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Comparative Analysis of Expressed Sequence Tags from *Flammulina velutipes* at Different Developmental Stages

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***Flammulina velutipes* is a popular edible basidiomycete mushroom found in East Asia and is commonly known as winter mushroom. Mushroom development showing dramatic morphological changes by different environmental factors is scientifically and commercially interesting. To create a genetic database and isolate genes regulated during mushroom development, cDNA libraries were constructed from three developmental stages of mycelium, primordium, and fruit body in *F. velutipes*. We generated a total of 5,431 expressed sequence tags (ESTs) from randomly selected clones from the three cDNA libraries. Of these, 3,332 different unique genes (unigenes) were consistent with 2,442 (73%) singlets and 890 (27%) contigs. This corresponds to a redundancy of 39%. Using a homology search in the gene ontology database, the EST unigenes were classified into the three categories of molecular function (28%), biological process (29%), and cellular component (6%). Comparative analysis found great variations in the unigene expression pattern among the three different unigene sets generated from the cDNA libraries of mycelium, primordium, and fruit body. The 19–34% of total unigenes were unique to each unigene set and only 3% were shared among all three unigene sets. The unique and common representation in *F. velutipes* unigenes from the three different cDNA libraries suggests great differential gene expression profiles during the different developmental stages of *F. velutipes* mushroom.**

Keywords: *Flammulina velutipes*, expressed sequence tag (EST), mushroom development, gene expression

Flammulina velutipes, known as winter mushroom, is a common white-rot basidiomycete and a popular edible mushroom in East Asia. Some components of this edible mushroom have been reported to possess various biological activities, such as immunomodulatory, antitumor, antiviral, antifungal, fibrinolytic, and cholesterol-lowering properties [6]. The basidiomycete *F. velutipes* shows morphological changes to an obvious fruiting body as a large characteristic tissue that produces many basidiopores, which is a higher fungus than other microorganisms in the developmental complexes. Although it had been reported that the production of the edible mushroom is morphologically controlled by environmental factors [16], the molecular mechanism of fruiting body formation is still unclear. Studies on the changes of gene expression in *F. velutipes* during mushroom development may lead to a better understanding of the fruiting formation process, thus providing new targets and strategies for efficient production by precise controls. Functional genomic approaches provide powerful tools for identifying expressed genes. The sequencing of expressed sequence tags (ESTs) has been the most widely used technique for characterizing genes, because of its relative simplicity and ease. To date, only a few basidiomycete edible mushroom EST studies have been reported and deposited in the dbEST GenBank, such as *Agaricus bisporus* [24], *Coprinopsis cinereus*, *Laccaria bicolor* [21], *Lentinula edodes* [29], *Pleurotus ostreatus* [11, 18], and *Schizophyllum commune* [9]. Recently, using the fluorescence differential display method, several genes involved in fruit body formation in *F. velutipes* were reported [31]. In the study, 75 genes were specifically isolated and developmentally characterized. Of these stage-specific genes, only 29 genes were identified as known proteins and the other 46 genes as unknown proteins. A pileus-specific gene encoding a cell-wall-associated protein was also isolated in *F. velutipes*, which was screened through two-dimentionals protein electrophoresis [27]. Furthermore, several studies for the genes specifically expressed at the fruit body stage were reported for

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basidiomycete mushrooms, such as FDS, FVFD30, and *fvh1* from *F. velutipes* [2, 5, 12], *priB* and *mfba* from *L. edodes* [7, 14], and lamellae-specific genes and PoMTP from *P. ostreatus* [10, 25].

Different methods have accumulated a large database of molecular genetic information about edible mushrooms. EST analysis is useful for collecting genetic information and profiling gene expressions during developmental processes. As a first step towards understanding the mechanism of mushroom development, we isolated large numbers of ESTs with their expression profiles and carried out the comparative analyses during *F. velutipes* development.

MATERIALS AND METHODS

Cultivation of Mushroom

F. velutipes (ASI4019) was vegetatively grown on potato dextrose agar. To obtain primordia and fruiting bodies, mycelial plugs were inoculated on a growth medium consisting of 80% poplar sawdust, 20% rice bran, and 65% water in a 1,000 cm^3 disposable bottle after sterilization [15]. Inoculated bottles were incubated at 20°C under the dark condition for 25 days, and then transferred to fruiting conditions, where 15°C temperature, 90% humidity, and continuous light were maintained. Primordia, which were 2–3 mm in diameter, budded at 10 days after physical stimulation and were collected. After budding of primordia, fruiting was stimulated by cold shock at 4°C for 3 days. At 7 days after cold stimulation, fruiting bodies were matured in 9°C and 75% humidity conditions and collected.

Construction of cDNA Library and DNA Sequencing

Three different cDNA libraries were prepared from mycelia (Mc), primordia (Pr) and fruit bodies (Fb) of *F. velutipes*. Approximately 0.5 g of fine powders of mushroom samples were used. Total RNA was extracted with an RNeasy plus mini kit (Qiagen) and mRNA was isolated by using an Oligotex mRNA kit (Qiagen) according to the manufacturer's instructions. cDNA libraries were constructed using a cDNA library construction kit with the vector of pCNS-D2 (Corebio) according to the manufacturer's instructions. Clones for plasmid isolation were derived from primary libraries containing 1.8×10^6 CFU/ml for mycelia, 2.2×10^6 CFU/ml for primordia, and 3.7×10^6 CFU/ml for fruit bodies without amplification. The clones were randomly selected and incubated overnight in a 96-well plate with 0.2 ml of Terrific broth containing ampicillin. Plasmid DNAs were isolated by using Multiscreen NA and FB filters with Wizard SV-96 Reagent (Promega), and a total of 7,488 plasmid DNAs were isolated and sequenced. DNA sequencing was performed with a T7 primer (5'-TAATACGACTCACTATAGGG-3') using an ABI Prism BigDye Terminator Cycle Sequencing kit (Applied Biosystems) on ABI prism 3100 and 3730XL sequencers (Applied Biosystems). The conditions for 25 times thermal cycling was 96°C for 10 s, 50°C for 6 s, and 60°C for 4 min.

Sequence Analysis

Base callings of EST sequence files were performed through the Phred program in a Linux system and then transferred to FASTA formats [8]. Crossmatch program was used to trim vector sequences and the sequences containing shorter than 100 bp. The trimmed sequences were assembled using the Cap3 program and contained numerous singletons or contigs consisting of more than two EST sequences. These unique sequences containing all of the contigs and singletons were determined to be unigenes [11]. All EST sequences were deposited at the laboratory server, Bioresource Information Center (BIC; http://www.mushgenome.com) with the local BLAST system to search genes.

Using BLASTX algorithm [1], *P. ostreatus* unigenes were surveyed against both a nonredundant (NR) database (comprising nonredundant sequences from GenBank CDS translations, PDB, SwissProt, PIR, and PRF databases) and a *Saccharomyces cerevisiae* database that is deposited in the BIC server from a database maintained by the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Unigenes showing significant similarity (E value $\leq1\times10^{-5}$) to characterized proteins were categorized as identified genes. Unigenes were classified into functional groups according to the putative functions using sequence comparison with gene ontology data, which were provided by the gene ontology (GO) database (http://www.geneontology.org) [4].

Table 1. Primer sequences for RT–PCR of the EST unigenes.

EST	Primer	Sequence (5'-3')
Contig 636	Forward	GGAATTGTCCTCCAGGATTT
	Reverse	ACAGCGGACCATGTAAAAGA
Contig 556	Forward	GAGAGAAGTTTCGCGATGAA
	Reverse	ATCGTCTCAGCAAAGTCCAG
Contig 414	Forward	CCAACATCACGTACATCGAA
	Reverse	AGCTCCAGTTCAGAACAACG
Contig 838	Forward	CACTCTCACCTCGACCAACT
	Reverse	GTCGTGTTAGATTGGGTGCT
Contig 774	Forward	GGACGAAACCAAGTTTGATG
	Reverse	ATCGATTTGCTGGTGGATAG
Contig 14	Forward	CGACGAGTATGTCGTCATTG
	Reverse	ACGGGTGGGCTCTTATAATC
Contig 587	Forward	AGAAGTCGCAGCTCTCGTTA
	Reverse	CAATGGGGTACTTCAAGGTG

RT–PCR Analysis

For quantitative RT–PCR analysis, cDNAs were synthesized using MMLV–reverse transcriptase from total RNAs of mycelia, primordia, and fruit bodies, as described previously [10]. These synthetic cDNAs were used as templates in PCR reactions with specific primers designed based on each EST sequence, as shown in Table 1. Specific primers for the actin gene of *F. velutipes*, which is constantly expressed during the developmental stages, were used as a PCR control.

RESULTS AND DISCUSSION

EST Sequence Analysis of Three cDNA Libraries from *F. velutios*

A total of 5,431 high-quality EST sequences from 7,488 sequenced clones were finally prepared from three cDNA libraries of mycelium, primordium, and fruit body. These 5,431 EST sequences were assembled into 2,442 (45%)

Table 2. Summary of EST sequences from the three different cDNA libraries.

	Total	Mycelium	Primordium	Fruit body
Sequenced clones	7,488	2,688	2,112	2,688
EST sequences	5,431	1,898	1,430	2,103
Unigenes (nonredundant ESTs)	3,332	1,343	991	1,519
Singletons	2,442	1,076	800	1,223
Contigs	890	267	191	296
Redundancy (%)	39	29	31	28
Unique (%)	61	71	69	72

singlets and 890 (16%) contigs (Table 2). This corresponds to a redundancy of 39%. The total number of unique sequences consisting of contigs and singlets from three cDNA libraries represents 3,332 putative unique genes (unigenes). The redundancy values were very similar among the three different stages of mycelium, primordium, and fruit body (from 28% to 31%). The percentages of unigenes between the three different stages of mushroom (from 69% to 72%) were also very similar and high. A total of 3,332 unigenes was assumed to represent 33–42% of the total expressed genes of *F. velutipes*, given that filamentous fungi have a total of 8,000–10,000 genes [17]. The total unigenes of *F. velutipes* were compared with the protein sequences derived from the genomic sequence databases of 11 eukaryotic species (seven fungi, two plants, and two animals). The highest sequence similarity to basidiomycete *F. velutipes* unigenes was found in the basidiomycete fungi of *Coprinopsis cinereus* (74%) and *Laccaria bicolor* (72%). These proportions decreased in the ascomycete fungi of *Aspergillus nidulans* (50%), *Magnaporthe grisea* (50%), and also in the yeasts of *Candida albicans* (38%) and *Saccharomyces cerevisiae* (38%). The *F. velutipes* unigenes displayed about 35% and 33% similarities to genomic sequences from plants (*Arabidopsis thaliana* and *Oryza sativa*) and animals (*Caenorhabditis elegans* and *Drosophila melanogaster*), respectively.

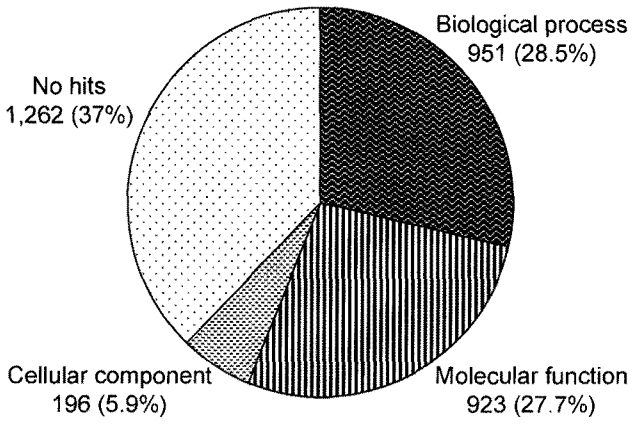

Fig. 1. Functional categories of the EST unigenes of *F. velutipes*.

Functional Annotation of EST Unigenes

The EST unigenes of *F. velutipes* were annotated functionally by homology similarity in the gene ontology database. A total of 3,332 unigenes were divided into the three principal categories: molecular function (28%), biological process (29%), and cellular component (6%) (Fig. 1 and Table 3). The remaining 37% did not show any sequence similarity. In the molecular function category to total unigenes, the catalytic activity contributed about 78%, followed by the binding, which contributed 17%. Transporter activity and transcription regulator activity were 12% and 10%, respectively. These distributions in the main ontology categories were very similar in the three unigene sets from mycelium, primordium, and fruit body stages. In the case of biological process and cellular component categories, similar gene distributions to total unigenes were observed in the three unigene sets derived from the three different stages of *F. velutipes*.

Comparative Expression Analysis of Unigenes at Different Developmental Stages

Comparison of the three unigene sets from the different stages of *F. velutipes* allows the identification of common and

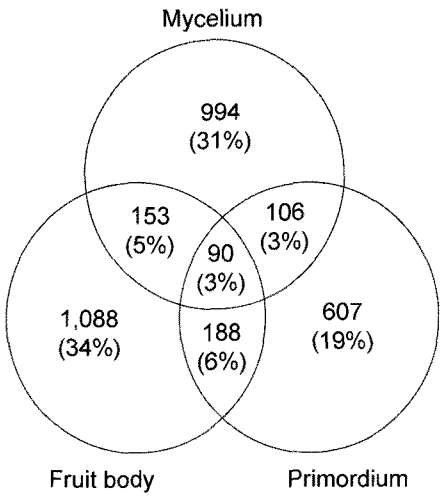

Fig. 2. Comparative analysis of three unigene sets derived from three cDNA libraries of mycelium, primordium, and fruit body. The diagram shows unique and common unigene numbers between different stages.

Table 3. Gene ontology (GO) functional assignment for the EST unigenes.

GO term	Total unigenes (%)		Stages [No. of unigenes (%)]					
			Mycelium		Primodium		Fruit body	
Molecular function	923	(100)	356	(100)	275	(100)	466	(100)
Catalytic activity	665	(72.1)	258	(72.5)	187	(68.0)	328	(70.4)
Hydrolase activity	27	(2.9)	9	(2.5)	7	(2.6)	18	(3.7)
Lyase activity	3	(0.3)	2	(0.6)	1	(0.4)	0	(0.0)
Ligase activity	2	(0.2)	0	(0.0)	1	(0.4)	2	(0.4)
Oxidoreductase activity	50	(5.4)	18	(5.1)	20	(7.3)	27	(5.8)
Transferase activity	18	(2.0)	7	(2.0)	4	(1.5)	11	(4.0)
Binding	155	(16.8)	63	(17.7)	49	(17.8)	76	(16.3)
Nucleic acid binding	8	(0.9)	1	(0.3)	3	(1.1)	6	(1.3)
Nucleotide binding	6	(0.7)	1	(0.3)	3	(1.1)	4	(0.9)
Protein binding	33	(3.6)	15	(4.2)	7	(2.6)	15	(3.2)
Transporter activity	109	(11.8)	46	(12.9)	32	(11.6)	51	(10.9)
Transcription regulator activity	89	(9.6)	33	(9.3)	38	(13.8)	43	(9.2)
Molecular transducer activity	40	(4.3)	13	(3.7)	17	(6.2)	19	(4.1)
Structural molecule activity	27	(2.9)	10	(2.8)	11	(4.0)	8	(1.7)
Enzyme regulator activity	10	(1.1)	3	(0.8)	3	(1.1)	8	(1.7)
Translation regulator activity	9	(1.0)	3	(0.8)	1	(0.4)	6	(1.3)
Antioxidant activity	4	(0.4)	1	(0.3)	2	(0.7)	3	(0.6)
Biological process	951	(100)	366	(100)	283	(100)	475	(100)
Metabolism	737	(77.5)	283	(77.3)	216	(76.3)	371	(78.1)
Cellular process	645	(67.8)	246	(67.2)	194	(68.6)	318	(67.0)
Localization	137	(14.4)	55	(15.0)	47	(16.6)	67	(14.1)
Biological regulation	96	(10.1)	36	(9.8)	39	(13.8)	51	(10.7)
Response to stimulus	68	(7.2)	29	(7.9)	22	(7.8)	34	(7.2)
Developmental process	30	(3.1)	7	(1.9)	9	(3.2)	21	(4.4)
Multi-organism process	16	(1.7)	7	(1.9)	6	(2.1)	10	(2.1)
Multicellular organismal process	12	(1.3)	2	(0.6)	2	(0.7)	9	(1.9)
Reproduction	8	(0.8)	3	(0.8)	2	(0.7)	3	(0.6)
Growth	4	(0.4)	1	(0.3)	1	(0.4)	2	(0.4)
Cellular component	196	(100)	85	(100)	65	(100)	94	(100)
Cell	195	(99.5)	85	(100)	63	(96.9)	94	(100)
Extracellular region	1	(0.5)	0	(0.00)	1	(1.5)	2	(2.1)

specific genes expressed during development. An overall analysis of specific and common sequences among the three unigene sets was performed (Fig. 2). Comparative analysis among the three unigene sets showed that 19–34% (607–1,088) of total unigenes (3,332) were specific to each unigene set, and only 3% (90 unigenes) of the total unigenes were shared among all three unigene sets. The limited number of common unigenes among the three unigene sets suggests that there are profound differences in gene expression during the different developmental stages of mushrooms.

It is expected that the redundancy values of cDNA sequences reflect the frequencies of mRNA expression. In *P. ostreatus* EST analysis, highly redundant ESTs were considered as the preferentially expressed genes at specific stages. These similar expression profiles were also confirmed by the quantitative RT–PCR analysis [11]. The most abundantly expressed genes from different developmental stages are listed in Table 4. We identified 14 unigenes with 10 or more redundancies, of which more than half (8 unigenes) were genes coding hypothetical or unknown proteins. The functionally unknown genes are omitted in Table 4's list. The top seven abundant genes during different developmental stages were FDS protein, hydrophobin, aspartic peptidase A1, transepithelial electrical resistance (TEER)-decreasing protein, FVFD16, elongation factor, and guanine nucleotide binding protein. As expected, a number of housekeeping genes were identified as translation elongation factor, ubiquitin, or ribosomal proteins. Some abundantly expressed genes were expressed at a specific developmental stage. FVFD16 protein [13] was highly represented in mycelia, but not in the developed tissues of primordium and fruit body. In contrast, FDS, TEER-decreasing protein [31], and aspartic peptidase were highly represented in the developed tissues, but not in mycelia. In a previous report, the

Table 4. The most abundantly expressed unigenes in three different cDNA libraries.

EST name	Redundancy	Proteins	Species	E-value	Stages		
					Mc	Pr	Fb
Total							
Contig 192	103	FDS protein	*F. velutipes*	6.00E-115	-	24	79
Contig 774	82	Aspartic peptidase A1	*L. bicolor*	2.00E-58	-	41	41
Contig 636	62	Hydrophobin (FV-Hyd1)	*F. velutipes*	1.00E-27	60	2	-
Contig 838	35	Hydrophobin (FV-Hyd2)	*F. velutipes*	6.00E-53	-	31	4
Contig 884	26	TEER-decreasing protein	*F. velutipes*	2.00E-129	-	17	9
Contig 482	19	FVFD16	*F. velutipes*	3.00E-18	18	-	1
Contig 446	18	Elongation factor 1-alpha	*S. commune*	0	7	7	4
Contig 804	12	Guanine nucleotide binding protein	*L. edodes*	2.00E-148	2	6	4
Mycelia stage							
Contig 636	62	Hydrophobin	*F. velutipes*	1.00E-27	-	2	0
Contig 482	18	FVFD16	*F. velutipes*	3.00E-18	-	0	1
Contig 446	7	Elongation factor 1-alpha	*S. commune*	0	-	7	4
Contig 611	6	Short-chain oxidoreductase	*S. aurantiaca*	1.00E-21	-	0	1
Contig 254	5	Ubiquitin	*S. domuncula*	3.00E-52	-	0	2
Contig 396	5	Ectomycorrhiza-regulated protein	*L. bicolor*	1.00E-42	-	0	0
Contig 544	5	Cu/Zn superoxide dismutase	*H. lixii*	8.00E-43	-	0	0
Contig 422	4	GTP-binding protein RAB5	*L. edodes*	1.00E-72	-	1	4
Contig 370	4	Peptidase S33, tricorn interacting factor 1	*P. syringae*	8.00E-59	-	0	0
Primordia stage							
Contig 774	41	Aspartic peptidase A1	*L. bicolor*	2.00E-58	0	-	41
Contig 838	31	Hydrophobin	*F. velutipes*	6.00E-53	0	-	4
Contig 192	24	FDS protein	*F. velutipes*	6.00E-115	0	-	79
Contig 884	17	TEER-decreasing protein	*F. velutipes*	2.00E-129	0	-	9
Contig 446	7	Elongation factor 1-alpha	*S. commune*	0	7	-	4
Contig 804	6	Guanine nucleotide binding protein	*L. edodes*	2.00E-148	2	-	4
Contig 303	4	S-phase specific ribosomal protein	*L. edodes*	4.00E-96	2	-	1
Contig 837	4	GTP-binding protein sar1	*L. bicolor*	3.00E-73	0	-	0
Fruiting body stage							
Contig 192	79	FDS protein	*F. velutipes*	6.00E-115	0	24	-
Contig 774	41	Aspartic peptidase A1	*L. bicolor*	2.00E-58	0	41	-
Contig 884	9	TEER-decreasing protein	*F. velutipes*	2.00E-129	0	17	-
Contig 446	4	Elongation factor 1-alpha	*S. commune*	0	7	7	-
Contig 804	4	Guanine nucleotide binding protein	*L. edodes*	2.00E-148	2	6	-
Contig 22	4	40S ribosomal protein S18	*C. cinerea*	3.00E-28	0	0	-

Mc, mycelium; Pr, primordium; Fb, fruit body.

genomic DNAs of FVFD [13] and FDS [5] were cloned from *F. velutipes*. These genes were expressed stage-specifically during development, but their functions were not known. A TEER-decreasing protein has also been found in *F. velutipes*, which was found to be identical to flammutoxin, a hemolytic pore-forming protein. The TEER decrease on the intestinal epithelial cell monolayer was accompanied by an increase in the absorption of materials [22]. A TEER-decreasing protein affecting the absorption of materials may be more necessary in the developed tissues of primordium and fruit body rather than at the mycelium stage. The aspartic proteinase is a proteolytic enzyme that is widely distributed among a variety of organisms [26]. This enzyme may be involved with the *F. velutipes* proteolytic system, more in the developed tissues rather than at the mycelium stage.

Two hydrophobin types (named Fv-Hyd1 and Fv-Hyd2) from *F. velutipes* were abundantly represented by redundancy analysis and were specifically expressed at the different development stages. The Fv-Hyd1 was abundantly expressed at the mycelium stage, and the Fv-Hyd2 in the developed tissues of primordium and fruit body. These two cDNA sequences of Fv-Hyd2 and Fv-Hyd1 corresponded with fvh1 and Fv-hyd1 cDNAs from *F. velutipes*, respectively [2, 31]. Different hydrophobins produced by the same organism could have their expression associated with a

specific mushroom structure or a slightly different function during different stages. Three hydrophobins were described in *P. ostreatus* [3, 20], four in *Schizophyllum commune* [28], and six in *Cladosporium fulvum* [23]. Hydrophobins are small secreted proteins that are unique for fungi and characterized by the presence of eight cysteine residues in conserved positions, but do not share significant sequence similarity [3]. They can play roles in diverse physiological processes including adhesion, development, and pathogenesis [19]. The deduced amino acid sequences of Fv-Hyd1 and Fv-Hyd2 have no homology, but the same general structures with eight cysteine residues in conserved positions are observed between two hydrophobins.

mRNA Expression Levels of Stage-Specific Unigenes

Seven genes with greater abundance at the specific stage of *F. velutipes* development were selected and subjected to semiquantitative RT–PCR analysis using the actin gene of *F. velutipes* as constitutive control (Fig. 3). The actin gene was expressed at all three stages of mycelium, primordium, and fruit body. The mRNA levels of hydrophobin Fv-hyd1 and two unknown unigenes (contigs 556 and 414) were expressed more strongly at the mycelium stage than at the fruit body stage, whereas the other three unigenes of hydrophobin Fv-Hyd2, aspartic peptidase, and one unknown protein were expressed more at the fruit body stage than at the mycelium stage. These results show the strong relationship between gene expression and unigene sequence redundancy as described previously.

Our EST unigene database generated from *F. velutipes* provides a significant resource for basic and applied research on mushrooms, although a high proportion of EST unigenes remains to be annotated as hypothetical proteins. A very small proportion (3%) of total unigenes was confirmed to be expressed at all stages of mycelium, primordium, and fruit body. The stage-specifically expressed unigenes in *F. velutipes* may be used to develop strategies to understand the differences in the morphology and the culture condition between mycelium growth and fruit body formation by using expression analysis methods such as DNA microarray.

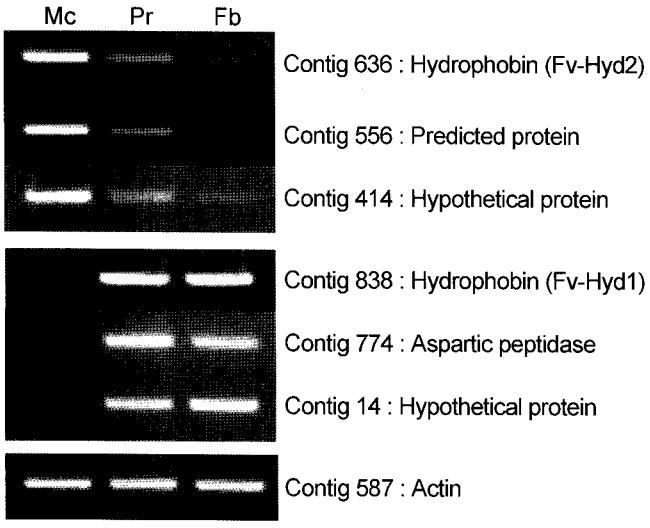

Fig. 3. RT–PCR analysis of unigenes showing high redundancy in the cDNA libraries at three different stages: mycelium (Mc), primordium (Pr), and fruit body (Fb).

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REFERENCES

1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, D. J. Miller, and M. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acid Res.* **25:** 3389–3402.
2. Ando, A., A. Harada, K. Miura, and Y. Tamai. 2001. A gene encoding a hydrophobin, fvh1, is specifically expressed after the induction of fruiting in the edible mushroom *Flammulina velutipes*. *Curr. Genet.* **39:** 190–197.
3. Asgeirsdóttir, S. A., O. M. de Vries, and J. G. Wessels. 1998. Identification of three differentially expressed hydrophobins in *Pleurotus ostreatus* (oyster mushroom). *Microbiology* **144:** 2961–2969.
4. Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, *et al.* 2000. Gene ontology: Tool for the unification of biology. *Nat. Genet.* **25:** 25–29.
5. Azuma, T., I. Yao, D. Kim, Y. Sakuma, Y. Kojima, and K. Miura. 1996. Sequence and characterization of the genomic clone of the FDS gene from the basidiomycete *Flammulina velutipes*. *Mokuzai Gakkaishi* **42:** 875–880.
6. Borchers, A. T., C. L. Keen, and M. E. Gershwin. 2004. Mushrooms, tumors, and immunity: An update. *Exp. Biol. Med.* (Maywood) **229:** 393–406.
7. Endo, H., S. Kajiwara, O. Tsunoka, and K. Shishido. 1994. A novel cDNA, priBc, encoding a protein with a Zn(II)2Cys6 zinc cluster DNA-binding motif, derived from the basidiomycete *Lentinus edodes*. *Gene* **139:** 117–121.
8. Ewing, B., L. Hiller, M. C. Wendl, and P. Green. 1998. Base-calling of automated sequencer traces using Phred. I. Accuracy assessment. *Genome Res.* **8:** 175–185.
9. Guettler, S., E. N. Jackson, S. A. Lucchese, L. Honaas, A. Green, C. T. Hittinger, Y. Tian, W. W. Lilly, and A. C. Gathman. 2003. ESTs from the basidiomycete *Schizophyllum commune* grown on nitrogen-replete and nitrogen-limited media. *Fungal Genet. Biol.* **39:** 191–198.
10. Joh, J. H., B. G. Kim, W. S. Kong, Y. B. Yoo, N. K. Kim, H. R. Park, B. G. Cho, and C. S. Lee. 2004. Cloning and developmental

expression of a metzincin family metalloprotease cDNA from oyster mushroom *Pleurotus ostreatus*. *FEMS Microbiol. Lett.* **239:** 57–62.

11. Joh, J. H., S. H. Lee, J. S. Lee, K. H. Kim, S. J. Jeong, W. H. Youn, *et al.* 2007. Isolation of genes expressed during the developmental stages of the oyster mushroom, *Pleurotus ostreatus*, using expressed sequence tags. *FEMS Microbiol. Lett.* **276:** 19–25.
12. Kim, D., T. Azuma, A. Harada, Y. Sakuma, A. Ando, Y. Tamai, and K. Miura. 1999. Cloning and sequence analysis of a cDNA for the gene FVFD30, specifically expressed during fruiting body development in *Flammulina velutipes*. *Mush. Sci. Biotech.* **7:** 95–99.
13. Kim, D. and T. Azuma. 2002. Sequence and characterization of the genomic clone of the FVFD16 and FVFD30 gene isolated from *Flammulina velutipes*. *Korean J. Mycol.* **28:** 26–31.
14. Kondoh, O., A. Muto, S. Kajiwara, J. Takagi, Y. Saito, and K. Shishido. 1995. A fruiting body-specific cDNA, mfbAc, from the mushroom *Lentinus edodes* encodes a high-molecular-weight cell-adhesion protein containing an Arg-Gly-Asp motif. *Gene* **154:** 31–37.
15. Kong, W. S., Y. H. Cho, C. S. Jhune, Y. B. Yoo, and K. H. Kim. 2004. Breeding of *Flammulina velutipes* strains adaptable to elevated-temperature. *Mycobiology* **32:** 11–16.
16. Kües, U. and Y. Liu. 2000. Fruiting body production in basidiomycetes. *Appl. Microbiol. Biotechnol.* **54:** 141–152.
17. Kupfer, D. M., C. A. Reece, S. W. Clifton, B. A. Roe, and R. A. Prade. 1997. Multicellular ascomyceteous fungal genomes contain more than 8000 genes. *Fung. Genet. Biol.* **21:** 364–372.
18. Lee, S. H., B. G. Kim, K. J. Kim, J. S. Lee, D. W. Yun, J. H. Hahn, *et al.* Yoo. 2002. Comparative analysis of sequences expressed during the liquid-cultured mycelia and fruit body stages of *Pleurotus ostreatus*. *Fungal Genet. Biol.* **35:** 115–134.
19. Linder, M. B., G. R. Szilvay, T. Nakari-Setälä, and M. E. Penttilä. 2005. Hydrophobins: The protein-amphiphiles of filamentous fungi. *FEMS Microbiol. Rev.* **29:** 877–896.
20. Ma, A., L. Shan, N. Wang, L. Zheng, L. Chen, and B. Xie. 2007. Characterization of a *Pleurotus ostreatus* fruiting body-specific hydrophobin gene, Po.hyd. *J. Basic Microbiol.* **47:** 317–324.
21. Martin, F., A. Aerts, D. Ahrén, A. Brun, E. G. Danchin, F. Duchaussoy, *et al.* 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature* **452**: 88–92.
22. Narai, A., H. Watanabe, T. Iwanaga, T. Tomita, and M. Shimizu. 2004. Effect of a pore-forming protein derived from *Flammulina velutipes* on the Caco-2 intestinal epithelial cell monolayer. *Biosci. Biotechnol. Biochem.* **68:** 2230–2238.
23. Nielsen, P. S., A. J. Clark, R. P. Oliver, M. Huber, and P. D. Spanu. 2001. HCf-6, a novel class II hydrophobin from *Cladosporium fulvum*. *Microbiol. Res.* **156:** 59–63.
24. Ospina-Giraldo, M. D., P. D. Collopy, C. P. Romaine, and D. J. Royse. 2000. Classification of sequences expressed during the primordial and basidiome stages of the cultivated mushroom *Agaricus bisporus*. *Fungal Genet. Biol.* **29:** 81–94.
25. Park, S. K., M. M. Peñas, L. Ramírez, and A. G. Pisabarro. 2006. Genetic linkage map and expression analysis of genes expressed in the lamellae of the edible basidiomycete *Pleurotus ostreatus*. *Fungal Genet. Biol.* **43:** 376–387.
26. Parr, C. L., R. A. Keates, B. C. Bryksa, M. Ogawa, and R. Y. Yada. 2007. The structure and function of *Saccharomyces cerevisiae* proteinase A. *Yeast* **24:** 467–480.
27. Sakamoto, Y., A. Ando, Y. Tamai, and T. Yajima. 2007. Pileus differentiation and pileus-specific protein expression in *Flammulina velutipes*. *Fungal Genet. Biol.* **44:** 14–24.
28. Schuren, F. H. J. and J. G. H. Wessels. 1990. Two genes specifically expressed in fruiting dikaryon of *Schizophyllum commune*: Homologies with a gene not regulated by mating-type genes. *Gene* **90:** 199–205.
29. Suizu, T., G. L. Zhou, Y. Oowatari, and M. Kawamukai. 2008. Analysis of expressed sequence tags (ESTs) from *Lentinula edodes*. *Appl. Microbiol. Biotechnol.* **79:** 461–470.
30. Watanabe, H., A. Narai, and M. Shimizu. 1999. Purification and cDNA cloning of a protein derived from *Flammulina velutipes* that increases the permeability of the intestinal Caco-2 cell monolayer. *Eur. J. Biochem.* **262:** 850–857.
31. Yamada, M., S. Sakuraba, K. Shibata, S. Inatomi, M. Okazaki, and M. Shimosaka. 2005. Cloning and characterization of a gene coding for a hydrophobin, Fv-hyd1, specifically expressed during fruiting body development in the basidiomycete *Flammulina velutipes*. *Appl. Microbiol. Biotechnol.* **67:** 240–246.