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RESEARCH LETTER

Isolation and analysis of genes specifically expressed during basidiomatal development in *Antrodia cinnamomea* by subtractive PCR and cDNA microarray

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Keywords
Antrodia cinnamomea; basidiomatal formation; subtractive PCR; cDNA microarray; differential expression.

Abstract

cDNAs specifically expressed at the basidiome stage were isolated by using PCR-selected cDNA subtraction in order to study gene regulation during porous-hymenium basidiomatal formation in *Antrodia cinnamomea*. BLASTX results suggested that most of the expressed sequence tags (52.4–69.5%) had no significant protein homology to genes from other published living things. cDNAs particularly expressed at different growing conditions were identified using cDNA microarray analysis. Reverse transcriptase PCR analyses confirmed that the clone putative to P-type ATPase, various cytochrome P450s and some unknown genes were abundant at natural basidiomes while endoglucanase was abundant at the tissue from artificial medium.

Introduction

Antrodia cinnamomea (T.T. Chang & W.N. Chou) (formerly *A. camphorata*) is a resupinate to effused-reflexed basidiomataycete with porous hymenium that is parasitic in the inner cavity of the endemic species *Cinnamomum kanehirai* Hay (Chang & Chou, 2004). *Antrodia cinnamomea* forms a traditional Chinese medicine and is well known under its Chinese name 'Chang-chih' in Taiwan. Recent studies have found that it possesses a wide range of biological functions, such as antioxidative activity (Hseu *et al.*, 2002; Hsiao *et al.*, 2003; Song & Yen, 2003; Shen *et al.*, 2006; Huang *et al.*, 2007), vasorelaxation (Wang *et al.*, 2003), anti-inflammatory activity (Shen *et al.*, 2004; Hseu *et al.*, 2005; Chen *et al.*, 2007), antitumor (Liu *et al.*, 2004; Nakamura *et al.*, 2004) and antihepatitis effects (Lee *et al.*, 2002; Lu *et al.*, 2007), hepatoprotective activity (Han *et al.*, 2006) and antiangiogenic activity (Cheng *et al.*, 2005). *Antrodia cinnamomea* grows very slowly in the wild is very slow, and it is thus difficult to cultivate in the greenhouse. As a result, the transfer from vegetative growth to basidiomatal formation is an important stage in both basic and applied fields of mycological research.

To gain global insight into the process of fruit body development, an expressed sequence tag (EST) analysis was used for *Agaricus bisporus* (Ospina-Giraldo *et al.*, 2000) and *Pleurotus ostreatus* (Lee *et al.*, 2002). In addition, suppressive subtractive hybridization (SSH) and differential display were also used for *Ophiostoma piceae* (Dogra & Breuil, 2004), *P. ostreatus* (Sunagawa & Magae, 2005) and *Flammulina velutipes* (Yamada *et al.*, 2006) to identity the genes differentially expressed during fruit body development. SSH utilizes PCR to provide rapid comparisons of the expression of mRNA from different samples and also to show the relative difference in the concentration of these molecules. Essentially, this technique removes common transcripts between two experimental samples while maintaining the uniqueness of those transcripts. This method not only reduces the number of highly abundant cDNAs in the library, it can also maximize opportunities to identify those low-abundance genes that may have played critical roles in the experimental samples of interest (Morse *et al.*, 2004).

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Thus, SSH is a simple and efficient method that can provide specific targeted genes among different samples.

The complexity of basidiomatal formation and biosynthesis of active components suggest that a large number of genes are involved. Differentiation of sexual fruit bodies from vegetative mycelia is an interesting process in basidiomataycetes from both the scientific and economic points of view. In particular, *A. cinnamomea* possesses a porous-hymenium basidiome without stipe. Although many mycologists have put much effort into identification of gene expression of cell regulation during fruit body formation, porous-hymenium basidiomatal formation and biosynthesis of active components at the molecular level have not yet been clarified. In order to identify genes that are differentially expressed in various sources to an experimental stimulus, we used SSH to identify well-expressed genes. Furthermore, genes obtained were arrayed and probed with cDNA libraries, and later confirmed by reverse transcriptase (RT)-PCR analysis from different cultures in this study.

Materials and methods

Strains and culture conditions

Antrodia cinnamomea strain TFRIB 479, obtained from rotten wood of *C. kanehirai* at Dawu and Taitung, was identified in the present study. The cultures were maintained as reported by Chang & Chou (2004). The natural basidiomes were obtained directly from the rotten wood. Liquid-cultured mycelia (AM), solid-cultured mycelia (AL), solid-cultured basidiomes (AF) and natural basidiomes (AT) were frozen in liquid nitrogen and stored at $-80\,^{\circ}\mathrm{C}$ until use.

Isolation of total RNA and mRNA

Total RNA of each tissue was prepared according to the method described by Chang *et al.* (1993) as modified by Chen *et al.* (2004). For PCR-selected DNA subtraction, mRNA was purified from total RNA using an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany).

cDNA library construction

Three SSHs were performed using a Clontech kit according to the manufacturer's instructions. cDNA from liquid-cultured mycelia was used as a tester and the cDNA from natural basidiomes was used as a driver. The SSH library used was M-T; by contrast, the cDNA from natural basidiomes used as a tester was T-M. In addition, the cDNA from solid-cultured basidiomes was used as a tester and the cDNA from liquid-cultured mycelia was used as a driver for the F-M SSH library. cDNAs were digested with RsaI and ligated to different adapters. Two rounds of hybridization and PCR amplification were processed to normalize and enrich the differentially expressed cDNAs. Products of the secondary PCR were cloned into the pGEM-T Easy vector system (Promega, Madison) following the protocol recommended for overnight ligation. The ligation mixture was transformed into High Efficiency DH5α Competent cells (Hopegen, Taichung, Taiwan) and then plated onto LB-ampicillin plates containing isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal).

DNA sequencing

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

Sequence analysis

Nucleotide sequences were excluded if they were <100 bp and the remaining sequences were subjected to removal of vector sequences the CHROMASPRO program (Technelysium Pty Ltd, Tewantin, Australia). Individual ESTs were assembled into contigs using thr CONTIGEXPRESS program within the Vector NTI Suite 8 (Invitrogen, Carlsbad) with parameters optimized for ESTs rather than genomic clones. The cDNA sequences were compared against nonredundant (nr) protein sequence databases at the National Center for Biotechnology Information (NCBI) using the BLASTX algorithm (Altschul *et al.*, 1997). *E*-value results obtained from the BLASTX search were categorized as follows: $\leq 10^{-10}$ represented significant homology and grouped according to putative function as a value $>10^{-10}$ implied no hit. A unique set with significant matches was annotated based on 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).

cDNA microarray preparation and analysis

The cDNA microarray was prepared according to Lin & Wu (2004). PCR products from each EST were arrayed from a 384-well plate onto CMTGAPS2-coated glass slides (Corning, New York) using an OmniGrid 100 microarrayer (GeneMachines, San Carlos, CA) according to the manufacturer's instructions. cDNA from the 18S rRNA gene of *A. cinnamomea* was also printed as an internal control. Total

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Table 1. Primer sequences

Gene name	Forward (5′–3′)	Backward (5′–3′)
P-type ATPase	CTTGGACCCGACGATAAATCCT	TATTCTGTATCTGCAGCCTCCC
Cytochrome P450-1	TACACAATATACTAAAGCGCCC	GACGAAACAAACCGAGAATACC
Cytochrome P450-2	TTCTGCCGGTGATACATTAGTG	ACGATCTGATATGGACATTCAC
Prolin iminopeptidase	CAATCAAGCAATTCCATGCGAC	CCGTGCATAGAACATGAGTGTG
β-Transducin protein	ACACTACTCTAGCTATCCTCGA	ACATCATCTCATAGATAGGCCG
Glucan 1,4-α-maltohexaosidase	CAGGAGTGCTATGATGACAGAG	ATGTAACAACCTGACTGTACCC
Endoglucanase	ATCGAGTTATTCTTCCGCCGAG	ACAATTGCCCCGTTTTCATTCC
Unknown protein-1	AACTACCTGAACGCATACCCTG	CAAGGAGAGTCTGTGTGAAGGT
Unknown protein-2	AGACAAGGGTGAGAGGACGCTACACGAT	CCGACCTGCTCAACGTCTTCGATATATGC
Unknown protein-3	CTCATACAACCACTCACATCACAAC	ACCGTTTCATGCGTAATAGGATTG

RNA prepared from different sources was reverse-transcribed and used as probes for expression profile analysis. The RNA was extracted according to Chang *et al.* (1993). The methods used for fluorescent probe preparation and hybridization were as described at http://www.botany.sinica.edu.tw/microarray/protocols.htm. In order to analyze cDNA microarrays, data files were imported to SMD, ASCC (http://www.bitora.sinica.edu.tw:7777/smd/MicroArray/SMD/) and GeneSpring 6.1 (Silicon Genetics, Redwood, CA) for further analysis. 'Default Computed Normalization' was applied for data normalization for SMD.

RT-PCR analysis

In order to detect differential expression by RT-PCR, total RNA was reverse-transcribed into first-strand cDNA following the protocol for SMART cDNA synthesis using the SMART cDNA library construction kit (Clontech, Mountain View). One microgram of total RNA was incubated at 42 °C for 1 h with 200 U of superscript^{TM} II reverse transcriptase in a 20-μL reaction mixture. Then, 0.3 μg cDNA of the first-strand cDNA products from each sample was used for PCR amplification to detect the transcripts. Twenty-five cycles of PCR were run under the following conditions: 1 min at 94 °C, 30 s at 58 °C and 30 s at 72 °C. The internal control was analyzed by a pair of primers, RT-18Sn (5′-ACTGTGAAACTGCGAATGGCTC-3′) and RT-18Sc (5′-GACTTG CCCTCCAATTGTTCCTC-3′), specific for the 18S rRNA gene of *A. cinnamomea*. Primers for the other genes are listed in Table 1. The RT-PCR products were detected with a UV transilluminator after undergoing 1.2% agarose gel electrophoresis and staining with ethidium bromide.

Results

Construction of the subtracted cDNA libraries

After eliminating vectors and unreadable sequences, the clones were submitted to dbEST (GenBank accession nos. EV472086–EV473050). The nucleotide sequences were excluded if they were < 100 bp, and a total of 945 high-quality clones were subjected to analysis. In total, 313 cDNA clones were obtained after applying subtractive hybridization via the M-T library. These clones were candidates for transcripts specific to or up-regulated for the M library. After sequence assembly, 111 ESTs were singletons, whereas the other 202 ESTs clustered into 39 contigs. Subtractive hybridization of the T-M and F-M libraries produced 370 and 262 cDNA clones, respectively. These clones were candidates for transcripts specific to or up-regulated for basidiomes. After sequence assembly, there were 192 singletons and 59 contigs in the T-M subtractive library, and 185 singletons and 31 contigs in the F-M subtractive library. These subtractive cDNA libraries of *A. cinnamomea* had insert sizes of 0.1–0.8 kb.

Functional classification of ESTs

The ESTs obtained were compared with the nr (all non-redundant GenBank CDS translations+PDB+Swissprot+PIR+PRF) database using the BLASTX algorithm. The database sequence matches were classified as either a hit (E-value $\leq 10^{-10}$) or no hit (E-value $> 10^{-10}$) (Chen *et al.*, 2004). In subtractive hybridization of the M-T library, a BLASTX search revealed that 164 (52.4%) ESTs showed no significant similarity to protein sequences described in the nr database. There were 231 (62.4%) and 182 (69.5%) ESTs in the T-M and F-M libraries without significant similarity to protein sequences described in the nr database. Except for ESTs for unknown function and no hit, the total expressed transcripts of these cDNA libraries are listed in Table 1. The database-matched ESTs were classified based on their putative function and were divided into seven different categories based on their cellular roles according to the MIPS FunCat scheme. In the mycelia library, the ESTs encoding endoglucanase were the most abundant. In addition, a group of genes associated with heat shock response were also abundant. However, P-type ATPase was the most abundant in natural basidiomes. Furthermore, various kinds of cytochrome P450s were also found in subtractive hybridization of the T-M library.

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Differential screening of various subtracted cDNA libraries

In order to recognize the relative level of these ESTs, these clones were analyzed using a cDNA microarray. Liquid-cultured mycelia labeled with Cys3, solid-cultured mycelia, solid-cultured basidiomes and natural basidiomes labeled with Cys5 were used. Each slide was hybridized with two probes, resulting in each labeled liquid-cultured mycelia coupled to each solid-cultured mycelia, solid-cultured basidiomes or natural basidiomes. Summarizing these three hybridization assemblies, we obtained seven expression types with ratios ≥ 4. Modes of expression in different growing conditions are shown in Fig. 1 and the expressed transcripts are detailed in Table 2. In modes A, B, F and G, the genes were expressed more abundantly in basidiomatal stages than in mycelial stages. However, their relative intensities were different. As shown in graph A of the gene tree, gene expression was much lower in the solid-cultured mycelia stage as compared with gene expression in graph B. P-type ATPase belonged to this group. In mode C, the expressed transcripts were lower in AF growing conditions. In contrast, the transcripts were higher in AF growing conditions in mode D. The reverse expression pattern was shown in mode E, i.e. transcripts were significant lower in AT growing conditions. The data were similar for SSH, except for type E with endoglucanase. Other gene types that were up-regulated in basidiomes included P-type ATPase and cytochrome P450. Only two kinds of genes, including heat shock protein 30 and NADPH dehydrogenase subunit 5, had ratio values ≤ 0.25 (data not shown).

Confirmation of differential screening

The highly expressed genes (expression ratio > 20), including endoglucanase, P-type ATPase, cytochrome P450, prolin iminopeptidase, β-transducin protein, glucan 1,4-α-maltohexaosidase and three unknown proteins, at different growing conditions were further examined by RT-PCR. In order to confirm the significant expression of these genes in various cultures as indicated SSH and cDNA microarray analysis, we designed specific primers to amplify the unique genes from RNA isolated from the liquid-cultured mycelia, solid-cultured mycelia, solid-cultured basidiomes and natural basidiomes. The gene expression profile via RT-PCR analysis is shown in Fig. 2. The gene encoding endoglucanase was significant in artificial medium, but very weak in natural basidiomes. However, P-type ATPase was significantly expressed in natural basidiomes, but not in liquid-cultured mycelia. In addition, two cytochrome P450s showed different expression patterns in different culture conditions. However, these two cytochrome P450s were both significantly expressed in natural basidiomes. Moreover, prolin iminopeptidase, glucan 1,4-α-maltohexaosidase and three unknown proteins were more abundant in natural basidiomes than in other cultured tissues.

Discussion

Various cultured cDNA libraries of *A. cinnamomea* were constructed by using PCR-selected cDNA subtraction. In summary, 945 high-quality clones were identified. The BLASTX analysis results suggested that most of the ESTs (52.4–69.5%) had no significant protein homology to genes from published databases. As the function for basidiomatal formation and biosynthesis of active components remain unknown, cDNA microarray analysis showing the expression patterns for each EST from different developing stages may offer important clues in understanding their functions.

It has been reported that several genes coding for the cytochrome P450 family of proteins were isolated by

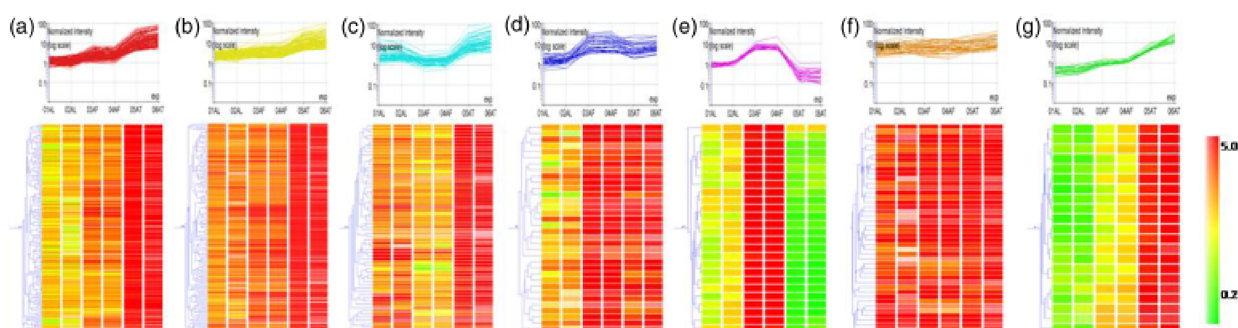

Fig. 1. Clusters of genes with various response kinetics to different growing conditions were analyzed using K-Means (GeneSpring): AL, solid-cultured mycelia; AF, solid-cultured basidiomes; AT, natural basidiomes. Only genes showing greater than fourfold expression changes in at least two conditions were included in the cluster analyses. Each condition has two biological replicates shown on the *x*-axis while the normalized intensity (the ratio of AL, AF and AT to the liquid-cultured mycelia condition) is shown on the *y*-axis. Clusters were subjected to gene tree analyses with the use of GeneSpring (below). The degree of change was color-coded (yellow, red and green represent no change, and up- and down-regulation, respectively).

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Table 2. Annotation of ESTs from subtractive libraries of *A. cinnamomea*

Category and putative function	Related taxon	Accession no.	M	F	T	S
Cell fate, cell cycle and DNA processing						
Actin	*Paxillus involutus*	AAT91279	–	1	–	–
ATP-dependent protein-binding protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46345	1	–	–	A
β-Tubulin	*Paxillus involutus*	AAT91243	–	–	2	–
Calmodulin	*Paxillus involutus*	AAT91341	–	1	–	–
Cell division control protein Cdc4	*Aspergillus fumigatus* Af293	EAL89143	1	–	–	–
Cyclin-binding protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43561	2	–	–	–
Cytokinesis-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42225	1	–	–	–
DNA-binding protein	*Schizophyllum commune*	AAK26659	–	1	–	B
DNA clamp loader	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41633	–	–	1	–
DNA supercoiling,	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43155	–	–	1	A
Golgi reassembly-stacking protein 2	*Gallus gallus*	XP_422002	–	1	–	–
Histone	*Agaricus bisporus*	CAC03460	–	–	2	C
Histone H2B	*Agaricus bisporus*	CAA63898	–	–	2	A
Histone H3	*Lentinula edodes*	BAD11819	–	1	–	B
TOM1 protein	*Neurospora crassa*	CAB92704	–	–	1	B
Tyrosine recombinase	*Coprinopsis cinerea*	DAA01992	–	–	1	A
Zeta DNA polymerase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41897	–	–	1	C
Cell rescue, defense, and virulence						
Catalase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45227	–	–	1	B
Chaperone activator	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40882	–	–	1	–
Heat-induced catalase	*Pleurotus sajor-caju*	AAK15159	–	–	1	B
Heat shock protein 30	*Coriolus versicolor*	BAA76591	23	–	–	–
HSP100	*Pleurotus sajor-caju*	AAF01451	5	–	–	–
Protein FDD123	*Trametes versicolor*	O74631	15	–	–	–
rds1	*Schizosaccharomyces pombe*	CAA54544	4	–	–	–
Small heat shock protein	*Laccaria bicolor*	AAM78595	16	–	–	–
Tetracycline efflux protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42686	1	–	–	–
Thiosulfate sulfurtransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43312	–	–	1	–
Cellular transport, transport facilitation and transport routes						
Allantoate permease	*Neurospora crassa*	CAE81935	–	–	1	C
Antho-RFamide neuropeptides type 2 precursor	*Anthopleura elegantissima*	Q16994	1	1	–	–
Amino acid transporter	*Amanita muscaria*	CAB38005	–	–	1	–
arp2/3 complex 16-kDa subunit	Cryptococcus neoformans var. neoformans JEC21	AAW40905	–	1	–	–
ATP-binding/permease protein	*Silicibacter pomeroyi* DSS-3	AAV95597	–	1	–	–
ATP-dependent permease	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44322	–	1	–	A
Calcium-transporting ATPase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46512	1	–	–	–
Carboxylic acid transport protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45996	–	–	1	D
CoA-binding protein	*Geobacter sulfurreducens* PCA	NP_952665	–	–	1	–
CRAL/TRIO domain protein	*Aspergillus fumigatus* Af293	EAL91315	1	–	–	–
GEF1	*Cryptococcus neoformans* var. *neoformans*	AAV98485	–	1	–	–
Golgi vesicle transport-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45310	–	1	–	–
GTPase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46779	1	–	–	–
Hydrogen-exporting ATPase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44429	1	–	–	–
isp4	*Schizosaccharomyces pombe*	CAC05511	–	1	–	B
Membrane transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46090	–	1	–	D
MFS transporter	*Aspergillus fumigatus* Af293	EAL85184	1	–	1	A
Mfs1.1	*Coprinus cinereus*	AAF01426	5	–	–	–
Myo-inositol transport protein ITR1	*Neurospora crassa*	CAB99236	–	–	2	–
Nucleoporin-interacting protein NIC96	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42380	–	1	–	–
P-type ATPase	*Aspergillus fumigatus* Af293	EAL86228	–	–	23	A
Peptide transporter MTD1	*Schizophyllum commune*	AAF26618	–	1	1	A
Protein transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45450	–	–	2	G

(*Continued*)

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Table 2. Continued.

Category and putative function	Related taxon	Accession no.	M	F	T	S
Tartrate transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45350	–	1	–	D
Transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40958	–	–	1	–
UDP-galactose transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46420	–	–	1	C
Urea transporter	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43008	–	–	1	–
Vacuolar ATP synthase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45529	–	–	2	A
Cellular communication/signal transduction						
β-transducin-like protein HET-E2C*4	*Podospora anserina*	AAL37299	–	2	–	F
G protein β subunit-like protein	*Homo sapiens*	AAP97225	–	2	–	B
Serine/threonine kinase	*Ustilago maydis*	AAM97788	–	–	3	F
Phosphoprotein phosphatase	*Lentinula edodes*	JC7206	–	–	1	–
Polyphenol oxidase	*Agaricus bisporus*	CAA59432	–	–	1	–
Two-component sensor molecule	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40853	–	–	1	A
Tyrosine phosphatase-like protein	*Mus musculus*	AAA87037	–	–	1	–
Metabolism						
Acetoacetyl-CoA synthetase	*Gallus gallus*	XP_415102	1	–	–	–
5-Methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46187	2	–	–	–
Acyl-CoA dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45980	–	1	–	F
Acyl-CoA oxidase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW47115	1	–	–	–
Alcohol dehydrogenase homolog Bli-4	*Neurospora crassa*	CAB99393	1	–	–	–
Aryl-alcohol dehydrogenase	Cryptococcus neoformans var. neoformans JEC21	AAW46369	1	–	–	–
Alcohol oxidase 2	*Pichia methanolica*	AAF02495	–	1	–	D
Carboxylase	*Pichia angusta*	AAL69566	–	1	–	B
cdc21	*Schizosaccharomyces pombe*	CAB76210	–	1	–	–
Citrate synthase	*Aspergillus niger*	CAB77625	1	–	–	–
Cytochrome P450	*Agaricus bisporus*	CAB85700	–	–	1	A
	Coprinopsis cinerea	BAA33717	1	–	1	–
	Coriolus versicolor	BAB59027	–	–	1	C
	Cryptococcus neoformans var. *neoformans* JEC21	AAW43969	–	8	4	A
	Heterobasidion annosum	AAV83804	–	–	2	D
	Lentinula edodes	BAD11817	–	1	3	–
	Phanerochaete chrysosporium	BAD94562	–	–	1	A
	Xenopus laevis	BAD02914	–	–	1	–
2-deoxy-D-gluconate 3-dehydrogenase	*Bacillus clausii* KSM-K16	YP_174541	–	–	1	B
D-arabinitol 2-dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42422	–	–	2	A
Dihydroxy-acid dehydratase	*Neurospora crassa*	CAD70774	–	–	2	–
Endoglucanase	*Phanerochaete chrysosporium*	AAU12275	27	–	–	E
Exo-β-1,3-glucanase	*Aspergillus phoenicis*	BAB83607	–	–	2	F
Farnesyltranstransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45467	–	1	–	D
Fatty acid β-oxidation-related protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41471	1	–	–	A
Flavin-containing monooxygenase	*Aspergillus fumigatus*	CAE47890	–	–	1	B
γ-Glutamyltransferase	*Neurospora crassa*	CAB91242	1	–	–	–
Glucan 1,4-α-maltohexaosidase	*Chlorobium phaeobacteroides* BS1	ZP_00533568	1	–	–	E
Glucanase	*Aspergillus fumigatus* Af293	EAL86910	–	–	1	–
1,4-Glucan-6-transferase	*Saccharomyces cerevisiae*	AAA34632	–	1	–	–
Glucose 1-dehydrogenase	*Nostoc* sp. PCC 7120	BAB75535	–	1	–	F
Glycerol dehydrogenase	*Symbiobacterium thermophilum* IAM 14863	YP_075096	2	–	–	–
Glycogen phosphorylase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44124	1	–	2	B
ich1 protein	*Aspergillus fumigatus* Af293	EAL89337	–	2	–	F
Isocitrate lyase	*Fomitopsis palustris*	BAD93181	1	–	–	–
Methionine synthase	*Aspergillus fumigatus* Af293	EAL92377	–	1	–	C
2-Methylcitrate dehydratase	*Burkholderia pseudomallei* K96243	YP_111731	–	–	2	–
Molybdopterin biosynthesis CNX1 protein	*Arabidopsis thaliana*	NP_197599	–	1	–	–
Nitrite reductase	*Hebeloma cylindrosporum*	CAB60008	1	–	–	B
2-Nitropropane dioxygenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44744	–	–	1	A
Nucleoside diphosphate kinase	*Paxillus involutus*	AAT91294	–	–	1	C

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Table 2. Continued.

Category and putative function	Related taxon	Accession no.	M	F	T	S
Oxoglutarate dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41534	–	–	1	–
Phytanoyl-CoA dioxygenase family protein	*Aspergillus fumigatus* Af293	EAL93882	1	–	–	–
Polynucleotide kinase 3′-phosphatase	*Rattus norvegicus*	NP_001004259	–	1	–	–
Pyruvate carboxylase	*Pichia angusta*	AAL69566	–	–	1	–
Quinone reductase	*Rubrobacter xylanophilus* DSM 9941	ZP_00187588	–	1	–	–
RAB-protein geranylgeranyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW47017	–	–	2	–
Riboflavin aldehyde-forming enzyme	*Lentinula edodes*	BAD11818	1	–	–	–
Ribose-phosphate diphosphokinase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44665	–	1	–	–
Saccharopine dehydrogenase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42771	–	–	1	G
ser/thr protein phosphatase	*Aspergillus fumigatus* Af293	EAL91474	–	1	–	–
Short-chain alcohol dehydrogenases	*Aspergillus fumigatus* Af293	EAL89916	–	3	–	F
Sniffer-like family member	*Caenorhabditis elegans*	NP_506407	–	1	–	–
Sphinganine-1-phosphate aldolase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44943	–	1	–	–
Succinate dehydrogenase/fumarate reductase	*Rubrobacter xylanophilus* DSM 9941	ZP_00188707	–	–	1	B
UDP-*N*-acetylglucosamine diphosphorylase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43995	–	–	1	C
UDP-xylose synthase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAM22494	–	–	1	A
Tyrosinase	*Lentinula edodes*	BAB71736	–	1	–	B
Protein fate and synthesis						
Asparaginyl-tRNA synthetase	*Desulfotalea psychrophila* LSv54	CAG36060	–	–	1	B
Aspartic protease precursor	*Phaffia rhodozyma*	AAC17105	–	2	1	A
Calnexin	*Aspergillus niger*	CAC82717	1	–	–	–
Chaperone	Cryptococcus neoformans var. neoformans JEC21	AAW42238	3	–	–	–
Coiled-coil protein	Cryptococcus neoformans var. neoformans JEC21	AAW44562	–	1	–	A
Cytosolic cyclophilin	*Arabidopsis thaliana*	AAB96833	–	–	1	–
Ecotropic viral integration site 5-like	*Homo sapiens*	NP_660288	–	–	1	–
Elongation factor 2	*Pichia pastoris*	AAO39212	–	–	1	B
Elongation factor 3	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42954	1	–	1	–
Eukaryotic translation initiation factor 3 subunit 7	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41507	–	1	–	F
Eukaryotic translation initiation factor 5A	*Candida albicans*	O94083	–	–	1	B
fuSed2 protease	*Aspergillus fumigatus*	CAE17674	–	–	4	A
fuSed3 protease	*Aspergillus fumigatus*	CAE46473	–	–	1	A
Lectin	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45608	1	–	–	–
Macrolide-binding protein FKBP12	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW41744	–	1	1	B
Methionine aminopeptidase 1	*Cryptosporidium hominis*	XP_668387	1	–	–	–
Methyltransferase-UbiE family	*Streptomyces avermitilis* MA-4680	BAC69591	2	–	–	–
Prefoldin subunit 3	*Neurospora crassa*	CAF05883	–	1	–	–
Presenilin associated	*Mus musculus*	XP_354952	1	–	–	–
Proline imino-peptidase	*Xanthomonas axonopodis* pv. citri str. 306	AAM37832	–	–	1	C
Proteasome component C2	*Neurospora crassa*	CAD70938	1	–	–	–
Protein phosphatase type 2A	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43622	–	–	2	–
Ribosomal protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42014	–	–	1	A
Ribosomal L22e protein family	*Aspergillus fumigatus* Af293	EAL92353	–	1	–	A
Ribosomal protein L41	*Filobasidiella neoformans*	AAG48930	–	–	1	–
RING and UBP finger domain protein	*Aspergillus fumigatus* Af293	EAL89752	2	–	–	–
rRNA intron-encoded homing endonuclease	*Pan troglodytes*	XP_523367	1	–	–	–
Serine/threonine/tyrosine interacting protein	*Homo sapiens*	AAH20265	–	–	1	–
Signalosome subunit 4	*Danio rerio*	Q6P0H6	–	1	1	B
Ubiquitin C-terminal hydrolase	*Aspergillus fumigatus* Af293	EAL89067	1	–	–	–
t-complex protein 1, α subunit	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42082	–	–	1	–

(*Continued*)

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Table 2. Continued.

Category and putative function	Related taxon	Accession no.	M	F	T	S
Tripeptidyl aminopeptidase	*Aspergillus oryzae*	AAU10333	–	–	2	F
Ubiquitin fusion degradation protein	*Aspergillus fumigatus* Af293	EAL88739	–	–	1	–
Ubiquitin fusion degradation protein-2	*Schizosaccharomyces pombe*	AAC80427	–	–	2	–
Transcription						
α-integrin-binding protein 80	*Homo sapiens*	CAB38231	–	–	1	–
Baker yeast's zinc finger protein RTS2	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW46711	–	1	–	–
b-zip transcription factor	*Schizosaccharomyces pombe*	T41672	–	–	1	–
p68-like protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43962	–	1	–	–
Pre-mRNA splicing factor	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW43637	–	–	2	–
Pre-mRNA splicing factor prp1	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40677	–	2	–	B
Polyadenylate-binding protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45527	–	–	1	B
Single-stranded nucleic acid binding protein	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW45689	–	1	–	–
Sm-like protein	*Dictyostelium discoideum*	AAO50813	–	–	1	B
Transcriptional repressor	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW44628	2	–	–	–
tRNA guanylyltransferase	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW42632	–	–	1	C
Zinc-finger protein zpr1	*Cryptococcus neoformans* var. *neoformans* JEC21	AAW40864	–	–	1	–

M, redundancy number of the sequences in liquid-cultured mycelia ESTs; F, redundancy number of the sequences in solid-cultured basidiomatal ESTs; T, redundancy number of the sequences in wild-type basidiomatal ESTs; S, mode of microarray analysis.

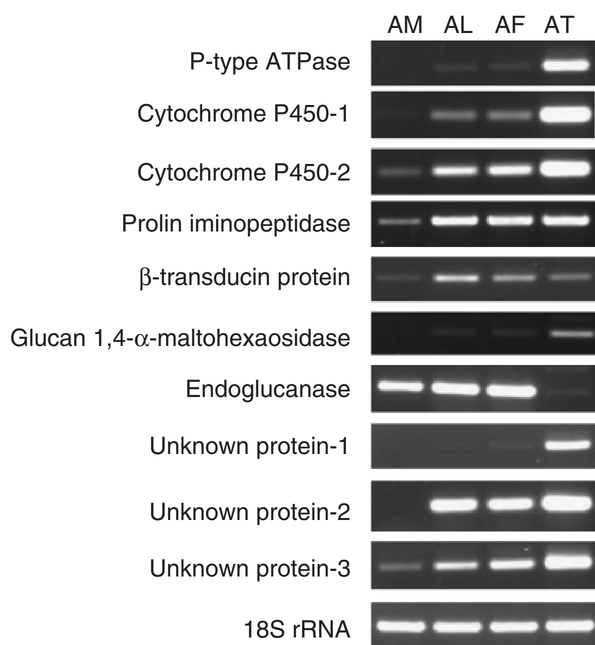

Fig. 2. RT-PCR analysis of gene expression in different growing conditions. Total RNAs isolated from different sources were RT-PCR amplified with gene-specific primers for each specific transcript of *Antrodia cinnamomea*. The 18S rRNA gene was the internal control. The RT-PCR products were subjected to electrophoresis on a 1.2% agarose gel. AM, liquid-cultured mycelium; AL, solid-cultured mycelium; AF, solid-cultured basidiomes; AT, natural basidiomes.

differential screening targeting genes specifically expressed during fruiting in the basidiomycete (De Groot *et al*., 1997; Muraguchi & Kamada, 2000; Ospina-Giraldo *et al*., 2000; Sunagawa & Magae, 2005; Yamada *et al*., 2006). In *A. cinnamomea*, various cytochrome P450s appeared during basidiomatal development. These results indicated that some oxidoreduction reactions driven by cytochrome P450 were necessary during fruiting in basidiomycetes.

PCR confirmed that the gene putative to P-type ATPase was abundant in natural basidiomes. Given that potassium is the most abundant cation in cells, plant-associated fungi and intracellular parasites are permanently or circumstantially exposed to high K^+, and they must avoid excessive K^+ accumulation activating K^+ efflux systems (Benito *et al*., 2002). Thus, high P-type ATPase expression is compatible in natural environments.

Comparing our data with results from other basidiomycetes, the identified genes were relatively different. Among basidiomycetes, hydrophobin is one of the most abundant genes in fruit bodies of basidiomycetes, as reported in *A. bisporus* (De Groot *et al*., 1997), *F. velutipes* (Ando *et al*., 2001) and *L. edodes* (Ng *et al*., 2000; Yamada *et al*., 2006). However, we did not isolate hydrophobin in basidiomatal development of *A. cinnamomea*. Hydrophobin was not detected in *P. ostreatus* also (Sunagawa & Magae, 2005). As *P. ostreatus* only has a short stipe and *A. cinnamomea* has no stipe, hydrophobin may be the only gene closely related to stipe formation.

cDNA microarray and PCR confirmed that several genes found in natural basidiomes had unknown function. It will be worthwhile investigated whether these genes are associated with basidiomatal formation or stress responses. Functional analysis of the novel genes identified in this study will be undertaken in future studies.

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