The revised manuscript for FEMS Microbiology Letters (Short Communication)

# Isolation and analysis of genes specifically expressed during fruiting body development in the basidiomycete *Flammulina velutipes* by fluorescence differential display

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*Keywords:* Fruiting body; Differential display; *Flammulina velutipes*; Primordium

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**Abstract**

Using fluorescence differential display, cDNAs specifically expressed at the primordial stage of fruiting body development were isolated from the basidiomycete, *Flammulina velutipes*. Seventy-five cDNAs were sequenced and compared to the amino acid sequences of proteins in the database by BLASTX search. Significant similarity was found for 29 cDNAs coding for proteins with known function: GTP-binding protein, growth factor, ubiquitin-proteasome, cytochrome P450, and hydrophobin; all of which would be associated with fruiting body development. Seventeen cDNAs were not similar to proteins in the database and may represent unique genes that play specific roles in the process of fruiting in *F. velutipes*.

## 1. Introduction

Edible mushrooms, the fruiting bodies of the basidiomycetes, are commercially cultivated worldwide. Some metabolites of mushrooms are medically important having anti-tumor or immunomodulating activities [1]. However, the precise mechanism of fruiting is still unclear at the molecular level. This is why only a limited number of species of basidiomycetes are artificially cultivated. In commercial production, fruiting is usually induced by controlling various environmental factors such as temperature, light, humidity, along with physical and chemical stimuli. These factors promote expression of regulatory gene(s) that trigger a set of genes required for the initiation of fruiting. The analysis of genes specifically expressed at the initial stage of fruiting would help to understand the mechanism of fruiting at the molecular level, and could improve artificial cultivation of mushrooms of various kinds of commercially important basidiomycetes.

Isolation of the genes specifically expressed during the development of fruiting bodies was reported for several basidiomycetes. Predominantly two methods have been used: a differential screening method used for *Schizophyllum commune* [2], *Agrocybe aegerita* [3],

*Agaricus bisporus* [4], and *Flammulina velutipes* [5]; and an expression sequenced tag (EST) analysis used for *A. bisporus* [6] and *Pleurotus ostreatus* [7]. In contrast to the above two methods, the fluorescent differential display (FDD) method includes a PCR step to amplify cDNAs and thus also allows to isolate cDNAs of weakly expressed genes. This approach was shown to be valuable in the isolation of genes specifically expressed during fruiting for *Lentinula edodes* [8] and *P. ostreatus* [9].

In this study, we used the FDD method to isolate genes specifically expressed at the early stage of fruiting in the basidiomycete, *F. velutipes*. *F. velutipes* is an edible mushroom popular in East Asia. In this fungus primordia of fruiting bodies appear in a well-synchronized manner. Therefore, *F. velutipes* is well-suited to study temporal changes in gene expression during fruiting body development. In this paper, we report on sequence analyses and temporal expression patterns of the isolated cDNAs at early stages of fruiting body differentiation, and discuss their possible roles in fruiting.

## 2. Materials and methods

### *2.1. Strain and culture condition*

A commercially cultivated *F. velutipes* dikaryotic strain MH092086 was used throughout this study. Mycelium plugs were inoculated on saw dust medium composed of *Cryptomeria* saw dust (17.5%), corncob meal (11%), rice bran (10%), and water (61.5%) contained in 850 ml-polypropylene culture bottles after sterilized at 120°C for 40 min. The cultures were incubated at 15°C in the dark for 30 days. Fruiting was induced by a combination of physical stimulation (scraping off the mycelial mat on the medium surface), addition of 20-30 ml of water, and exposure to continuous light (white fluorescence light, 0.6 μmol $m^{-2}$ $s^{-1}$) at 15°C (90% humidity). After ten days, primordia (2-5 mm in length) appeared on the surface of the medium (Fig. 1). To conduct maturation of fruiting bodies, the bottles were subsequently kept at 8°C under continuous white fluorescence light (0.6 μmol $m^{-2}$ $s^{-1}$) for 20

days. Cells were collected from the bottles at different stages, immediately frozen in liquid nitrogen, and stored at –80°C until use.

*2.2 Fluorescent differential display*

Total RNAs were prepared from vegetative mycelia before induction (Fig. 1, M) and primordia at day 10 after induction (Fig. 1, P). The cells were homogenized under liquid nitrogen, and total RNA was prepared using TRI reagent (Molecular Research Center, Inc. Montgomery, OH). First strand cDNA synthesis, PCR amplification, and detection of amplified cDNAs on polyacrylamide gel electrophoresis were done using an FDD kit (Takara Bio, Kusatu, Japan) according to the manufacture's protocol. Briefly, the first cDNAs were synthesized using one of the nine downstream primers (D1-D9): D1: 5'oligo-($dT_{13-16}$)AA3', D2: 5'oligo-($dT_{13-16}$)AC3', D3: 5'oligo-($dT_{13-16}$)AG3', D4: 5'oligo-($dT_{13-16}$)CA3', D5: 5'oligo-($dT_{13-16}$)CC3', D6: 5'oligo-($dT_{13-16}$)CG3', D7: 5'oligo-($dT_{13-16}$)GA3', D8: 5'oligo-($dT_{13-16}$)GC3', D9: 5'oligo-($dT_{13-16}$)GG3', which were labeled by fluorescein isothiocyanate (FITC) at 5'-termini. The resulting first cDNAs were used as a template for PCR using the same downstream primer of the 1st cDNA synthesis in combination with one of the 12 upstream primers (U1- U12): U1: 5'GATCATAGCC3', U2: 5'CTGCTTGATG3', U3: 5'GATCCAGTAC3', U4: 5'GATCGCATTG3', U5: 5'CTTGATTGCC3', U6: 5'AGGTGACCGT3', U7: 5'GATCATGGTC3', U8: 5'TTTTGGCTCC3', U9: 5'GTTTTCGCAG3', U10: 5'GTTGCGATCC3', U11: 5'GATCTGACAC3', U12: 5'CTGATCCATG3'. Accordingly, a set of 108 PCR (9 downstream primers x 12 upstream primers) was used for each RNA sample according to the manufacture's protocol. The PCR products were size-fractionated by polyacrylamide gel (4% (w/v)) electrophoresis in the presence of 7 M urea, and FITC-labelled cDNA bands were detected by a fluorescent scanner, FM-BIO II (Takara Bio). The cDNA bands, which appeared in the lane of samples from primordia but not from mycelia, were eluted and stored at –20 °C until use. Each of the

isolated cDNAs was named by the number of downstream primer, the number of upstream primer, and the decreasing order of size (alphabetical) in this order. For example, the name of 110b denotes the product obtained from the PCR with a combination of D1 and U10 primers, and the second large fragment among cDNA bands distinctly detected.

*2.3 Cloning and nucleotide sequence analysis*

The cDNAs eluted from the gel were used as a template for second PCR using the same upstream primer of the first PCR and non-labelled downstream primer. The amplified product was ligated onto a TA cloning vector pT7Blue (Novagen, Darmstadt, Germany), and propagated in *E. coli* JM109. Nucleotide sequence of the insert cDNA was determined with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) and a big dye terminator sequencing kit (Applied Biosystems). The determined nucleotide sequences have been deposited in the DDBJ database under accession numbers **AB201060-AB201106**.

*2.4 Northern blot analysis and reverse transcript-PCR*

For northern blot analysis, total RNAs (10 μg) were denatured, fractionated by agarose (1.5%)-formaldehyde gel electrophoresis, and transferred onto GeneScreen Plus membrane (NEN Life Science Products, Boston MA). DNA probes were labelled with alkaline-phosphatase by AlkPhos direct labeling and detection system (Amersham Bioscience, Tokyo, Japan). Chemifluorescent signals derived from ECF substrate (Amersham) were detected by a fluorescent imaging analyzer Storm 860 (Amersham). Size of transcripts was estimated by a migration distance of RNA molecular weight markers, RNA molecular weight marker I (Roche, Mannheim, Germany) in the same gel. To detect the transcripts whose signals could not be found in the northern blot analysis, reverse transcription (RT)-PCR was done for total RNAs prepared from primordia. First strand cDNA synthesis and PCR amplification were conducted using an RNA PCR kit (Takara Bio) using primers designed

based on the nucleotide sequences of the cDNAs initially isolated.

## 3. Results and discussion

*Isolation of genes specifically expressed at the primordial stage of fruiting*

In this study, we used a commercially cultivated strain of *F. velutipes* that develops fruiting bodies in a highly synchronized manner after induction (Fig. 1). Total mRNAs used in the FDD screening were prepared from cells at two different stages: vegetative mycelium and primordium. The primordial stage, where young fruiting bodies (2-5 mm in length) appear on the surface of the medium, represents a very early stage of fruiting because emerging primordia can be easily dedifferentiated into mycelia by a cessation of continuous light exposure required for the development of fruiting (data not shown). We obtained approximately 600 cDNAs (average size, 520 bp), which were specifically amplified from mRNAs derived from primordia but not from vegetative mycelia. Nucleotide sequences were determined for 75 cDNAs that showed a distinct and reproducible pattern of amplification. A BLASTX search showed 29 cDNAs (39%) had a significant similarity (E value $< 10^{-4}$) to amino acid sequences of proteins deposited in the database; and 19 cDNAs (23%) showed no similarity (E value $\geq 10^{-4}$) (Table 1). The remaining 29 cDNAs were redundant having nucleotide sequences identical to those of other cDNAs. These redundant cDNAs resulted from the same species of mRNA by PCR amplification with different upstream primers.

*Northern blot analysis*

Forty-six unique cDNAs were used for northern blot analysis. Total RNAs were prepared from vegetative mycelium (just before induction), and from cells 4 hours, and 2, 6, 10 (primordia), and 16 days (mature fruiting body) after induction (Fig. 1). Seventeen cDNAs showed strong hybridization signals with total RNAs prepared from primordia but not from

vegetative mycelia (Table 1), suggesting that primordium-specific cDNAs were successfully selected by FDD. These cDNAs were grouped, based on their temporal expression patterns (Fig. 2). Type A represents transcript peaking at primordium formation and then decreasing at formation of mature fruiting body. Type B transcript began to increase in primordia and peaked in mature fruiting body. These different patterns suggested that the corresponding genes play specific roles at different stages in fruiting. Another four cDNAs (type C) hybridized with RNAs from both vegetative mycelia and primordia; however, a much higher intensity of signals in primordia shows that these genes were expressed at much higher levels in primordia as compared to vegetative mycelia. The remaining 25 cDNAs did not exhibit differentiation-specific expression although they could be amplified by RT-PCR (data not shown).

*Analysis of genes specifically expressed during fruiting*

The BLASTX search showed that 29 cDNAs had a significant similarity (E value < $10^{-4}$) to amino acid sequences of proteins deposited in the database (Table 1). The functions ascribed to these homologous proteins led us to estimate a role of the gene products in association with a process of fruiting:

(1) Signal transduction pathway

The deduced amino acid sequence of 58i cDNA showed a similarity to that of the α subunit of G proteins that link to the plasma membrane receptor and respond to many different signal molecules [10, 11]. Northern blot analysis showed three distinct signals of 1500, 800, and 500 nt in size (Fig. 2). They resulted from a cross-hybridization with the 58i cDNA because the nucleotide sequences of genes encoding α subunits are highly conserved. A variation in the relative intensities of each signal at primordia and mature fruiting bodies suggests that different species of the G proteins function during fruiting. In *S. commune,* light exposure, one of the fruiting induction factors, is known to increase the intracellular

concentration of cyclic AMP via activation of adenylate cyclase [12], and the resulting cAMP induces fruiting through a signal transduction driven by G proteins [13]. Signal transduction mediated by G proteins plays a critical role at the process of fruiting in *F. velutipes*.

The 410c cDNA was fond to encode a protein homologous to MAPK organizer 1 that regulates cell development through the MAPK cascade [14, 15]. This gene product may be involved in signal transduction during a process of fruiting. MAPK gene (*LeMapK*) that was specifically expressed during fruiting was also isolated from *L. edodes* by differential display [8].

(2) Growth factor-like protein

The 44b cDNA was found to code for a protein whose amino acid sequence is similar to adenosine deaminase growth factor (ADGF) reported from various organisms [16, 17]. ADGF has been intensively studied in the flies, *Sarcophaga peregrina* [18, 19] and *Drosophila melanogaster* [20]. Adenosine deaminase activity is indispensable for promoting cell growth although a precise mechanism has not yet been clarified. ADGF-like genes have not been previously reported from basidiomycetes. We are now examining if this ADGF-like protein is related to cell proliferation during fruiting in *F. velutipes*.

(3) Ubiqutin-proteasome pathway

The gene products of six cDNAs showed similarity to members of proteins involved in the ubiqutin-proteasome pathway that selectively degrade ubiqutin-tagged proteins within cells. 72b, 811b, and 79o cDNAs correspond to ubiquitin activating protein (E1), ubiqutin binding protein (E2), and ubiquitin ligase (E3), respectively. The other three cDNAs (42c, 47h, and 48e) code for different subunits of the proteasome [21]. The ubiquitin proteasome system plays an important role in cellular processes for the control of the signal transduction pathway, cell development, and cell differentiation to degrade target proteins at appropriate temporal and spatial patterns [22]. Therefore, degradation of unnecessary proteins through the ubiquitin-proteasome pathway is presumably linked with fruiting body development in

basidiomycetes. Kanda *et al*. reported on ubiquitin-mediated protein degradation during fruiting in *Coprinus cinereus* [23, 24].

(4) Cytochrome P450

Two cDNAs (82f and 810i) are presumed to encode proteins homologous to cytochrome P450. In the basidiomycete, *C. cinereus*, cytochrome P450 (encoded by *eln2*) is associated with elongating the stipes of fruiting bodies [25]. In *A. bisporus*, several genes coding for the cytochrome P450 family of proteins were isolated by differential screening targeting genes specifically expressed during fruiting [4, 6]. Some oxidoreduction reactions driven by cytochrome P450 may play an important role during fruiting in basidiomycetes.

(5) Hydrophobin

Two genes (45c and 69q) were found to code for hydrophobin-like proteins. Hydrophobins are widely distributed in filamentous fungi and highly related to the morphology of mycelia and fruiting bodies [26, 27, 28]. The full-length gene (*fv-hyd1*) of *F. velutipes* corresponding to the 45c cDNA was previously isolated and characterized [29]. The *fv-hyd1* is specifically expressed from the primordial stage to the formation of mature fruiting body.

In this study, we isolated various genes specifically expressed during fruiting in *F. velutipes* using FDD. Differential screening was previously reported to isolate genes expressed during fruiting in *F. velutipes*; however, only a few genes (hydrophobin and oxidoreductase) were observed [5, 30]. In addition, we isolated a number of cDNAs for genes coding for proteins involved in signal transduction (58i) and growth regulation (44b), which are usually expressed at low levels. Some of the cDNAs isolated in this study did not show similarities to the proteins deposited in the database, and may be unique genes that play specific roles in the process of fruiting in *F. velutipes.*

**Acknowledgements**

This work was supported by Grant-in-Aid for 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology. We are indebted to the Division of Gene Research, Research Center for Human and Environmental Sciences, Shinshu University for providing facilities. We are also grateful to Seiji Horie, Takara Shuzo Co., Ltd. for technical support and for the analyses using FMBIO II.

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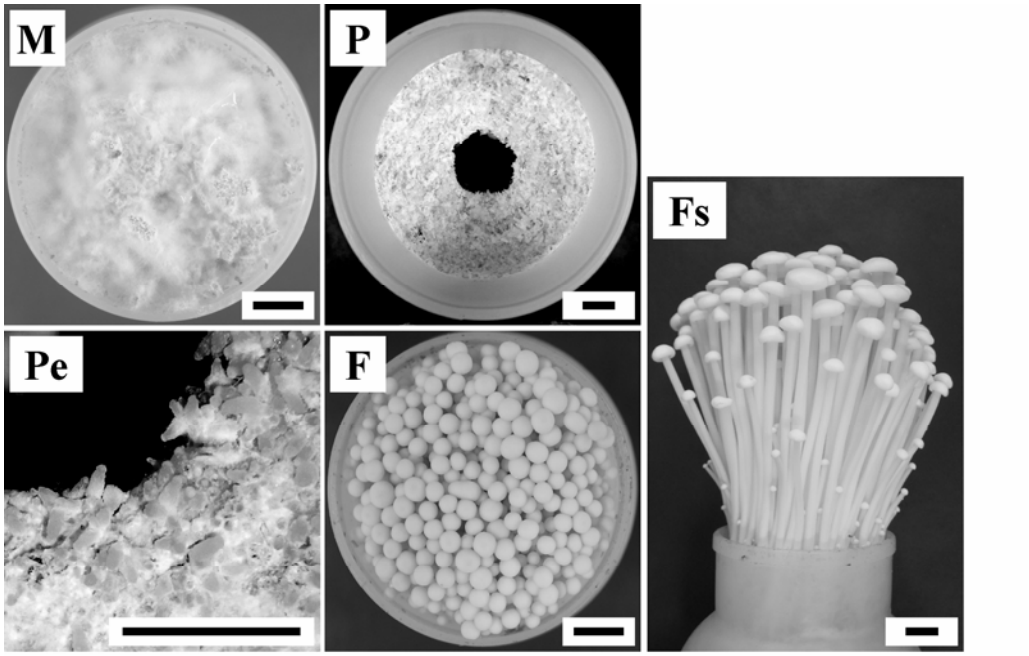


Fig. 1 Yamada *et al*.

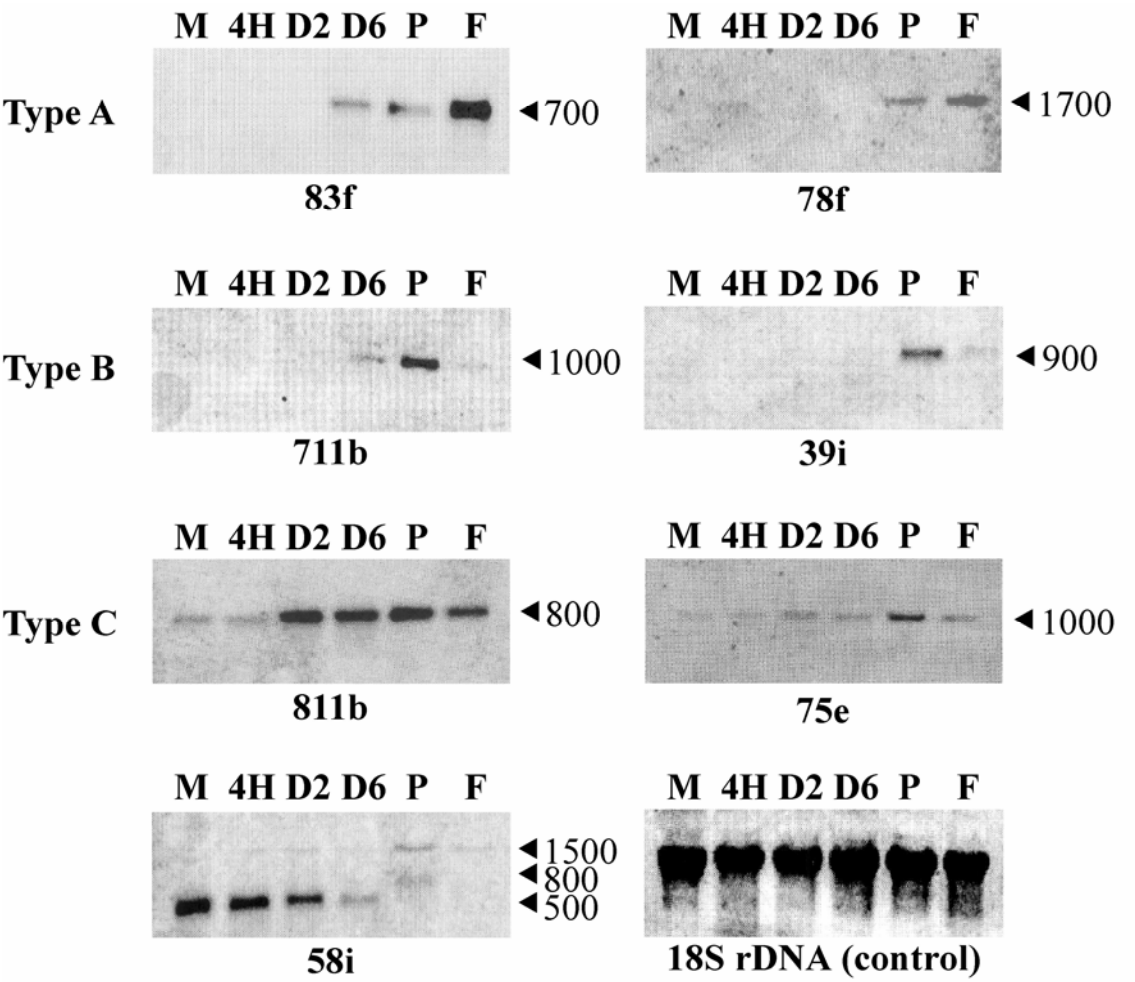


Fig. 2 Yamada *et al*.

Figure legends

Fig. 1 Development of fruiting bodies in *F. velutipes*. Vegetative mycelium just before induction (M), at 10 days (primordia, P), and 16 days (mature fruiting body, F) after induction. A portion of (P) is enlarged to show an appearance of primordia (Pe). A side view of mature fruiting bodies at 20 days after induction (Fs). Scale bars, 1 cm.

Fig. 2 Northern blot analyses of the transcripts encoded by each of the cDNAs. Equal amounts (10 μg each) of the total RNAs prepared from cells at different stages (as shown in Fig. 1) were loaded in each lane. Representatives of the three types (type A, B, and C) of temporal expression patterns are shown. The cDNAs used as a probe are indicated at the bottom of the photos. To confirm an equal loading, 18S rDNA was used as a control probe. The numerals indicate the sizes (nt) of transcripts. M, vegetative mycelium just before induction; 4H, 4 hours; D2, 2 days; D6, 6 days; P, primordia (10 days); F, fruit body (16 days) after induction

**Table 1 Homology search analysis of the sequenced cDNAs obtained by FDD**

| cDNA | Acssesion No. | Blastx search result[a] | E Value | Plausible function | Redundancy[b] | Northern blot anarysis[c] | | Expression type | Transcript size (nt) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mycelium | Primordium | | |
| 110a | **AB201060** | *Schizosaccharomyces pombe* spbc2a9.05c hypothetical protein | 8.00E-09 | unknown function | 2 | - | - | | |
| 110a6 | **AB201061** | *Aspergillus fumigatus* afu6g09590 alcohol dehydrogenase, putative | 6.00E-20 | alcohol dehydrogenase | 1 | - | - | | |
| 22d | **AB201062** | *Caenorhabditis elegans* w02d9.4 hypothetical protein | 0.260 | | 2 | - | + | B | 1000 |
| 24b | **AB201064** | *Magnaporthe grisea* mg06585.4 hypothetical protein | 0.34 | | 1 | - | - | | |
| 24c | **AB201065** | *Zygosaccharomyces rouxii* ketoreductase protein | 0.022 | | 1 | - | - | | |
| 26b | **AB201066** | *Cryptococcus neoformans* cnn00270 deacetylase, putative | >1 | | 4 | - | - | | |
| 27i | **AB201067** | *Cryptococcus neoformans* cnb02240 hypothetical protein | 6.00E-10 | unknown function | 1 | - | - | | |
| 29f | **AB201068** | *Candida albicans* cao19.72 potential copper transport protein | 8.00E-07 | copper transport protein | 2 | - | - | | |
| 32a | **AB201069** | *Burkholderia pseudomallei* bpsl2176 ABC transport system, putative | 0.63 | | 2 | - | ++ | B | 1200 |
| 39e | **AB201070** | *Cryptococcus neoformans* cnk00380 membrane transporter | 7.00E-32 | membrane transporter | 3 | - | + | A | 1500 |
| 39f | **AB201071** | *Cryptococcus neoformans* cnj01360 carboxylic acid transporter | 1.00E-41 | carboxylic acid transport protein | 4 | - | + | B | 5000 |
| 39h | **AB201072** | *Ustilago maydis* um04576.1hypothetical protein | >1 | | 1 | - | - | | |
| 39i | **AB201073** | *Dictyostelium discoideum* limA protein | 0.022 | | 1 | - | +++ | A | 900 |
| 410c | **AB201074** | *Mus musculus* 1500041n16rik hypothetical G-protein beta | 2.00E-10 | MAPK cascade scaffold protein | 1 | - | - | | |
| 410l | **AB201075** | *Ustilago maydis* um00976.1hypothetical protein | 4.00E-28 | EF hand protein | 1 | + | ++ | C | 600 |
| 42c | **AB201076** | *Cryptococcus neoformans* cnm01550 endopeptidase protein, putative | 1.00E-95 | regulatory subunit of 26S proteasome | 1 | - | + | A | 1600 |
| 44b | **AB201077** | *Ustilago maydis* um01794.1hypothetical protein | 6.00E-12 | adenosine deaminase | 1 | - | - | | |
| 45c | **AB201078** | *Pleurotus ostreatus* vmh2-1hydrophobin 2 protein | 6.00E-15 | hydrophobin | 3 | - | +++ | A | 800 |
| 47h | **AB201079** | *Arabidopsis thaliana* rpn8a 26S proteasome subunit protein | 9.00E-06 | 26S proteasome subunit | 1 | - | + | A | 1700 |
| 48e | **AB201080** | *Ustilago maydis* um00585.1 hypothetical protein | 3.00E-35 | 26S proteasome regulatory subunit | 1 | - | + | A | 1500 |
| 56j | **AB201081** | *Dictyostelium discoideum* ddb0188664 hypothetical protein | >1 | | 1 | - | - | | |
| 58i | **AB201082** | *Cryptococcus neoformans* cnc04930 cytoplasm protein, putative | 1.00E-58 | GTP-binding protein | 2 | - | + | -[d] | 500<br>800<br>1500 |
| 612e | **AB201083** | *Trametes versicolor* aad arylalcohol dehydrogenase | 1.00E-55 | arylalcohol dehydrogenase | 1 | - | - | | |
| 62f | **AB201084** | No hits found | | | 1 | - | ++ | A | 800 |
| 62k | **AB201085** | *Aspergillus fumigatus* afu6g05070 isopentenyltransferase, putative | 8.00E-06 | isopentenyltransferase | 1 | - | - | | |
| 69q | **AB201086** | *Coprinopsis cinerea* CoH1 protein | 3.00E-08 | hydrophobin | 1 | - | - | | |

**Table 1-*Continued***

| cDNA | Acssesion No. | Blastx search result[a] | E Value | Plausible function | Redundancy[b] | Northern blot anarysis[c] | | Expression type | Transcript size (nt) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Mycelium | Primordium | | |
| 69u | **AB201087** | *Cryptococcus gattii* 163.m06341protein | 3.00E-05 | unknown function | 2 | - | - | | |
| 710g | **AB201088** | *Cryptococcus neoformans* cnb00110 hypothetical protein | 0.001 | | 2 | - | - | | |
| 711b | **AB201089** | *Flammulina velutipes* fvfd16 protein | 4.00E-16 | unknown function | 4 | - | +++ | A | 1000 |
| 72b | **AB201090** | *Ustilago maydis* um04880.1 hypothetical protein | 6.00E-29 | ubiquitin activating E1-like enzyme | 2 | - | - | | |
| 72c | **AB201091** | *Drosophila polymorpha* omb optomotor blind protein | 0.044 | | 1 | - | + | A | 800 |
| 73d | **AB201092** | *Ustilago maydis* um02351.1 hypothetical protein | 1.00E-09 | Ca-dependent solute carrier protein | 1 | - | - | | |
| 73g | **AB201093** | *Plasmodium yoelii yoelii* methionine aminopeptidase, putative | >1 | | 2 | - | ++ | B | 1700 |
| 75e | **AB201094** | *Ustilago maydis* envelope um02701.1 predicted protein | 0.077 | | 2 | + | +++ | C | 1000 |
| 77eb | **AB201095** | *Cryptococcus neoformans* cnbg0590 hypothetical protein | 4.00E-05 | unknown function | 2 | - | - | | |
| 78b | **AB201096** | *Yarrowia lipolytica* yali0D03267g protein | 2.00E-08 | tRNA nucleotidyltransferase protein | 1 | + | ++ | C | 1800 |
| 78f | **AB201097** | *Trametes versicolor* oxalate decarboxylase protein | 7.00E-31 | oxalate decarboxylase | 2 | - | +++ | B | 1700 |
| 79o | **AB201098** | *Candida albicans* cao19.7497 hypothetical protein | 1.00E-04 | | 1 | - | - | | |
| 79r | **AB201099** | No hits found | | | 1 | - | - | | |
| 810h | **AB201100** | *Cryptococcus neoformans* cnb00110 hypothetical protein | 5.00E-04 | | 1 | - | - | | |
| 810i | **AB201101** | *Aspergillus nidulans* an8615.2 hypothetical protein | 8.00E-12 | cytochrome P450 protein | 1 | - | - | | |
| 811b | **AB201102** | *Neurospora crassa* ncu02289.1 hypothetical protein | 1.00E-34 | ubiquitin conjugating protein | 1 | + | +++ | C | 800 |
| 82f | **AB201103** | *Coprinus cinereus* eln2 cytochrome P450 protein | 2.00E-29 | cytochrome P450 protein | 3 | - | - | | |
| 82h | **AB201106** | *Gahnia deusta* ndhF NADH dehydrogenase subunit F protein | >1 | | 1 | - | - | | |
| 83f | **AB201104** | *Flammulina velutipes* immunomodulatory protein FIP-Fve | 3.00E-08 | immunomodulatory protein | 2 | - | +++ | B | 700 |
| 86e | **AB201105** | *Cryptococcus neoformans* cnj02750 mms2 protein, putative | 6.00E-76 | ATPase | 2 | - | ++ | A | 600 |

[a] The names of species and gene product showing the highest similarity.

[b] The number of cDNAs with identical sequence out of the 75 cDNAs initially sequenced.

[c] The relative intensity of signals is indicated: -, no signal; +, weak signal; ++, moderate signal; +++, strong signal.

[d] Three signals were detected.