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RESEARCH ARTICLE

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Expression on wood, molecular cloning and characterization of three lignin peroxidase (LiP) encoding genes of the white rot fungus *Phlebia radiata*

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Abstract Lignin peroxidase (LiP) is the first enzyme connected to oxidative breakdown of the aromatic plant heteropolymer lignin and related xenobiotics. However, this extracellular enzyme has been described in only a few species of wood-decaying basidiomycetous fungi. The white rot basidiomycete *Phlebia radiata* 79 readily produces a versatile set of lignin-oxidizing enzymes including lignin and manganese peroxidases (LiPs and MnPs) and laccases. Here we describe genomic and primary structure of two new LiP-encoding genes, *Pr-lip1* and *Pr-lip4*, and genomic characterization for isozyme LiP3/LIII of *P. radiata*, encoded by the gene depicted *Pr-lip3*. *Pr-lip1* and *Pr-lip4* code for 370- and 361-amino-acid long proteins beginning with 26- and 24-amino-acid secretion pre-propeptides, respectively. Translated LiP1 and LiP4 share the highest protein sequence identity (74 and 86%) with *P. radiata* LiP3, and 70% identity with the one deduced LiP from *Bjerkandera adusta*. The three *P. radiata* LiP sequences form a coherent phylogenetic cluster, which is further supported by similarities within gene organization interrupted by 11-introns. To find out the significance of LiP upon fungal growth on natural lignocellulose, such as wood, we studied ligninolytic gene expression on hardwood (milled alder) and softwood (spruce chips). All the LiP-encoding genes were expressed on wood with predominance of *Pr-lip3* transcript abundance, in particular on spruce wood chips, where also time-dependent expression of the multiple *lip* genes was observed.

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Introduction

Lignin peroxidase (LiP; EC 1.11.1.14, diarylpropane peroxidase) is an extracellular oxidative fungal enzyme, first found in cultures of *Phanerochaete chrysosporium*, and connected to direct degradation of the plant polymer lignin and lignin-like aromatic model compounds (Tien and Kirk 1984; Renganathan et al. 1985; Paszczynski et al. 1986). Catalytic activity of LiP leads to aromatic ring oxidation and cleavage, and C–C bond disruption within dimeric model compounds (Kirk and Farrell 1987; Gold et al. 1989; Lundell et al. 1993). The crystal structure of native LiP shows a globular α-helical fold with the heme (protoporphyrin IX) prosthetic group embedded between two domains of the monomer including at least one glycosyl unit (Piontek et al. 1993; Poulos et al. 1993).

Lignin peroxidase performes the typical peroxidase catalytic cycle with three enzyme redox-intermediate stages (Gold et al. 1989; Lundell et al. 1993). The enzyme shows remarkably high oxidation capacity for aromatic non-phenolic reductants (Schoemaker et al. 1994). Site-directed mutagenesis on a solvent-exposed tryptophan (W171 in LiP-H8 of *P. chrysosporium*) has revealed the crucial role of this amino-acid residue in oxidation of the preferred aromatic substrate compound veratryl (3,4-dimethoxybenzyl) alcohol, which is a natural substrate for LiP (Blodig et al. 2001). Enzyme kinetic studies suggest, however, another binding site at the surface of the enzyme for long-range electron transfer from larger substrates such as dye-compounds (Doyle et al. 1998) and synthetic lignin (DHP) (Johjima et al. 1999).

Manganese peroxidase (MnP; EC 1.11.1.13) is structurally similar to LiP (Gold et al. 2000; Martínez 2002) and produced by almost all genera of lignin-degrading basidiomycetes (Hatakka 1994; Hofrichter

2002; Martínez 2002). On the contrary, active LiP isozymes have been described for only a few, wood–lignin degrading white rot fungi, that is in *Phanerochaete chrysosporium*, *Phlebia radiata*, *Merulius (Phlebia) tremellosus*, *Trametes (Coriolous) versicolor* and *Bjerkandera adusta* (Hatakka 1994; Martínez 2002). Potential *lip* genes were demonstrated in *Phanerochaete sordida* and *Ceriporiopsis subvermispora* but no LiP-activity was detected in the culture fluid of the latter fungus (Rajakumar et al. 1996).

Current information on the total number of peroxidase genes in *P. chrysosporium* is ten *lip* and five *mnp*, with one additional, at deduced protein sequence level different (hybrid-type) peroxidase gene (Martinez et al. 2004). The additional peroxidase is not homologous to either LiP or MnP or the so called versatile peroxidase (VP) (A. T. Martínez, personal communication). VP shares catalytic properties with MnP while structurally obtaining more similarity with LiP. The versatile peroxidase was first characterized for *Pleurotus eryngii* (Martínez 2002).

Due to the low degree of enzyme secretion and difficulties in the isolation from lignocellulosic substrates, in particular from wood, only in a few cases has it been possible to indicate, which ligninolytic enzymes are expressed on these substrates. Datta et al. cultivated *P. chrysosporium* on softwood pulp and were able to isolate MnP but no LiP (Datta et al. 1991). Later, it was observed that *lip* genes are also transcribed when the fungus is grown on spruce wood chips (Janse et al. 1998). Recently, proteomic analysis revealed that predominantly cellulolytic and hemicellulolytic enzymes, and two LiP isozymes but no MnP were detected when *P. chrysosporium* was cultivated on red oak wood chips (Abbas et al. 2005).

In order to clarify the significance of LiP in lignin biodegradation on natural lignocellulose substrates, we studied gene expression of the diverse ligninolytic peroxidases produced by *Phlebia radiata* on wood and in liquid cultures. Active isoforms of LiP, MnP and laccase were previously identified from wheat straw cultures of the fungus (Vares et al. 1995). *P. radiata* is a well-studied, lignin-degrading basidiomycete (Hatakka et al. 1983, 1991) naturally causing white rot in hardwood and softwood. *P. radiata* and *P. chrysosporium* are taxonomically related corticioid fungi (Dresler-Nurmi et al. 1999) and show similarities in degradation of synthetic lignin and lignin model compounds (Lundell et al. 1990; Hatakka et al. 1991; Moilanen et al. 1996). However, physiological properties such as hyphal growth pattern in wood, morphology, growth temperature range (Hatakka et al. 1983; Hakala et al. 2004), and secretion of laccase (EC 1.10.3.2) (Lundell et al. 1990; Niku-Paavola et al. 1990) differentiate *P. radiata* clearly from *P. chrysosporium*.

Transcripts of two divergent MnPs are present in *P. radiata* cultures supplemented with milled hardwood (Hildén et al. 2005). In our present study, two new lignin peroxidase genes and their phylogeny are described, and active transcription of three different *lip* genes of *P. radiata* is demonstrated when the fungus is cultivated on spruce wood chips. Our results are consistent with the latest findings on lignocellulose substrates and strongly point to the significance of LiP for fungal colonization of wood and breakdown of wood lignin.

Materials and methods

Fungal cultures and LiP activity

Phlebia radiata strain 79 (ATCC 64658) was maintained on malt-extract agar plates, and grown in 50 ml of malt extract (2% wt/vol) medium, or in 75 ml of low-nitrogen asparagine-succinate medium (LN-AS), pH 4.5 (Moilanen et al. 1996), supplemented with either 2 g (dw) fine milled alder (*Alnus incana*) wood (semisolid MAW cultures) or 0.5% (wt/vol) glucose (liquid cultures) as carbon source. The stationary flask cultures were incubated in the dark at +28°C for 4–13 days. *P. radiata* 79 was also cultivated in 100 ml conical flasks containing 2 g (dw) of Norway spruce (*Picea abies*) wood sticks of approx. 2.5 cm×0.3 cm×0.3 cm in size, on water-agar (1% wt/vol agar–agar, Biokar) (solid SWC cultures). The flasks were inoculated with 2 ml of mycelial suspension obtained from 7-day malt extract medium cultures and incubated at +28°C for 14–21 days. LiP activity was determined at pH 3.0 by using the veratryl alcohol oxidation method in the presence of H_2O_2 (Tien and Kirk 1984).

Extraction of RNA and DNA

The mycelial mats from liquid LN-AS and MAW cultures were harvested, frozen to −80°C and homogenized in liquid nitrogen with mortar and pestle. Frozen wood sticks from SWC cultures were milled in liquid nitrogen using Polymix Analysenmühle A10 Kinematica. Total RNA was extracted with CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) buffer, purified with chloroform:isoamylalcohol (24:1) and precipitated with LiCl (Chang et al. 1993). DNA was removed by RNase-free DNaseI (Invitrogen). Further purification of total RNA was performed with RNeasy Plant kit (Qiagen). After spectrophotometric quantitation at 260 nm, the RNA was used for cDNA synthesis. DNA was isolated from homogenized mycelium with CTAB-containing buffer and purified (Hildén et al. 2005). DNA and RNA were resuspended in nuclease-free water, and stored at -20°C.

RT- and RACE- (Rapid Amplification of cDNA Ends) PCR

cDNA synthesis, the following RT-PCR and RACE-PCR were performed as described in Hildén et al. (2005). A 168-bp (base pair) fragment of *P. radiata lip4* cDNA was amplified with degenerative primers (sense: 5′- GT/

CC TT/CG TC/G/TC CC/A/GG AGC CA/C/GT TCC-3′ (Rajakumar et al. 1996) and antisense: 5′-GGG AGT NGA GTC GAA GGG-3′) designed for LiP-encoding target cDNA. Amplified fragments were then A-tailed with 1.25 U Taq DNA polymerase (Invitrogen) at 72°C for 15 min for T/A cloning. To amplify the 5′ and 3′ cDNA ends of the transcript of *lip4*, we performed RT reaction with 1 μg of purified total RNA using the Smart® RACE cDNA Amplification kit (Clontech) (Hildén et al. 2005). Primary amplification was performed with the universal primer mix (UPM) and the *lip* antisense primer described above. An aliquot of the primary 5′-RACE-PCR was reamplified using the nested universal primer and inner *lip*-transcript specific antisense primer (5′-AAA CAG TCT CGA GCT CAT CGA AT-3′). The 3′-RACE products were amplified with primers designed according to the cloned and sequenced 5′-RACE fragments (*lip1* sense: 5′-CTT GAG GCT CAG GGT AAA TTC G-3′ and *lip4* sense: 5′-CAT GAT GTC GAC ACG ATC CTT-3′) and the UPM as antisense primer. The respective nested sense primers (5′-CAT TGT CAT CTT CTC CGA TGT C-3′ for *lip1* and 5′-CTC GAG ACT GTT TGG TTC TTG AT-3′ for *lip4*) and nested universal primer were used in the second round of PCR.

Cloning, sequencing and transcript size analysis

Full-length cDNA and genomic clones of the *Pr-lip* genes were amplified with gene-specific primers designed according to the nucleotide sequence data obtained applying RACE-PCR and the published cDNA sequence of *P. radiata lip3 (lgp3)* (Saloheimo et al. 1989). PCR was performed and the amplification products of correct size were purified, subcloned into pCR2.1TOPO (Invitrogen) and sequenced using ABI Prism 310 DNA Analyzer (Applied Biosystems) as described before (Hildén et al. 2005). For Northern blot analysis, glyoxylated mRNA (3 μg) or total RNA (10 μg) was size-fractioned by 1.5% agarose gel electrophoresis and blotted to Hybond-N^+ membranes (Amersham International) that were treated with *lip1* and *lip3* cDNA-amplified [α-^{32}P]-labeled probes and analyzed as described in Hildén et al. (2005).

RT-PCR with *Pr-gpd*

To determine the relative abundance of *Pr-lip* and *Pr-gpd* transcripts in liquid cultures, cDNA-specific primers designed for each transcript (according to coding sequence) were used in RT-PCR (Table 1). The 25-μl PCR mixture contained 0.5 μl of the cDNA as template (corresponding 0.04 μg of total RNA), 0.3 mM dNTP mixture (Fermentas), 0.4 μM of 5′ sense and 3′ antisense primers, 1 × amplification buffer and 1 U DynazymeII DNA polymerase (Finnzymes). Initial denaturation occurred at 94°C for 30 s, followed by 35 cycles performed as follows: denaturation at 98°C for 10 s, annealing at 57°C for 30 s, elongation at 72°C for 15 s, and 10 min final extension at 72°C. The PCR products were electrophoresed on 1% agarose gel, then visualized with ethidium bromide under UV-light. Gel image files were acquired with Kodak EDAS 290 digital camera and analyzed with Kodak 1D Image Analysis software.

Competitive RT-PCR

Competitive RT-PCR was performed to study the transcript levels of *Pr-lip1*, *lip3* and *lip4* when the fungus was growing on spruce wood (SWC cultures). Transcript-specific primers for *Pr-lip* and *Pr-gpd* genes were designed according to the nucleotide sequences of full-length cDNA and gDNA (genomic DNA) clones. The 25-μl PCR reactions contained 0.5 μl of the cDNA template, 0.3 mM dNTP mixture, 0.4 μM of 5′ and 3′ primers, 1 × PCR amplification buffer and 1 U DynazymeII DNA polymerase (Finnzymes). Full-length genomic clones of *Pr-lip1*, *lip3* and *lip4* in pCR2.1TOPO were used as competitive templates in dilution series of known concentrations (0.1–15 pg) of the plasmid in the appropriate amplification reaction mixture. Initial denaturation occurred at 94°C for 30 s, followed by 25 cycles performed as follows: denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and elongation at 72°C for 15 s, followed by a 10 min final extension at 72°C. The amplification products were run on 2% agarose gels, visualized and analyzed as described above. Introns within the competitive genomic amplicons gave longer PCR products resulting with shorter migration lengths on the agarose gel, thus allowing their separation from the PCR products of the cDNA (transcript) amplicons. Amount of each transcript was estimated by determining concentration of the plasmid containing the competitive gDNA amplicon at the point of equivalent intensities of the gDNA and cDNA PCR products (Gilliland et al. 1990). The identities of the PCR products were confirmed by sequencing.

Sequence accessions

Nucleotide sequence accessions for the *P. radiata* genes *Pr-lip1*, *Pr-lip3* and *Pr-lip4* are AY743218, AY749105 and AY745250, respectively, cloned and described in this work. Uniprot translated sequence accessions retrieved by SRS (www.ebi.ac.uk) are as follows: *Phanerochaete chrysosporium* LIG3 (P21764), LIGH8 (P06181), LIG1 (Q01775), LIG2 (P49012), LIGA (P31837), LIG6 (P50622), LIG5 (P11543), LiPJ (Q9UW80), LiPH2 (P11542); *Bjerkandera adusta* LiP (E51135); *Phlebia radiata* LiP3 (P20010), MNP3 (Q96TS6); *Trametes versicolor* LPGVI (Q99057), LPGIII (Q12435), LPGI (Q7LHY3), LPGIV (CAA83228, allele to LPGI), PGV (CAA54398), MnP2 (Q99060), MPGI (Q99058); *Bjerkandera sp* RBPa (Q874A6).

Table 1 Oligonucleotides used as primers for competitive RT-PCR

Gene	5′ Primer (5′-3′)	3′ Primer (5′-3′)
Pr-lip1	CCCCCACTCACTACCACACCT	CGTTCCGAACCGGAGAACT
Pr-lip3	CTTGATCGACTGCTCTGACG	CAGGAAAACAGAGCGCAATC
Pr-lip4	CTCGGATGTTCTGCCGACTC	TCAGGCCAGACCTTATACTCAG
Pr-gpd	GAGTCCACCGGTGTCTTCAC	AAGTTGTCGTGGATGACCTTG

Results

Amplification and cloning of LiP-encoding cDNAs and genes

RT-PCR-based strategy was used to clone and identify new LiP-isozyme encoding genes expressed in the mycelium of *P. radiata* upon growth in the LN-AS-liquid medium supplemented with glucose. A 168-bp amplification product was obtained with the LiP-degenerative oligonucleotide primers. Subcloning and sequencing pointed to three distinct gene products, two of which were different from the previously cloned cDNA of *Pr-lip3*/*lgp3*, and were thereby designated as *Pr-lip1* and *Pr-lip4* corresponding to distinct isozymes, LiP1 and LiP4, respectively. The lacking 5′ and 3′ ends of the open reading frame were amplified by applying RACE-PCR strategy. Full-length coding sequence of *Pr-lip1* and *Pr-lip4* resulted in cloning the 1,107 and 1,083-bp fragments, respectively. A single transcript of ca. 1.4 kb in length was detected with Northern hybridization for each of the *lip* gene (data not shown) corresponding to the sizes of the cloned full-length cDNA fragments. With *P. radiata* DNA as target in PCR, the *lip1*, *lip3* and *lip4* genes were then amplified from start to stop codon resulting in 1,708, 1,681 and 1,659-bp PCR products that were subcloned and sequenced.

Characterization of the *Pr-lip* genes

The exon sequences in the genomic clones were identical to the coding sequences of the cDNA clones of *Pr-lip1*, *Pr-lip3* and *Pr-lip4*, and each *lip* gene was interrupted by 11 introns (48–61 bp in length) with similar positioning (Fig. 1). Within the three *lip* genes, introns 1 and 8 were differentially positioned in *lip1* whereas all the introns within *lip3* and *lip4* were at identical positions with only small variations in length.

Primary structure of the LiP isozymes

Predicted from the full-length cDNA clones, the *Pr-lip1* and *lip4* genes code for 370-aa (amino acid) and 361-aa long polypeptides, respectively (Fig. 2). The amino-terminae in the translated LiP1 and LiP4 begin with secretion signal leader peptide, in accordance with the previously characterized LiP3 (LIII) isozyme of *P. radiata* (Saloheimo et al. 1989). Prediction of the signal peptide cleavage site with SignalP (V2.0, www.cbs.dtu.dk) suggested that the Pr-LiP proteins are synthesized as pre-proenzymes. LiP1 and LiP4 are preceded by 21 and 18-aa secretion signal peptides, respectively, which is followed by an intermediate 5–6-aa propeptide including dibasic motif (K/RR) for cleavage. The amino-acid residues essentially involved in heme peroxidase structure and catalysis are all conserved in Pr-LiP1 and LiP4, the most important of which being the proximal histidine (H203, H199, respectively), the distal histidine (H74, H70) and arginine (R70, R66) (Fig. 2). The protein surface-exposed tryptophan (W171 in *P. chrysosporium* LiPH8) found in LiPs and VPs (Martínez 2002) and involved in veratryl alcohol and aromatic substrate oxidation (Blodig et al. 2001; Mester et al. 2001) is also present in Pr-LiP1, LiP3 and LiP4. Only one potential *N*-glycosylation site with the aa-sequence *N*-X-S/T is obviously found in Pr-LiP1 whereas in Pr-LiP4, two N-glycosylation sites may be recognized. The latter is seen at similar location (N268) both in Pr-LiP3 and LiP4. At amino acid sequence level, the predicted full-length polypeptides of Pr-LiP1 (74% identity) and Pr-LiP4 (86% identity) were the most related to Pr-LiP3 (LIG_PHLRA, protein accession P20010) of *P.*

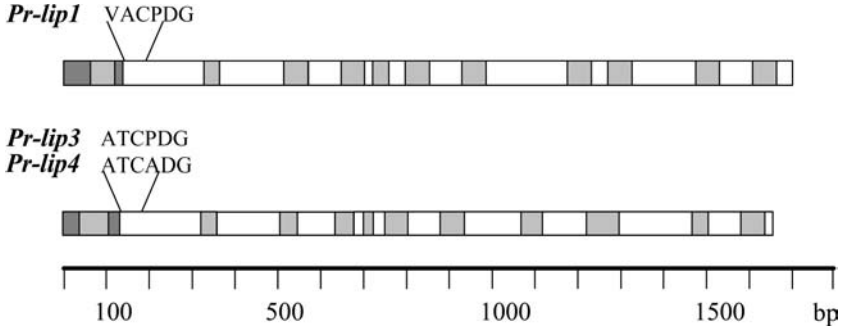

Fig. 1 Intron–exon structure of the *lip* genes of *Phlebia radiata*. The exons are indicated with *white boxes* whereas *grey* boxes correspond to the introns. *Dark grey box* indicates the 5′ leader region encoding the pre-propeptide including secretion signal peptide. The deduced amino-terminal beginning of the mature LiP polypeptides is indicated

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Pr-LiP1  MAFKQLFTVVTVALALNVANAAITKRVACPDGINTATNAACCALFPVRDDLNKNLFNGGQ  60
Pr-LiP3  MAFKQLLSAVTLALAASAA--SVTRRATCPDGT-QLMNAECCALLAVRDDLQNNMFNN-E  56
Pr-LiP4  MAFKQLIGAVALALAASAA--TVNRRATCADGT-QLVNAECCALIKVRDDLQKNMFRN-E  56

                  ¤    *
Pr-LiP1  CNDEAHEAFRLTFHDAIAISPALEAQGKFGGGGADGSIVIFSDVETKFHPNVGLDEVIAI 120
Pr-LiP3  CGDEAHEALRLTFHDAIAISPAMEATGQFGGGGADGSIMIFSDIETKFHPNIGLDEVVES 116
Pr-LiP4  CGDEAHEALRLTFHDAIAISPALEAEGKFGGGGADGSIMIFSKTETAFHPNIGLDEVVES 116

Pr-LiP1  QKPFLQRSNLSVADFIQFAGAVGISNCPGTPPLPAFVGRKDATQPAPDGLVPEPFHTVDT 180
Pr-LiP3  FRPFQQRSGMGVADFIQFSGAVGTSNCPGAPTLNAFIGRKDATQAAPDGLVPEPFHDVNT 176
Pr-LiP4  FRPFQERSGMGVADFIQFSGAVGTSNCPGAPVLNVSIGRTDATRPAPDGLVPEPFHDVDT 176

                         ¤    *      ¤
Pr-LiP1  ILARFNDAGGFDELETVWFLIAHTVAAQNDIDPSIPRTPFDSTPDVFDGQFFIETQLRGT 240
Pr-LiP3  ILARFNDAGDFDELETVWFLIAHSVAAQNDIDPAVSHAPFDSTPSVMDGQFFIETQLRGV 236
Pr-LiP4  ILARFADAGEFDELETVWFLIAHSVAAQNDIDPTVSHAPFDSTPEIMDGQFFIETQLRGE 236

Pr-LiP1  LFPGKGGIQGTVQSPLKGEFRLQSDHELARDSRTACEWQSFGTDQAKLQNRFQFIFGAMG 300
Pr-LiP3  EFIGSGGIEGVAESPVKGEFRLMSDQQIARDNRTACEWQSFGTDQAKLQNRFQFIFEAMG 296
Pr-LiP4  GFIGKAGIEGVAKSPVRGEFRLQSDELLARDNRTACEWQSFGTDQAKLQNRFQFIFQAMG 296

Pr-LiP1  QLGHDPNDLIDCSEVLPNPPPLTTTPHFPAGKSLADVEQACAETPFPALRSDPGPQTAVP 360
Pr-LiP3  QLGTDPTTLIDCSDVLPVPPPLSTVPHFPAGITINDVEPACAETPFPTLPTDPGPATAVA 356
Pr-LiP4  ELGTDPTTLIDCSDVLPTPPDLTTVPHFPAGVSINDVQAACAETPFPTLPTDPGPATAVA 356

Pr-LiP1  AVPSSPVRNA  370
Pr-LiP3  AVPRD-----  361
Pr-LiP4  PVPRD-----  361
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Fig. 2 Comparison of translated amino-acid sequences of *P. radiata* LiP1, LiP3 and LiP4 lignin peroxidases with ClustalW multiple alignment. The distal and proximal histidines (*) and other catalytically important conserved residues ( ) are indicated. Deduced leader pre-propeptide is shaded in *dark grey* and potential glycosylation sites in *light grey*

radiata (Fig. 3). Based on phylogenetic grouping, *P. radiata* LiP polypeptides form a cluster with all the other so far cloned and sequenced LiPs from *P. chrysosporium* and *T. versicolor*, and are the nearest related to the only characterized LiP from *B. adusta*.

Expression of *lip* transcripts on spruce wood

Phlebia radiata was cultivated on spruce wood chips (SWC cultures) for 21 days. The amount of mRNA in these cultures was too low to be detected by Northern hybridization, and thereby competitive RT-PCR was used to quantify relative abundance of the three *Pr-lip* transcripts (Fig. 4). Gene specific primer pairs (Table 1) were used to amplify the transcripts from cDNA and respective regions of the genomic plasmid templates of known concentrations. No genomic DNA contamination was detectable among the RT-PCR products derived from total RNA. Expression levels of the transcripts from *Pr-lip1* and *Pr-lip4* were less than 0.1 pg (in comparison to the amount of respective genomic plasmids) after 21-days of cultivation on spruce chips. In contrast, the amount of the *Pr-lip3* transcript was at that time remarkably high (over 150-fold higher) pointing to more intense level of expression.

Effect of Mn on expression of *lip* transcripts

Nitrogen-limited semisolid cultures were supplemented with fine-milled alder wood (MAW cultures) and Mn^{2+} (240 μM or 480 μM Mn^{2+}). From day 4 to day 13, total RNA was extracted from mycelia grown in cultures without Mn^{2+}. Relative abundance of the three *lip* transcripts were analyzed by using RT-PCR (Fig. 5) with *Pr-lip* and *Pr-gpd* gene-specific primer pairs (Table 1). On day 4, low levels of *Pr-lip1* and *Pr-lip4* transcripts were detected whereas no amplification of *Pr-lip3* transcript was observed (Fig. 5a). No extracellular LiP enzyme activity was detected until day 11 (only 5.5 nkat/L) and on day 13 all three *lip* genes were expressed. On day 11, relative abundance of the *Pr-lip1* and *Pr-lip4* transcripts were clearly increased with 240 μM Mn^{2+}-

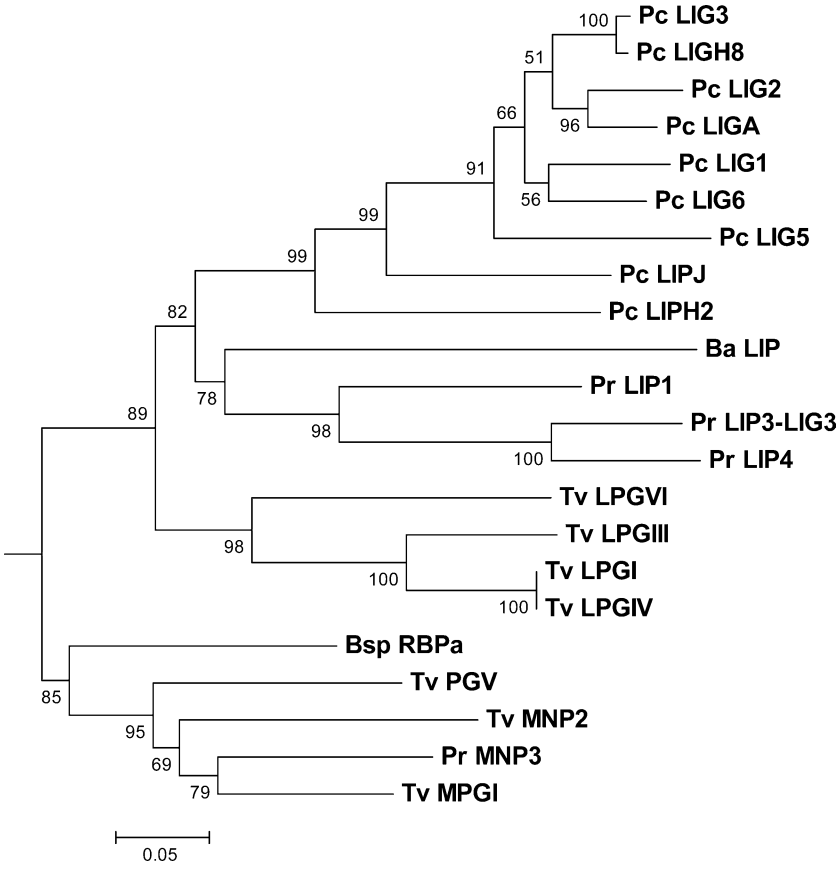

Fig. 3 Phylogeny of class II fungal lignin peroxidases (LIG, LIP, LPG) depicted in unrooted neighbor-joining subtree. The related manganese peroxidases (MNP, MPG) and versatile peroxidases (RBPa, PGV) are included. ClustalW multiple alignment with Gonnet250 distance matrix of translated full-length coding sequences was transferred to Mega 2.1 software for tree creation and molecular evolutionary calculation. Bootstrap values (as percentage) for the branches were obtained with 1000× replication. The bar length corresponds to 5% amino-acid dissimilarity. Peroxidase sequences were retrieved from nucleotide sequence data banks with SRS (www.ebi.ac.uk) and they are as follows: Pc (*Phanerochaete chrysosporium*) LIG3 (P21764), LIGH8 (P06181), LIG1 (Q01775), LIG2 (P49012), LIGA (P31837), LIG6 (P50622), LIG5 (P11543), LiPJ (Q9UW80), LiPH2 (P11542); Ba (*Bjerkandera adusta*) LiP (E51135); Pr (*Phlebia radiata*) LiP1 (this work, gene accession AY743218), LiP3 (P20010, gene accession AY749105), LiP4 (this work, gene accession AY745250), MNP3 (Q96TS6); Tr (*Trametes versicolor*) LPGVI (Q99057), LPGIII (Q12435), LPGI (Q7LHY3), LPGIV (CAA83228, allele to LPGI), PGV (CAA54398), MnP2 (Q99060), MPGI (Q99058); Bsp (*Bjerkandera sp.*) RBPa (Q874A6)

treatment (Fig. 5b). In contrast, accumulation of the *Pr-lip3* transcripts was somewhat repressed under these conditions, and with addition of 480 μM Mn^{2+}, complete inhibition of expression of *lip3* occurred.

Discussion

Isozymes of LiP, MnP and laccase are produced by *P. radiata* 79 both in synthetic liquid cultures (Lundell et al. 1990; Niku-Paavola et al. 1990; Hatakka et al. 1991; Moilanen et al. 1996) and in solid-state cultures on straw (Vares et al. 1995). We describe here the genomic and primary structure of two new *lip* genes, *Pr-lip1* and *Pr-lip4*, and genomic sequence of the previously described *Pr-lip3 (lgp3)* cDNA of *P. radiata* (Saloheimo et al. 1989). Our study shows at molecular level that besides the two recently cloned and described, divergent *mnp* genes coding for two structurally different enzymes, Pr-MnP2 and Pr-MnP3 (Hildén et al. 2005), the three *P. radiata lip* genes described here are also expressed when the fungus is growing on wood, irrespective of the type of wood, either softwood (spruce) or hardwood (alder).

Primary structures of the three predicted LiP-polypeptides were compared to the amino-terminal sequences of LiP isozymes isolated from bioreactor cultures of *P. radiata* (T. Lundell et al., unpublished data). According to the amino-terminal peptide sequences, two of the purified isozymes, Pr-LiP1 and Pr-LiP3, corresponded the predicted mature protein products of two distinct *lip* cDNA clones, and the respective genes were named as *Pr-lip1* and *Pr-lip3*. One of the *lip* clones differed in this respect and was designated *Pr-lip4*. Prediction of the signal peptidase cleavage site suggests that the three Pr-LiP proteins are synthesized as preproenzymes composed of ca. 20-aa secretion prepeptide followed by a 5–6-aa short propeptide ending with KEX2-protease specific dibasic motif (-KR-,-RR)

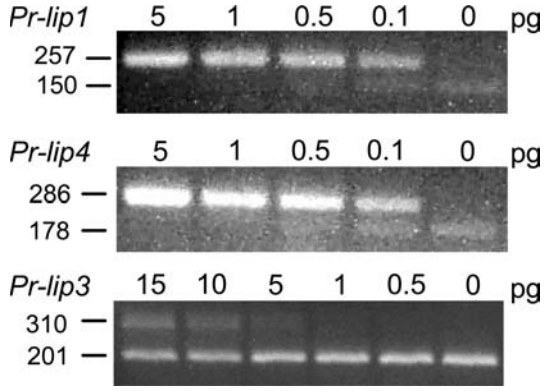

Fig. 4 Competitive RT-PCR of 21-day spruce chip cultures of *P. radiata* for estimation of abundance of the *lip1*, *lip3* and *lip4* transcripts. The gene specific primers used in PCR are shown in Table 1. Concentration of the competitive genomic amplicon is indicated in picograms of the corresponding plasmid, and comparative amount of each transcript was estimated according to the equivalence point of the intensities between the competitive genomic and targeted cDNA product. Sizes of the PCR products are indicated (as base pairs) on the left

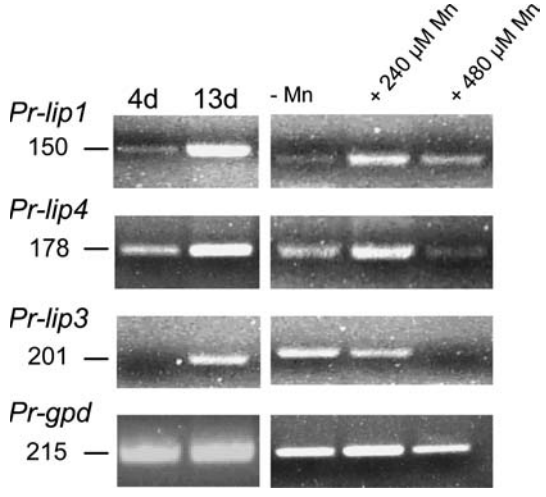

Fig. 5 RT-PCR analysis on the *lip* and *gpd* transcripts of *P. radiata* amplified from cDNA obtained from semi-solid liquid MAW cultures amended with milled alder wood as carbon source. **a** Expression levels of the *lip* transcripts on day 4 and 13 of cultivation. **b** Effect of Mn^{2+} supplementation on the levels of the *lip* transcripts in 11-day cultures. In both cases, the constant intensity of the *gpd* RT-PCR product justifies comparison with the intensities of the PCR products obtained for *lip* transcripts

resembling the leader peptide structure described for deduced *P. chrysosporium* LiPs (Ritch et al. 1991; Cullen 1997).

The intron–exon structure within the three *P. radiata lip* genes shows a high degree of conservation with 11-intron splicing. In other basidiomycetes, over ten short introns are also present in e.g., the *B. adusta lip* gene (Kimura et al. 1991), *T. versicolor pgv* gene (12 introns; sequence accession X77154), in *P. radiata mnp3* gene (11 introns; Hildén et al. 2005) and in the versatile peroxidase (VP) encoding genes of *Pleurotus eryngii* and *P. ostreatus* (15 introns; Martínez 2002). However, gene organization is significantly different with lower intron number in most of the cloned *lip* genes, that is from *P. chrysosporium* (8–9 introns) (Gold and Alic 1993; Cullen 1997) and *T. versicolor* (5–6 introns) (Johansson and Nyman 1996; Martínez 2002), suggesting different evolutionary lineages for the *lip* genes according to individual basidiomycete species. Interestingly, the exon-intron organization of *Pr-lip3* and *Pr-lip4* was very similar to the structure of the *Pr-mnp3* gene coding for the short-type of MnP in *P. radiata* (Hildén et al. 2005).

Similar evolutionary inheritance of the three *lip* genes and the *mnp3* gene of *P. radiata* is supported by similarities in predicted polypeptide length and cleavage of the amino-terminal pre-propeptide, maybe resulting from early duplication of an ancestral heme peroxidase gene. However, at protein phylogeny level, *P. radiata* LiPs 1–3 form a coherent cluster with all the other fungal LiPs characterized so far (Fig. 3).

LiPs as well as VPs and short MnPs form the diverse group A recently presented by us within the family of fungal secretory class II heme peroxidases (Hildén et al. 2005). The present subtree in Fig. 3 illustrates detailed and well-supported branching of the group A to several fungal species-based LiP clusters: Pc-LiP(LIG) cluster, Pr-LiP cluster including the *P. radiata* and *B. adusta* LiPs, and Tv-LiP/LPG cluster (Fig. 3). These main branches are more related to each other than to the VP-short-hybrid-MnP (Tv-MnP-VP-Pr-MnP3) cluster. Molecular evolutionary analysis of the fungal lignino-lytic peroxidases supports their grouping accordingly on enzyme catalytic properties (Hildén et al. 2005).

The discrepancy between gene structure and protein product identity may be explained by conservation of the intron–exon structure for a functional eukaryotic gene within one basidiomycete species, thereby allowing evolutionary changes for the coding sequences but conserving intron positioning. The importance of intron positioning relative to the coding sequence is supported by the estimation that 17–18% of fungal introns match to animal or plant intron positions in orthologous genes (Fedorov et al. 2002). In addition, it has been shown recently that at least one intron is required for efficient expression of green fluorescent protein in basidiomycetes (Lugones et al. 1999), and the intron length affects expression levels e.g., in RNA silencing in filamentous fungi (Nakayashiki et al. 2005).

Thus far, nothing has been known of the expression and regulation of *P. radiata lip* genes when the fungus is cultivated on lignocellulose. In this study, we detected *Pr-lip3* as the major transcript in abundance in three weeks, when the fungus was grown on spruce wood (SWC cultures) whereas transcript levels of *Pr-lip1* and *Pr-lip4* were very low. In contrast, in semisolid cultures of *P. radiata* supplemented with milled alder wood (MAW cultures), *Pr-lip1* and *Pr-lip4* transcripts were observed early (on day 4) whereas *Pr-lip3* transcripts were not then detectable by RT-PCR. After 13 days, however, transcripts of all three *lip* genes were present in

good amount. These results suggest endogenous time-dependent regulation of expression of the *P. radiata lip* genes, which is in accordance with previous studies on *P. chrysosporium lip* gene expression (Broda et al. 1995; Bogan et al. 1996; Janse et al. 1998).

In our present work, the different composition of the wood used, either hardwood (alder) or softwood (spruce), may also have affected *lip* gene expression. We noted previously at protein level that LiP3 was the most abundant LiP isozyme produced when lignocellulosic materials were used as carbon source in nitrogen-limited liquid cultures of *P. radiata* (Niku-Paavola et al. 1990). In contrast, on wheat-straw based solid-state cultures of *P. radiata*, LiP3 was not detectable whereas LiP2 and two minor LiP isoforms were produced together with MnP and laccase (Vares et al. 1995).

Consistent with our data, studies on *P. chrysosporium* have also demonstrated differential regulation of expression of *lip* and *mnp* genes in response to culture conditions (Gold et al. 1989, 2000; Gold and Alic 1993; Cullen 1997; Martínez 2002). Substantial substrate-dependent effect on *lip* transcript levels was observed in *P. chrysosporium* cultivated on aspen wood chips (Janse et al. 1998), in defined liquid medium (Broda et al. 1995; Stewart and Cullen 1999) and in soil cultures (Lamar et al. 1995; Bogan et al. 1996). Low levels of LiP but significant amount of MnP activity were detected in cultures of *P. chrysosporium* grown on softwood pulp (Datta et al. 1991) whereas recently, in oak wood chip cultures, LiP isozymes but no MnP was identified while *P. chrysosporium* readily produced cellulolytic enzymes (Abbas et al. 2005).

In our present study, influence of Mn^{2+} addition was also investigated in the semisolid MAW cultures of *P. radiata*. Clear increase on accumulation of *Pr-lip1* and *Pr-lip4* mRNAs was observed with 240-μM Mn^{2+} supplementation whereas the twice higher addition of Mn^{2+} had no effect on the transcript levels. It is possible that moderate concentrations of Mn^{2+} ions may stabilize fungal transcripts (Manubens et al. 2003). In contrast, the 240-μM Mn^{2+} treatment caused no change on expression of *Pr-lip3*, which was, however, completely inhibited with 480-μM Mn^{2+}. Accordingly, relatively less LiP3 isozyme was observed in liquid bioreactor cultures of *P. radiata* amended with high-Mn^{2+} whereas the quantities of Pr-MnP2 and MnP3 were somewhat enhanced (Moilanen et al. 1996).

Putting these data together, high amount of soluble manganese ions obviously depress expression of the predominant LiP3 isozyme. Further analysis is needed to identify regulatory sequences at promoter regions, which obviously play critical roles in orchestrating expression of the several *lip* genes in *P. radiata*.

These results support that all the three *Pr-lip* genes are transcribed and further processed to active, secreted enzymes by *P. radiata* when growing on wood, although in different amounts depending possibly on the nature of the wood substrate and concentration of Mn^{2+}. *P. radiata* 79 mineralizes ^{14}C-labelled guaiacyl-type synthetic lignin (DHP), ^{14}C-lignin labelled fir and spruce to $^{14}CO_2$ (Hatakka et al. 1983; Lundell et al. 1990; Moilanen et al. 1996), and grows well on softwood causing some delignification (Hakala et al. 2004). Three *lip* (this work), two divergent *mnp* (Hildén et al. 2005) and laccase genes (Mäkelä M. et al., unpublished data) are expressed by *P. radiata* during growth on wood. This set of ligninolytic enzymes is functionally more versatile than is found in the white rot model fungus *P. chrysosporium* (Cullen 1997; Martinez et al. 2004, Abbas et al. 2005).

Moreover, our current data puts more value to the role of LiP-type peroxidases to be expressed, together with the MnPs and laccases, for efficient degradation of wood by white rot basidiomycetes. Variation on the level of *lip* transcripts observed here upon growth of *P. radiata* on wood substrates indicates that time-dependent regulation for expression of each *lip* gene also occurs on natural substrates.

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