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Gene expression studies of the dikaryotic mycelium and primordium of *Lentinula edodes* by serial analysis of gene expression

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ABSTRACT

Lentinula edodes (Shiitake mushroom) is a common edible mushroom that has high nutritional and medical value. Although a number of genes involved in the fruit of the species have been identified, little is known about the process of differentiation from dikaryotic mycelium to primordium. In this study, serial analysis of gene expression (SAGE) was applied to determine the gene expression profiles of the dikaryotic mycelium and primordium of *L. edodes* in an effort to advance our understanding of the molecular basis of fruit body development. A total of 6363 tags were extracted (3278 from the dikaryotic mycelium and 3085 from the primordium), 164 unique tags matched the in-house expressed sequence tag (EST) database. The difference between the expression profiles of the dikaryotic mycelium and primordium suggests that a specific set of genes is required for fruit body development. In the transition from the mycelium to the primordium, different hydrophobins were expressed abundantly, fewer structural genes were expressed, transcription and translation became active, different genes became involved in intracellular trafficking, and stress responses were expressed. These findings advance our understanding of fruit body development. We used cDNA microarray hybridization and Northern blotting to verify the SAGE results, and found SAGE to be highly efficient in the performance of transcriptome analysis. To our knowledge, this is the first SAGE study of a mushroom.

Introduction

Lentinula edodes, commonly known as the Shiitake mushroom or Xianggu, is a popular edible mushroom due to its taste and high medicinal value. As *L. edodes* is a heterothallic homobasidiomycete, it can be easily cultivated in laboratories, and therefore, used in studies of mushroom genetics and physiology. *L. edodes* has a similar life cycle to other basidiomycetes (Kues 2000; Kues & Liu 2000; Moore 1998; Wessels 1993, 1994): the dikaryotic mycelia propagate vegetatively in the presence of adequate nutrients, and under certain environmental stresses, they aggregate to form primordia, which gradually develop into mature fruit bodies with specialized mushroom tissues. In mature fruit bodies, nuclear fusion occurs in the basidia, which are located in the mushroom gills. Within each basidium, meiosis generates four genetically unique haploid basidiospores, which are then dispersed to repeat the life cycle.

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The transition from dikaryotic mycelium to primordium is critical for fruit. Certain environmental conditions can induce the formation of the primordium, which then develops into a mature fruit body. Therefore, the identification of differentially expressed genes through the comparison of the gene expression profiles of the dikaryotic mycelium and primordium should help us to understand fruit body initiation. A number of differentially expressed genes at various developmental stages of *L. edodes* have been cloned and studied, for example, cAMP (Takagi *et al.* 1988), *priA* (Kajiwara *et al.* 1992), *priBc* (Endo *et al.* 1994), *mfbAc* (Kondoh & Shishido 1995), *uck1* (Kaneko *et al.* 1998), *lac*1 and *lac*2 (Zhao & Kwan 1999), *Le.paa* (Ishizaki *et al.* 2000), *Le.hyd1* (Ng *et al.* 2000), and *Le.rnr2c* (Kaneko & Shishido 2001). In addition, various molecular techniques have been applied to study fruit body development in *L. edodes*. Thirteen differentially expressed genes were found in the fruit body using RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) (Leung *et al.* 2000), and six fruit body specific genes were identified by differential screening (Hirano *et al.* 2004). Using cDNA representational difference analysis (cDNA-RDA), 105 genes were isolated from the vegetative mycelium, primordium, and mature fruit body of *L. edodes* (Miyazaki *et al.* 2005). In addition, about 800 expressed sequence tags (ESTs) from the primordium were recently identified in our laboratory. Although these techniques provide valuable information on the molecular mechanisms of fruit body development in *L. edodes*, they do not generate high throughput results and comprehensive data.

High-throughput techniques, such as the serial analysis of gene expression (SAGE) and microarray hybridization, have been developed for transcriptome analysis. SAGE was first proposed by Velculescu *et al.* (1995) to analyse global expression profiles. The technique allows the simultaneous comparative and quantitative analysis of transcripts (Yamamoto *et al.* 2001) based on the acquisition of short tag sequences (9–13 bp) from a defined position in the mRNA. Multiple tags are concatenated before sequencing, and each tag contains sufficient information for identification (Velculescu *et al.* 1995, 1997). SAGE can be used to study any organisms for which the genomes have yet to be sequenced, because the transcript prerequisite is not necessary (Huang *et al.* 2005; Velculescu *et al.* 2000). SAGE is effective in providing quantitative and comprehensive profiles (Vedoy *et al.* 1999; Velculescu *et al.* 2000), and has, therefore, been used in genome-wide studies of yeast (Varela *et al.* 2005; Velculescu *et al.* 1997) and rice (Matsumura *et al.* 1999). It has also been used to compare expression profiles at different developmental stages or conditions in species, such as in *Cryptococcus neoformans* under different temperatures (Steen *et al.* 2002) and *Arabidopsis* pollen under cold stress (Lee & Lee 2003). It has also been used for diagnosis and therapeutics, and especially for studying cancers, such as melanoma (Weeraratna *et al.* 2004), and thyroid tumours (Diehl *et al.* 2005).

In this study, SAGE was used to produce mRNA expression libraries of the dikaryotic mycelium and primordium of *L. edodes*. SAGE tags were quantitatively recorded and matched to our in-house EST database. A comparison of the SAGE libraries for the two developmental stages revealed different gene expression profiles, and allowed genes potentially related to fruit to be identified. The expression patterns of the selected tags were validated by cDNA microarray hybridization (Duggan *et al.* 1999) and northern blotting. Some of the unknown abundant transcripts were confirmed to be differentially expressed in the primordium, and these genes will be invaluable in further investigations of fruit body development. Through the comparison of SAGE data from various developmental stages of *L. edodes*, a more comprehensive analysis of the developmental processes of the species can be obtained (Chum & Kwan 2005). To our knowledge, this is the first SAGE study of a mushroom.

Materials and methods

Mushroom cultivation and RNA extraction

Lentinula edodes strain L54 was cultivated on artificial logs (80 % dry sawdust and 20 % dry wheat bran) at 25 °C for one month to allow mycelium growth. Once the artificial logs were fully covered with mycelia, they were subject to a cold shock at 4 °C for pinning, followed by cultivation at 8 °C for several weeks to allow primordium initiation. RNA was extracted from the mycelium and primordium using TRI Reagent® (Molecular Research Center). Poly (A^+) RNA was isolated using an mRNA isolation kit (Boehringer Mannheim). The mycelial mRNA was extracted when the whole artificial logs were almost completely covered with mycelium and the primordial mRNA was extracted when the primordium was around 2–6 mm diam, which is similar to stage 1 primordia in the basidiomycete *Coprinopsis cinereus* (Kues 2000).

Generation and analysis of the SAGE library

An I-SAGE™ kit (version A; Invitrogen™, Life Technology) was used to produce the SAGE libraries for the dikaryotic mycelium and primordium. The total RNA from the dikaryotic mycelium and primordium was used to generate the SAGE libraries with the I-SAGE™ kit according to the manufacturer's instructions. The mRNA was bound to oligo(dT) magnetic beads. SuperScript™ II reverse transcriptase was used to synthesize cDNA from the mRNA–bead complexes. 3′ cDNA-beads were digested by the 4 bp cut anchoring enzyme *Nla*III and divided into two pools. Two pairs of primers were used for the amplification of NAD^+ dependent 15-hydroxprosprostrglandin dehydrogenase (5′-GCAATTACCCATGGCCTGAT-3′ and 5′-GTG GCAAGCGTTTCCTCT-3′) and glyceraldehydes-3-phosophate dehydrogenase (5′-GGCAGCAAACACCAAGAG-3′ and 5′-GGAT TTGCTGGTGCAGTA-3′) of *Lentinula edodes* to verify whether the cDNA synthesis and *Nla*III digestion had been successful. Each cDNA pool was ligated with two *ca* 40 bp adapters (A & B) using T4 DNA ligase. The ligation products were digested with tagging enzyme *Bsm*FI, which recognizes linker sequences and cleaves 10–14 bp downstream. The magnetic beads were discarded and the released linker-tags were blunted with T4 DNA polymerase. Two pools of linker-tags were subjected to a Klenow reaction to fill in the 5′ overhangs and then ligated to form *ca* 100 bp ditags. The ditags were diluted to 1:40 and amplified by PCR for 32 cycles (95 °C for 30 s, 55 °C for 1 min, 70 °C for 1 min), and were then purified from the PCR products by 12 % polyacrylamide gel electophoresis.

The purified PCR products were digested by *Nla*III again to obtain 26 bp ditags. The released ditags were separated from the linkers by 12 % polyacrylamide gel electrophoresis, and the purified ditags were then concatemerized and size fractionated from 8 % polyacrylamide gel. Concatemers of different sizes were inserted into pZero®-1 plasmid and transformed into One Shot® TOP 10 Electrocomp™ *Escherichia coli* by electroporation. Zeocin-resistant clones were PCR screened using M13 (−20) forward and reverse primers. The PCR products were resolved in 1 % agarose gel, and clones with an insert length of >400 bp were subjected to sequencing. The sequences were analysed using SAGE™ Analysis Software (version B, Invitrogen) to extract and quantify the tags from the concatemers. The tags from both SAGE libraries were matched against the *L. edodes* EST database that was produced in our laboratory. The difference between the expression of the tags at the different developmental stages was analysed using the Fisher's exact test.

cDNA microarray hybridization

Most in-house ESTs and RAP-PCR clones (about 500 clones) were robotically printed on a chip in triplicate for hybridization analysis after PCR amplification and purification. The total RNA extracted from the dikaryotic mycelium and primordium was used to generate the cDNA for probe synthesis. The cDNA generated from the dikaryotic mycelium and primordium was labelled with Cy3 and Cy5, respectively, and reversed in repeated experiments. Following hybridization, the probes were detected by using TSA from a MICROMAX™ TSA™ Labelling and Detection Kit (NEN® Life Science Products). The microarray images were scanned by a ScanArray® 4000 (Biochip® Technologies, Packard BioScience) and analysed using QuantArray® Microarray Analysis Software (Biochip® Technologies, Packard BioScience). In the normalization, the intensity for each spot was calculated by the following: (1) calculation of the total intensity (I_t) of all spots in the image *via*

$$I_t = \sum (I_a - I_b) \quad \text{Eq. (1)}$$

where I_a was measured intensity for each spot and I_b was the background intensity for the spot. (2) Calculation the normalization factor (N_c) for each channel using

$$N_{c2} = I_{t2}/I_{tc} \quad \text{Eq. (2)}$$

where I_{tc} and I_{t2} were the total intensity for hybridization samples either from mycelium or primordium. (3) Calculation of the normalized intensity of a data point (I_n) using

$$I_n = I_a(N_{c2}) \quad \text{Eq. (3)}$$

Hydrophobin genes (PEL0163 and PEL1258) were used as controls for the cDNA microarray analysis. There expression profiles at dikaryotic mycelium and primordium were consistent with previous results (Ng *et al.* 2000). Student's t-test from the SPSS software was applied to examine the statistical significance ($P > 0.05$) of the results obtained from the various slides.

Northern blotting analysis

The total RNA from the dikaryotic mycelium and primordium was transferred to nylon membranes (Hybond-N+, Amersham). Selected EST clones were then amplified by T3 and T7 primers with Dig PCR labelling solution (Boehringer Mannheim) and hybridized to the membranes. After washing, the signals were detected through chemiluminescence by exposure to X-ray films (Kodak). The film images were quantified using the Kodak ID 3.5.3 image analyses program.

Results

Dikaryotic mycelial and primordial SAGE tag analysis and annotation

Two SAGE libraries were constructed and sequenced to produce RNA expression profiles for *Lentinula edodes* at the dikaryotic mycelium and primordium stages (Table 1). All of the tags were listed along with their abundance on the NCBI Gene Expression Omnibus (GEO) website (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi). Of 3545 unique tags, 759 were recorded twice or more, whereas the remaining tags were detected only once in either stage. As there is no annotated genome sequence for *L. edodes*, we matched all of the SAGE tags with our in-house EST database. One hundred and sixty-four unique tags showed homologies to in-house ESTs and tags that did not match any of the EST sequences were designated as unknown. To examine the transcriptome difference between the dikaryotic mycelium and primordium, statistical analysis was performed using Fisher's exact test. Tags with two-fold or greater differences ($P < 0.05$) in count numbers were taken as differentially expressed in the two developmental stages. Thirty-four tags showed differential expression in the dikaryotic mycelium, and 40 unique tags showed differential expression in the primordium (Tables 2

Table 1 – Summary of SAGE libraries for dikaryotic mycelium and primordium of *Lentinula edodes*

	Dikaryotic mycelium library	Primordium library	Combination of dikaryotic mycelial and primordial libraries
Total tags[a]	3278	3085	6363
Unique tags[b]	1984	1895	3545
Unique tags with a copy no. ≥3	184	191	293
Identified unique tags[c]	108	113	164
Total identified unique tags with a copy no. ≥3	66	78	93

Due to the inevitable sequencing errors, the results should be regarded as a slight overestimation of the true numbers. (Velculescu *et al.* 1995, 2000; Zhang *et al.* 1997).
a Total number of tags in the SAGE libraries.
b Number of unique tags in the SAGE libraries, tags that appeared in both libraries were counted as one unique tag.
c Number of unique tags that matched the in-house EST database, tags that appeared in both libraries were counted as one unique identified tag.

Table 2 – SAGE tags with higher abundance in dikaryotic mycelium *versus* primordium of *Lentinula edodes*

Tag sequence[a]	Copies[b]		Homology (accession no.)[c]	Organism[d]	E-value[e]	EST clone (accession no.)[f]	P-value[g]	Gene ontology
	Myc	Pri						
GCTTGACGAG	83	0	–	–	–	–	2.20E-16	–
GAAGACGAAA	61	11	–	–	–	01B02 (CO501287)	3.66E-09	–
TACATACATA	14	3	40s ribosomal protein S12 (O14062)	*Schizosaccharomyces pombe*	4.2E-41	PEL0481 (CN605955)	1.33E-02	–
TTTTCTATAT	56	0	–	–	–	–	2.20E-16	–
AAGTTCACCA	15	5	Hydrophobin-1 precursor (P52748)	*Pisolithus tinctorius*	7.86E-24	17D01 (CO501757)	4.30E-02	–
TCCCCGTACA	23	10	–	–	–	–	3.74E-02	–
CCACTCCTTT	23	1	–	–	–	HKLC15319 (HKLC15319)	3.52E-06	–
CAGTTCAAAC	60	3	Hydrophobin-3 precursor (O13300)	*Agaricus bisporus*	5.09E-19	10E01 (CO501611)	2.95E-14	–
GATATTGTCT	40	0	Hydrophobin-3 precursor (O13300)	*A. bisporus*	1.42E-17	03G08 (CO501401)	2.94E-12	–
CAATAAATGT	17	1	–	–	–	HKLC00862 (HKLC00862)	1.59E-04	–
CTAATGGTGT	17	1	–	–	–	HKLC15167 (HKLC15167)	1.59E-04	–
GAGTCTTCGA	17	0	–	–	–	HKLC15181 (HKLC15181)	1.69E-05	–
GATCCAGATG	7	0	Isopenicillin N epimerase (Q03046)	*Amycolatopsis lactamdurans*	1.01E-07	LedoSEQ11299 (EB009543)	1.59E-02	GO:0008152
CTCTTAGTCT	18	0	Lectin (BAD16585)	*Pleurotus cornucopiae*	3.0E-13	12A02 (CO501640)	8.55E-06	–
TCTCTCGAAA	16	0	Mannose-P-dolichol utilization defect 1 protein (Q60441)	*Cricetulus griseus*	3.87E-32	LedoSEQ7045 (EB016041)	3.34E-05	GO:0005515
GGTGTACAAT	13	0	–	–	–	HKLC06022 (HKLC06022)	2.59E-04	–
GGGCTACTGG	12	0	–	–	–	–	5.14E-04	–
CGACCGGTGG	9	0	Polyporopepsin (P17576)	*Irpex lacteus*	3.00E-38	HKLC10296 (HKLC10296)	4.02E-03	GO:0006508, GO:0007586
AATTTAAGAG	11	0	–	–	–	HKLC10230 (HKLC10230)	1.02E-03	–
GTGAAAATGC	11	0	–	–	–	HKLC15593 (HKLC15593)	1.02E-03	–
TATGTTGTAC	11	0	–	–	–	–	1.02E-03	–
AATAACGGCT	22	7	Phosphatidylethanolamine binding protein (NP_061346)	*Mus musculus*	2.0E-10	PEL1123 (CN606240)	8.83E-03	GO:0005515
TCTTCTTATG	10	0	–	–	–	06C02 (CO501483)	2.02E-03	–
TTCTTCGCTC	10	0	–	–	–	HKLC15722 (HKLC15722)	2.02E-03	–
TACCAATAGT	11	1	Phosphatidylserine decarboxylase proenzyme 2 precursor (P53037)	*Saccharomyces cerevisiae*	2.25E-15	HKLC10043 (HKLC10043)	6.58E-03	GO:0006656
GGCGCTAATA	9	0	–	–	–	–	4.02E-03	–
GTCTCATAGA	8	0	–	–	–	–	7.98E-03	–
TAGTTTTCAA	10	2	Hydroxyproline-rich glycoprotein (XP_666323)	*Cryptosporidium hominis*	5.24E-27	LedoSEQ1029 (EB008797)	5.00E-29	GO:0010404
CCGAAACAGA	7	0	–	–	–	HKLC01465 (HKLC01465)	1.59E-02	–
GTTTCTTGGT	7	0	–	–	–	HKLC07375 (HKLC07375)	1.59E-02	–
GAAAGGACAT	6	0	–	–	–	–	3.16E-02	–

(*continued on next page*)

Table 2 – (continued)

Tag sequence[a]	Copies[b]		Homology (accession no.)[c]	Organism[d]	E-value[e]	EST clone (accession no.)[f]	P-value[g]	Gene ontology
	Myc	Pri						
GTGAAGACGG	6	0	–	–	–	HKLC06708 (HKLC06708)	3.16E-02	–
TACTATAAAG	6	0	–	–	–	LedoSEQ7777 (EB016592)	3.16E-02	–
TATGCAGTAG	6	0	–	–	–	HKLC02227 (HKLC02227)	3.16E-02	–

a SAGE tag is 10 bp sequence downstream of the *Nla*III site (CATG).
b Number of tags in the SAGE libraries.
c Putative homologous gene product of the EST that matched the SAGE tag. Different SAGE tags may match with different ESTs that have same homology. Homology searches were performed using BLASTX (Altschul *et al.* 1997). The best homology from Swiss-prot and NCBI RefSeq, with highest identity score, found in each search is shown. SAGE tags matching to ESTs that could not be matched to genes in GenBank are referred to as unknowns. Those without entry are SAGE tags that could not be matched to any *Lentinula edodes* EST or gene in GenBank.
d E-value (expected value) is that reported by BLASTX.
e Organism of the best homology reported by BLASTX.
f Accession no. of the matched *Lentinula edodes* EST or gene in the GenBank.
g P-value (the probability of differential expression) comparing tag abundances in dikaryotic mycelium and primordium by using Fisher's exact test. Tags with P-value smaller than 0.05 were shown.

and 3). In both developmental stages, about 60 unique tags had similar expression levels ($P > 0.05$; Table 4), but only those with more than two copies in either stage were analysed.

Gene expression profiles of the mycelium and primordial stages

We found great differences in the gene expression profiles of the dikaryotic mycelium and primordium. Many genes were expressed abundantly in one stage but at a very low level in the other stage (Fig 1). Hydrophobin was a typical example. The tag representing *Le.hyd*1 (hydrophobin 1) was found in the primordium but not in the dikaryotic mycelium, whereas tags representing *Le.hyd*2 and other hydrophobins were observed in the dikaryotic mycelium only. The cellular roles of the matched unique tags were categorized using the Expressed Gene Anatomy Database (EGAD) of the Institute for Genomic Research (TIGR) (http://www.tigr.org/egad/), and only tags with ≥3 counts in either developmental stage were analysed.

Tags with a greater abundance in the dikaryotic mycelial stage

The most differentially expressed transcripts among the identified tags in the dikaryotic mycelial stage were genes that encode the structural proteins hydrophobins 3. Other highly expressed transcripts correspond to proteins that function in stress response, metabolism, and cellular growth, such as lectin, phosphatidylethanolamine binding protein (PEBP), isopenicillin N epimerase, hydroxyproline-rich glycoprotein, phosphatidylserine decarboxylase proenzyme 2 precursor, mannose-P-dolichol utilization defect 1, and polyporopepsin.

Tags with a greater abundance at the primordial stage of Lentinula edodes

The gene that encodes for signal transduction proteins, the priA precursor, was differentially expressed in the primordium. The tag representing another type of hydrophobin, *Le.hyd*1, was also specifically expressed in the primordium (Table 3). Genes encoding ribosomal proteins were, in general, more abundant in the primordium than in the dikaryotic mycelium (Table 3). The acyl carrier protein encoding gene, which is involved in metabolism, was up-regulated at the primordium stage, and the genes that encode BiP and metallothionin for stress response were differentially expressed in the primordium. An unclassified gene that encodes protein riboflavin aldehyde-forming enzyme was also found to be highly expressed in the primordium.

Comparative analysis of the SAGE and cDNA microarray hybridization results

SAGE tags matched about 30 % of the cDNA microarray hybridization clones. About 30 % had count numbers more than 2. The expression levels of the ESTs that matched the SAGE tags were determined by cDNA microarray hybridization. Two repeats of cDNA microarray hybridizations with reverse labelling dyes were performed on mycelium and

Table 3 – SAGE tags with higher abundance in primordium *versus* dikaryotic mycelium of *Lentinula edodes*

Tag sequence[a]	Copies[b]		Homology (accession no.)[c]	Organism[d]	E-value[e]	EST clone (accession no.)[f]	P-value[g]	Gene ontology
	Myc	Pri						
CCAATCCCAG	0	83	Hydrophobin 1 (AAF61065)	*Lentinula edodes*	1.0E-33	PEL0163 (CN605708)	2.20E-16	–
TCCCTATTAA	4	30	–	–	–	–	2.13E-06	–
ATGGGGCATA	0	29	–	–	–	–	7.05E-10	–
CCAATGACAG	4	24	40S ribosomal protein S27a (P62978)	*Cavia porcellus*	2.16E-25	HKLC10859 (HKLC10859)	7.19E-05	GO:0006412
CGCTTAGGTT	3	22	–	–	–	HKLC15204 (HKLC15204)	5.94E-05	–
ACATCTTGGG	7	18	–	–	–	HKLC10894 (HKLC10894)	2.56E-02	–
GGTGCATCCT	2	16	–	–	–	–	5.30E-04	–
TACGCCGTGA	2	16	–	–	–	HKLC10931 (HKLC10931)	5.30E-04	–
GGCTTCGGTC	2	16	60S acidic ribosomal protein P2 (Q9C3Z5)	*Podospora anserina*	1.02E-36	LedoSEQ10397 (EB008872)	5.30E-04	GO:0006412,GO:0006414
ATCGCTCGTC	0	16	60S ribosomal protein L37a (Q9VMU4)	*Drosophila melanogaster*	1.01E-30	HKLC06595 (HKLC06595)	9.08E-06	GO:0006412
TCAAGCTCCG	1	15	40S ribosomal protein S14 (P14130)	*D. melanogaster*	5.69E-67	LedoSEQ1170 (EB009865)	1.89E-04	GO:0006412
TCGGGCTCGG	1	12	40S ribosomal protein S2 (P31009)	*D. melanogaster*	6.54E-100	LedoSEQ2474 (EB012674)	1.37E-03	GO:0006412
AATCGTGTAC	0	12	Uncharacterized protein Afu6g02800 precursor (Q4WCX9)	*Aspergillus fumigatus*	1.33E-11	LedoSEQ4549 (EB014188)	1.66E-04	–
TATTCCTCGC	3	11	–	–	–	–	3.06E-02	–
GAACTGCACG	1	10	40S ribosomal protein S3aE (P40910)	*Candida albicans*	9.56E-99	LedoSEQ6537 (EB015655)	5.05E-03	GO:0006412
CTATTCTAAC	0	10	–	–	–	HKLC06768 (HKLC06768)	7.10E-04	–
TTGCTCGTGC	2	9	Protein priA precursor (Q01200)	*L. edodes*	5.37E-45	AF146050 (AF146050)	3.35E-02	–
CTTCTGATAT	1	9	Riboflavin aldehyde-forming enzyme (AAB62250)	*Schizophyllum commune*	1.80E-04	PEL11063 (CN606187)	9.59E-03	–
AAATGCTTAC	1	8	40s ribosomal protein S12 (P46405)	*Sus scrofa*	1.2E-44	PEL0097 (CN605688)	1.81E-02	GO:0006412
CCAGCCATTG	0	8	60S ribosomal protein L23 (P04451)	*Saccharomyces cerevisiae*	1.62E-28	HKLC06887 (HKLC06887)	3.03E-03	GO:0006412
CACACAGAAT	1	8	–	–	–	–	1.81E-02	–
TAAACGAGGT	1	8	–	–	–	–	1.81E-02	–
TTGTTAGAAT	0	8	–	–	–	–	3.03E-03	–
ACAAACTGCG	0	7	60S ribosomal protein L35-3 (Q9M3D2)	*Arabidopsis thaliana*	2.96E-14	HKLC00403 (HKLC00403)	6.26E-03	GO:0006412
CTTCTGTACT	1	7	60S ribosomal protein L6-2 (Q9C9C6)	*A. thaliana*	7.57E-55	LedoSEQ3613 (EB013503)	3.38E-02	GO:0006412
AAGCGTCTGG	1	7	Cell division control protein 12 (P32468)	*S. cerevisiae*	2.34E-19	HKLC07444 (HKLC07444)	3.38E-02	GO:0007047
GCCGTTCGCT	0	7	-	–	–	–	6.26E-03	–
CACCCCCAAC	0	7	Hydrophobin-3 precursor (O13300)	*Agaricus bisporus*	3.15E-13	PEL0059 (CN605671)	6.26E-03	–
AGGAAGCCCG	0	6	40S ribosomal protein S22-B (Q3E7Y3)	*S. cerevisiae*	4.03E-24	HKLC04139 (HKLC04139)	1.29E-02	GO:0006412
GTCGCCGATG	0	6	Glucose-regulated protein homolog precursor (P78695)	*Neurospora crassa*	4.17E-08	HKLC10349 (HKLC10349)	1.29E-02	GO:0051084
TTCCCATAAG	0	6	Metallothionein (AAS19463)	*Paxillus involutus*	3.00E-06	PEL0202 (CN605733)	1.29E-02	GO:0016209
AAATTGAAAA	0	6	–	–	–	–	1.29E-02	–
AACAAGCCAG	0	6	–	–	–	HKLC00325 (HKLC00325)	1.29E-02	–

(*continued on next page*)

Table 3 – (continued)

Tag sequence[a]	Copies[b]		Homology (accession no.)[c]	Organism[d]	E-value[e]	EST clone (accession no.)[f]	P-value[g]	Gene ontology
	Myc	Pri						
GTCATTGTGA	0	6	–	–	–	HKLC07149 (HKLC07149)	1.29E-02	–
ATGTCGATCC	0	5	60S ribosomal protein L8-A (P17076)	*Saccharomyces cerevisiae*	1.85E-86	LedoSEQ2638 (EB012793)	2.67E-02	GO:0006412
GCAGTAGAAG	0	5	Acyl carrier protein (Q7VKH6)	*Haemophilus ducreyi*	3.55E-12	HKLC01303 (HKLC01303)	2.67E-02	GO:0006633
ACCGACCCGA	0	5	–	–	–	HKLC14980 (HKLC14980)	2.67E-02	–
CGATATGCCC	0	5	–	–	–	–	2.67E-02	–
GTTGATCCTT	0	5	–	–	–	–	2.67E-02	–
TGTACTCTAC	0	5	–	–	–	HKLC07234 (HKLC07234)	2.67E-02	–

a SAGE tag is 10 bp sequence downstream of the NlaIII site (CATG).
b Number of tags in the SAGE libraries.
c Putative homologous gene product of the EST that matched the SAGE tag. Different SAGE tags may match with different ESTs that have same homology. Homology searches were performed using BLASTX (Altschul *et al.* 1997). The best homology from Swiss-prot and NCBI RefSeq, with highest identity score, found in each search is shown. SAGE tags matching to ESTs that could not be matched to genes in GenBank are referred to as unknowns. Those without entry are SAGE tags that could not be matched to any *Lentinula edodes* EST or gene in the GenBank.
d *E*-value (expected value) is that reported by BLASTX.
e Organism of the best homology reported by BLASTX.
f Accession no. of the matched *L. edodes* EST or gene in the GenBank.
g *P*-value (the probability of differential expression) comparing tag abundances in dikaryotic mycelium and primordium by using Fisher's exact test. Tags with *P*-value smaller than 0.05 were shown.

primordium cDNA samples to verify the variance between the data obtained from the two hybridization experiments. Student's *t*-test was used to compare the expression levels across the slides, and revealed no significant difference ($P > 0.05$) in the data obtained from any two slides. The relative expression level of the cDNA clone of each transcript was correlated with the abundance of each unique SAGE tag. The hybridization results were generally consistent with the SAGE results, but the consistency of the expression profiles obtained by the SAGE and cDNA microarray hybridizations was affected by the ESTs sequences and the count numbers of the SAGE tags. ESTs with repeating sequences, such as serine rich proteins and cysteine rich proteins, showed inconsistent results in SAGE and microarray hybridization. The expression profiles of the more abundant SAGE tags (count number > 40) were the same as those of the cDNA hybridization clones. The consistency of the expression profiles of the SAGE tags and the cDNA microarray hybridization clones decreased with the count number of the SAGE tags, only about 25 % consistency was observed when the SAGE tags copy number was below 3.

Validation of the SAGE results by northern blotting analysis

The SAGE expression patterns of five selected transcripts were confirmed by northern blotting. The transcripts were PEL0196, PEL0525, PEL0549, PEL0710, and PEL1063 (Fig 2). These transcripts had similar expression ratios to those in the SAGE, cDNA microarray hybridization, and northern blotting results.

Discussion

We report the first SAGE studies of gene expression in basidiomycetes. SAGE is an effective technique for quantitatively analysing both complete gene expression profiles in isolation and across different profiles (Tuteja & Tuteja 2004; Velculescu *et al.* 1995, 1997). We aimed to use SAGE to examine the gene expression profiles at the initiation of fruit body development. SAGE tags from the dikaryotic mycelial and primordial stages of a basidiomycete would also allow a comparison with SAGE tags obtained from other developmental stages or conditions to generate a more comprehensive transcriptome analysis of *Lentinula edodes*. A total of 3545 unique tags were obtained from the dikaryotic mycelium and (stage I) primordium of *L. edodes*. About 26 % of these tags matched to our in-house EST database including cDNA fragments obtained from EST, cDNA microarray, and sequencing-by-synthesis (Margulies *et al.* 2005). The results showed that SAGE yielded different sets of genes from those obtained with other methods, and that compared with EST, SAGE is more efficient in detecting low-abundance transcripts (Sun *et al.* 2004). The transition from dikaryotic mycelium to primordium is an essential step in the fruit of mushrooms, and genes that are differentially expressed in either stage may play a crucial role in the initiation of the fruit body. The SAGE results showed the difference in gene expression profiles between the two developmental stages (Fig 1), and revealed that most of the highly expressed genes in one stage were suppressed in the other stage.

Table 4 – SAGE tags with similar abundance in dikaryotic mycelium and primordium of *Lentinula edodes*

Tag sequence[a]	Copies[b]		Homology (accession no.)[c]	Organism[d]	E-value[e]	EST clone (accession no.)[f]	P-value[g]	Gene ontology
	Myc	Pri						
CAGATCTTCG	19	8	Ubiquitin (CAA80851)	*Phanerochaete chrysosporium*	3.00E-85	05G12 (CO501478)	5.47E-02	–
TAGCTGGTCG	16	12	Probable glycosidase crf1 precursor (Q8J0P4)	*Aspergillus fumigatus*	6.05E-10	HKLC15289 (HKLC15289)	5.76E-01	GO:0005975
CCACGTAACT	7	1	Zinc-regulated transporter 2 (Q12436)	*Saccharomyces cerevisiae*	3.57E-35	LedoSEQ8790 (EB017442)	7.12E-02	–
GATATCAAGG	7	2	Surfeit locus protein 4 (O57590)	*Takifugu rubripes*	9.34E-46	LedoSEQ1780 (EB012196)	1.81E-01	–
TAGAGCTAAT	7	3	N-methyl-D-aspartate receptor glutamate-binding chain (S19586)	*Rattus norvegicus*	7.60E-02	PEL0153 (CN605705)	3.46E-01	–
TTCGGCAAGG	6	3	ADP, ATP carrier protein (P02723)	*Neurospora crassa*	6.90E-16	HKLC06722 (HKLC06722)	5.09E-01	GO:0006810
ATGAATTCAC	5	0	Streptavidin V1 precursor (Q53532)	*Streptomyces violaceus*	1.49E-20	HKLC00216 (HKLC00216)	6.30E-02	–
ATTTGAGTAG	5	0	Cytochrome b-245 heavy chain (O46522)	*Bos taurus*	3.19E-30	HKLC15419 (HKLC15419)	6.30E-02	GO:0006118, GO:0006811
CATCGGATAA	4	0	Probable transporter PB10D8.01 (Q9C0V8)	*Schizosaccharomyces pombe*	3.17E-27	LedoSEQ1080 (EB009178)	1.26E-01	–
GAGACGGACC	4	0	Phosphatidylglycerol/ phosphatidylinositol transfer protein precursor (Q5KIR9)	*Filobasidiella neoformans*	1.12E-43	LedoSEQ1060 (EB009027)	1.26E-01	–
TTGAGCTGTG	4	0	Hydrophobin-3 precursor (O13300)	*Agaricus bisporus*	1.59E-18	18D03 (CO501793)	1.26E-01	–
TCCGCACCTC	4	0	Basic salivary proline-rich protein 2 (P02812)	*Homo sapiens*	2.42E-12	LedoSEQ4526 (EB014168)	1.26E-01	GO:0000004
GGTTCTTCCT	4	2	S-adenosylmethionine synthetase (P48466)	*Neurospora crassa*	1.01E-120	LedoSEQ4602 (EB014225)	6.88E-01	GO:0006730
TTGTATGACT	4	3	Histone H3 (Q9HDN1)	*Mortierella alpina*	1.22E-62	LedoSEQ12343 (EB010337)	1.00E-00	GO:0006334, GO:0007001
GGCCCGAGTG	4	4	Ubiquitin-conjugating enzyme E2-17 kDa 11 (P35134)	*Arabidopsis thaliana*	1.30E-50	HKLC15563 (HKLC15563)	1.00E-00	–
AATGCCAAAC	3	0	Transitional endoplasmic reticulum ATPase (Q01853)	*Mus musculus*	1.94E-39	LedoSEQ7926 (EB016714)	2.51E-01	–
TGGTGTTCAA	3	0	Glycine-rich protein A3 (P37705)	*Daucus carota*	8.04E-07	LedoSEQ3383 (EB013346)	2.51E-01	–
AATCCTCTGC	3	0	Cellular nucleic acid-binding protein (O42395)	*Gallus gallus*	1.55E-21	LedoSEQ11024 (EB009336)	2.51E-01	GO:0006350, GO:0006355
GCTTCATTGT	3	0	ADP, ATP carrier protein (P02723)	*N. crassa*	1.79E-95	03F02 (CO501389)	2.51E-01	GO:0006810
CTAGTGTCCA	3	1	Peroxiredoxin-6 (O35244)	*R. norvegicus*	1.24E-15	HKLC07246 (HKLC07246)	6.26E-01	–
AACGAATGGT	3	1	GTP-binding protein SAS2 (P20791)	*Dictyostelium discoideum*	1.80E-24	16E05 (CO501729)	6.26E-01	–
GGTATGGGCA	3	1	Mitochondrial phosphate carrier (AAC47174)	*Plasmodium falciparum*	2.00E-19	PEL0338 (CN605837)	6.26E-01	–
AAGCTAAATC	3	2	Heat shock protein SSB (Q75E44)	*Eremothecium gossypii*	2.03E-47	HKLC13262 (HKLC13262)	1.00E-00	–
ATCAAGGGTA	3	2	Glycoprotein FP21 precursor (P52285)	*D. discoideum*	1.64E-25	HKLC13704 (HKLC13704)	1.00E-00	–
CATACAGCCA	3	3	A-agglutinin attachment subunit precursor (P32323)	*S. cerevisiae*	5.66E-08	HKLC15640 (HKLC15640)	1.00E-00	–
TAAATTGACC	3	3	40S ribosomal protein S23 (Q873W8)	*Aspergillus fumigatus*	2.97E-54	HKLC05448 (HKLC05448)	1.00E-00	GO:0006412
TCGTGCGCGC	3	6	Cytochrome b5 (Q9HFV1)	*Rhizopus stolonifer*	1.79E-22	LedoSEQ1625 (EB012090)	3.30E-01	–
TCATTCACTA	3	8	60S ribosomal protein L2 (Q75AP7)	*Eremothecium gossypii*	9.70E-109	LedoSEQ13626 (EB011334)	1.35E-01	GO:0006412
CCCTGTGTTA	3	10	60S ribosomal protein L36 (Q9HFR7)	*Trichoderma hamatum*	2.79E-25	LedoSEQ7633 (EB016475)	5.10E-02	GO:0006412
GCTCGAACAC	2	3	Stress protein DDR48 (P18899)	*S. cerevisiae*	5.55E-54	HKLC06618 (HKLC06618)	6.78E-01	–
TACGCGTATA	2	4	60S ribosomal protein L27 (P38706)	*S. cerevisiae*	6.68E-46	LedoSEQ3939 (EB013734)	4.40E-01	–
GGGGGAGTAT	2	5	60S ribosomal protein L38-1 (Q9USR7)	*Schizosaccharomyces pombe*	9.24E-21	LedoSEQ8806 (EB017456)	2.75E-01	–

(*continued on next page*)

Table 4 – (*continued*)

Tag sequence[a]	Copies[b]		Homology (accession no.)[c]	Organism[d]	E-value[e]	EST clone (accession no.)[f]	P-value[g]	Gene ontology
	Myc	Pri						
TTTCGTCTAT	2	6	Ubiquitin (CAB16209)	*S. pombe*	2.50E-62	PEL0328 (CN605827)	1.68E-01	–
ACCAGTATGA	2	7	60s ribosomal protein L11 (Q10157)	*S. pombe*	4.00E-59	PEL1096 (CN606219)	9.97E-02	–
TCATTTTTGT	2	8	Hesp-379 (ABB96272.1)	*Melampsora lini*	0.00000005	PEL1275 (CN606301)	5.83E-02	–
TGCATTACCA	1	3	60S ribosomal protein L3 (Q8NKF4)	*Aspergillus fumigatus*	7.15E-29	HKLC04400 (HKLC04400)	3.60E-01	GO:0006412
GTTCCGAAAC	1	3	60S ribosomal protein L20 (P47913)	*Saccharomyces cerevisiae*	1.60E-64	LedoSEQ12117 (EB010181)	3.60E-01	–
CCAAGCTTTT	1	3	Guanine nucleotide-binding protein alpha subunit Gpa (P30675)	*Coprinus congregatus*	1.50E-28	PEL0557 (CN606020)	3.60E-01	–
CTGCCGCTAT	1	3	Merozoite surface protein 2 (AAC02237)	*Plasmodium falciparum*	1.90E-17	PEL0198 (CN605730)	3.60E-01	–
CTATCTCCGA	1	4	Histone H2B.1 (P02293)	*S. cerevisiae*	2.00E-36	HKLC06644 (HKLC06644)	2.05E-01	–
TTCTTCTACA	1	4	60S ribosomal protein L17 (Q9HE25)	*Neurospora crassa*	4.63E-49	LedoSEQ8465 (EB017163)	2.05E-01	GO:0006412
CCTTTATGCA	1	4	60S ribosomal protein L10a (Q755D9)	*Eremothecium gossypii*	1.48E-79	LedoSEQ8862 (EB017503)	2.05E-01	GO:0006412
GTTCAGGTCA	1	5	Actin-1 (Q9Y702)	*Schizophyllum commune*	1.15E-72	LedoSEQ1808 (EB012217)	1.14E-01	–
CGCTGGTGCC	1	5	60S ribosomal protein L10 (Q9XSI3)	*Bos taurus*	6.26E-50	HKLC07323 (HKLC07323)	1.14E-01	GO:0006412
CTTTGTCGCG	1	5	40S ribosomal protein S6 (P02365)	*Saccharomyces cerevisiae*	1.37E-28	HKLC06706 (HKLC06706)	1.14E-01	–
CTTTTTTCGT	0	3	Tubulin beta-4 chain (P45960)	*Oryza sativa*	1.17E-23	HKLC06885 (HKLC06885)	1.14E-01	–
AAGATTGAGA	0	3	THO complex subunit 4 (Q86V81)	*Homo sapiens*	1.81E-19	LedoSEQ4456 (EB014115)	1.14E-01	GO:0000398, GO:0006406, GO:0006457, GO:0006810
CAGATCAGGC	0	3	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16 (O60231)	*Homo sapiens*	8.46E-65	LedoSEQ6102 (EB015330)	1.14E-01	GO:0000074, GO:0000398, GO:0008380
TGGTGGTAAT	0	3	Protein yop1 (Q4WTW3)	*Aspergillus fumigatus*	1.08E-26	LedoSEQ1483 (EB011996)	1.14E-01	–
TCCAAGTCAC	0	3	Protein transport protein SEC9 (Q752V4)	*Eremothecium gossypii*	2.03E-16	LedoSEQ8882 (EB017519)	1.14E-01	–
ATGGACTCTA	0	3	Protein NipSnap2 (Q9PU58)	*Danio rerio*	7.10E-35	LedoSEQ7209 (EB016165)	1.14E-01	–
GCAGAGGGCG	0	3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 (Q95KV7)	*Bos taurus*	1.79E-16	LedoSEQ2236 (EB012508)	1.14E-01	GO:0006606, GO:0006915, GO:0008624, GO:0030262, GO:0030308, GO:0045892
TCGACCAGCT	0	3	Cyclopentanone 1,2-monooxygenase (Q8GAW0)	*Comamonas sp. NCIMB 9872*	1.58E-40	LedoSEQ12630 (EB010562)	1.14E-01	GO:0006118
TACGTAGTCG	0	3	ADP-ribosylation factor (Q7RVM2)	*Neurospora crassa*	2.27E-51	HKLC01473 (HKLC01473)	1.14E-01	GO:0006364, GO:0007046, GO:0007264
TGGCAAACAA	0	3	Cytochrome p450 (I48130)	*Coprinopsis cinereus*	1.50E-03	PEL0218 (CN605746)	1.14E-01	–
GACCCTGACG	0	4	Translationally-controlled tumor protein (Q95VY2)	*Branchiostoma belcheri*	1.04E-41	LedoSEQ6472 (EB015604)	5.51E-02	–
TAATTTAAAT	0	4	Synaptobrevin homolog YKT6 (Q757A4)	*Eremothecium gossypii*	1.69E-09	HKLC12895 (HKLC12895)	5.51E-02	GO:0006810, GO:0016192
CCATCTCTGA	0	4	Histone H2B (Q4WWC5)	*Aspergillus fumigatus*	4.66E-28	HKLC06552 (HKLC06552)	5.51E-02	GO:0006334, GO:0007001
CTCGAAGCCG	0	4	Farnesyl pyrophosphate synthetase (P08524)	*Saccharomyces cerevisiae*	4.70E-53	LedoSEQ221 (EB012488)	5.51E-02	–
CCCGAAGATG	0	4	Adenosylhomocysteinase (Q27580)	*Drosophila melanogaster*	1.63E-128	LedoSEQ121 (EB010166)	5.51E-02	–
TACCGTATAT	0	4	60S ribosomal protein L30 (Q752U5)	*Eremothecium gossypii*	9.03E-32	10B12 (CO501604)	5.51E-02	GO:0006412

a SAGE tag is 10 bp sequence downstream of the *Nla*III site (CATG).

b Number of tags in the SAGE libraries.

c Putative homologous gene product of the EST that matched the SAGE tag. Different SAGE tags may match with different ESTs that have same homology. Homology searches were performed using BLASTX (Altshcul *et al*. 1997). The best homology from Swiss-prot and NCBI RefSeq, with highest identity score, found in each search is shown. SAGE tags matching to ESTs that could not be matched to genes in GenBank are referred to as unknowns. Those without entry are SAGE tags that could not be matched to any *Lentinula edodes* EST or gene in the GenBank.

d E-value (expected value) is that reported by BLASTX.

e Organism of the best homology reported by BLASTX.

f Accession no. of the matched *Lentinula edodes* EST or gene in the GenBank.

g P-value (the probability of differential expression) comparing tag abundances in dikaryotic mycelium and primordium by using Fisher's exact test. Tags with P-value equal or larger than 0.05 were shown.

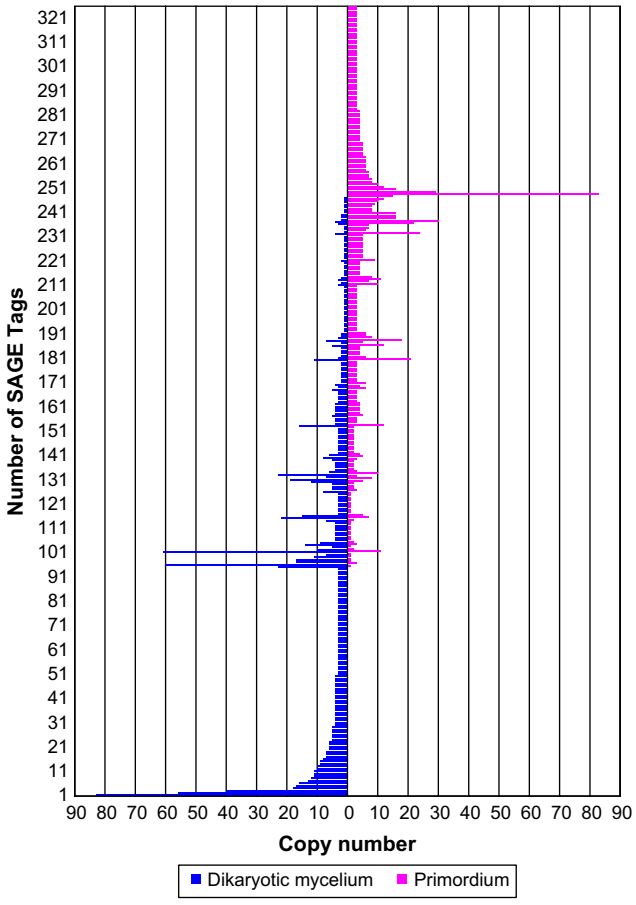


Fig 1 – Graph of transcription expression for SAGE candidates with copy number ≥3 in either dikaryotic mycelial or primordial stage of *Lentinula edodes*. The *x*-axis represents the copy number of the SAGE tags; the *y*-axis represents the number of SAGE tags. The SAGE tags expression in both developmental stages was clustered into five groups. (A) Tags only expressed at the primordium stage. (B) Tags more highly expressed at the primordium stage. (C) Tags expressed at the same level in both the dikaryotic mycelium and primordium stages. (D) Tags more highly expressed at the dikaryotic mycelium stage. (E) Tags expressed only at the dikaryotic mycelium stage.

Hundreds of differentially expressed genes were identified from the SAGE data. In the following section, we discuss the differentially expressed genes that have tag count number of more than or equal to 3. Tags with a count number of less than 3 are considered to be less reliable. Based on analysis using Fisher's exact test, tags with $P < 0.05$ were defined as significantly different in either the dikaryotic mycelium or primordium, and were designated to represent differentially expressed genes.

Thirty-four tags were differentially expressed in the dikaryotic mycelium (Table 2). Eleven of these matched ESTs that encode various putative proteins, including three hydrophobins, isopenicillin N epimerase, mannose-P-dolichol utilization defect 1 protein, polyporopepsin, phosphatidylethanolamine binding protein, phosphatidylserine decarboxylase proenzyme 2 precursor, lectin, and hydroxyproline-rich glycoprotein (Table 2). Forty genes were differentially expressed in the primordium (Table 3). Twenty-one of these matched ESTs that encode putative proteins, including glucose-regulated protein homologue precursor, acyl carrier protein, cell division control protein 12, methallothionein, protein priA precursor, hydrophobin 1, riboflavin aldehyde-forming enzyme, and various ribosomal proteins. It can be inferred that most of the differentially expressed genes are involved in fruit body development, and their potential functions in this process are discussed in the following sections.

Developmental processes

Most of the abundant genes in the dikaryotic mycelium and primordium of *Lentinula edodes* are proteins that are related to morphogenesis and cellular structural components, including hydrophobins and lectin. Hydrophobins are essential for morphogenesis and pathogenesis in fungi and fruit development in mushrooms (Kershaw & Talbot 1998). Hydrophobin-encoding genes have been isolated from several fungi, such as *Schizophyllum commune* (Wessels *et al.* 1991), *Agaricus bisporus* (De Groot *et al.* 1996), and *Pleurotus ostreatus* (Penas *et al.* 1998). Their expression in *L. edodes* is developmentally regulated (Ng *et al.* 2000). Two hydrophobin-encoding genes (*Le.hyd1* and *Le.hyd2*) were isolated from *L. edodes* and characterized by differential expression in different developmental stages (Ng *et al.* 2000). Various lectins have been shown to have developmental stage-specific expression in *Pleurotus cornucopiae*. One of the lectins (PCL-M) is only expressed in the mycelium, thus revealing its crucial role in primordium formation (Oguri *et al.* 1996). This mycelial lectin is abundantly located on the surface of solid-medium grown dikaryotic mycelia, and exhibits haemagglutinating activity that stimulates the formation of primordia in *P. cornucopiae* by causing the cohesion of hyphae (Oguri *et al.* 1996). In this study, genes that encode three hydrophobins and a lectin were differentially expressed in the dikaryotic mycelium, whereas genes that encode another two hydrophobins were differentially expressed in the primordium of *L. edodes*. Different types of hydrophobins thus appear to be required to initiate morphological changes in different developmental stages. Lectin may cooperate with hydrophobins to maintain cellular structure and aid the cohesion of hyphae in the dikaryotic mycelium, thus promoting primordium initiation. In addition, phosphatidylethanolanine binding protein (PEBP), which is the prototype of the serine protease inhibitors that are involved in normal development and homeostasis (Hengst *et al.* 2001), was differentially expressed in the dikaryotic mycelium. The high expression of the PEBP gene indicates its importance in cellular growth in the dikaryotic mycelium.

Once the primordium has been initiated, cellular growth slows and rapid cellular differentiation occurs. Fruit body development in *Coprinopsis cinerea* shows a dramatic change in the pattern of growth from a loose mesh of free, undifferentiated hyphae to a compact multihyphal structure of different cell types through hypha–hypha interactions (Kues 2000; Moore 1998). Similar to *C. cinerea*, *L. edodes* develops from undifferentiated mycelium to multihyphal primordium under suitable environmental conditions. In this study, various ribosomal proteins were up-regulated from the dikaryotic

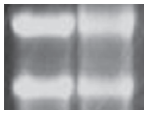
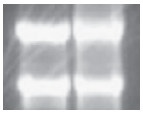
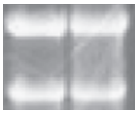
Stages[a]	Myc	Pri	Myc	Pri	Myc	Pri	Myc	Pri	Myc	Pri
EST I.D.[b]	PEL0196		PEL0525		PEL0549		PEL0710		PEL1063	
Hybridization result[c]										
RNA loading control[d]										
Northern[e]	E		2.10		1.29		1.69		2.09	
SAGE[f]	0:29		3:22		2:16		4:24		1:9	
Microarray[g]	6.93		1.08		4.74		1.61		2.05	

Fig 2 – Northern blotting results of some selected genes with their relative expressions in cDNA microarray hybridization and tag counts in SAGE. (A) The dikaryotic mycelial (Myc) and the primordial (Pri) stages of *Lentinula edodes* were analysed by northern blotting. (B) The EST identities of the selected genes for northern blotting analysis. (C) The northern blotting hybridization results. (D) The ethidium bromide (EtBr)-stained gels showing 18S and 28S rRNA molecules for loading controls. (E) The expression folds of the selected genes in primordium/the expression folds of the selected genes in dikaryotic mycelium in northern blotting. (F) The copy number of tags for the selected genes in dikaryotic mycelium: the copy number of tags for the selected genes in primordium. (G) cDNA microarray hybridization ratio showed the expression level of selected transcripts in the primordial stage/the expression level of selected transcripts in dikaryotic mycelial stages.

mycelium to the primordium, which implies that an increase in protein varieties is needed for cell differentiation in the primordium. It also indicates that substantial translation occurs to produce ample proteins for the development in the fruit body of *L. edodes*. A gene that encodes a putative signal transduction-related protein, priA precursor, is also up-regulated in the primordium. The *PriA* gene encodes a DNA-binding transcription factor and has a higher expression in primordia and immature fruit bodies than in preprimordial mycelia and mature fruit bodies, and may thus play a role in fruit body initiation (Kajiwara *et al.* 1992). The SAGE results imply that in the developmental process of fruit body initiation, genes related to morphogenesis and signal transduction gene are up-regulated to initiate cell differentiation.

Environmental challenges

In making the transition from dikaryotic mycelium to primordium, *Lentinula edodes* faces various stresses. At the mycelial stage, *L. edodes* competes with other microorganisms for the space and nutrients that it requires to sustain its rapid growth rate. Hydroxyproline-rich glycoproteins (HRGPs) are responsible for numerous cellular processes in plants, including development (Sommer-Knudsen *et al.* 1998). HRGP is one of the most important structural wall glycoproteins in plant cells, and is highly expressed in plant tissues that are subject to fungal and bacterial infection or that have been treated with elicitors obtained from pathogens (Bradley *et al.* 1992; Brownleader *et al.* 1995; Kang & Buchenauer 2003; Shailasree *et al.* 2004). The expression of proline/hydroxyproline-rich glycoprotein (P/HRGP) purified from pearl millet coleoptiles was found to be affected by pathogen infection or environmental stresses, such as elicitor treatment and wounding (Deepak *et al.* 2007). In this study, HRGP was differentially expressed at the mycelial stage, which suggests that it may be associated with hydrophobins and lectin and may act as a structural component of mycelial hyphae in response to pathogen attack.

Under certain environmental stresses, dikaryotic mycelia aggregate to form primordium, which marks the beginning of fruit body development. A glucose-regulated protein homologue precursor and metallothionein are differentially expressed in the primordium. In *Neurospora crassa*, glucose-regulated protein 78 (GRP78), which belongs to the heat shock protein 70 (*hsp70*) gene family, is induced by starvation or elevated temperatures (Techel *et al.* 1998), whereas metallothionein is a metal chaperon and scavenger of the reactive oxygen type (Mir *et al.* 2004) and is related to anti-oxidative stresses and mycobacterial infection (Regala & Rice 2004). These findings imply that dikaryotic mycelia aggregate to form primordium as environmental challenges increase. Following this aggregation, a protective mechanism is turned on to protect the developing fruit body from these environmental stresses.

Provision of building blocks for growth

Genes involved in different metabolic pathways were differentially expressed in the dikaryotic mycelium and primordium. Genes that encode phosphatidylserine decarboxylase proenzyme 2 precursor and polyporopepsin were highly expressed in the dikaryotic mycelium, whereas acyl carrier protein was highly expressed in the primordium. More genes involved in metabolic processes were found to be differentially expressed in the dikaryotic mycelium, which suggests a higher energy demand for hyphal growth or storage during primordium development.

In both eukaryotic and prokaryotic organisms, phosphatidylserine is converted to phosphatidylethanolamine by

phosphatidylserine decarboxylase (Bishop & Bell 1988; Carman & Henry 1989; Raetz & Dowhan 1990). Studies of this gene are important because of its potential role in regulating phospholipid biosynthesis and the inter-organelle trafficking of phosphatidylserine in eukaryotic cells. PSD gene encoding phosphatidylserine decarboxylase has been studied in *Escherichia coli* to examine its role in phospholipid metabolism (Dowhan *et al.* 1974; Hawrot & Kennedy 1975; Satre & Kennedy 1978). However, the disruption of the PSDI gene in *Saccharomyces cerevisiae* was reported to reduce phosphatidylserine decarboxylase activity, no loss in viability or any major alterations in phospholipids composition was found (Clancey *et al.* 1993). Therefore, alternative genes or mechanisms may complement the function of PSDI in phospholipid composition. Genes that are homologous to phosphatidylserine decarboxylase proenzyme 2 precursor, which is highly expressed in mycelia, may be related to phosphatidylserine decarboxylase activity and thus be involved in phospholipid biosynthesis for hyphal growth.

Polyporopepsin (aspartic proteinase) is an important degrading enzyme that obtains amino acids by cleaving proteins into small peptides or amino acids. Although it provides nutrition to fungi, aspartic proteinase is also associated with fungal virulence. Aspartyl proteinase secreted by the pathogenic yeast *Candida albicans* has been shown to be a potential virulence factor (Naglik *et al.* 2003; Staib 1965). However, *L. edodes* is saprophytic, not pathogenic, and thus the high expression of the gene that encodes polyporopepsin may be needed to obtain the nutrients during hyphal cell growth.

Acyl carrier protein (ACP) is important for fatty acid synthesis in bacteria (Alberts *et al.* 1964; Majerus *et al.* 1964), plants (Overath & Stumpf 1964), and mammalian systems (Alberts *et al.* 1964; Larrabee *et al.* 1965; Wakil *et al.* 1964). Acetoacetyl-ACP is a substrate for the condensation reaction of fatty acid synthesis in yeasts (Rudney *et al.* 1966). Fatty acid synthases (FASs) systems are likely to be involved in metabolic, structural, and endocrinic functions, and are thermoreceptors in *C. utilis* (Ito *et al.* 1986). Δ9 and Δ12 fatty acid desaturases are related to stress responses in the primordium and mature fruit body of *L. edodes* (Sakai & Kajiwara 2003, 2005). The abundant expression of the gene that encodes ACP suggests that the active synthesis of fatty acids, which can be a source of energy, structural components, and lipo-proteins, is needed to support active growth in the primordium. It may also imply the importance of FAS system for stress response during initial fruit body formation.

Intracellular trafficking

In this study, two genes that code for intracellular trafficking-related proteins, mannose-P-dolichol utilization defect 1 and cdc 12, were highly expressed in the dikaryotic mycelium and primordium, respectively. Mannose-P-dolichol utilization defect 1 (MPDU1) protein is essential for the efficient use of dolichol-P-mannose and dolichol-P-glucose within the rough endoplasmic reticulum (rER). The mutation of MPDU1 in humans causes defects in the mannosylation and glucosylation of glycans in the lumen of the rER, which results in defective dolichylpyrophosphate-linked oligosaccharides biosynthesis (Kranz *et al.* 2001). The high expression of MPDU1 in the dikaryotic mycelium suggests that dolichylpyrophosphate-linked oligosaccharides biosynthesis is related to hyphal growth at the mycelium stage. In contrast, the high expression of the cdc12 gene in the primordium may be the result of microtubule and actin-dependent movement in cells during fruit body development. Mutants of *Schizosaccharomyces pombe* cdc12 cause a severe defect in the formation of the medial ring and septum, suggesting that cdc12p is required for actin ring formation and cell division in this species (Chang *et al.* 1996, 1997). Chang (2000) found that cdc12p is important for microtubule and actin-dependent movement in fission yeasts.

Riboflavin aldehyde-forming enzyme

A riboflavin aldehyde-forming enzyme catalyses the oxidation of 5′-hydroxymethyl of riboflavin to a formyl group and produces aldehyde and carboxylic acid derivatives of riboflavin (Tachibana & Oka 1981). The differential expressions of this enzyme in the primordium and mature fruit body of *Lentinula edodes* have been reported (Miyazaki *et al.* 2005). It was also reported to be more associated with morphogenesis of *Agaricus bisporus* rather than specific post-harvest processes such as stress response (Sreenivasaprasad *et al.* 2006). From our SAGE data, a high transcript level of this riboflavin aldehyde-forming enzyme was observed in the primordium, which indicates that the riboflavin derivatives may play important roles in primordium formation. Although the transcript (PEL11063) showed low homology to *Schizophyllum commune* riboflavin aldehyde-forming enzyme (AAB62250), it was homologous (E-value: 1e-32) to *Lentinula edodes* riboflavin aldehyde-forming enzyme (BAD11818.1) and also homologous (E-value: 9e-07) to *Agaricus bisporus* riboflavin aldehyde-forming enzyme (CAH69601). Riboflavin aldehyde-forming enzyme may also be involved in protection against other microbes through the removal of riboflavin, which is an essential cell component.

Validation of the SAGE results

Gene expression profiles of SAGE tags were validated by cDNA microarray hybridization (Supplementary Material Table S1). Some of the homologues shown in Tables 2–4 were not identical to their corresponding microarray clones. It is because all in-house ESTs were BLASTx searched anew by filtering out the homologies with low-complexity regions. Besides, only Swiss-Prot (sp) sequences or NCBI RefSeq (ref) sequences with the highest homology to the ESTs were selected from BLASTx results. This also explained why less in-house ESTs shown in the Results section were homologous to genes from other basidiomycetes. Although many in-house ESTs were similar to genes from other basidiomycetes, their homologues were uninformative and not characterized by gene ontology (GO) so were omitted from this analysis. The relative expression of the genes detected by cDNA microarray hybridization was mostly consistent with the SAGE results. However, we observed that the results for the transcripts with a low abundance from the SAGE and cDNA microarray hybridization were often inconsistent, with the consistency decreasing when the count number of the SAGE tags decreased. In the

study of human corneal endothelial cells using SAGE and affymetrix microarray, the authors also found that transcripts with a SAGE tag abundance of greater than 50 had a more consistent expression when detected by microarray than medium and low abundance transcripts (Hackam *et al.* 2004). In this study, there was also inconsistency between the SAGE and cDNA microarray hybridization results for genes with repeated sequences, such as serine-rich proteins and hypothetical proteins that contain repeated sequences. This implies that the accuracy of cDNA microarray hybridization is also affected by the repeated sequence of the transcript, and thus the cross-hybridization of transcripts that contain repeat sequences may lead to overestimation. Intramolecular folding, which renders probes unable to hybridize with the hybridization clones, easily occurs within long cDNA probe sequences (Southern *et al.* 1999). In general, we found the results of the northern blotting to be more consistent with the SAGE data (Fig 2).

Some of the differentially expressed genes that were found in the SAGE do not match any known ESTs. These genes can be identified by the generation of longer cDNA fragments from the SAGE tag (Chen *et al.* 2000, 2002).

In conclusion, from our SAGE data, we observed the gene expression profiles of dikaryotic mycelium and primordium to be quite different. Genes with a high level of expression in the dikaryotic mycelium were usually down-regulated in the primordium. Conversely, genes with high transcript levels in the primordium often had low expression levels in the dikaryotic mycelium (Fig 1). This shows unique expression patterns in the stages of vegetative growth and initial fruit body development. The differentially expressed genes that were obtained from the SAGE provide valuable information on the process of fruit body development. A high expression of genes that encode structural proteins, proteins that afford stable growth, and enzymes that catalyse energy production, all of which support vegetative hyphal growth, were observed in the dikaryotic mycelium. The formation of fruit bodies may be initiated by a process that is characterized by the presence of certain environmental challenges that stimulate specific signal transduction, which causes the initiation of the transition of the dikaryotic mycelium to the primordium by inducing the expression of certain special genes. These gene products may regulate fruit body development by active intracellular trafficking. Finally, the reconstruction of proteome occurs *via* protein degradation, protein modification, and protein synthesis.

The SAGE technique has provided valuable data in this study on the gene expression profiles of *L. edodes* at the dikaryotic mycelium and primordium stages, which serves to advance our knowledge of the biology of mushrooms. The SAGE data that we obtained is also suitable for conducting a comparison of the different developmental stages in the life-cycle of fungi, such as the monokaryotic mycelium and mature fruit body stages, to obtain a more comprehensive gene expression profile of mushroom development.

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Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.mycres.2008.01.028.

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Supplementary Table 1.

Tag sequence[a]	Copies[b] Myc	Pri	Homology[c]	EST Clone[d]	Accession no.[e]	Ratio[f]	Consistency[g]
CCAATCCCAG	0	83	Hydrophobin 1	PEL0163	CN605708	6.24	Yes
ATGGGGCATA	0	29	Unknown	PEL0196	CN605728	6.93	Yes
CCAATGACAG	4	24	Ubiquitin	PEL0710	CN606067	2.54	Yes
CGCTTAGGTT	3	22	Hypothetical protein	PEL0525	CN605996	1.08	No
ATCGCTCGTC	0	16	60S ribosomal protein L37A	PEL0213	CN605742	3.58	Yes
TACGCCGTGA	2	16	Pyridoxal phosphate dependent aminotransferase	PEL0549	CN606013	5.27	Yes
TCAAGCTCCG	1	15	40S ribosomal protein S14	PEL0289	CN605792	4.04	Yes
AATCGTGTAC	0	12	Merozoite surface protein 2	PEL0199	CN605731	0.46	No
CCCTGTGTTA	3	10	60S ribosomal protein L36	PEL0224	CN605751	4.61	Yes
CTTCTGATAT	1	9	B2-aldehyde forming enzyme	PEL11063	CN606187	2.23	Yes
TCATTTTTGT	0	8	Sericin MG-1 - g	PEL1275	CN606301	1.06	No
AAATGCTTAC	1	8	40S ribosomal protein S12	PEL0097	CN605688	4.81	Yes
ACAAACTGCG	0	7	60S ribosomal protein L35	PEL0208	CN605738	6.38	Yes
ACCAGTATGA	0	7	60S ribosomal protein L11	PEL1096	CN606219	4.91	Yes
AATAACGGCT	0	7	Unknown	PEL1123	CN606240	0.74	No
TAGATGCGCG	5	6	Unknown	PEL1268	CN606297	1.48	Yes
CCACTAGTTG	4	6	T-cell receptor	PEL0313	CN605814	0.79	Yes
TTTCGTCTAT	0	6	Ubiquitin	PEL0096	CN605687	2.11	Yes
TTCCCATAAG	0	6	Cadmium-binding metallothionein	PEL0202	CN605733	4.81	Yes
GATGGATCTA	1	5	Unknown	PEL1215	CN606254	5.53	Yes
TGTATGCTTC	1	5	40S ribosomal protein S19	PEL0267	CN605775	6.95	Yes
CTTTGTCGCG	1	5	40S ribosomal protein S6	PEL0322	CN605822	5.54	Yes
ATGTCGATCC	0	5	60S ribosomal protein L7	PEL0358	CN605854	6.58	Yes
TGTACTCTAC	0	5	40S ribosomal protein S27	PEL0528	CN605998	5.32	Yes
AGTTCACGTT	6	4	Unknown	PEL0337	CN605836	0.33	Yes
TACCGTATAT	0	4	60S ribosomal protein L30	PEL0091	CN605683	6.06	Yes
CGCTGGTGCC	1	5	60S ribosomal protein L10	PEL0172	CN605712	6.25	Yes
ATGGTTGAAT	2	4	Probable membrane protein	PEL0177	CN605717	2.66	Yes
GCTGTCCGAT	0	4	ATP-synthase J chain	PEL0317	CN605818	0.58	No
TTCTTCTACA	0	4	60S ribosomal protein L17	PEL0524	CN605995	5.10	Yes
TTGTATGACT	4	3	Histone H3	PEL0185	CN605723	4.26	No
CATACAGCCA	3	3	B2-aldehyde forming enzyme	PEL0293	CN605796	0.49	No
CCGTTCTGGA	0	3	Unknown	PEL1285	CN606311	3.25	Yes
CTGCCGCTAT	1	3	Hypothetical protein	PEL0198	CN605730	4.71	Yes
TGGCAAACAA	0	3	Cytochrome p450	PEL0218	CN605746	2.87	Yes
ATCACATTTT	1	3	ATP synthase regulatory factor	PEL0298	CN605801	2.65	Yes
AATGCTTGCT	0	3	60S ribosomal protein L12	PEL0316	CN605817	0.58	No
CCAAGCTTTT	1	3	Guanine nucleotide-binding protein	PEL0557	CN606020	1.11	No
TACGTAGTCG	0	3	ADP-ribosylation factor 1	PEL0597	CN606044	4.38	Yes
TGCCGTTGCC	0	3	Hypothetical protein	PEL0706	CN606063	1.50	Yes
ACGTCTGCAG	0	3	Unknown	PEL1060	CN606184	2.23	Yes
CAGTTCAAAC	60	0	Hydrophobin 2	PEL1258	CN606292	0.18	Yes
GATATTGTCT	40	0	Hydrophobin3	PEL0608	CN606049	0.15	Yes
TAGCTGGTCG	16	0	Merozoite surface protein 2	PEL0199	CN605731	0.75	No
TCTTCTTATG	10	1	Hypothetical serine rich protein	PEL0466	CN605944	1.98	No
ACCTGACGAC	5	0	Super cysteine rich protein	PEL0480	CN605954	2.59	No
GAGGAGGCAA	5	0	Unknown	PEL0751	CN606086	0.36	Yes
TCCTTATCAC	3	0	Unknown	PEL1216	CN606255	3.97	No
AAGCTAAATC	3	0	Heat shock protein 70	PEL1224	CN606263	0.97	No
ATGCTGAAAA	3	1	NADH dehydrogenase	PEL1080	CN606204	0.65	No