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Basidiomatal formation in *Antrodia cinnamomea* from the perspective of gene expression

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ABSTRACT. In order to understand the phenomenon of morphological differentiation in medicinal fungus *Antrodia cinnamomea*, two complementary DNA (cDNA) libraries were constructed from the liquid-cultured mycelia (AM) and natural basidiomes (AT) produced from the infested wood. Using single-passed sequencing of cDNA clones, 821 and 993 high-quality expressed sequence tags (ESTs) were generated from the liquid-cultured mycelial and wild basidiomatal cDNA libraries, respectively. The results from BLASTX search revealed that only 32.5% to 33.7% of ESTs showed significant similarity to protein sequences in public databases (*E values* $\leq 10^{-10}$). The cDNAs encoding genes related to metabolism were found to be most abundant, followed by genes involved in protein fate and protein synthesis in each category. Genes related to the "Cell fate, cell cycle, and DNA processing" category showed the greatest difference between the liquid-cultured mycelial and wild basidiomatal cDNA libraries, followed by genes involved in metabolism. The results from this study have provided valuable sequence information, which may lead to improved production of basidiomes of *A. cinnamomea* and regulation of metabolites in the future.

Keywords: *Antrodia cinnamomea*; Expressed sequence tags; Medicinal fungus; Morphological differentiation.

INTRODUCTION

Antrodia cinnamomea is a resupinate to effused-reflexed basidiomycete with porous hymenium (Chang and Chou, 2004). The basidiomes of *Antrodia cinnamomea* (Chinese name, chang-chih) are well-known in Taiwan as a highly-prized folk medicine. This medicine has been used to treat drug intoxication, diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer (Tsai and Liaw, 1982). *Antrodia cinnamomea* grows in the inner cavity of a decayed tree trunk of *Cinnamomum kanehirai* Hay (Lauraceae), and its large-scale cultivation by artificial means has been unsuccessful so far. Also, due to overexploitation, *C. kanehirai,* an endemic species of evergreen tree, is becoming rare. Hence, it is being conserved by the Taiwan government. The basidiomes of *A. cinnamomea* are becoming a scarce and high priced folk medicine in Taiwan, due to the species' host specificity and rarity in nature and to the failure of artificial cultivation (Chang and Chou, 2004).

Investigation of the differential mechanism of *A. cinnamomea* would be beneficial for the development of a new technique for the regulation of porous-hymenium basidomatal formation. However, no molecular characterization of the fruiting bodies from *A. cinnamomea* has been reported so far. Expressed sequence tag (EST) analysis has been successfully applied to the study of gene expression in animals, plants, and fungi subjected to various stresses or in different stages of development, as is evident from the increased number of reports on ESTs (Adams et al., 1991; Cooke et al., 1996; Yamamoto and Sasaki, 1997; Lee et al., 2002). In addition to the identification of pathogenicity of fungi, fundamental aspects of fungal development have also been examined using the EST-guided approach (Lee et al., 2002; Li et al., 2004). In edible mushrooms, the process of fruiting body formation is very important from a scientific and commercial point of view, and many scientists have therefore studied specifically expressed genes in the fruiting bodies of *Lentinula edodes*, *Schizophyllum commune*, and other basidiomycetes (Wessels, 1992; Kondoh and Shishido, 1995; Fernandez Espinor and Labarere, 1997). The EST-based approach investigated in the present study may lead to a fundamental understanding of basidiomatal formation in *A. cinnamomea*.

In the present study, two complementary DNA (cDNA) libraries from liquid-cultured mycelia (AM) and natural basidiomes (AT) were established in order to characterize the gene expression during porous-hymenium basidiomatal formation in *A. cinnamomea.* The potential functions of the EST clones were determined by comparing them with the sequences of other fungi and by obtaining specific genes for *A. cinnamomea*. The results obtained in the study may lead to the identification of genes and analysis

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of gene expression during basidiomatal formation in *A. cinnamomea*.

MATERIALS AND METHODS

Strains and culture conditions

Antrodia cinnamomea strain TFRIB 479 obtained from the rotten wood of *C. kanehirai* at Dawu, Taitung was identified in the study. The cultures were maintained as reported earlier (Chang and Chou, 2004). The natural basidiomes were obtained from the infested wood. Liquid-cultured mycelia (AM) and natural basidiomes (AT) were frozen in liquid nitrogen and stored at -80°C until use.

cDNA library construction

Total RNA for cDNA library construction was prepared following the method described by Chang et al. (1993) and modified by Chen et al. (2004). Poly $(A)^+$ RNA was then purified with Oligotex mRNA Mini Kit (Qiagen). The single strand cDNA was synthesized by SuperScript™ II Reverse Transcrpitase (Invitrogen) while the double strand cDNA was amplified using PCR Plus Master Mix Kit (GeneMark) (Sized-fractionation > 100 bp). Purification of the cDNA was performed using a DNA Clean/ Extraction Kit (GeneMark). The resulting cDNA was cloned into the pGEM-T Easy vector system (Promega) following the protocol for an overnight ligation. The ligation mixture was transformed into High Efficiency DH5α Competent cells (Hopegen) and then plated onto LB-ampicillin plates containing IPTG and X-gal.

DNA sequencing

After a cDNA library was plated onto LB media plates, the transformed white colonies were transferred into test tubes containing 5 mL LB-amp medium. Plasmid DNA was isolated from overnight cultures by the Plasmid Miniprep Purification Kit (GeneMark). Sequencing was performed with an ABI 377 automatic sequencer (Perkin Elmer) using a T7 sequencing primer.

Sequence analysis

Nucleotide sequences of less than 100 bp were excluded, and the remaining ones were analyzed. The leading vector and poor quality sequences were removed manually or via ChromasPro software (Technelysium Pty Ltd). Individual EST was assembled into contigs using the ContigExpress program of Vector NTI Suite 8 (InforMax, Inc.) with parameters optimized for ESTs rather than for genomic clones. The cDNA sequences were compared to non-redundant (nr) protein sequence databases at the National Center for Biotechnology Information (NCBI) (Altschul et al., 1997). *E*-value results obtained from the BLASTX were categorized into $\leq 10^{-10}$, which represented significant homology, and $> 10^{-10}$, which implied no hit. The former results were then grouped according to putative function. A unique set with significant matches was annotated on the basis of their most similar functions following the general rules from the Functional Catalogue by the Munich Information Center for Protein Sequences (MIPS) (www.mips.biochem.mpg.de/proj/yeast/catalogues/funcat/index.html) and with the aid of the Gene Ontology Consortium (www.geneontology.org).

RESULTS

Sequencing and assembling of ESTs sequences

Total RNAs were isolated from liquid-cultured mycelial (AM) and natural basidiomes (AT) of *A. cinnamomea* for the construction of cDNA libraries. Initially, a total number of 1823 clones were randomly picked and subjected to 5′ end single-pass sequencing from the libraries. The leading vector, tailing of the sequence, and poor-quality sequences were excluded. After excluding fragments shorter than 150 nucleotides, 1,809 high-quality ESTs were submitted to dbEST (GeneBank accession nos. DV629596-DV631404). Most of the ESTs were 300-700 bp in length with an average of 538 bp. Among 817 clones from the mycelia library, 537 clones assembled into 104 contigs while 180 ESTs were unigenes. In the basidiomatal library, with its 992 clones, 742 clones assembled into 162 contigs while 250 ESTs remained as unigenes. As a result of the contig analysis, 604 different genes (unigene) could be obtained out of 1,809 analyzed cDNA clones. Among the 604 unigenes, 512 (84.8%) expressed differently in the mycelial and basidiomatal libraries.

Characterization of the cDNA library and the ESTs sequences

In this study, the putative function of the cDNAs, which is based on BLASTX's result, was assigned to those with E-values less or equal to 10^{-10} (Sterky et al., 1998). In AM and AT libraries, only 267 (32.7%) and 325 (32.8%) ESTs, respectively, showed similarity when these were compared with the public sequence databases. A total of 1809 ESTs matched with 592 distinct sequences in the non-redundant protein database and were assigned to 259 functional genes from both the libraries. The developmental stage specificity and redundancy of these 259 unigenes were analyzed to characterize the developmental gene expression from the EST data (Table 1). The results indicated that lanosterol 14-alpha-demethylase, and nucleolar protein nhp2 occurred more frequently in the basidiomatal library than in the mycelial library. On the other hand, peroxiredoxins, ATP-binding cassette transporter ABC1, phytase, and H/ACA snoRNP component occurred more redundantly in the mycelial library. The potential roles of these in basidiomatal formation in *A. cinnamomea* will be explored in the future.

Cellular roles of ESTs in *A. cinnamomea*

To obtain an overview of how *A. cinnamomea* functions at the cellular level, the transcripts identified with putative functions were assigned nine putative cellular roles based on those assigned them by the MIPS FunCat scheme

Table 1. Annotation of ESTs from liquid-cultured mycelia (AM) and wild basidiomes (AT) of *A. cinnamomea.*

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
I. Cell fate, cell cycle and DNA processing				
Anaphase promoting complex subunit 10	*Oryza sativa*	AAU10698	1	
ATP-dependent DNA helicase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43036		1
Brefeldin a resistance protein	*Schizosaccharomyces pombe*	CAB44765	1	4
Bud site selection-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43110		1
Cell cycle regulatory protein	*Ustilago maydis*	AAN10186		1
DNA topoisomerase I	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45618		1
Fibrillarin (NOP1)	*Neurospora crassa*	CAC18188	1	
Flap endonuclease-1	*Coprinopsis cinerea*	BAD14303		1
Microtubule end-binding protein EB1	*Coturnix japonica*	AAU12573		1
Opa-interacting protein OIP2	*Cryptococcus neoformans* var. *neoformans* B-3501A	EAL23044	1	
Prohibitin PHB1	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40684	1	
RAB18	*Mus musculus*	BAC32402	1	1
Ras1p	*Suillus bovinus*	AAF65465		1
Topoisomerase I	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45618		1
Tubby-like protein 2	*Arabidopsis thaliana*	AAK98801		1
II. Cell rescue, defense, and virulence				
Catalase	*Campylobacter jejuni*	CAA59444	1	
Cytochrome P450, alkane-inducible	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43969	1	1
Cytochrome P450 monooxygenase	*Coriolus versicolor*	BAB59027	1	2
Cytochrome P450 oxidoreductase	*Coriolus versicolor*	BAB83588		1
Glutathione reductase	*Onchocerca volvulus*	CAA72516		1
Heat shock protein HSS1	*Puccinia graminis* f. sp. *tritici*	AAB93665	1	
Hob1p	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40855		1
Lanosterol 14-alpha-demethylase	*Phanerochaete chrysosporium*	AAU01160	3	17
Methylenetetrahydrofolate reductase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41236		5
O-methylsterigmatocystin oxidoreductase	*Neurospora crassa*	CAE76231	1	1
Osmotic stress-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41050	1	3
Peroxiredoxin Q	*Triticum aestivum*	AAV66923	1	
Peroxiredoxins	*Phanerochaete chrysosporium*	AAV53576	19	9
Protoplast regeneration and killer toxin resistance gene	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44305		1
Snodprot-FS	*Gibberella pulicaris*	AAV83793	8	3
WD-repeat protein-like	*Arabidopsis thaliana*	BAB09052		1
III. Cellular transport, transport facilitation and transport routes				
Allantoate permease	*Neurospora crassa*	CAE81935		1
ATPase of the ABC class	*Thermoanaerobacter tengcongensis*	ZP_00178674		1
ATP-binding cassette transporter ABC1	*Venturia inaequalis*	AAK62810	11	1
ATPase of the CDC48/PAS1/SEC18 (AAA) family	*Saccharomyces cerevisiae*	NP_013501		1
ATP synthase gamma chain	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43492		2

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Carnitine/acyl carnitine carrier	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41054	15	12
ER to Golgi transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC211	AAW44532	3	3
GTPase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46779	1	
Intracellular transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41812		1
Late endosome to vacuole transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42196	2	
Membrane zinc transporter	*Aspergillus fumigatus*	AAT11930		1
Nucleoporin	*Ustilago maydis*	CAG26761	1	
Phosphate transporter	*Pholiota nameko*	BAB43910		2
Protein-vacuolar targeting-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40999	1	
P-type cation-transporting ATPase	*Blastocladiella emersonii*	CAA04499		1
Sugar transporter	*Neurospora crassa*	CAB88582		1
Synaptobrevin (v-SNARE) homolog Bos1	*Schizosaccharomyces pombe*	CAB77004	1	1
Transmembrane transporter Liz1p	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43513		1
Tricarboxylate transport protein	*Schizosaccharomyces pombe*	CAB10116	2	
Triose phosphate translocator	*Cryptococcus neoformans* var. *neoformans*	AAR82906		1
Vacuolar ATP synthase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45529	1	
V-ATPase subunit A	*Fundulus heteroclitus*	BAB62103		1
Vesicle-mediated transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW47173	1	
Urea transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43008		1
IV. Cellular communication/signal transduction				
Calcium ion binding protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44613	1	1
Guanine nucleotide-binding protein alpha-4 subunit	*Ustilago maydis* 521	EAK86634		1
Sensory histidine kinase	*Mesorhizobium loti*	NP_103743	2	3
Serine kinase (hPAK65)	*Homo sapiens*	AAA75468		1
Rho GTPase activator	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44732		1
V. Metabolism				
Acetamidase	*Schizosaccharomyces pombe*	AL023592		1
Acid sphingomyelinase	*Neurospora crassa*	CAD70921		1
Acyl-CoA transferases/carnitine dehydratase	*Ralstonia metallidurans* CH34	ZP_00273947	1	
Agmatinase precursor	*Schizosaccharomyces pombe*	NP_593990	1	
Aldo-keto reductase	*Cryptococcus neoformans* var. *neoformans* B-3501A	AAW46629	2	3
Allantoicase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43136		2
Amidophosphoribosyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42423		1
Aryl-alcohol dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46369	1	1
Beta-1,3-mannanase	*Paecilomyces lilacinus*	BAD06516	1	
Chorismate synthase	*Schizosaccharomyces pombe*	T41268		3
Cycle propane fatty acid synthase	*Coprinopsis cinerea*	AAL73238		1
Dihydrokaempferol 4-reductase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43891		4

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Dolichyl-phosphate beta-glucosyltransferase	*Schizosaccharomyces pombe*	NP_596707	2	1
Exo-1,3-beta-glucanase	*Agaricus bisporus*	CAA63536		1
Endo-1,4-β-xylanase A	*Phanerochaete chrysosporium*	AAG44993	1	
Flavin-containing monooxygenase	*Aspergillus fumigatus*	CAE47890		2
Glucosidase 1	*Caenorhabditis elegans*	NP_502053	1	
Glutamate synthase (NADH)	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46054		1
Glycine dehydrogenase-like protein	*Pleurotus djamor*	AAS46734		2
Homoserine O-acetyltransferase	*Saccharomyces pastorianus*	Q06736	1	
Hydrolases	*Pseudomonas syringae* pv. *syringae* B728a	ZP_00126253		1
3-hydroxyanthranilate 3,4-dioxygenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41998	1	1
3-hydroxyisobutyrate dehydrogenase	*Novosphingobium aromaticivorans* DSM 12444	ZP_00305232		1
Hydroxymethylglutaryl-CoA lyase	*Pseudomonas mevalonii*	AAA25896	1	
IMP dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40949	1	
Laminarinase	*Phanerochaete chrysosporium*	BAC67687	1	
Lysosomal alpha-N-acetyl glucosaminidase	*Gallus gallus*	XP_418147		1
Mannosyl-oligosaccharide glucosidase	*Schizosaccharomyces pombe*	NP_594106		1
Nicotinate-nucleotide diphosphorylase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42323		1
Oxidoreductase	*Agrobacterium tumefaciens* str. C58	AAL43649	9	9
Phenol 2-monooxygenase	*Neurospora crassa*	CAF06102	1	
Phospho-2-dehydro-3-deoxyheptonate aldolase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46670	1	1
Phosphatidylinositol phosphate phosphatase	*Schizosaccharomyces pombe*	NP_596431		1
Phosphoglycerate mutase GPM2	*Mycobacterium tuberculosis* H37Rv	CAE55568	1	3
Phosphoribosyl-5-amino-1-phosphoribosyl-4-imidazolecarboxiamide isomerase	*Saccharomyces cerevisiae*	NP_012244	1	2
Phosphotyrosyl phosphatase activator	*Oryza sativa*	BAD37240		2
Phytase	*Trametes pubescens*	CAC48234	46	4
Polygalacturonase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42245		1
Polyketide biosynthesis associated protein	*Agrobacterium tumefaciens* str. C58	NP_532458	2	
Prenyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42863		1
Riboflavin aldehyde-forming enzyme	*Lentinula edodes*	BAD11818		1
Short-chain alcohol dehydrogenases	*Ralstonia metallidurans* CH34	ZP_00271907	1	3
Urate oxidase	*Aspergillus flavus*	CAA43896	1	1
UTP-glucose-1-phosphate uridylyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42292		1
Zinc-binding dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42578	1	
VI. Protein fate and synthesis				
Alanine-tRNA ligase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44283	1	
Arginyl tRNA synthetase	*Candida albicans* SC5314	EAK99505		1
Aspartyl-tRNA synthetase	*Saccharomyces cerevisiae*	NP_015221		1
Aspartic proteinase precursor	*Botryotinia fuckeliana*	AAG43236	6	6
ATP-dependent protease proteolytic subunit ClpP	*Arabidopsis thaliana*	BAC43126	1	

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Calmodulin-dependent protein kinase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45617		1
Carboxypeptidase C	*Saccharomyces cerevisiae*	S46008		1
Carboxypeptidase Y	*Trichophyton rubrum*	AAS76668		1
Chaperone regulator	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42057		2
Dipeptidyl aminopeptidases/ acylaminoacyl-peptidases	*Microbulbifer degradans* 2-40	ZP_00315190	1	
Elongation factor 1-gamma	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40932	3	7
Endopeptidasee	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43542	1	4
Eukaryotic translation initiation factor SUI1 family protein	*Arabidopsis thaliana*	NP_177291	1	
FKBP-type peptidyl-prolyl cis-trans isomerases 1	*Rubrivivax gelatinosus* PM1	ZP_00245218		1
Glutaminyl-trna synthetase	*Schizosaccharomyces pombe*	NP_596745		1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	*Schizophyllum commune*	P32638		1
Insulin degrading enzyme	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46588	2	2
Leucine aminopeptidase	*Coprinopsis cinerea*	BAB87833	1	
Nucleolar peptidyl-prolyl cis-trans isomerase (PPIase)	*Saccharomyces cerevisiae*	NP_013637	2	3
Polyubiquitin 6	*Gracilaria verrucosa*	S53719	1	
20S proteasome alpha-type subunit	*Saccharomyces cerevisiae*	NP_014604		3
Ribosomal large subunit assembly and maintenance-related protein	*Cryptococcus neoformans* var. *neoformans* B-3501A	AAW41360		2
Ribosomal protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42014		3
40S ribosomal protein S8	*Schizophyllum commune*	AAC69196	5	6
Ribosomal protein l15 homologue	*Aspergillus fumigatus*	CAE47918		1
50S ribosomal protein L22	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43715	1	
60s ribosomal protein l5-b	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42426		1
60s ribosomal protein l27	*Cryptococcus neoformans* var. *neoformans* JEC21	EAK83063	1	
LSU ribosomal protein L1P	*Thermus thermophilus* HB27	YP_005708	1	
RING/C3HC4/PHD zinc finger-like protein	*Cucumis melo*	AAO45753	1	
t-complex protein 1, beta subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40957	1	1
t-complex protein 1, delta subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44504	6	6
t-complex protein 1, theta subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42275		2
Translation initiation factor	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43820	2	1
Ubiquitin-like protein	*Plasmodium yoelii*	EAA18158		1
Ubiquitin-like protein 5	*Drosophila melanogaster*	Q9V998		1
Ubiquitin carboxyl-terminal hydrolase	*Schizophyllum commune*	AF077976	1	
VII. Transcription				
Cofilin	*Pichia angusta*	AAK85273	1	1
DNA-directed RNA polymerase I	*Schizosaccharomyces pombe*	NP_594382		1
Glia maturation factor beta	*Cyprinus carpio*	BAA95482	1	1
H/ACA snoRNP component	*Candida albicans* SC5314	EAK98515	16	
Hexamer-binding protein HEXBP	*Leishmania major*	A47156	1	1

Table 1. (Continued.)

Category and putative function	Related taxon	Accession No.	N_M[a]	N_T[b]
Leucine zipper protein	*Oryza sativa*	BAD38167	1	1
Mammalian swi/snf complex 60 kda subunit homolog	*Schizosaccharomyces pombe*	T50184		2
mRNA processing-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43157		2
nhp2	*Schizosaccharomyces pombe*	AL158056	3	7
Nucleus protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40929	1	3
Reptin	*Apis mellifera*	XP_395860	1	
Pre-mRNA splicing factor RNA helicase PRP28	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41184	1	6
TATA box binding protein (TBP)	*Homo sapiens*	AB010067	2	
tRNA dihydrouridine synthase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45443	1	
trp-asp repeats containing protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46166	1	
VIII. Energy				
Cytochrome-b5 reductase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45007	1	
Cytochrome c1	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44407	1	2
Glyceraldehyde 3-phosphate dehydrogenase	*Arabidopsis thaliana*	NP_172801	2	
NADP-dependent oxidoreductases	*Pseudomonas syringae* pv. *syringae* B728a	ZP_00124416	3	8

[a] Redundancy number of the sequences in liquid-cultured mycelia ESTs.
[b] Redundancy number of the sequences in wild-type basidiomatal ESTs.

(Figure 1). A major portion (79 ESTs) in the AM library represented transcription, which is in the metabolism category, corresponding to 9.6% of all ESTs. The next one was involved in protein fate and protein synthesis. Except for the "unknown function" category, ESTs related to metabolism were found to be the most abundant, followed by the category "protein fate and protein synthesis" categories in the AT library. In these categories, 40S ribosomal protein, S8, and the delta subunit of t-complex protein 1 (tcp-1-delta) were found in both libraries. In the metabolism category, there were 23 and 33 unigenes in the AM and AT libraries, respectively (Table 1). However, in the differential expression during the mycelial and basidiomatal stages, the cell fate, cell cycle, and DNA processing category was found to be the most divergent, followed by those involved in cellular transport, transport facilitation, and transport routes (Table 2). There were 15 kinds of ESTs in the cell fate, cell cycle, and DNA processing category while only two unigene ESTs occurred in both stages. The difference between the mycelial and basidiomatal stages was 86.7%.

DISCUSSION

From a scientific and economic point of view, differentiation of sexual fruiting bodies from mycelia at vegetative is an interesting phenomenon in homobasidiomycetes, especially while *A. cinnamomea* possesses a porous-hymenium basidiome without stipe. Although several mycologists have made efforts to study gene expression during the formation of fruit bodies (Lee et al., 2002; Sunagawa and Magae, 2005; Yamada et al., 2006), porous-hymenium basidiomatal formation and biosynthesis of active components at the molecular level have not yet been explained.

High-throughout single-run partial sequencing, generation, and analysis of ESTs have proven to be a rapid and efficient approach to obtaining information on mRNA expression (Adams et al., 1991). To study the gene regulation during porous-hymenium basidiomatal formation in *A. cinnamomea*, cDNA clones were randomly sequenced at different stages of the culture of *A. cinnamomea*. In total, 1,809 ESTs matched with 592 distinct sequences in the non-redundant protein database and assigned to 259 functional genes from all libraries. Although the number of ESTs in this study is low, one may obtain some useful information from the genes encoded by the *A. cinnamomea* genome. The cDNA libraries and the accompanying database are valuable resources for researchers hoping to understand the genetic control of basidiomatal formation and secondary metabolism in *A. cinnamomea*.

It is customary in genome annotation to establish a cutoff for "statistically significant" database hits. By correlating an observed alignment score with the expected distribution, we can quantify statistical significance in

Table 2. Divergency analysis of unigenes in each category from mycelia and basidiomatal stages.

Category	I	II	III	IV	V	VI	VII	VIII
Total[a]	15	16	24	5	45	37	15	4
Both[b]	2	7	4	2	10	9	7	2
Divergency (%)[c]	86.7	56.3	83.3	60	77.8	75.7	53.3	50

(Mention all the categories in Table 1)
[a]Total number of independent unigenes derived from two libraries.
[b]Number of independent unigenes appearing in both stages.
[c]Percentage of unigenes appearing only in mycelia or basidiomatal stage.

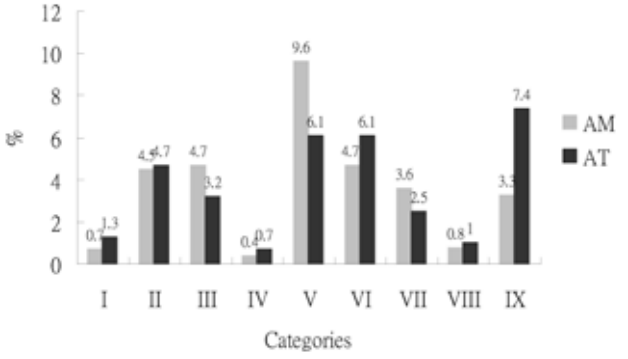

Figure 1. Functional categories of ESTs from different stages of *A. cinnamomea*. I: Cell fate, cell cycle and DNA processing; II: Cell rescue, defense and virulence; III: Cellular transport, transport facilitation and transport routes; IV: Cellular communication/signal transduction; V: Metabolism; VI: Protein fate and protein synthesis; VII: Transcription; VIII: Energy; IX: Unknown. AM: liquid-cultured mycelia; AT: wild basidiomes of *A. cinnamomea*. The number listed on top of the bar indicates the percentage of EST.

the form of an *E* value. It can be expressed in terms of the false-positive expectation value for the BLAST searches and is set routinely at values such as $E = 0.001$ or $E = 10^{-5}$. The problem with this approach is that the distribution of similarity scores for evolutionarily and functionally relevant sequence alignments is very broad, and a considerable fraction of them may fail the *E*-value cutoff, resulting in undetected relationships and missed opportunities for functional prediction (false negatives) (Galperin and Koonin, 2001). In the present study, to minimize the false-positive rate, it was critical to set the *E* values at less or equal to 10^{-10} while filtering low-complexity sequences. BLASTX analysis revealed that only 22.1% and 33.7% of ESTs showed significant similarity to published protein sequences in the AM and AT libraries, respectively. The absence of these sequences from public databases might indicate specific roles for these proteins in *A. cinnamomea*. Similarly, with E values less than or equal to 10^{-5} as the cutting point, only 39.5% to 40.8% ESTs showed no significant similarity to any known proteins in the existing databases.

In redundant ESTs analysis, most ESTs occurred one to four times while only few occurred several times in the cDNA libraries. In this study, 16 unigenes occurred more than five times in the libraries. There were 46 instances when cDNAs were identified as phytase among these unigenes. These unigenes showed high expression in the liquid-cultured mycelial library compared to the basidiomatal library. BLASTX analysis showed that these ESTs of phytases had 62~67% identity with *Trametes pubescens* phytase. Moreover, cDNA was identified as lanosterol 14-alpha-demethylase 17 times in the basidiomatal library, but only thrice in the liquid-cultured mycelia library. The lanosterol 14-alpha-demethylase belongs to the CYP51 family of the cytochrome P450 superfamily, and it is notable that lanosterol 14-alpha-demethylase is the only member of the cytochrome P450 family. Also, this is true in all biological kingdoms (Revankar et al., 2004). In addition, the ESTs identified as putative methylenetetrahydrofolate reductase were found five times in the basidiomatal libraries only. This suggests the possibility that this gene was expressed specifically at the basidiomatal stage and may be related to basidiomatal formation.

According to the number of unigenes in each category, cDNAs encoding for the genes related to "metabolism" were found to be the most abundant. Among the 45 unigenes isolated from these two libraries, 22 unigenes (48.9%) were expressed only in the basidiomatal library. In another study, riboflavin aldehyde-forming enzyme specifically and abundantly was expressed in the mature basidiomes of *Lentinula edodes* (Hirano et al., 2004). Thus, the promoter region of the riboflavin aldehyde-forming enzyme may be the emitter of the expressing signal of the vector for gene manipulation in a mature fruiting body. In our study, the putative riboflavin aldehyde-forming enzyme appeared only in the wild-type *A. cinnamomea.* This result indicates that the promoter of the aldehyde-forming enzyme may be the vector regulator when the mature basidiome is being manipulated.

Regardless of the cultural conditions or different expression patterns during the mycelial and basidiomatal stages, the group of ESTs tentatively assigned to the cell fate, cell cycle, and DNA processing-related category varied the most. Among the 27 unigenes isolated from these two libraries, only 18 unigenes (66.7%) were expressed in the basidiomatal library. On comparing our results with the sequence expression of *P. ostreatus* in the same category, the putative Ras 1p gene also appeared only at the fruiting body stage (Lee et al., 2002).

The EST database established in this study provides the sequence information of genes expressed during basidomatal formation at the whole-gene level. Combining the analysis with metabolomics, proteomics, microarray analysis, transformation system, and bioinformatics, we hope to explore the genes and to understand how basidiomatal formation can be regulated in the future.

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樟芝子實體形成過程基因表現之解析

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為了釐清樟芝不同型態之分化過程，我們分別自液態培養的樟芝菌絲體及野生之樟芝子實體建構二個互補股 DNA 的基因資料庫。經由單向之解序結果，分別穫得了 821 及 993 個高品質之表現序列標籤，續由 BLASTX 的分析結果顯示，與現有已知序列的資料庫之相似度僅有 32.5%-33.7% (以期望值 $\leqq 10^{-10}$ 為界)。不論是菌絲體或是子實體的資料庫，其中，屬於代謝相關的基因數目最多，接著是屬於與蛋白質的表現時期和蛋白質生合成有關之基因數目次多，而兩組互補股 DNA 的基因資料庫之間則以「細胞時期、細胞週期及 DNA 生成」一組所表現的基因差異最大，接著是與代謝有關之基因數目次多。藉由本研究所建構的序列資訊，將有助於克服樟芝所面臨難以促進子實體形成之難題，並可提供未來調控代謝物研究時之重要參考訊息。

關鍵詞：樟芝；基因表現標籤；藥用真菌；型態分化。