

Cloning and characterization of two hydrophobin genes differentially expressed during fruit body development in *Lentinula edodes*

W.L. Ng^a, T.P. Ng^a, H.S. Kwan^{a,b,*}

^a Department of Biology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, PR China

^b Department of Food and Nutritional Sciences Programme, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, PR China

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Abstract

Hydrophobins play important roles in morphogenesis and pathogenesis in fungi and fruit development in mushrooms. Two genes encoding hydrophobins (*Le.hyd1* and *Le.hyd2*) were isolated during sequencing of random clones from a primordial cDNA library of *Lentinula edodes*. The nucleotide sequences of these two genes were determined. These two genes are 760 and 738 bp in length and the deduced amino acid sequences are homologous to various fungal hydrophobins with characteristic cysteine spacing. These hydrophobin genes are Class I hydrophobins judging by their conserved domains and hydropathy patterns. The transcript level of *Le.hyd1* is high in primordium and that of *Le.hyd2* is high in dikaryotic mycelial tissues. Poor expression of these two genes in monokaryotic parents indicates that these two genes are under mating-type regulation. We thus suggest that differential expression of these two *L. edodes* hydrophobins during fruit development may contribute to their distinct roles in fruiting of this mushroom. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Shiitake mushroom; Expressed sequence tag; Differential expression; Dikaryon

1. Introduction

Lentinula edodes (Shiitake or Xianggu mushroom) is a popular cultivated mushroom in the world. Some molecular studies of this mushroom have been reported recently [1,2,3], but the basic molecular biology of this mushroom is still poorly understood. Molecular studies of this mushroom have been focused on identifying genes involved in the regulation of fruit body development. The beta sub-unit of mitochondrial processing peptidase β -MPP expression was found to be expressed higher in the fruit body of the mushroom than in mycelium, suggesting that higher mitochondrial activities, and thus more mitochondrial biosynthesis, are required during fruiting [4]. Several other genes of this mushroom were developmentally regulated such as a cell-adhesion protein [5], the DNA-binding pro-

tein priBc [6] and priA [7]. However, up to now, the molecular events that occur during fruiting of this mushroom are still unknown. To add more informative genes to the gene collection of this mushroom, we have been sequencing random cDNA clones to generate an expressed sequence tag (EST) from a cDNA library of primordium, the initial stage of fruiting.

Hydrophobins are moderately hydrophobic small proteins with characteristic cysteine spacing [8]. Hydrophobins were found to play important roles in morphogenesis and pathogenesis in various fungi [9]. They were first isolated as genes activated during the onset of aerial hyphae formation in *Schizophyllum commune* [10]. Subsequently, hydrophobins were isolated from many fungi and their expressions were found to be developmentally regulated. These genes include *SC1*, *SC3* and *SC3* in *S. commune* [11], *CoH1* and *CoH2* in *Coprinus cinereus* [12], *ABH1*, *ABH2* and *ABH3* in *Agaricus bisporus* [13,14,15] and *Fbh1* in *Pleurotus ostreatus* [16]. However, hydrophobins in *L. edodes* have not yet been described and their roles in the development of this mushroom are therefore unknown. During the course of an EST study of this mushroom, two cDNA clones encoded an amino acid sequence homologous to hydrophobins of other fungi. We report

* Corresponding author. Tel.: +85 (2) 2609 6285;
Fax: +85 (2) 2609 5745; E-mail: hoishankwan@cuhk.edu.hk

Abbreviations: β -MPP, beta sub-unit of mitochondrial processing peptidase; ds cDNA, double strain cDNA; EST, expressed sequence tag; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate

here the cloning and the differential expression analyses of these two hydrophobin genes in *L. edodes*. These two hydrophobin genes may have distinct roles in fruiting.

2. Materials and methods

2.1. Construction of primordial cDNA library of *L. edodes*

Total RNA was extracted from primordia of *L. edodes* strain L54 growing on sawdust compost using TRIAGENT® (Molecular Research Center) as described by the manufacturer. Purified total RNA was converted into double stranded cDNA by SMART™ PCR cDNA Library Construction Kit (Clontech Laboratory). The resulting double strand (ds) cDNA was cloned into ZAP Express™ vector and packaged with Gigapack III Gold Packaging Extract (Stratagene). The primary cDNA library was amplified once. Phagemids pBK-CMV were mass excised from the amplified cDNA library using ExAssist helper phage and XL0LR strain according to manufacturer protocols.

2.2. Random sequencing of mass-excised cDNA clones

Colonies were randomly selected from the mass-excised library and the sizes of inserts were determined by PCR using T3 and T7 primers which flank the multiple cloning site of the pBK-CMV phagemid vector. Bacterial colonies were picked and placed into a reaction mix which contained 50 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, 1.5 mM MgCl₂, 0.2 mM each dNTPs, 0.2 μM each primers and 0.2 units *T7* DNA polymerase (Epicentre Technology) in a 20 μl volume. The PCR reaction was performed with 35 cycles of 94°C, 30 s; 58°C, 30 s, and 72°C, 2 min. Colonies carrying plasmids with an insert size greater than 0.5 kb were selected for the purification of plasmids using Wizard™ Plus Miniprep DNA Purification System (Promega). 3'-End nucleotide sequences of the cDNA inserts were determined using ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). dT25(dN) primer and an annealing temperature of 45°C were used in the sequencing reaction as a modification of the manufacturer's protocol. Sequence specific primers were designed based on the information of partial sequences and used for subsequent full-length cDNA sequence analysis.

2.3. Nucleotide and protein sequence analyses

Sequence data were edited using SeqEd version 1.0.3 (PE Applied Biosystems) and used to homology search using BlastX at the National Center for Biotechnology Information at the National Institute of Health (URL: <http://www.ncbi.nlm.nih.gov>) [17]. Multiple sequence

alignment was performed by ClustalW. Hydrophobicity plots were generated with GeneJockey (Biosoft) based on the Kyte–Doolittle algorithm [18].

2.4. Northern blot hybridization

Total RNAs were isolated from mycelium, primordium and mature fruit body of *L. edodes* strain L54 growing on sawdust compost as described in Section 2.1. Equal amounts (20 μg) of purified total RNAs from different developmental stages were resolved on a formaldehyde-denaturing agarose gel and transferred to Hybond-N membrane (Amersham) [19]. cDNA probes of hydrophobins were prepared by the Megaprime random primer labeling system (Amersham). The blotted membranes were hybridized with denatured probes in 5× SSPE, 5× Denhardt solution, 0.5% SDS, 50% formamide and 20 μg ml⁻¹ denatured salmon sperm DNA at 42°C. The signals were detected by autoradiography.

2.5. Quantitative RT-PCR of hydrophobin genes

Total RNAs from two monokaryotic parents (i.e. parent A and parent B) growing on PDA medium were isolated as described above. Total RNAs from parent A, parent B and dikaryotic mycelium, primordium and mature fruit body of *L. edodes* strain L54 were treated with RNase free DNaseI (Gibco BRL) to eliminate the contaminated genomic DNA. Two μg of each treated RNA sample was used to synthesize the first strand cDNA using SuperScript™ II RNase H-Reverse Transcriptase (Gibco BRL) with oligo dT_{12–18} primer (Gibco BRL) following the manufacturer's protocol. Two μl of each first strand cDNA sample was PCR-amplified with 10 μl of 10× PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 6 μl of 25 mM MgCl₂, 2 μl of 10 mM dNTP mix, 5 units of *Taq* DNA polymerase (Gibco BRL), 2 μl of each 10 μM hydrophobin primer and autoclaved distilled water to a final volume of 100 μl. PCR amplification was performed at 95°C for 1 min; 55°C for 30 s and 72°C for 1 min. At 20, 25, 30, 35 and 40 PCR cycles, 12 μl of PCR products was withdrawn from the PCR mixture. For PCR products at each interval, 5 μl was resolved with 2% agarose gel and stained with ethidium bromide. A pair of primers designed from an EST clone, encoding the ribosomal protein S15, were used as a PCR control in quantitative RT-PCR experiments.

3. Results and discussions

3.1. Isolation of hydrophobin genes by random sequencing of cDNA clones

Information about the molecular mechanism governing fruiting of *L. edodes* is still scarce. Zhang et al. [4] used

A *Le.hyd1*

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1      ggggagaacaaaaactccctgtttctactccagtaatcgctcaaccactaacatcttttcaacaaa
Met Leu Ser Leu Leu Ser Lys Ala Val Ser Leu Ala Ile Leu Val Thr Ala   17
66    ATG CTT TCT TTG CTC TCC AAA GCA GTC TCC CTT GCC ATC CTC GTC ACC GCT
Val Val Ala Ser Pro Ala Gly Asn Gly Val Glu Trp Ser Gly Gly Gly Thr   34
117   GTT GTT GCT AGC CCT GCT GGT AAT GGT GTC GAA TGG AGC GGT GGA GGA ACC
Thr                                                                           Thr Val   37
168   ACG tacgggtttattcaatgactgtctgggtccgctgctgttactaacgctgtacagg ACC GTC
Thr Val Thr Ala Ser Ser Pro Thr Thr Thr Val Thr Gln Ser Gln Cys Ser   54
232   ACC GTC ACC GCC TCT TCT CCT ACT ACT ACA GTC ACA CAG AGT CAA TGC AGC
Thr Gly Asp Gln Lys Cys Cys Gln Ser Val Gln Asn                             66
283   ACT GGA GAC CAA AAA TGC TGC CAG TCT GTG CAG AAT gtaggcatttcagtaattg
Ser Ser Ala Ala Gly Val Ser Ser                                           74
339   atcagtcctatgacagagttgatgacagcgtttacg TCG AGT CTT TTG GGG CTT TTG GGT
Leu Leu Gly Leu Leu Gly Ile Val Leu Ser Gly Thr Asp Val Pro Val Gly     91
397   GCC GCA GGG GTT TCC TCT ATC GTT CTC TCG GGC ACC GAT GTT CCT GTC GGG
Leu Thr Cys Leu Pro Ile Val Gly Gly Ala Cys Gln Ser Gln Ala Val Cys     108
448   CTC ACT TGC CTG CCC ATC GTT GGG GGT GCA TGC CAA TCC CAG GCC GTT TGT
Cys Thr Asp Asn Ser Tyr                                                     114
499   TGC ACC GAT AAC TCT TAT gtaaggattttctctcctcctcctgcaggatattttgagtaact
Gly Asn Leu Ile Ser Leu Gly Cys Ser Pro Leu Gln Leu                       127
560   aatgagaatttcgaag GGC AAC CTC ATC TCC CTT GGC TGC AGT CCC TTG CAA CTC
STOP
615   TAG ttgagcagcaccagatgctcgatcagctcgtgggcaactggggaatttctactagcatccta
681   agtagtagtagtacttgttcaaggaccatttaaataatgatggcagcattccgtatttctcaatacacgtt
748   attctttttgtca

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B *Le.hyd2*

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1      actcgaacaaccaacaattgtcgtttcaagtattccccagtttcatc
Met Gln Phe Lys Leu Ala Phe Val Ser Ile Ala Leu Ala Thr Leu Ala Val
48    ATG CAG TTC AAA CTT GCC TTT GTC TCC ATC GCT CTT GCT ACT CTT GCG GTC
Ala Thr Pro Ala Pro Arg Gly Glu Pro Ala Ser Ser Cys Ser Thr Gly Asp
99    GCC ACA CCT GCT CCT CGT GGT GAG CCG GCT AGC AGC TGC AGT ACA GGC GAC
Leu Gln Cys Cys Asn Thr Val Glu Pro Ala Ser Ser Pro Ser Ala Ser Thr
150   CTT CAG TGT TGC AAC ACC GTC GAG CCG GCC TCC AGT CCC TCG GCC AGT ACG
Ile Leu Gly Leu Leu Gly Ile Val Ile Gln Gly Val Asp Val Leu Val Gly
201   ATT CTT GGT CTC CTC GGT ATC GTC ATC CAA GGC GTT GAC GTT CTG GTC GGC
Leu Thr Cys Ser Pro Ile Thr Val Ile Gly Leu Glu Ser Gly Gly Cy
252   CTC ACT TGC TCT CCC ATA ACC GTT ATT GGT CTT GAA AGT GGT GGC TG gtaa
s Ser Ala Gln Ala
303   gtttatctatcccgtattttcggggtgtgtgctaacgatggccacag C TCT GCG CAG GCC
Val Cys Cys Thr Asp Asn Ser Asn G
363   GTC TGC TGC ACT GAC AAC AGT AAC G gtataatgatacctttcaattttggaccctacttgt
ly Gly Leu Ile Ser Ile Gly Cys Leu Pro Val
424   tttcggttctgatgcttgttgacag GT GGA CTC ATT TCC ATT GGA TGT CTT CCC GTC
Thr Leu Stop
481   ACT CTC TGA gcacctgtacctcgcgggctccatacacgaacagctcacctagcaggttgg
gggaaagaaagtgcacctgtggctttgatcagtcgacggatgttgagctgtagaatgaactttctt
612   cgcatttgtgtctatacgaataatgtttctatataacacaaaaaaagtagatgttgggtatgctt
679   tctagtagaacgcactgtgggttctgtgatgtgtcaggcctgaacatacaaacctca

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Fig. 1. Nucleotide sequences and deduced amino acid sequences of *Le.hyd1* (Accession No. AF217807) and *Le.hyd2* (Accession No. AF217808) of *L. edodes*. The coding nucleotides are in upper-case letters; non-coding nucleotides and introns are in lower-case letters. Nucleotide and amino acid sequences are numbered on the left- and right-hand sides, respectively.

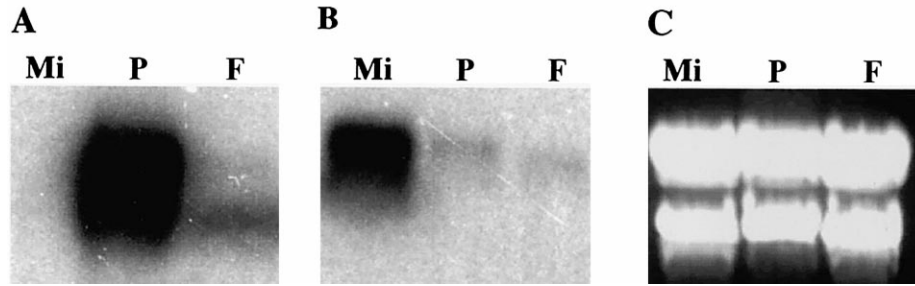


Fig. 4. Northern blotting analysis of two hydrophobin transcripts at different developmental stages of *L. edodes*. Equal amounts of total RNAs from induced dikaryotic mycelium (Mi), primordium and fruit body (F) were hybridized with ^{32}P -labeled cDNA of *Le.hyd1* (A) and *Le.hyd2* (B). (C) Equal amounts of total RNA samples were visualized by RNA gel stained with ethidium bromide before blotting.

sequences preceding the first cysteine residue were omitted in the comparison because they would contain the signal sequences of variable lengths [8] and would be specific for each hydrophobin. All hydrophobins investigated, including *Le.hyd1* and *Le.hyd2*, have a limited sequence homology with each other but possess conserved cysteine spacing. ABH2 is exceptional in that it only contains seven cysteine residues, while eight cysteine residues are conserved among all other hydrophobins. These conserved

cysteine residues are important for the general structures and functions of hydrophobins [9]. Although with limited sequence homology, the hydrophathy patterns of *Le.hyd1* and *Le.hyd2*, determined by Kyte–Doolittle algorithm [18] with a six amino acid window, were very similar to that of SC3 of *S. commune* (Fig. 3). The conserved domains and the hydrophathy patterns of *Le.hyd1* and *Le.hyd2* indicated that they belong to the Class I hydrophobins proposed by Wessels [21].

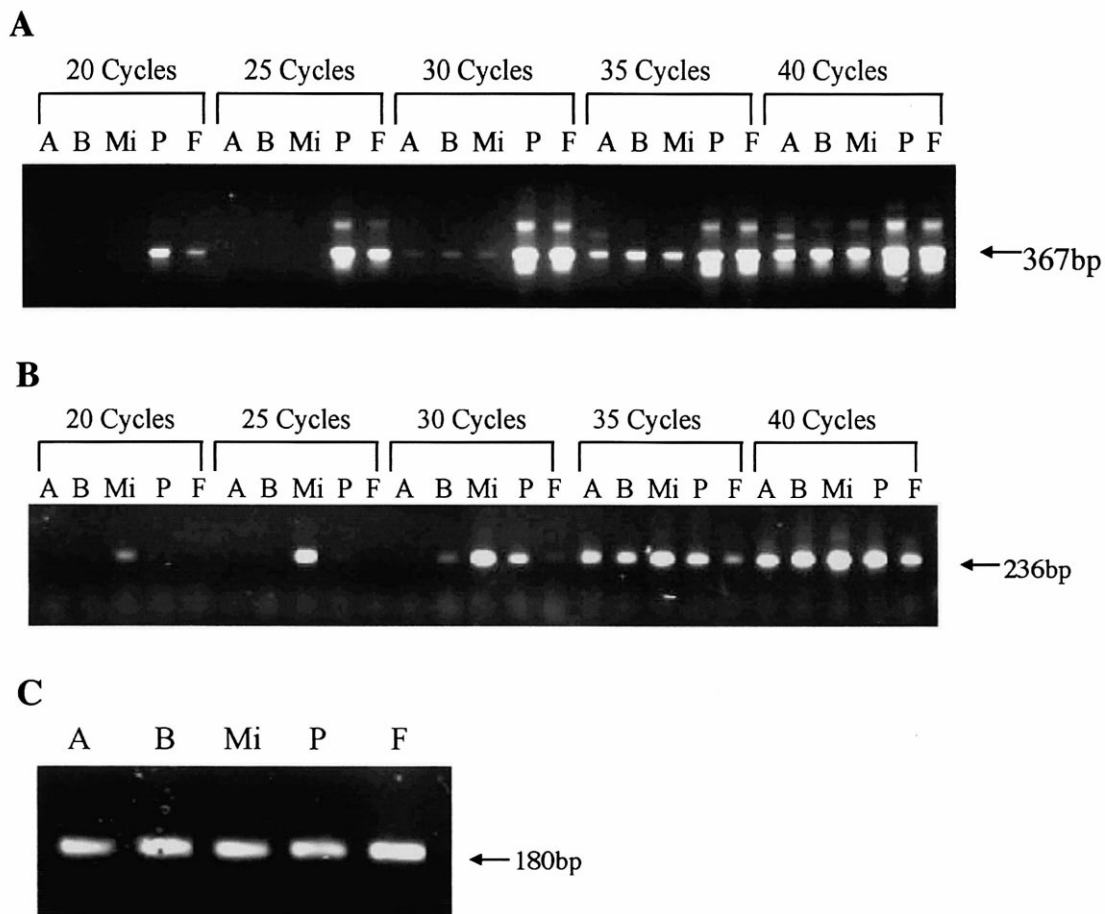


Fig. 5. Quantitative RT-PCR of two hydrophobin transcripts of *L. edodes*. The expression patterns of *Le.hyd1* (A) and *Le.hyd2* (B) at different developmental stages were analyzed by quantitative RT-PCR. The arrows indicate the sizes of hydrophobin fragments generated by specific hydrophobin primers. (C) Control of RT-PCR was carried out by using a pair of primers from ribosomal protein S15. Lane A, monokaryotic parent A; lane B, monokaryotic parent B; lane Mi, induced dikaryotic mycelium; lane P, primordium and lane F, fruit body.

3.2. Differential expression of *Le.hyd1* and *Le.hyd2*

Hydrophobins in several fungi are developmentally regulated. For examples, CoH1 and CoH2 of *C. cinereus* are highly expressed in monokaryotic mycelia [20], ABH3 and ABH1 of *A. bisporus* are highly expressed in vegetative homokaryon and fruit body, respectively [13–15], and SC3 of *S. commune* is highly expressed in both monokaryons and dikaryons while SC1 and SC4 are only highly expressed in fruiting dikaryons [11]. The expression profiles of *Le.hyd1* and *Le.hyd2* in dikaryotic mycelium, primordium and fruit body of *L. edodes* were analyzed by Northern blotting analysis (Fig. 4). *Le.hyd1* transcript was highly detected in the primordia with the molecular size of about 1.0 kb. The lower mass transcript appearing in panel A of Fig. 4 could not be found for the replicate experiment (date not shown). The *Le.hyd2* transcript (~0.9 kb), on the other hand, was only detected in the dikaryotic mycelia. Differential expression of these two hydrophobin genes during the development of fruit bodies suggest that the roles played by *Le.hyd1* and *Le.hyd2* in mushroom development are distinct from one another.

To find out whether these two hydrophobins are specifically expressed in the heterokaryotic fruiting process or monokaryotic mycelial stage, we designed primers to amplify the coding sequence of each hydrophobin cDNA sequence, a 367-bp fragment was amplified from *Le.hyd1* and a 236-bp fragment from *Le.hyd2* (Fig. 5A and B). The expression levels of these two hydrophobin genes in monokaryotic parents, dikaryotic mycelium, primordium and mature fruit body were analyzed using quantitative RT-PCR. The reproducible results were also obtained (data not shown). The Q-RT-PCR results for developmental stages agreed with the results from Northern blotting analysis. Highest expression levels of the *Le.hyd1* fragment in the primordium and the *Le.hyd2* fragment in mycelium were detected after 20 PCR cycles (Fig. 5A and B). A *L. edodes* EST, ribosomal protein S15, was used as the control for RNA loading in the RT reactions (Fig. 5C). Both hydrophobin genes showed low transcript levels in monokaryotic parents, corresponding hydrophobin PCR fragments were detected only after 35 PCR cycles.

To successfully produce a fruit body, the fungal mycelia first grow on the substrate in order to accumulate nutrients. During the mycelial growth, the fungal mycelia have to develop some ways to securely attach to the substrate. Wosten et al. [22] demonstrated that SC3p hydrophobins of *S. commune* can self-assemble and form an SDS-insoluble protein membrane coating the aerial hyphae that can attach to a Teflon surface. By analogy, in *L. edodes*, *Le.hyd2* may allow the dikaryotic mycelia to attach to the hydrophobic surface of the substrate. Higher expression of *Le.hyd2* in dikaryotic mycelia than in monokaryotic mycelia indicates that dikaryons require more *Le.hyd2* hydrophobin than the monokaryons, presumably for a higher rate of hyphal growth. The expression of

Le.hyd2, however, is different from SC3, which are abundant in both monokaryon and dikaryon [11,23].

In mushrooms, several hydrophobins are fruit-body-specific, such as the Fbh1 hydrophobin of *P. ostreatus* [16], SC1 and SC4 hydrophobins of *S. commune* [11,23] and the ABH1 of *A. bisporus* [13]. Abundant expression of *Le.hyd1* in the primordial stage suggests that it is important in *L. edodes* fruit body initiation rather than in mature fruit body maintenance.

Dikaryotic mycelia in *L. edodes* form a compact mat of hyphae on sawdust compost before fruiting occurs. In the presence of environmental cues such as cold shock, which often stress the mycelium, the hyphae form tiny primordia which may subsequently develop into fruit bodies if the environment is favorable [24]. Similar morphological changes occur in *S. commune*, in which SC4 hydrophobin was found lining air spaces within the fruit bodies and it was proposed to play important roles in organizing the complex structure of the fruit bodies and preventing collapse of the air channels during cycles of drying and wetting [21]. We think that the function of *Le.hyd1* during fruit development may be similar to SC4.

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