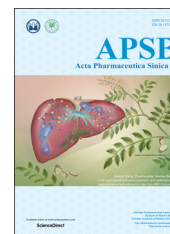




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ORIGINAL ARTICLE

Cloning and expression of three thaumatin-like protein genes from *Polyporus umbellatus*



Mengmeng Liu^{a,b}, Dawei Zhang^{a,b}, Yongmei Xing^{a,*}, Shunxing Guo^{a,*}

^aKey Laboratory of Bioactive Substances and Resources Utilization of Chinese Herbal Medicine, Ministry of Education, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China

^bInstitute of Bioinformatics and Medical Engineering, School of Electrical and Information Engineering, Jiangsu University of Technology, Changzhou 213001, China

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KEY WORDS

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Gene cloning;
Expression

Abstract Genes encoding thaumatin-like protein (TLPs) are frequently found in fungal genomes. However, information on TLP genes in *Polyporus umbellatus* is still limited. In this study, three TLP genes were cloned from *P. umbellatus*. The full-length coding sequence of *PuTLP1*, *PuTLP2* and *PuTLP3* were 768, 759 and 561 bp long, respectively, encoding for 256, 253 and 187 amino acids. Phylogenetic trees showed that *P. umbellatus PuTLP1*, *PuTLP2* and *PuTLP3* were clustered with sequences from *Gloeophyllum trabeum*, *Trametes versicolor* and *Stereum hirsutum*, respectively. The expression patterns of the three TLP genes were higher in *P. umbellatus* with *Armillaria mellea* infection than in the sclerotia without *A. mellea*. Furthermore, over-expression of three PuTLPs were carried out in *Escherichia coli* BL21 (DE3) strain, and high quality proteins were obtained using Ni-NTA resin that can be used for preparation of specific antibodies. These results suggest that *PuTLP1*, *PuTLP2* and *PuTLP3* in *P. umbellatus* may be involved in the defense response to *A. mellea* infections.

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*Corresponding authors.

E-mail addresses: meimary@163.com (Yongmei Xing), sxguo1986@163.com (Shunxing Guo).

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1. Introduction

Polyporus umbellatus, belonging to Polyporaceae in the class of basidiomycetes, was used as a traditional Chinese medicine for more than two thousand years in China¹. In our previous study, we found that the growth of *P. umbellatus* sclerotia depended on a symbiotic relationship with *Armillaria mellea*². *A. mellea* would invade the sclerotia of *P. umbellatus* when the symbiotic relationship had been formed. Meanwhile, *P. umbellatus* would take a series of effective measures to prevent the invasion of *A. mellea*, such as forming a cavity structure in the medullar tissue of sclerotia and isolating *A. mellea* in cavity structure². Although the morphological characteristics of the defensive structures in *P. umbellatus* had been fully studied, no genes that may involve in the forming of the defensive structures have been identified and characterized up to now.

Thaumatocin-like proteins (TLPs) are proteins with molecular weight of 21–26 kDa that commonly found in various organisms, such as plants, nematodes, arthropods and fungi³. However, most of the TLPs expressed by *Puccinia graminis* were defined as small TLPs (sTLPs) with molecular mass of 16–17 kDa³. Plant TLPs, which belongs to the pathogenesis related-5 (PR-5) proteins family, were most studied as they played key roles in the plant defense system. In recent years, more and more attention has been paid on the research of fungal TLPs. The TLPs are reported to have β -1,3-glucans and glucose polymers degrading activities in fungal cell walls^{4,5}. It has been reported that the TLP TLG1 expressed by *Lentinula edodes* possessed glucanase activity, indicating that TLG1 involved in cell wall degradation and remodeling⁶. Interestingly, a protein expressed by the oomycete pathogen *Phytophthora parasitica*, named OPEL, which contained a thaumatocin-like domain, was an elicitor of plant defense system⁷.

To our knowledge, the study of the functional genes of *P. umbellatus* is still in its infancy. A full-length cDNA library was constructed by SMART technology, but no TLPs gene was found⁸. In our previous study, we constructed a suppression subtractive hybridization (SSH) cDNA library of *P. umbellatus* and none of TLPs were found⁹. In recent study, we found three TLPs genes in the transcriptome of *P. umbellatus*¹⁰. Due to the glucanase activity of fungal TLPs, we decided to investigate the presence of TLPs in *P. umbellatus* and elucidate the response of *P. umbellatus* TLPs to *A. mellea* stress. In this study, we reported the identification of three TLP genes in *P. umbellatus* (*PuTLPs*). In addition, *PuTLPs* expression profiles in *P. umbellatus* during *A. mellea* infection were assessed. Furthermore, we expressed and purified the recombinant three *PuTLPs* in *Escherichia coli* for preparation of specific antibodies in the future. The results will provide experimental materials and theoretical basis for future research to identify the precious function of these three genes.

2. Materials and methods

2.1. *P. umbellatus* sclerotia collection

P. umbellatus sclerotia were sampled from Liuba (N33°37'9.85", W106°54'59.63"), Shaanxi province in June 2015. The sclerotia were firstly surface sterilized by dipping in 75% ethanol for 1 min. The sclerotia were then cut in half and 100 mg of medullar tissue was obtained. The tissue without *A. mellea* infection was regarded as the control group (CK), while the part invaded by the *A. mellea* rhizomorphs was treated as the test group (CT) (Fig. 1). Each

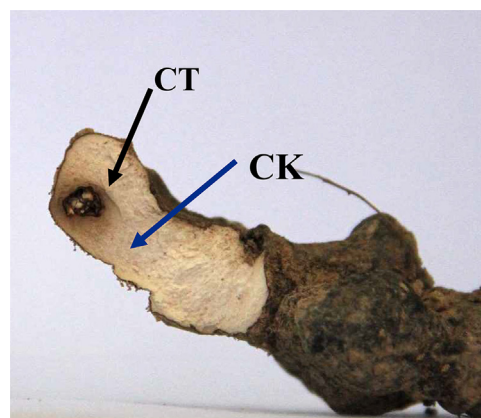


Figure 1 The internal part of *P. umbellatus* sclerotium. The black region (CT) surrounding the area invaded by rhizomorphs of *A. mellea* (black arrow) and the none-invading region (CK) was white in color (blue arrow).

sample was repeated triple, and all the samples were frozen in liquid nitrogen immediately after collection and stored at -80°C before RNA extraction.

2.2. Total RNA extraction and first strand cDNA synthesis

Total RNA from each tissue was extracted and treated with RNase-free DNase with an Easy spin Plus Total RNA Kit (Aidlab, Beijing, China) according to the manufacturer's instructions. The integrity of RNA was determined by using a 1% agarose gel. The quality and concentration of RNA were measured by NanoDrop 2000 (Thermo Fisher Scientific, USA). The RNA was stored at -80°C for further use, and cDNA was synthesized using a PrimerScript™ RT Master Mix (Takara, Japan) following the manufacturer's protocol.

2.3. Polymerase chain reaction (PCR)

The full sequences of the three subunits of TLP were obtained from our previous transcriptome library of *P. umbellatus*¹⁰. The primers for the full sequences of the three genes were designed (Table 1). PCR was carried out in a T100 thermal cycler (Bio-Rad, CA, USA) using Phanta Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China). The cycling conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 3 min.

PCR products were separated by electrophoresis on a 1.2% agarose gel and then ligated into the pTOPO vector (Aidlab, China), and transformed into DH5 α competent cells (Tiangen, China), following the manufacturer's instructions. Transformed cells were cultured overnight on Luria-Bertani (LB) agar plates contained 50 $\mu\text{g}/\text{mL}$ ampicillin. Clones were randomly selected and cultured in LB medium containing 50 $\mu\text{g}/\text{mL}$ ampicillin for 4 h. The recombinant clones were sequenced by Beijing GENEWIZ (Beijing, China). DNASTar software (DNASTar Inc., USA) was used to analyze and assemble the sequences.

2.4. Sequences and phylogenetic analysis

The full-length cDNA sequences and the deduced amino acid sequences of these tree genes were analyzed using the National

Table 1 Primers for RT-PCR and q-PCR.

Primer type	Gene	Primer sequence (5'–3')
PCR	Comp11397	Forward: ATGAAGCTCTCTATTGCAACTGCTCTTGTC Reverse: TCAGGGCGGCGGGCAGAAGGTCAAT
	Comp33782	Forward: ATGAAGGGTTTAgTTGCTGTTGTTGCAC Reverse: TCAAGGGCAGAATGTGATAGTGTAGTCC
	Comp30274	Forward: ATGAAGAACGTCGCTCTCTC Reverse: TTAGGGGCAGAAAGTGTGA
qPCR	Comp11397	Forward: GGTGTACAACGGCTGTCTT Reverse: GTGTGTGGATCGCATTGAAG
	Comp33782	Forward: CGAACAAATTGGAGGTCTGGT Reverse: TCCACCAAGGACACATCGTA
	Comp30274	Forward: ATCCGCTAGTTTTGGTGTGCG Reverse: CCGCCATACTGTCCAAAGTT
	β -Tublin	Forward: CCTTCCTTGGCAACTCGACA Reverse: TCGTCCATACCCTCTGTGT
Plasmid	Comp11397	<u>CCGCGCGGCAGCCATATGATGAAGCTCTCTATTGCAACTGCTCTTGTC</u> <u>GGCTGCTAACAAAGGATCTTCAGGGCGGCGGGCAGA AGGTCAAT</u>
	Comp33782	<u>CCGCGCGGCAGCCATATGATGAAGGGTTTAgTTGCT GTTGTTGCAC</u> <u>GGCTGCTAACAAAGGATCTTCAGGGCAGAATGTGATAGTGTAGTCC</u>
	Comp30274	<u>CCGCGCGGCAGCCATATGATGAAGAACGTCGCTCTCTC</u> <u>GGCTGCTAACAA GGA TCCTTAGGGGCAGAAAGTGTGA</u>

Center for Biotechnology Information BLAST programs (<http://www.ncbi.nlm.nih.gov/BLAST/>). The molecular weight (MW) and theoretical isoelectric point (pI) were predicted using the ExPASy ProtParam tool (http://www.expasy.org/tools/pi_tool.html). Multiple sequence alignments were carried out using the ClustalW program (<http://www.ebi.ac.uk/clustalW>). A phylogenetic analysis was performed using the neighbor-joining (NJ) method in MEGA software (version 6.0)¹¹. Node robustness was assessed by the bootstrap method ($N = 1000$ pseudoreplicates).

2.5. Quantitative real-time PCR

The qPCR primers were designed according to the three full-length cDNA sequences using Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) (Table 1). PCR was performed with a specific set of qPCR primers and cDNA as a template in a final volume of 15 μ L containing 7.5 μ L of SYBR Green Premix Ex TaqTM (2 \times) (TaKaRa, Dalian, China), 0.3 μ L of 10 mmol/L gene-specific forward and reverse primers, 2 μ L of cDNA template and 4.9 μ L of PCR grade H₂O. The β -tubulin gene was used as a reference control. The reaction was performed using the following conditions: denaturation at 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 5 s; 60 °C for 34 s). Each plate was repeated for three times in independent runs for all reference and selected genes. Gene expression was evaluated by the $2^{-\Delta\Delta Ct}$ method¹².

2.6. Construction of protein expression plasmids

The coding sequence of these three TLP genes was amplified by PCR from the pTOPO vector using the specific oligonucleotides (Table 1), which contained *Nde*I and *Bam*H I restriction sites, respectively. The PCR amplification product was cloned into the pET15b (Novagen, USA) expression vector using the In-Fusion HD Cloning Kit (Clonetech, Japan) following the manufacturer's

instructions, which were added a N-terminal six-histidine tag to the coding sequence. The presence of base substitutions in the recombinant plasmid was determined by DNA sequencing method.

2.7. Recombinant proteins expression and purification

The tree *TLP* genes were expressed in the *E. coli* BL21 (DE3) strain. The cells grown at 37 °C with shaking at 180 rpm in 250 mL of LB broth, containing 50 μ g/mL Ampicillin until an OD600 reached 0.8. The expression of the recombinant protein was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) at the final concentration of 0.1 mmol/L, followed by cultivation for 24 h at 15 °C with 160 rpm (High speed tabletop centrifuge, Sorvall ST8, Thermo Fisher Scientific Corporation, MA, USA). The culture was harvested by centrifugation (10,000 $\times g$ for 15 min at 4 °C), and the cell pellets were re-suspended in 30 mL of buffer A (50 mmol/L Tris-HCl pH 8 and 100 mmol/L NaCl) and were incubated for 30 min on ice. The cells were disrupted by sonication, and the insoluble fraction was collected by centrifugation (14,400 rpm for 60 min at 4 °C).

The three protein purification was performed in a single gravity flow chromatography step using a column packed with 1 mL of Ni-NTA resin (smart-lifesciences, China) and was equilibrated with buffer A. The purified protein was eluted using a five-step gradient of imidazole (10, 20, 80 and 300 mmol/L) in buffer A, each step containing 10 mL of the respective buffer. The purity of the protein samples was estimated by SDS-PAGE and stained with Coomassie brilliant blue G-250.

2.8. Western blot analysis

Western blot analysis was performed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrotransferred onto a polyvinyl Di-fluoride

(PVDF) membrane (Millipore, USA), which was then blocked with a blocking solution containing 5% non-fat powdered milk for 4 h. The PVDF membrane was blotted with the anti-his antibody (Abcam, England) in PBS. After incubation overnight at 4 °C, the membrane was washed in PBST for 3 times. The PVDF membrane was then incubated with peroxidase-linked anti-mouse IgG antibodies for 2 h, developed using an enhanced chemiluminescence detection kit (TransGen Biotech, China), and then analyzed with a Gel Doc™ XR+ imaging system (Bio-Rad, USA).

2.9. Statistical analysis

For each sample, three technical replicates of the qRT-PCR assay were used with a minimum of three biological replicates. Results were expressed as means ± standard deviation (SD) of the number of experiments. Differences were regarded as statistically significant at $P < 0.05$ and extremely significant at $P < 0.01$.

3. Results

3.1. cDNA cloning, sequence characterization of PuTLP1, PuTLP2 and PuTLP3

We have cloned the complete open reading frame of PuTLP1, PuTLP2 and PuTLP3, three new TLP from *P. umbellatus*. The full-length coding sequence of PuTLP1 was 768 bp in length and encoded 256 amino acid (aa) residues. The predicted molecular weight of the deduced proteins was 27.06 kDa and the isoelectric point was 4.35. The PuTLP1 cDNA sequence was deposited in GenBank and the accession number was KU179178. The result of SignalP 4.1 Server showed that the mature protein of PuTLP1 had an N-terminal cleavable signal sequence of 18 aas with the probable cleavage site between 18th and 19th aa, LAR and TF.

The full-length coding sequence of PuTLP2 contained 759 bp and encoded 253 aa residues. The calculated MW of PuTLP2 was

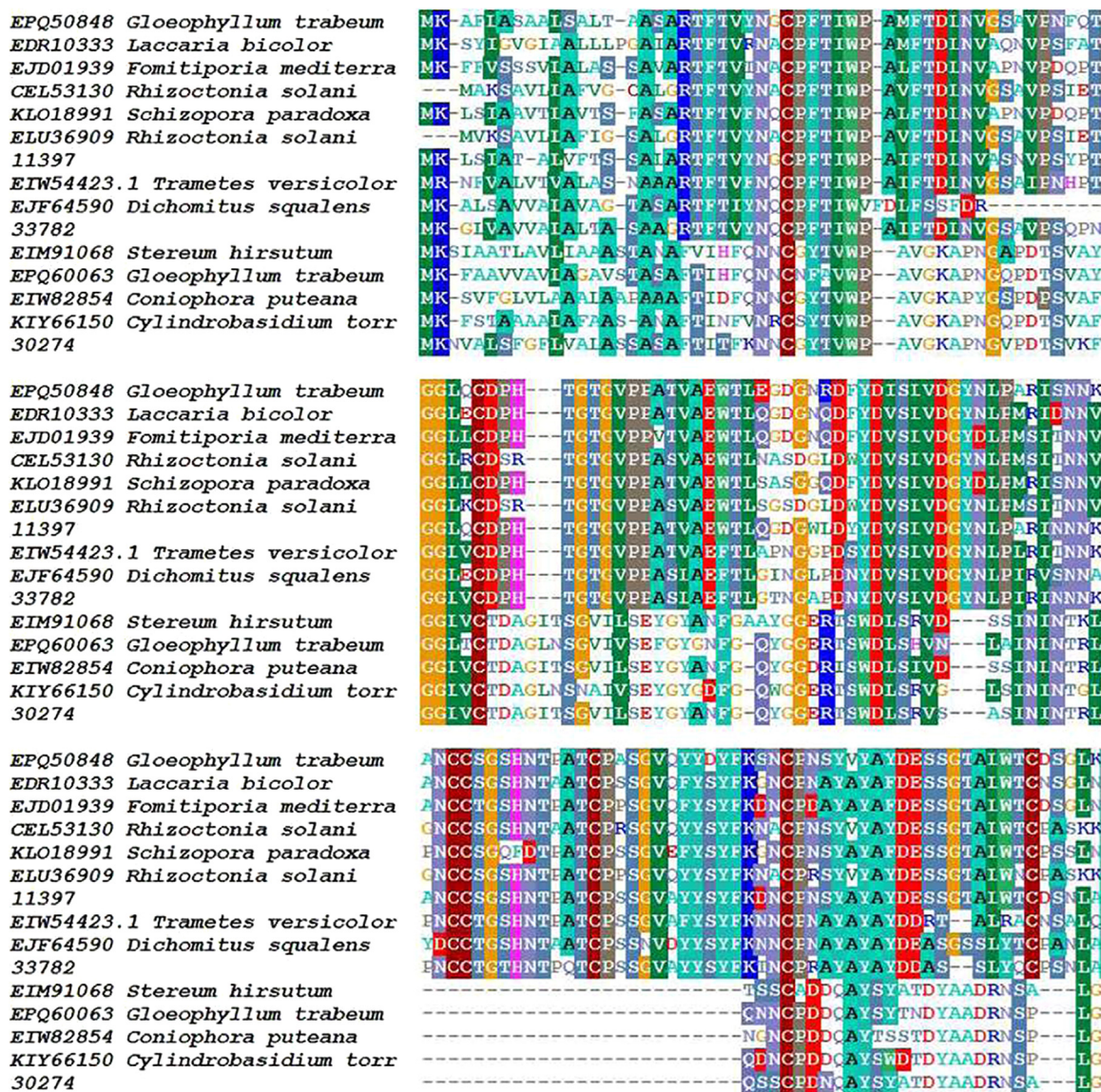


Figure 2 Amino acid alignment of PuTLP from *P. umbellatus* sclerotia with twelve other known sequences of the TLP.

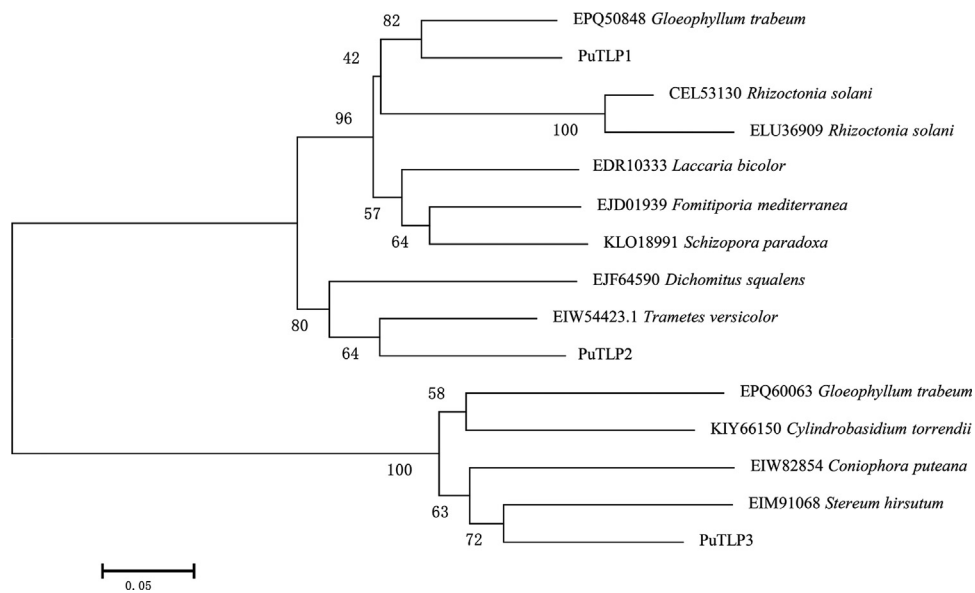


Figure 3 A phylogenetic tree of PuTLP was constructed with the neighbor-joining method by the program MEGA 6.0.

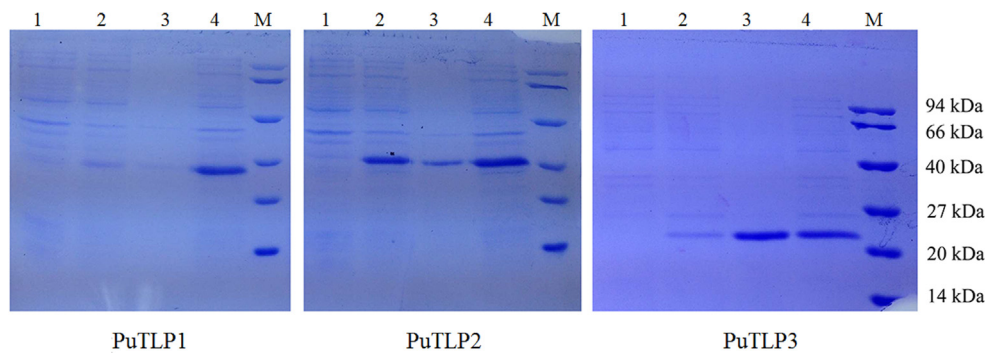


Figure 4 SDS-PAGE analysis of recombinant PuTLP overexpressed in *E. coli* BL21. M: Low molecular protein marker; 1, The total expressed proteins before induced; 2, The soluble expressed proteins induced with 0.1 mmol/L IPTG 24 h at 15 °C; 3, Insoluble expressed proteins induced with 0.1 mmol/L IPTG 24 h at 15 °C; 4, The total expressed proteins induced with 0.1 mmol/L IPTG 24 h at 15 °C.

26.5 kDa and the predicted pI was 5.5. The GenBank accession number of *PuTLP2* was KU179180. The result of SignalP 4.1 Server has shown that mature protein of *PuTLP1* has an N-terminal cleavable signal sequence of 19 aas with the probable cleavage site between the 19th and 20th aa, AGR and TF.

The completed coding sequence of *PuTLP3* was 561 bp. The deduced PuTLP3 protein sequence contained 187 aas, with the estimated molecular mass of 19.26 kDa and the isoelectric point of 6.02. The GenBank accession number for *PuTLP3* is KU1791798. The result of SignalP 4.1 Server has shown that the mature protein of *PuTLP1* has an N-terminal cleavable signal sequence of 20 aas, with the probable cleavage site between the 20th and 21st aa, SAF and TI.

3.2. Homology and phylogenetic analyses

Amino acid sequences of TLP from several fungal species were downloaded to carry out the homologous and phylogenetic analyses. BLAST analysis suggested that *P. umbellatus* PuTLP1

had high sequence homology similarities with other fungal species: *Gloeophyllum trabeum* (87% identity) and *Laccaria bicolor* (80% identity); PuTLP2 from *P. umbellatus* had high sequence homology similarities with other fungal species: *T. versicolor* (83% identity) and *Dichomitus squalens* (74% identity). The amino acid sequence of PuTLP3 in *P. umbellatus* shared the highest identity 80.5% and 74% with *Stereum hirsutum* and *G. trabeum*, respectively (Fig. 2).

Phylogenetic trees were constructed by analyzing the amino acid sequences of PuTLP with those from other fungal species. *P. umbellatus sclerotia* PuTLP1 was clustered with sequences from *G. trabeum*. PuTLP2 of *P. umbellatus* showed a close phylogenetic relationship with that of the *T. versicolor* and PuTLP3 was closest to the corresponding *S. hirsutum* (Fig. 3).

The numbers at each branch indicate the percentage bootstrap values. The amino acid sequences were downloaded from NCBI: *G. trabeum* (EPQ50848.1), *Rhizoctonia solani* (CEL53130.1), *R. solani* (ELU36909.1), *L. bicolor* (EDR10333.1), *Fomitiporia mediterranea* (EJD01939.1), *Schizopora paradoxa* (KLO18991.1), *Dichomitus squalens* (EIJF64590.1), *T. versicolor*

(EIW54423.1), *G. trabeum* (EPQ60063.1), *Cylindrobasidium torrendii* (KIY66150.1), *Coniophora puteana* (EIW82854.1) and *S. hirsutum* (EIM91068.1).

3.3. Expression of PuTLPs

Expression of pET-15b-PuTLP was induced with 0.1 mmol/L isopropyl- β -D-galactopyranoside (IPTG) at 15 °C for 24 h. After sonication and centrifugation, the bacterial supernatants were analyzed by 12% SDS-PAGE (Fig. 4). PuTLP protein expressed by pET-15b-PuTLP /BL21 (DE3) stains showed a SDS-PAGE band with molecular mass about 19–27 kDa (Fig. 4).

Crude extract was applied to the Ni-NTA column provided in QIA express Ni-NTA spin kit (Qiagen), and the His6-tagged recombinant PuTLP was eluted from column under 250 mmol/L imidazole in 50 mmol/L Tris-HCl buffer (pH 7.8). The purified PuTLP was used for Western Blot analysis to further confirm the purification result.

In order to identify the purified His-PuTLP protein more accurately, anti-his antibody was used to determine the purified His-PuTLP protein. The result has shown that the His-PuTLP protein can combine with specific anti-his antigen with obvious band (Fig. 5).

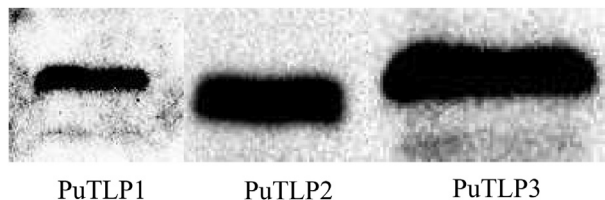


Figure 5 His-PuTLP protein verification by Western blotting.

3.4. Tissue-specific expression of PuTLPs

The abundance of *PuTLP* transcripts in different part of *P. umbellatus* sclerotia was determined by qPCR, using β -tublin for normalization. The qPCR results showed that *PuTLP1*, *PuTLP2* and *PuTLP3* were observed in all examined sclerotia, including the sclerotia without and without *A. mellea*. The mRNA expression levels of the *PuTLP1*, *PuTLP2* and *PuTLP3* were significantly higher ($P < 0.05$) in sclerotia with *A. mellea* infection than in the sclerotia without *A. mellea* (Fig. 6).

4. Discussion

4.1. Sequence analysis of PuTLPs

TLPs are found in diverse eukaryotes, such as plants, nematodes, arthropods and fungi¹³. As the plant TLPs were considered as fungal inhibitors, they have drawn even greater attention. In this study, we have successfully cloned and characterized three kinds of TLPs from *P. umbellatus*. Overall, two genes are similar both in size and in gene structure, except for *TLP3*. For instance, *PuTLP3* coding sequence (CDS) contains 561 bp, while *PuTLP1* and *PuTLP12* have 768 and 759 bp, respectively. In fact, *PuTLP3* could be regarded as the smallest TLPs, like sTLPs from *pucciniales* fungi^{3,14}. It is very common for fungi that the similar genes encoding proteins involved in the same pathway like the genes taking part in fungal secondary metabolism¹⁵.

PuTLPs are rich in Cys residues. Among the three PuTLP genes, *PuTLP1* and *PuTLP2* both have 16 Cys residues while the *PuTLP3* only has 8 Cys residues. All of the PuTLPs have acidic PIs. Signal peptide sequences were calculated in all PuTLPs which means that these three proteins are secreted. It has been reported that majority of the MpTLPs in *Moniliophthora perniciosa* have a five highly conserved amino acids (REDDD)¹⁶. However, the

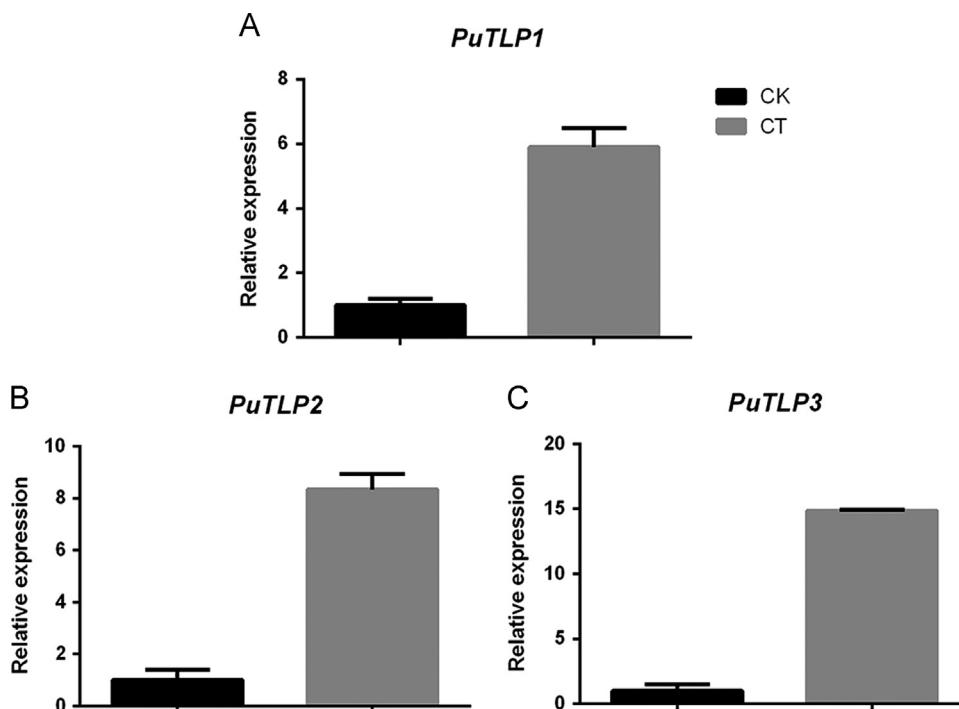


Figure 6 Relative levels of *PuTLP1* (A), *PuTLP2* (B) and *PuTLP3* (C) mRNA transcripts in different larval stages of *P. umbellatus* sclerotia. All values represent the means \pm S.E. ($n=3$ biological replicates). $P < 0.05$.

three PuTLPs in the present study does not contain all of these amino acids which may suggest that the TLPs are diverse among different kinds of fungus.

A neighbor-joining phylogenetical tree was constructed to gain insight into the evolutionary relationship of these three *PuTLP* genes. Interestingly, all the three genes are located in the different phylogenetic clades, but all the three genes show a close phylogenetic relationship with basidiomycete fungi.

4.2. Expression of *PuTLPs*

The signal peptide will be removed from the mature protein, so we constructed the pET-15b-TLPs without the signal peptide sequences. After transformation of the pET-15b-TLPs into *E. coli* BL21 (DE3) cells, the *PuTLP*-His6 protein was induced with 0.1 mmol/L IPTG at 15 °C for 24 h. The level of protein expression was evaluated by comparing pre and post induction samples of the *PuTLP*-His6 by 12% SDS-PAGE. The results indicated the expression of protein was consistent with the molecular weights of *PuTLP*-His6 (19–27 kDa apparent molecular weight). Cell lysis showed that the protein was highly expressed, but with low level of soluble protein. The protein concentration was calculated by the Bradford protein assay kit (cwbiotech, Beijing, China). BSA was used to construct the standard curve. The concentration of *PuTLP*1, *PuTLP*2 and *PuTLP*3 is 0.406, 0.122 and 0.584 mg/mL, respectively.

4.3. Expression of *PuTLPs* is induced by biotic stress

PuTLPs expression patterns were assessed by RNAseq data from *P. umbellatus* transcriptome data⁸ and the expression profiles of these three genes were further validated using qPCR. Based on qPCR data, all the three genes were all detected higher expression in the sclerotia with *A. mellea* infection.

It is well known that the TLPs have antifungal activities¹². It has been reported that the glucanases can degrade the fungal cell walls which was secreted by *Filamentous* fungi^{17,18}. The research has indicated that these glucanases are similar to the plant TLPs. Thus, it has been suggested that the TLPs secreted by basidiomycetes serve multiple functions that can adapt to interspecific competition. It has been determined that the TLP TLG1 of *Lentinula edodes* degrades lentinan, a β -1,3-glucan present in its own cell wall, likely being involved in cell wall degradation and remodeling⁶. In our previous study, we found that *P. umbellatus* sclerotia has formed the cavity structure in the medullar tissue and isolate *A. mellea* to prevent further intrusion¹⁹. In order to form the cavity, the *P. umbellatus* sclerotia cell wall must firstly be hydrolyzed and then fused with other cells. Therefore, we assume that these three *TLPs* play key roles in the defense response to *A. mellea* infection.

In this study, the expression pattern of *PuTLPs* in biotic stress was determined, and the results provided the understanding about the level of *PuTLPs* expression under the biotic stress. In the further project, we will focus on the study of gene expression under abiotic stresses.

5. Conclusions

For the first time, we have cloned, expressed, and characterized three new *PuTLPs* from *Polyporus umbellatus*. These *PuTLPs* genes contain N-terminal cleavable signal sequence and are rich in

Cys 8–16 residues. Changes in TLP protein levels after *Armillaria mellea* infection in *P. umbellatus* indicated that TLP did play a role in the defense action. The discovery of genes encoding the three TLP in *P. umbellatus* will shed light on further investigations of defense reactions in *P. umbellatus*. The purified protein with high quality will be the raw material for the production of antibodies to further define the specific function of *PuTLPs*.

Acknowledgments

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