Mycobiology 34(4): 209-213 (2006)

Genes Expressed During Fruiting Body Formation of *Agrocybe cylindracea*

Sung Mi Shim, Sang Beom Kim, Hey Young Kim, Hyun-Su Rho[1], Hyun Sook Lee[1], Min Woong Lee[2], U Youn Lee, Kyung Hoan Im and Tae Soo Lee*

Department of Biology, University of Incheon, Incheon 402-749, Korea
[1]Department of Microbiology, Gyeongsang National University, Jinju 660-701, Korea
[2]Department of Biology, Dongguk University, Seoul 100-715, Korea

(Received December 4, 2006)

***Agrocybe cylindracea*, an edible mushroom belonging to Bolbitiaceae, Agaricales, is widely used as invaluable medicinal material in the oriental countries. This study was initiated to find the genes expressed during the fruiting body formation of *A. cylindracea*. The cDNAs expressed differentially during fruiting body morphogenesis of *A. cylindracea* were isolated through subtractive hybridization between vegetative mycelia and fruiting bodies. The cDNAs expressed in the fruiting body morphogenesis of *A. cylindracea* were cloned and twenty genes were identified. Eleven were homologous to genes of known functions, three were homologous to genes in other organism without any function known. Six were completely novel genes specific to *A. cylindracea* so far examined. Some genes with known functions were a pleurotolysin, a self-assembling pore-forming cytolysins; Aa-Pril and Pir2p, specifically induced genes during fruiting initiation of other mushroom, *Agrocybe aegerita*; an amino acid permease; a cytochrome P450; a MADS-box gene; a peptidylprolyl isomerase; and a serine proteinase. For other clones, no clear function was annotated so far. We believe the first report of the differentially expressed genes in fruiting process of *A. cylindracea* will be great helps for further research.**

KEYWORDS: ***Agrocybe cylindracea*, cDNA clones, Differential screening, Gene expression**

A. cylindracea is an edible mushroom widely cultivated and consumed all over the world. Recently its consumption is sharply increasing due to its delicious taste and unique texture. In addition to its nutritional value, it has long been used for folk remedies even without any knowledge of which component are responsible. The scientific evidences of this mushroom as multi-purpose medicines on various human diseases have been accumulated. Especially the fruiting bodies of *A. cylindracea* are popularly used to combat human diseases. It has anti-tumor (Kiho *et al.*, 1989), decreasing sugar level in blood (Kiho *et al.*, 1989), immuno-stimulating (Yoshida *et al.*, 1996), and lipid peroxidation inhibitory activities (Lee *et al.*, 1998). Its polysaccharide extracts have anti-mutagenic activity against direct-acting mutagens such as [4-nitro-o-phenylenediamine (NPD) and sodium azide (NaN(3))] and indirect-acting mutagens [2-aminofluorene (2-AF) and benzo[a]pyrene (B[a]P)]. And its anti-mutagenic activity is co-related with anti-tumor activity by inducing expression of detoxifying enzymes such as quinone reductase and glutathione S-transferase (Shon and Nam, 2001). In addition to exopolysaccharides which are responsible for anti-tumor and immuno-stimulating activities, antifungal proteins can be used for medical use. An antifungal protein named as agrocybin has been isolated in *A. cylindracea* by Ngai *et al.* (2005). The agrocybin isolated from fruiting bodies further shows the inhibiting activity of HIV-1 reverse transcriptase (Ngai *et al.*, 2005), possible being used for retarding or healing AIDS disease.

Due to multiple medicinal effects of *A. cylindracea* on human diseases, efforts getting mycelia and exopolysaccharide without inducing fruiting bodies has also been tried to get much more biomass in short time compared to induction of fruiting bodies (Kim *et al.*, 2005). But still the fruiting bodies of *A. cylindracea* are widely consumed as health food and therapeutic medicines for various diseases. Thus, study on its differentiating process is worthy for further research.

Until now, no molecular or genetic datum is available about this important mushroom. Although the genetics on the formation of fruiting bodies in other mushroom have been studied in *Schizophyllum commune*, *Pleurotus ostreatus* and *Lentinus edodes* (Hoge *et al.*, 1982; Mulder and Wessels, 1986; Endo *et al.*, 1994; Kajiwara *et al.*, 1992), no one ever tried to find genes involved in fruiting body differentiation. In *Lentinus edodes*, expressions of several genes which are developmentally regulated during formation of fruiting body are elucidated (Endo *et al.*, 1994; Kajiwara *et al.*, 1992). An EST database containing more that 2000 clones was recently set up for the other edible mushroom, *Pleurotus ostreatus* with the aim of providing information about transition of vegetative mycelium to fruiting body (Lee *et al.*, 2002). In other basidiomycetes, cDNAs which show down- or up-regula-

*Corresponding author <E-mail: tslee@incheon.ac.kr>

tion during the development of fruiting body have been characterized for the *Agrocybe aegerita* and *Agaricus bisporus* (Salvado and Labarere 1991; De Groot *et al.*, 1997).

In this research, we tried to identify the genes expressed during the developmental processes of fruiting body in *A. cylindracea* using suppression subtractive hybridization. We found some important genes specifically induced during the process of fruiting body differentiation. We expect these findings should contribute to a better understanding of gene function in the developmental process of fruiting body as well as in the process of useful metabolite formation.

Materials and Methods

Materials and culture condition. The *A. cylindracea* culture stock was obtained from Gyeyang mushroom cultivation company (Korea). An agar disk of 5 mm in diameter was cut from the culture that was grown on potato dextrose agar (potato 200 g, dextrose 20 g and agar 20 g/ distilled water 1 *l*) medium at 25°C in the dark. To get mycelia, the disk was inoculated in potato dextrose broth and cultured with shaking at 25°C for 7 days. To get fruiting bodies, the culture was placed in a sawdust and wheat bran (8 : 2) medium at 65% absolute humidity and 25°C for 45 days in dark, then transferred to a culture room that was under continuous illumination by a fluorescent lamp at 15°C to induce fruiting bodies. The mycelium and fruiting bodies (Fig. 1) of *A. cylindracea* were harvested separately and kept in liquid nitrogen for RNA extraction.

RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol and the polysaccharides were removed from the crude RNA by the method of Asif *et al.* (2000). Poly A+ RNA was isolated from the mycelia and fruiting body using the PolyATtract mRNA Kit (Promega).

Suppression subtractive hybridization (SSH). SSH was performed with 2 μg of poly A+ RNA using the PCR-SelectTM cDNA Subtraction Kit (Clontech). Mycelial mRNA was used as a driver, and the mRNA of fruiting bodies was used as a tester. The PCR-amplified DNAs selected as differentially expressed in fruiting body were cloned into the TA vector (Invitrogen).

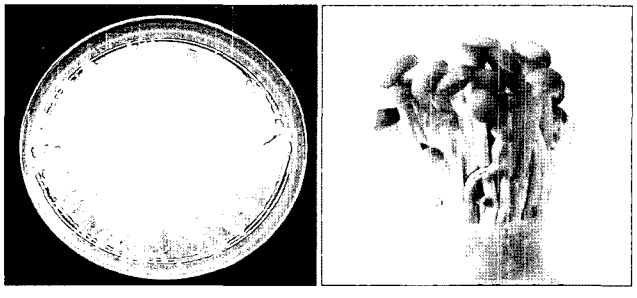

Fig. 1. Mycelia and fruiting bodies of *Agrocybe cylindracea* used for SSH.

Reverse Northern blot analyses. The subtracted cDNA clones were digested with *Eco*RI and separated on a 1% agarose gel. The gel was blotted onto duplicate Hybond-N+ nylon membranes (Amersham Pharmacia Biotech), and each blot was screened with alkaline phosphatase-labeled (Amersham Pharmacia Biotech) cDNA probes prepared from the total RNAs of mycelia and fruiting bodies, respectively. The hybridization was carried out at 55°C in a hybridization buffer (Amersham Pharmacia Biotech). The membranes were washed under highly stringent conditions (0.2 × SSC at 65°C for 30 min) as described by Sambrook *et al.* (1989) and exposed to X-ray film for autoradiography.

Reverse transcript-mediated polymerase chain reaction (RT-PCR). Total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's guidelines. The extracted RNA was treated with DNase to completely remove residual genomic DNA contamination. First strand cDNA was synthesized from total RNA (5 μg) by reverse transcription using the oligo (dT)-primer as the antisense primer. The first strand reaction was used for subsequent PCR reactions to detect gene expression using gene-specific primers designed from the coding sequence of each gene. RT-PCR was performed for 15 to 40 cycles at 5-cycle intervals, and the best RT-PCR result showing non-saturating levels of amplified gene expression was chosen to quantify the transcript level of each gene. The rRNA RT-PCR was used as the internal standard. The primer sets for RT-PCR were shown in Table 1.

DNA sequence analyses. Sequences were determined using a DNA sequencer (Applied Biosystems). DNA and predicted amino acid sequences were searched against DNA and protein databases using the BLAST program available at the NCBI website.

Results and Discussion

Isolation and sequence analysis of differentially up-regulated genes during fruiting body formation. To clone genes differentially expressed in fruiting bodies including stipes and pilei of *A. cylindracea*, a subtraction cDNA library was constructed from fruiting bodies and mycelia by SSH. Approximately 100 cDNA clones were isolated after subtraction (Fig. 2). The cDNA clones were subsequently screened by reverse Northern analysis (Fig. 3). The 20 clones that exhibited differential expression in

Table 1. The primer sets used for RT-PCR of cDNA clones

cDNA clones	forward primer (5'-3')	reverse primer (5'-3')
Agf 2	ATATGCACTACCGGCGTCGT	GCAATCTGCGTCGAGTCCTC
Agf 7	CAGCACGCCATTCAGCAAAG	GTAGCCTGTTGCATTCCTGC
Agf 10	ATCCTGGGTGTGAGCAGAGAG	ACAGCTCGACCTGGACGAG
Agf 16	CGAGGACGAGTTTGACATTG	CATAGCACCAACCAAATCAG
Agf 19	CGACGGCAACTACAGCAAGAC	CTGCCACCAGCTCGAATACCA
Agf 20	GAACACAAGTCAACTGACACGAG	AATCCCTTCGACGCTTGCCTAAC
Agf 23	TGCCAGCAGCTCTCCCACTGC	CTCGACAAGCTTGAGCTTCAAG
Agf 25	AGAGAAAGTGTCAAACCCGCT	CAGGCGATGACATTGCTGGT
Agf 34	GGCAATACGATGCGCTTACA	CTCGATCACCAGATAGTCCA
Agf 37	CTCGTTCAAGTCAAGGGGCA	CGCTGAAGCATTCCAGAAGG
Agf 38	GGTGTAGATGGCAGCGTGGTT	CTTGACCGTGTCCAGAGACGT
Agf 40	CACACCCCAGGAATTCCCGT	ACGACATGGGCCCTGTATGC
Agf 46	AATACGTGGACACGGCGGT	GCGACAAATTGAGCTTCATAGG
Agf 51	TTCGGAAGAACTGGGGGC	ACGACGAAGGGCAAGGACTT
Agf 53	AAGAGGGCCTTGGGAAAGC	GCCGTCCCTTCTCGACAG
Agf 54	TCCTCAGCACCCCAGGATG	CGTTGCGGAAGCTCGAGT
Agf 56	GCGTAGGGATGCGCTGTTAC	CAGATTGCGTTATTTAAGACGAGA
Agf 62	AAGCGCTAACGAAGAGCCCA	ACTCCTCCTCCCAATGGCTC
Agf 63	TCTTGCGCGTCTGGAGAATGC	ATGATAGGTCCCAGGCTCGAG
Agf 64	AACGACGAGCAAGCCAGTG	TGTTAATGACACAGCGAAGGG
Agf 66	CCTGGATTGGAAACGACAAC	GGGAATCCCACTGAGGACC
rRNA	AGATCATTGCAAATTGTTG	CACCTACGGAAACCTTGT

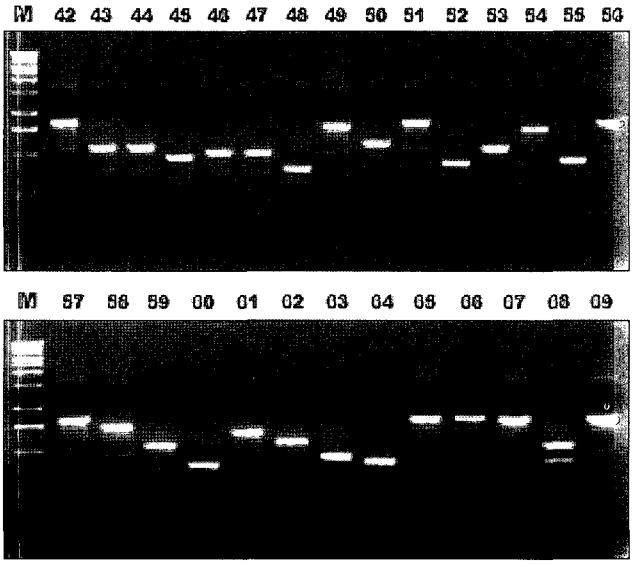

Fig. 2. Examples of putative fruiting body-specific genes cloned by SSH. All genes represented as PCR bands in this figure were used subsequently for reverse Northern analyses. Each numbered lane contains PCR product from positive clones selected by SSH. M; 100 bp DNA size marker.

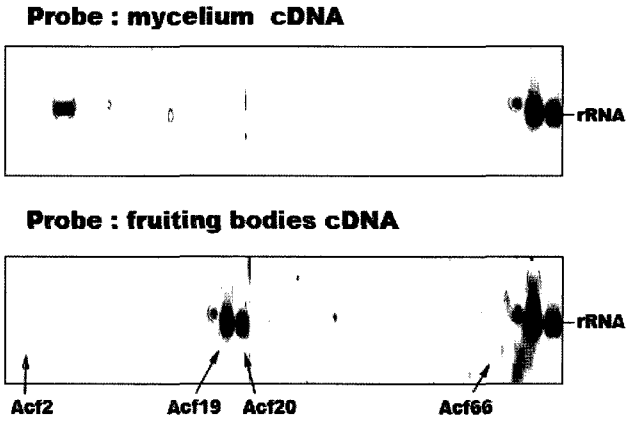

Fig. 3. Identification of differentially expressed genes by reverse Northern analysis. Arrows indicate differentially expressed genes in the fruiting bodies. Numbers on the panels indicate clone numbers. rRNA were used an internal control to check equal loading.

the fruiting bodies were isolated, and their nucleotide sequences were determined by single-run sequencing. The sequence information was searched against the database using the BLASTn and BLASTx programs. The differentially expressed cDNA clones are summarized in Table 2.

Of the 20 cDNA clones, 11 were homologous to genes with known function, three were homologous to genes which have been cloned from other organisms but whose functions have been unknown. Six were revealed as novel genes.

Among 11 genes with known function, the translated polypeptides of *Agf 10* and *Agf 64* were highly homologous to the pleurotolysin, a self-assembling pore-forming cytolysins (Bernheimer and Avigad, 1979). *Agf 19* and *Agf 20* were homologues of *Aa-Pril* and *Pir2p*, respectively. Both *Aa-Pril* and *Pir2p* are known to be specifically expressed during fruiting initiation of a basidiomycete,

Table 2. Partial cDNA clones of genes up-regulated in the fruiting bodies of *A. cylindracea*

cDNA clones	Putative functions (acc. No./Organism)	Accession number
Agf 2	hypothetical protein (EAK84949/*Ustilago maydis*)	BP999626
Agf 7	Unknown	BP999627
Agf 10	pleurotolysin B (BAD66667/*Pleurotus ostreatus*)	BP999628
Agf 16	Unknown	BP999629
Agf 19	Aa-Pri1 (AAC02265/*Agrocybe aegerita*)	BP999630
Agf 20	Pri2p (AAD4122/*Agrocybe aegerita*)	BP999631
Agf 23	Unknown	BP999632
Agf 25	Unknown	BP999633
Agf 34	Hypothetical protein (EAK81305/*Ustilago maydis*)	BP999634
Agf 37	Hypothetical protein (AAW41683/*Cryptococcus neoformans*)	BP999635
Agf 38	General amino acid permease (AAN52080/*Hebeloma cylindrosporium*)	BP999636
Agf 40	Cytochrome P450 (AAW43969/*Cryptococcus neoformans*)	BP999637
Agf 46	Pistillara homolog Rfpl-1 (AAC42573/*Ranunculus ficaria*)	BP999638
Agf 51	Peptidylprolyl isomerase (BAB10690/*Arabidopsis thaliana*)	BP999639
Agf 53	Splicing factor SR1B (AAD52610/*Arabidopsis thaliana*)	BP999640
Agf 56	Unknown	BP999641
Agf 62	Unknown	BP999642
Agf 63	putative phospholipid transfer protein (EAL66379/ *Dictyostelium discoideum*)	BP999643
Agf 64	pleurotolysin B (BAD66667/*Pleurotus ostreatus*)	BP999644
Agf 66	Serine proteinase (CAA74137/*Agaricus bisporus*)	BP999645

Agrocybe aegerita (Fernandez Espinar and Labarere, 1997; Salvado and Labarere, 1991).

Agf 38 showed a characteristic peptide of a general amino acid permease, which is assumed to play a role in uptake of organic nitrogen from the soil. *Agf 40*, a homologue of a *cytochrome P450* was also cloned. The *cytochrome P450* is a member of xenobiotic-metabolizing enzyme family (Choudhary *et al.*, 2004). They are believed to metabolize most foreign compounds including carcinogens and drug (Choudhary *et al.*, 2004). *Agf 46* was found to belong to a MADS-box gene family. It showed high similarity to *APETALA3* and *PISTILLATA*, which are required for petal and stamen identity in Arabidopsis (Krizek and Meyerowitz, 1996). *Agf 51* was identified as a peptidylprolyl isomerase that catalyzes the isomerization of proline residues. *Agf 53*, a serine-arginine (SR)-rich splicing factors and *Agf 66*, a serine proteinase were cloned.

Expression pattern of early fruit development-regulated mRNAs. We examined the expression of cloned genes in mycelia and fruiting bodies using semi-quantative RT-PCR (Fig. 4). The genes whose expressions were more than 4 times up-regulated in fruiting bodies compared to the mycelia were as follows; *Acf 10*, *19*, *20*, *23*, *38*, *46*, *64*, and *66*. The moderately up-regulated genes were *Acf 2*, *7*, *16*, *25*, *34*, *37*, *40*, *51*, *53*, *56*, *62*, and *63*.

Agf 10 and *Agf 64*, homologues of a pleurotolysin, a self-assembling pore-forming cytolysins were firstly found to be up-regulated in fruiting bodies developing process. Considering pleurotolysin has been isolated from fruiting bodies of another edible mushroom, *Pleurotus ostreatus*

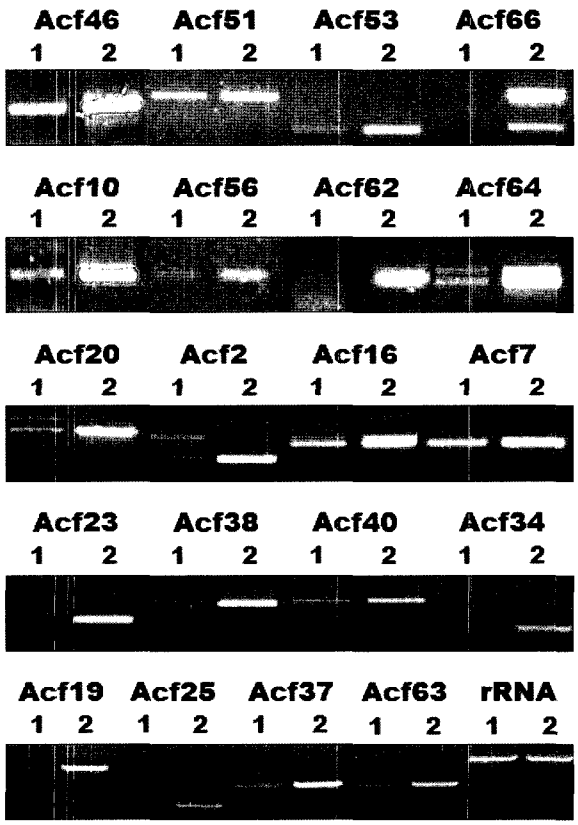

Fig. 4. RT-PCR showing up-regulation of gene expression in the fruiting bodies. Numbers over the panels indicate clone numbers. The rRNA RT-PCR was used as the internal standard. 1, Mycelia; 2, Fruiting bodies.

(Bernheimer and Avigad 1979), the up-regulation of the gene in *Agrocybe cylindracea* indicates the induction of

pleurotolysin gene expression and protein are not confined in *Pleurotus ostreatus*. It might be produced in the fruiting process of *Agrocybe cylindracea* and play a certain role common in the process of fruiting in both types of mushrooms. *Agf 19* and *Agf 20* whose functions are unknown, were already known to be specifically expressed during fruiting initiation of a basidiomycete, *A. aegerita* (Fernandez Espinar and Labarere, 1997). Thus, its up-regulation in *A. cylindracea* in fruiting initiation indicates that 1) this gene expression is related with the fruiting process in both mushrooms and 2) the SSH methods applied in this study and RT-PCR were correctly done. We think a few multiple bands in RT-PCR (Fig. 4) may indicate that some genes belong to gene family in *A. cylindracea*. The similar intensity of RT-PCR bands of rRNA from mycelia and fruiting bodies shows that RT-PCR method for detecting the differences of gene expression in both samples was reliable.

In conclusion, we identified 20 genes differentially expressed in the process of fruiting in *A. cylindracea*. Among them, 6 were completely novel genes and specific to this mushroom based on the DNA sequence data so far deposited. We believe that these data will provide basic molecular information about further physiological and biochemical changes during fruiting bodies formation in *A. cylindracea*. Although functions of each up-regulated gene during fruiting process can not be predicted by the data we provided, we believe we provide the first molecular and genetic data of this important mushroom. Further cloning of the full size cDNAs and functional characterization of each gene will be followed based on the data presented in this study.

Acknowledgments

This research was supported by Ministry of Science and Technology and Korea Science and Engineering Foundation(KOSEF) through CCWM(Culture Collection of Wild Mushroom Species) in Department of Biology, University of Incheon.

References

Asif, M., Dhawan, P. and Nath, P. 2000. A simple procedure for the isolation of high quality RNA from ripening banana fruit. *Plant Mol. Biol. Rep.* **18**: 109-115.

Bernheimer, A. W. and Avigad, L. S. 1979. Cytolytic protein from the edible mushroom, *Pleurotus ostreatus*. *Biochim. Biophys. Acta.* **585**: 451-61.

Choudhary, D., Jansson, I., Sarfarazi, M. and Schenkman, J. B. 2004. Enobiotic-metabolizing cytochromes P450 in ontogeny: evolving perspective. *X Drug Metab. Rev.* **36**: 549-68.

De Groot, P. W. J., Schaap, P. J., Van, G. L. J. and Visser, J. 1997. Isolation of developmentally regulated genes from the edible mushroom *Agaricus bisporus*. *Microbiology* **143**: 1993-2001.

Endo, H., Kajiwara, S., Tsunoka, O. and Shishido, K. 1994. A novel cDNA, *priBc*, encoding a protein with a ZnII. 2Cys 6 zinc cluster DNA-binding motif, derived from the basidiomycete *Lentinus edodes*. *Gene.* **139**: 117-121.

Fernandez Espinar, M. T. and Labarere, J. 1997. Cloning and sequencing of the Aa-Pri1 gene specifically expressed during fruiting initiation in the edible mushroom *Agrocybe aegerita*, and analysis of the predicted amino acid sequence. *Curr. Genet.* **32**: 420-424

Hoge, J. H. C., Springer, J. and Wessels, J. G. H. 1982. Changes in complex RNA during fruit-body initiation in the fungus *Schizophyllum commune*. *Exp. Mycol.* **6**: 233-243.

Kajiwara, S., Yamaoka, K., Hori, K., Miyazawa, H., Saito, T., Kanno, T. and ShiShido, K. 1992. Isolation and sequence of a developmentally regulated putative novel gene, *priA*, from the basidiomycete *Lentinus edodes*. *Gene.* **114**: 173-178.

Kiho, T., Yoshida, I., Nagai, K., Ukai, S. and Hara, C. 1989. (1→3)-α-D-Glucan from an alkaline extract of *Agrocybe cylindracea*, and antitumor activity of its O-carboxymethylated derivatives. *Carbohydr. Res.* **189**: 273-279.

Kim, H. O., Lim, J. M., Joo, J. H., Kim, S. W., Hwang, H. J., Choi, J. W. and Yun, J. W. 2005. Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. *Bioresour. Technol.* **96**: 1175-1182.

Krizek, B. A. and Meyerowitz, E. M. 1996. The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. *Development.* **122**: 11-22

Lee, I. K., Yun, B. S. and Yoo, I. D. 1998. A nucleotide with lipid peroxidation inhibitory activity from *Agrocybe cylindrace*. *Kor. J. Appl. Micbiol. Biotechnol.* **26**: 558-61.

Lee, S. H., Kim, B. G., Kim, K. J., Lee, J. S., Yun, D. W., Hahm, J. H., Kim, G. H., Lee, K. H., Suh, D. S., Kwon, S.T., Lee, C. S. and Yoo, Y. B. 2002. Comparative analysis of sequences expressed during the liquid-cultured mycelia and fruit body stages of *Pleurotus ostreatus*. *Fungal. Genet. Biol.* **35**: 115-134.

Mulder, G. H. and Wessels, J. G. H. 1986. Molecular cloning of RNAs differentially expressed in monokaryons and dikaryons of *Schizophyllum commune* in relation to fruiting. *Exp. Mycol.* **10**: 214-227.

Ngai, P. H., Zhao, Z. and Ngai, T. B. 2005. Agrocybin, an antifungal peptide from the edible mushroom *Agrocybe cylindracea*. *Peptides.* **26**: 191-196.

Salvado, J. C. and Labarere, J. 1991. Isolation of transcripts preferentially expressed during fruit body primordium differentiation in the basidiomycetes *Agrocybe aegerita*. *Curr. Genet.* **20**: 205-210.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual, *Cold Spring Harbor Laboratory Press.*

Shon, Y. H. and Nam, K. S. 2001. Antimutagenicity and induction of anticarcinogenic phase II enzymes by basidiomycetes. *J. Ethnopharmacol.* **77**: 103-109.

Yoshida, I., Kiho, T., Usui, S., Sakushima, M. and Ukai, S. 1996. Polysaccharides in fungi. XXXVII. Immunomodulating activities of carboxymethylated derivatives of linear 1-->3.-alpha-D-glucans extracted from the fruiting bodies of *Agrocybe cylindracea* and *Amanita muscaria*. *Biol. Pharm. Bull.* **19**: 114-21.