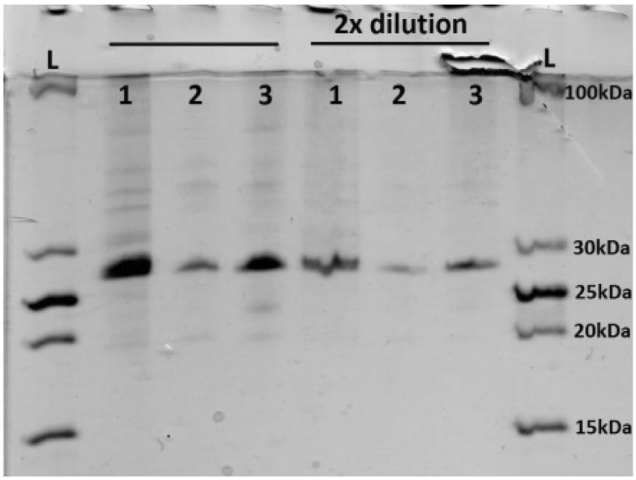


Fig. 5 Purification of Ni-NTA eluted TLP with Sephadex G-50 to remove imidazole: 1—TLP without enterokinase cleavage; 2—imidazole removal by Sephadex G-50 and TLP cleavage by enterokinase for 24 h; 3—TLP incubated with enterokinase for 24 h. Size of TLP with the N-terminal tag is 27.61 kDa. Predicted TLP size after enterokinase cleavage is 24.61 kDa

Table 1 In vitro antifungal activity of the purified *P. sylvestris* TLP against various fungal species

	Concentration of in vitro expressed TLP					
	20 μg	30 μg	50 μg	100 μg	200 μg	300 μg
Amylostereum areolatum	–	–	+	+	++	+++
Sistotrema brinkmannii	+	+	++	++	++	+++
Bjerkandera adusta[a]	–	–	+	++	+++	+++
Resinicium bicolor	+	+	++	++	++	+++
Climacocystis borealis	+	+	+	+	+	++
Gloeophyllum sepiarium	–	+	++	++	+++	+++
Stereum sanguinolentum	–	–	+	+	++	+++
Heterobasidion annosum 50	–	–	–	+	++	+++
Heterobasidion annosum V Ma15	–	–	+	+	++	+++
Heterobasidion parviporum 17	–	+	++	+++	+++	+++
Heterobasidion parviporum 162	–	–	+	+	++	+++
Lophodermium seditiosum[b]	–	–	–	+	+	++
Lophodermium conigenum[b]	–	–	+	++	++	+++
Trichoderma sp.[a]	–	–	–	+	++	+++

Inhibition of mycelial growth was recorded after 48 h of incubation at 25 °C or when mycelium reached the edge of control wells. Data are presented as the mean of three independent experiments. Mycelial growth inhibition was scored as the following: − no inhibition of mycelial growth; + inhibition zone width 1–2 mm; ++ inhibition zone 2–3 mm; +++ inhibition zone > 3 mm

[a] Fast growing fungi

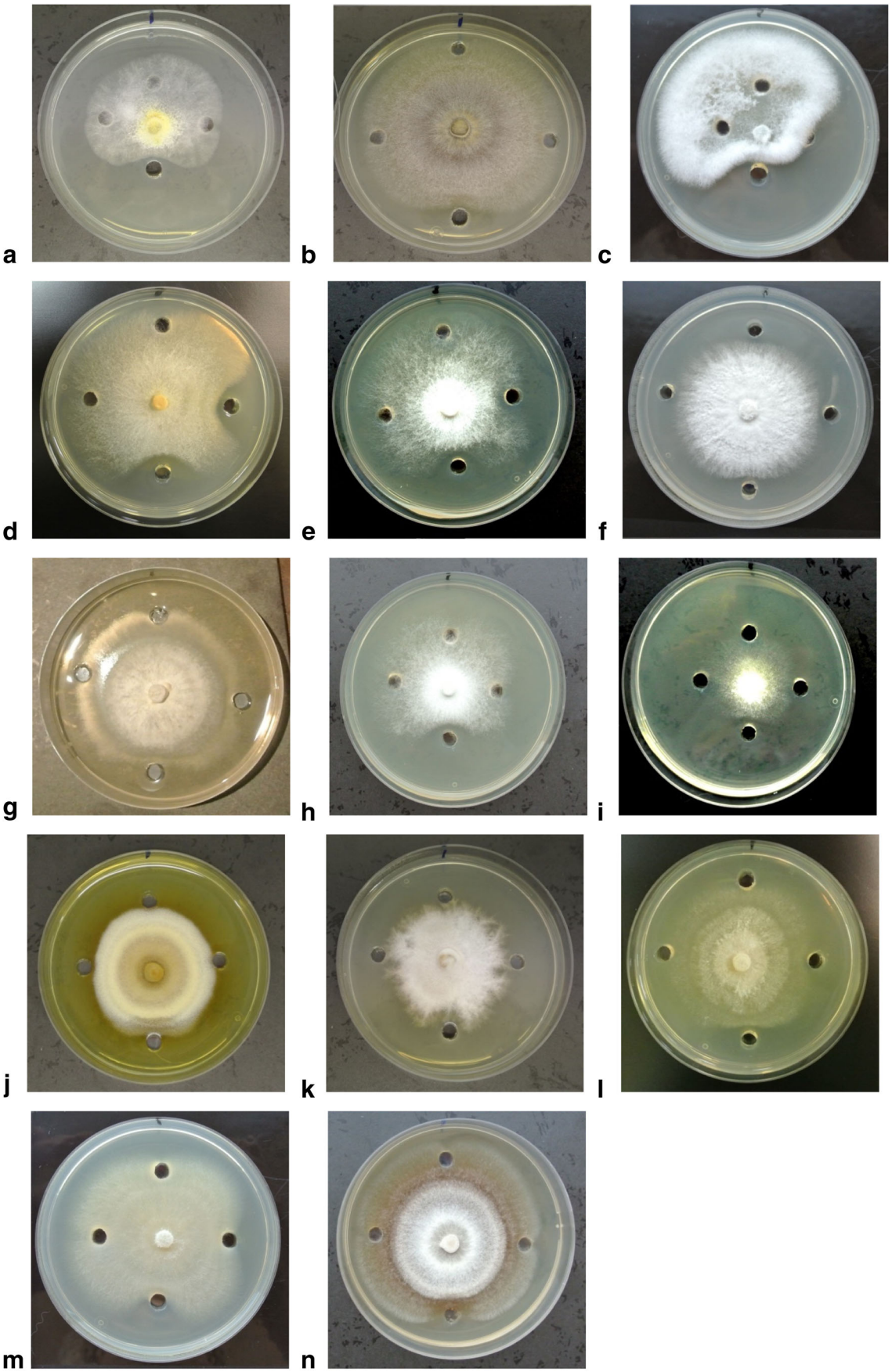
[b] Slowly growing fungi

Exogenous applications of MeJA and inoculation with *H. annosum* increase TLP gene expression in *P. sylvestris* seedlings but transcript accumulation levels differed between individuals, which could be due to differential stresses or conditions in the seedling nursery, or the differing genetic background of the utilized plant material, e.g., having differing copy numbers of the *TLP* gene (Šķipars et al. 2011; Šķipars et al. 2017). According to molecular mass, TLPs in *P. monticola* have been categorized into small (S-) type and large type (L-) TLPs, with varying levels of transcript accumulation after fungal (*Cronartium ribicola*) infection (Liu et al. 2010a). Sequence analysis of the predicted *Pinus sylvestris* TLP indicated that it belongs to L-type TLPs and contained the sequence features common with other TLPs with antifungal activity. The *P. sylvestris* TLP sequence had the five conserved amino acid residues (R, E, D, D, D) forming the acidic cleft that is believed to be involved in binding to β-1,3-glucan in the fungal cell wall, resulting in a targeted interaction between the host TLP and the fungal cell, leading to permeabilization of the fungal cell membrane and disruption of the osmotic balance inside hyphal cells (Osmond et al. 2001).

Undoubtedly, additional TLP gene family members will be identified and fully characterized in other *Pinus* species as a result of further studies and improved gene annotation and sequence coverage. The three *P. monticola* genes most similar to the *P. sylvestris* TLP gene discussed previously have pairwise sequence similarities of over 90%. TLP gene copy number polymorphism has been reported (Šķipars et al. 2017), and differences in copy numbers were detected when analyzing the 5′, central and 3′ regions of the gene. One possibility is that these discrepancies between these gene regions reflect differences between closely related gene family members. Additional sequencing of full-length genes in *Pinus sylvestris* will enable better characterization of the TLP gene family.

TLPs form large gene families, and they are involved in resistance and a wide range of developmental processes in fungi, plants, and animals. Under un-induced conditions, TLPs are expressed at low levels in certain organs at specific developmental stages (Futamura et al. 2006; Dafoe et al. 2010; Liu et al. 2010a) and are particularly abundant in the leaves, where they can amount to 5–10% of total leaf proteins (van Loon 1999), for example, TLP is found in large quantities in sorghum leaves (Chou and Huang 2010). Many PR-5 group proteins are up-regulated only in response to abiotic and biotic stimuli (Hu and Reddy 1997; Piggott et al. 2004; Futamura et al. 2006; Joosen et al. 2006; Deihimi et al. 2013). MeJA treatment has been utilized to induce defense responses in conifers, and responses over a range of time periods have been reported, ranging from a few hours to several weeks (Heijari et al. 2005;

Fig. 6 Inhibition of fungi growth by recombinant *TLP*. **a** *Trichoderma sp.*, **b** *A. areolatum*, **c** *B. adusta*, **d** *H. parviporum 17*, **e** *H. parviporum 162*, **f** *C. borealis*, **g** *G. sepiarium*, **h** *H. annosum 50*, **i** *H. annosum V Ma15*, **j** *L. seditiosum*, **k** *L. conigenum*, **l** *R. bicolor*, **m** *S. brinkmannii*, **n** *S. sanguinolentum*. Clockwise from top: 20 μg; 50 μg; 300 μg of protein; control (Ni-NTA elution buffer)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Heijari et al. 2008; Moreira et al. 2009; Piggott et al. 2004). Synthesis of mono-and sesquiterpenes and pinenes peaks approximately 7–15 days after treatment but volatile terpenes in needles are produced just a few hours after treatment (Martin et al. 2002; Martin et al. 2003; Miller et al. 2005; Gould et al. 2009). In *Pinus monticola*, TLP accumulation started in the wounded needles after 4 days (Piggott et al. 2004). Seasonal regulation has been observed for some *P. monticola* TLP genes, for example, TLP-L3 transcript levels increase in shoots and stems during winter, while both TLP-L2 and -S2 transcripts were up-regulated in needles but TLP-S2 transcripts were down-regulated in other organs (shoots, stems, and roots) during winter (Liu et al. 2010a). Expression of TLP-L2, -L4, -L6, and -S1 was inhibited during winter. Expression of the *P. sylvestris* gene is induced in stems and needles in response to inoculation with *H. annosum* and MeJA treatment, in contrast to some of the *P. monticola* TLP genes (Liu et al. 2010a). The results from this study indicate that the induction of expression is relatively long term (1–2 weeks after treatment). A more detailed analysis of tissue and temporal specificity should be undertaken in *P. sylvestris*, in conjunction with a comprehensive analysis of the TLP gene family. This will enable a better characterization of germplasm in the Latvian Scots pine breeding program. The *P. sylvestris* TLP that was expressed in vitro was active against a range of forest fungal species, confirming the prediction from the analysis of conserved sequence motifs.

Expressing recombinant TLP, we found that expression of the recombinant TLP was toxic to the *E. coli* cells (data not shown). Similar results were reported for the in vitro expression in BL21 cells of the soluble NP24I protein (belonging to the TLP family) from tomato fruit (Mohamed et al. 2011) and the TLP-1, TLP-3 proteins from *Arabidopsis* (Hu and Reddy 1997), where the recombinant proteins were obtained in an insoluble form as inclusion bodies. Overexpression of proteins that are toxic to the host cells can be difficult. Cell lysis and purification of protein from whole cell lysates can be complicated; problems may arise because of aggregation or degradation of proteins within the cells. Aggregation of recombinant proteins is probably due to a limiting amount of chaperones at high levels of recombinant gene expression (Thomas and Baneyx 1996). The aggregation reaction is in general favored at higher temperatures. A direct consequence of temperature reduction is the partial elimination of heat shock proteases that are induced under overexpression conditions. Also several environmental conditions such as high temperatures and media acidification stimulate inclusion body formation (Strandberg and Enfors 1991).

Recombinant His-tagged proteins expressed in *E. coli* and purified by IMAC are commonly co-eluted with native *E. coli* proteins, especially if the recombinant protein is expressed at a low level and/or in stress conditions. IMAC is very sensitive to the presence of metal chelators, and the *E. coli* lysate contains many unspecific weak chelators such as dicarboxyl acids from the citric acid cycle. Under stress conditions, *E. coli* can also produce highly specific metal chelators, metallophores (Neilands 1995; Magnusdottir et al. 2009). Bolanos-Garcia and Davies (2006) described 17 *E. coli* IMAC contaminants (*E. coli* host proteins that routinely co-purified during IMAC procedures and display high affinity to divalent metal binding sites), 15 were reported to elute from Ni-NTA at an imidazole concentration of 55 mM, a concentration which is higher than advised for most IMAC column washing procedures. Thus, most of these proteins are eluted only when the imidazole concentration is increased to a level that elutes the histidine-tagged protein of interest. As described before, recombinant TLP expression in BL21 cells was toxic to bacterial cells, and therefore, the cells were growing in high stress conditions, which may cause elution of non-target proteins in all purification steps. Magnusdottir et al. (2009) recommends removing periplasmatic material with osmotic shock treatment before cell lysis, suggesting that low-molecular weight components of *E. coli* cell lysates such as siderophores that reduce the binding capacity of IMAC columns are associated with the periplasm.

Because it is difficult to predict the effect that a His-tag may have on the activity of a given protein, the tag can be removed by enterokinase. Site-specific proteases that cut within the recognition sequences leave extra amino acids in the cleaved peptide products, but digestion with enterokinase produces a 100% native protein sequence and structure from recombinant protein. The expressed recombinant TLP with N-terminal tag containing a 6× His-tag was purified using Ni-NTA and imidazole. However, in our experiments, enterokinase showed poor cleavage efficiency or no cleavage at all. A disadvantage of using imidazole is that it inhibits enterokinase activity and the presence of imidazole often results in protein aggregates (Hefti et al. 2001). Addition of imidazole adds more salts (the storage buffer contains 200 mM NaCl), resulting in protein precipitation. An excess amount of imidazole (> 50 mM) and/or NaCl (> 250 mM) reversibly interferes with and inhibits enterokinase activity (Lu et al. 1999), and the Ni-NTA elution buffer contains 250 mM imidazole and 300 mM NaCl. To avoid this problem, imidazole and NaCl need to be removed from the eluted protein through buffer exchange, such as dialysis, ultrafiltration, or size-exclusion chromatography. We attempted to remove imidazole with Sephadex G-50 using Multiscreen Filter plates (Millipore); however, this did not successfully remove imidazole as enterokinase did not cleave the His-tag after purification. This might be due to the amount of Sephadex utilized for purification; we used plates while previous reports utilized columns (Wang et al. 2008). Therefore, for further antimicrobial activity assays, the recombinant TLP with the 6× His-tag was used. A similar approach was described with recombinant TLP from tomato fruit (Mohamed et al. 2011). It cannot be excluded that the affinity

tag may interfere with protein activity (Wu and Filutowicz 1999), but the relatively small size and charge of the 6× His-tag rarely influences protein activity (Carson et al. 2007). The initial problems with protein expression using *E. coli* cells (a large amount of expressed recombinant protein with the 6× His-tag was toxic for *E. coli*) support the fact that the expressed TLP with the 6× His-tag has antimicrobial activity. Optimization of the 6× His-tag cleavage protocols will allow more precise determination of the effect (if any) of the tag on the antimicrobial activity of the expressed TLP.

Most of the PR proteins have wide spectrum antifungal activities, suggesting that target recognition may be determined by their interaction with pathogen cell surface components. In this study, approx. 20–100 μg of TLP were required to inhibit fungal growth. Hu and Reddy (1997) reported that a TLP from Arabidopsis (ATLP-3) showed antifungal activity against *Candida albicans* even at a concentration of only 5 μg of protein (while 2 μg of protein did not inhibit mycelial growth). Similar results were reported for an in vitro synthesized rice TLP that inhibited the mycelial growth of *Fusarium oxysporum f. sp. cubense*, *F. graminearum*, *Botrytis cinerea*, *Drechslera oryzae*, *Sarocladium oryzae*, *Curvularia lunata*, *Rhizoctonia solani* at 5 μg of protein per disc (Jayaraj et al. 2004). These differences in inhibition threshold could be due to differences in experimental methodology or to differing intrinsic activity of the expressed TLPs from different species, for example, TLP isolated from *Cassia didymobotrya* (Fres.) demonstrated varying antimicrobial activity against different *Candida* species (Vitali et al. 2006). These differences in inhibitory activity may be due to differences in fine structural features of the (1,3)-ß-D-glucans involved in recognition, for example, if different fungi produce cell wall ß-1,3-glucans with different degrees of ß-1,6-glucosyl substitution or with different degrees of branching, the plant might have coevolved different PR5 proteins capable of binding the evolving fungal cell wall (1,3)-ß-D-glucans (Osmond et al. 2001). Some reports have suggested that the antifungal properties of TLPs may be also attributed to the inhibition of fungal enzymes such as ß-glucanase, α-amylase, xylanase, and trypsin (Grenier et al. 1999; Schimoler-O'Rourke et al. 2001; Fierens et al. 2007), or that TLP may act by inducing programmed cell death in fungi. Osmotin is a PR-5 protein that binds to the plasma membrane receptor proteins and induces programmed cell death in *Saccharomycetes cerevisiae* by signaling suppression of cellular stress responses via RAS2/cAMP. It is a homolog of mammalian adiponectin and controls apoptosis in yeast through a homolog of mammalian adiponectin receptor (Narasimhan et al. 2005).

As use of fungicides in forests is forbidden and TLP has antifungal activity against wide range of fungi, it could protect seedlings against various fungal pathogens and could be used in disease control of forest trees. However, the practical and economic feasibility of using this as an antimicrobial treatment have not been examined, particularly considering the broad range of fungal species whose growth is inhibited. However, the exogenous application of MeJA can also induce chemical and anatomical changes, including induction of expression of the TLP gene, that lead to a reduction of disease incidence in plants. In addition, inoculation with the root rot fungus *H. annosum* also induced expression of this gene. The recombinant TLP demonstrated antimicrobial activity against 12 fungal species commonly found in Latvian forest ecosystems. The obtained results (antifungal activity of the protein and increase of gene expression after stress) concur with a proposed role for TLP in *P. sylvestris* resistance against fungal pathogens, which can be further investigated for tree breeding for increased resistance and the induction of resistance mechanisms in seedlings.

Data archiving statement The DNA sequence of the *P. sylvestris* TLP gene has been deposited in GenBank (Acc. No. JX461338).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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