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Several Genes Expressed During Morphogenesis of *Lentinus edodes* (ImHyup-1)

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Differential display of reverse transcription (DDRT)-PCR was conducted to have a profile of the differentially expressed genes during the formation of fruiting body of *Lentinus edodes*. The lines of *L. edodes* (ImHyup-1) employed were cultivated in the artificial blocks of sawdust, and the fruiting body was induced from the mycelia or the mass protruded from the brown surface of the sawdust blocks. RNAs were prepared from the four different developmental stages; mycelial, primordial, and stipes and pileus of fruiting body. The fragments of cDNA were synthesized from the combinations of the arbitrary primers and 3' one anchored Oligo-dT primer. Twelve combinations using the primers have been tested, and among them nineteen bands were identified as differentially expressed. Those genes were further analyzed by DNA sequencing and followed by homology search. Characterization of one clone was conducted as a preliminary data and more are under investigation.

KEYWORDS: DDRT-PCR, *Lentinus edodes*, Morphogenesis

The mushroom of *Lentinus edodes* (shiitake) is a wood rotting edible fungus belonging to the family of Tricholomataceae, and the most popular cultivated mushroom in the world (Chang and Miles, 1991). The cultivation of this mushroom is now rapidly expanding because of its flavor, textures, and nutritional value (Park *et al.*, 1997). In recent years, new cultivars or isolates of *L. edodes* have been hybridized and screened for the high production (Lee *et al.*, 1997).

The traditional cultivation of *L. edodes* mainly uses natural logs, but its production depends on the climatic events, and a whole cycle of production is very long for approximately 5 years (Hiromoto, 1991). As the cultivation of other mushroom, the sawdust blended with the rice bran is developed for a new technique. After sterilization, each bags of substrate mentioned above are inoculated with the spawn and cultivated for 3-4 months (Diehle and Royse, 1991; Lee *et al.*, 2000). The mycelia of *L. edodes* form a compact mat on the sawdust solid substrate. The initiation of fruiting body is critical in the sawdust media than in the natural log cultivation.

Many studies on *L. edodes* have been focused on the artificial cultivation under the sawdust medium (Kim *et al.*, 1987), the physiology and improvement of substrate (Park *et al.*, 1992), selective breeding (Bak *et al.*, 1996), and cultivation in liquid media (Song *et al.*, 1987). Moreover, molecular biological techniques have been applied to the study of *L. edodes*, including the classification of isolates (Chiu *et al.*, 1996; Lee *et al.*, 1997; Park *et al.*, 1997), isolation of the differentially expressed genes (Leung *et al.*, 2000; Zhang *et al.*, 1998), and identifying the genes involved in the regulation of fruit body development (Ng *et al.*, 2000). Environmental stresses such as temperature, humidity and light induce the hyphae to form primordium, which may subsequently develop into the fruit bodies (Leung *et al.*, 2000; Yoo *et al.*, 1998). However, information about the molecular mechanism governing the fruiting bodies of *L. edodes* is still scarce (Ng *et al.*, 2000), but is considered to be very important for sawdust cultivation. Current methods to distinguish mRNA in comparative studies would rely largely on subtractive hybridization (Lee *et al.*, 1991). This technique, although it has been used successfully in isolating a number of important genes, is rather difficult to establish, irreproducible, and requires large amounts of RNA (Liang *et al.*, 1994). Differential display of reverse transcription (DDRT)-PCR was developed as the new method to identify and analyze the altered gene expression at the mRNA level in various cells or under the altered conditions (Liang and Pardee, 1992). DDRT-PCR method would allow not only to identify new genes but also the diagnosis of any changes in gene expression involved in a particular cellular process (Bauer *et al.*, 1993). Also, this method has been successfully used to isolate the differentially expressed gene of yeast for heavy metal stress (Hong *et al.*, 1997), low temperature induced genes of rice (Choi *et al.*, 1997) and plant-fungus interactions (Benito *et al.*, 1996).

Here we attempted the DDRT-PCR of mRNA isolated from three growth stages of *L. edodes* including mycelium, primordium, and two tissues (stipe and pileus) of fruiting body, to analyze the genes differently expressed during the morphological development.

Materials and Methods

Fungal culture. A line of *L. edodes* (ImHyup-1; very commonly produced in the oak logs in Korea) was obtained from National Forestry Cooperatives Federation

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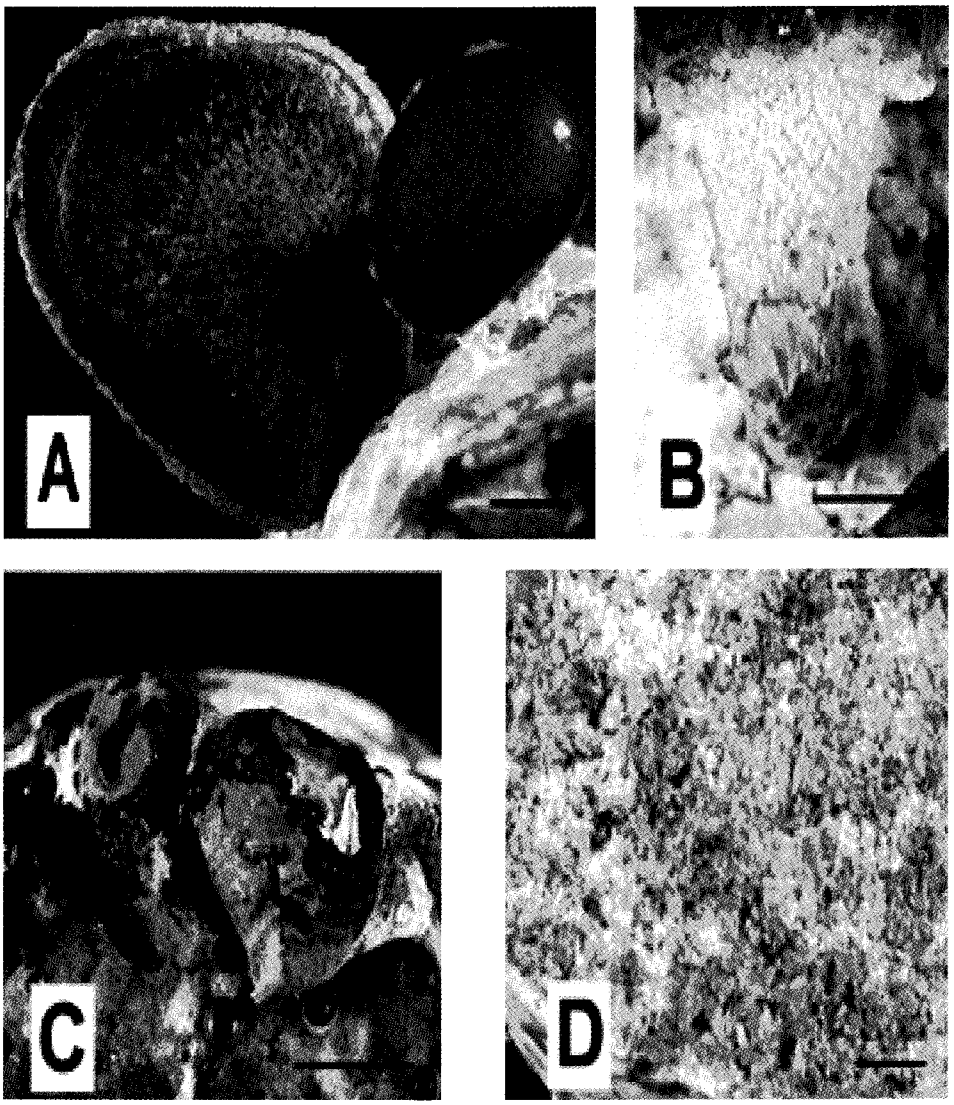

Fig. 1. The different growth stages of *Lentinus edodes* (ImHyeup-1). A, Formation of fruiting body (Pileus); B, Fruiting body stipe; C, Primordium formations from the surface of the sawdust; D, Mycelia colonized in the sawdust media (each scale = 1 cm).

(NFCF) in Korea used for this purpose. The cultures of *L. edodes* were maintained on potato dextrose agar (PDA, Difco, USA) at 25°C. The blocks of PDA grown by *L. edodes* were cultured in synthetic liquid medium (glucose 20 g, yeast extracts 2 g, tryptone 2 g, KH_2PO_4 1 g, $MgSO_4$ 0.75 g, $FeSO_4$ 0.02 g, $ZnSO_4$ 0.02 g, H_2O 1 *l*) at 25°C by shaking incubator for the liquid spawn. The mycelia cultured in the liquid broth were inoculated to the 1~1.5 kg of the sterilized oak sawdust (with rice bran by the ratio 8 : 2) moistened by the approximately 60% before sterilization. The *L. edodes* was grown on sawdust substrate at 25°C in the dark rooms for three months until the white mycelia were covered in whole surface. To induce fruiting, the substrate blocks were kept in moist and cool room (at 15°C, for 2 days) under the dim light (Lee *et al.*, 2000). The four stages of mycelial growth were collected for this work: (A) Formations of fruiting body (Pileus); (B) the Stipe of Fruiting body; (C) Primordium formations from the surface of the sawdust; (D) The mycelia colonized in the sawdust media shown in Fig. 1.

RNA isolation. The total RNA's from the fungal tissues mentioned above were extracted by the based SDS-Phenol extraction method (Ausbel *et al.*, 1999). Ten ml of extraction buffer (0.18 M Tris, pH 8.7, 90 mM LiCl, 4.5 mM EDTA, 1% SDS) was added to 0.5 g~1 g of grounded sample. All samples (5 g of the fungal tissue and 15 g of the mycelia mixed with the sawdust) were ground in liquid nitrogen using mortars and pestles. An equal volume of acidic phenol (pH 4.3) was added and homogenized at 8000 rpm for 1 min. The homogenate was extracted several times with an equal volume of phenol and chloroform. The 800 μl of the top phase was re-transferred to a new eppendorf tube and an equal volume

of 4 M LiCl was added to precipitate RNA for overnight at 4°C. The RNA was precipitated by centrifugation at 12,000 rpm for 15 min. The pellet was washed with the 70% ethanol and dried, dissolved in 50 μl of RNase free water. Finally, the RNA concentrations were measured by spectrophotometer and confirmed by the electrophoresis method, using the formaldehyde agarose gel (Ausbel *et al.*, 1999; Sambrook *et al.*, 1989).

DDRT-PCR. To obtaining representative pools of cDNA fragments from polyA-tract to arbitrary primer, 1 of total RNA was reverse transcribed with oligo-dT primer according to the protocols provided by the supplier (Bioneer, Korea). The reverse transcription was performed at 42°C for 1 h and the reaction was stopped by incubation at 94°C for 5 min. The cDNA synthesized was stored at -20°C for a subsequent PCR reaction. One tenth of the cDNA was then amplified in a total 20 μl volume of the PCR mixtures contained 1U Taq DNA polymerase, the 250 mM dNTP of each, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, stabilizer, tracking dye (Bioneer, Korea), 20 pmole of 3' anchored oligo-dT primers and 20 pmole arbitrary primers (Table 1). The amplification reaction was done for 1 cycle with 94°C for 5 min, 40°C for 30 sec and 72°C for 30 sec, and 30 cycles with 94°C for 30 sec, 40°C for 30 sec and 72°C for 1 min, and an additional extension cycle with 94°C for 30 sec, 40°C for 30 sec and 72°C for 5 min. The 5 μl of the PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide for confirmation. The band was photographed with Digital Camera (Kodak, USA).

Electrophoresis. The PCR products were resolved on a 6% poly-acrylamide sequencing gel (Econo Sequencer I™ Electrophoresis System) according to the manuals provided by the supplier (Bioneer, Korea). The gel was stained by silver staining method using a Silverstar™ staining kit according to the protocols provided by the supplier (Bioneer, Korea). For further characterization, individual bands representing the cDNA differentially expressed were excised, transferred into tubes for DNA elution and DNA was re-amplified by PCR using same set of 3' one anchored oligo-dT primers in combinations with the arbitrary primer (AP), and followed by cloning of amplified products. The re-amplified cDNA fragments were resolved on a 1.5% agarose gel and stained with ethidium bromide. The bands were photographed with Digital Camera (Kodak, USA) and estimated their sizes with the standard markers.

Table 1. List of primers used for DDRT-PCR in this study

Primers (Length)	Nucletide sequences
oligo dT/G (16 mer)	5'-AAGCTTTTTTTTTTG-3'
oligo dT/C (16 mer)	5'-AAGCTTTTTTTTTTC-3'
oligo dT/A (16 mer)	5'-AAGCTTTTTTTTTTA-3'
AP-1 (13 mer)	5'-AAGCTTGATTGCC-3'
AP-2 (13 mer)	5'-AAGCTTCGACTGT-3'
AP-3 (13 mer)	5'-AAGCTTTGGTCAG-3'
AP-4 (13 mer)	5'-AAGCTTCTCAACG-3'

DNA sequencing. The re-amplified cDNAs were cloned into the pRIP-T™ vector (Bioneer, Korea) and transformed in to *E. coli* DH 12S (Bioneer, Korea). DNA sequencing was performed by the dideoxy sequencing method (Ausbel *et al.*, 1998). The data of the cDNA sequence were further homology searched using BLASTX at the National Center for Biotechnology Information and at the National Institute of Health (URL: http://www.ncbi.nlm.nih.gov). The expression pattern of cloned gene was confirmed by a modified Northern Hybridization using unfractionated RNA immobilized by the slot blotting (Sambrook *et al.*, 1989).

Results

Growth. The ImHyup-1 line was selected from more than 20 lines of *L. edodes* known, being commercially sold in Korea. This line was developed for cultivation of *L. edodes*, for more than 20 years, because the rapid growth takes place in the log cultivation and morphogenesis at the high temperate as compared with other lines of *L. edodes*. Also, this line was known to be traditionally cultivated in the oak or other tree logs. The liquid spawns grown were inoculated in the sawdust solid blocks and the cultivation was made at 25°C under laboratory ways. The white mycelia grew for 1 or 1.5 months in the dark room and covered the whole surface of sawdust blocks (Fig. 1D). To induce fruiting body (primordium), the sawdust substrate was kept in the moist and cool room under dim light. The white mycelia turned to the black brown color (Fig. 1C) and made to produce several harden mass (primordium called in Fig. 1C) in the surfaces. This mass was projected out for a week in the cooled room, showing in Fig. 1B. Conclusively. more than ten basidiocarps were protruded from the mass on the surfaces of the black brown block (Fig. 1A). The fungal tissues shown at the four different stages represented in Fig. 1 were collected under the artificial conditions and stored in the deep freezer for the extracts of mRNA; the mycelia, primordium, fruit body pileus and stipe protruded.

RNA bands. The DDRT-PCR was displayed after reacted with the equal amounts of RNA (1) extracted from each of the three developmental stages and different tissue types using three 3' one anchored oligo-dT primer combinations with the four arbitrary primers (Figs. 2 and 3). When the primer of AP-1 was reacted with three different anchored oligo-dT primers, four fragments (in the

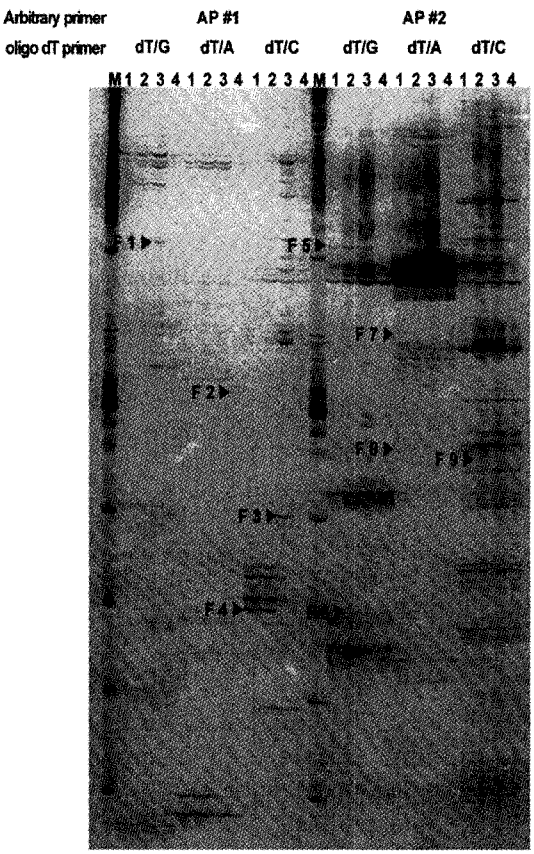

Fig. 2. Differential display of total RNA from the three development stages and different tissue types of *Lentinus edodes* using the arbitrary primer AP-1 and AP-2. The DDRT-PCR bands were displayed on 6% polyacrylamide gel stained with the silver staining method as described in Materials and Methods. M, size marker (100 bp ladder); 1, pileus; 2, stipe; 3, primordia; 4, mycelia.

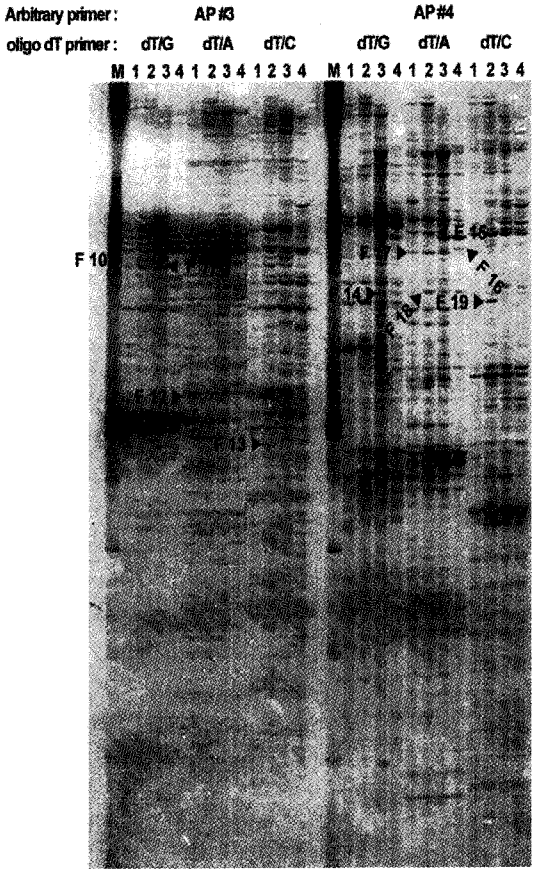

Fig. 3. Differential display of total RNA from the three development stages and different tissue types of *Lentinus edodes* using arbitrary primer AP-3 and AP-4. The DDRT-PCR products were resolved on 6% polyacrylamide gel and stained with the silver staining method. M, size marker (100 bp ladder); 1, pileus; 2, stipe; 3, primordia; 4, mycelia.

left of Fig. 2) were distinguished. The bands of F1 originated from the tissues of primordium and mycelia were distinguished from those originated from the others. The band of F2 was expressed on only the tissue of mycelium. The bands of F5, F9 to F11, and F17 were found in the fungal tissues of pileus, stipe, and primordium, but not in the fungal tissues of mycelia. The 19 bands marked in Figs. 2 and 3 were listed in Table 2. The three bands of DNA (F14, F18 and F3) differentially expressed in three fungal tissues were selected for further works.

Sequence. The 19 fragments of DDRT-PCR were excised from the gel and amplified with the two different primers mentioned above. The re-amplified cDNAs of the fragments were sized about 110 to 330 bps in the length of DNA (Fig. 4). Out of the 19 fragments, only three fragments made from the DDRT-PCR (F3, F14 and F18) were selected and sequenced (Table 3). The four DNA sequences were searched for further information by BLAST search program on the WWW server at the internets (BLASTX; http://www.ncbi.nlm.nih.gov). The DNA sequences of ours were checked to be not matched with any DNA sequences provided, but to be partially matched at the ranged of 15 to 25 bps.

Northern blot analysis. To confirm the expression patterns of each genes isolated by DDRP-PCR, F-3 fragment was reevaluated by a modified Northern blot analysis and the result was consistent with those of the DDRT-PCR (Fig. 5).

Discussion

The line of ImHyeop-1 *L. edodes* were known to be the fungus grown rapidly in the high temperature. The mycelia were inoculated by the liquid spawn and cultured in the sawdust blocks for 1 or 1.5 month (Fig. 1). After then, the mycelia were observed to turn to dark brown color and, after then, to several protruding mass (2 or 3 cm diameters) in the surfaces (Fig. 1C). The mRNAs induced

Table 2. List of fragments identified by DDRT-PCR

Fragments	Primer combination		Sizes[a]	Expression stage and tissue			
	Arbitrary primer	Oligo-dT primer		Pileus	Stipe	Primordia	Mycelia
F 1	AP 1	dT/G	-[b]			○	○
F 2	AP 1	dT/A	-				○
F 3	AP 1	dT/C	150			○	
F 4	AP 1	dT/C	110	○	○		
F 5	AP 2	dT/G	-	○	○	○	
F 6	AP 2	dT/G	-		○		
F 7	AP 2	dT/A	-	○			
F 8	AP 2	dT/A	-	○	○		
F 9	AP 2	dT/C	160	○	○	○	
F 10	AP 3	dT/G	320	○	○	○	
F 11	AP 3	dT/G	-	○	○	○	
F 12	AP 3	dT/A	240	○	○		
F 13	AP 3	dT/C	230		○		
F 14	AP 4	dT/G	300			○	○
F 15	AP 4	dT/A	-	○	○		
F 16	AP 4	dT/A	160				○
F 17	AP 4	dT/A	330	○	○	○	
F 18	AP 4	dT/A	300		○	○	○
F 19	AP 4	dT/C	300		○		

[a]Re-amplified Molecular weight.
[b]Re-amplification band was not shown.

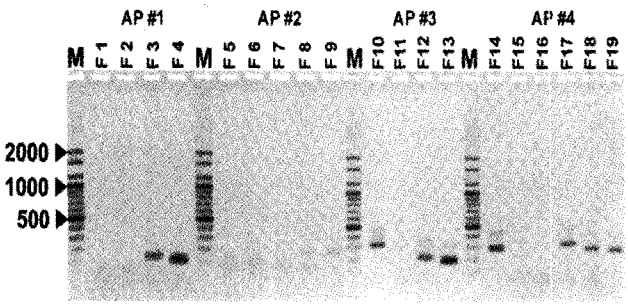

Fig. 4. The re-amplified band-products on 1.5% agarose gel. The differentially expressed bands were picked up from the gel, and amplified by PCR. M, size marker (100 bp ladder).

by the four different morphogenesis stages of *L. edodes* were collected, conducted DDRT-PCR using the arbitrary primers. Many genes were expressed in the DDRT-PCR, when the three arbitrary primers of AP-2, 3, and 4 were reacted with mRNA rather than the other arbitrary primer AP-1. Particularly, the many genes were detected when the AP primers were reacted with the oligo-dT primers T/C. The many bands or fragments were observed on the electrophoresis gels when the arbitrary primers AP-2 or AP-4 reacted with the oligo-dT primers T/C. The gene fragments not expressed in only a fungal tissue were observed in the preparations of stipe more than the others, so less expressed in the preparations of mycelia (Table 2, and Figs. 2 and 3). Generally, so many genes would be expected to be expressed in the fungal tissues of basidiocarps (pileus and stipe), because more complexity of structure was found in the basidiocarps than in the mycelia. The fragments (F3, F14, and F18) observed in

Table 3. DNA sequences of the bands obtained from DDRT-PCR

Clones	Sequences	
	Length (bps)	The sequences determined
F 3	140	aagcttgattgccaatgtgaggaagggtttcctggcaattgtattaatccaggagtaagtcgatcttaaaaccagtcctaaagggtcaataaggccatatatt attaaatttatatattcctgatggttgaaaaaaaaaa
F 14-1	143	ggttgatgagagctatttctcttgtttcaagcacatatgctaaccgtatacttaatgcataaatattgcttgaccatatttatccaaatttggacactacttgtcaat aaattactcctgatgtatgccaaaaaaaaaagctt
F 14-2	165	aaagaagctatggatgaaggggaattcgatgaagatgagcatgacgaatatgactatgacgacgacgacgatgagcggtgtccggaggtggacacaa ggttgatgagagctatttctcttgtttccaacacatattctaaccgtatacttaaatgcataaatatt
F 18	199	gaattcactagtgattaagcttctcaacgtgctgcctcagctttcgttatccttacaggaactctcctatacacttatgtcaaatctcgggagagtggcccatcc aacccctcatcaggaaccaattctgcacctttgaaggatttggaatctcagtctacaaaacaggaacaggtggatgaaaagccgctcacttaag

The poly A tails underlined. The black box represented 3' UTR region. Direction 5'-3'.

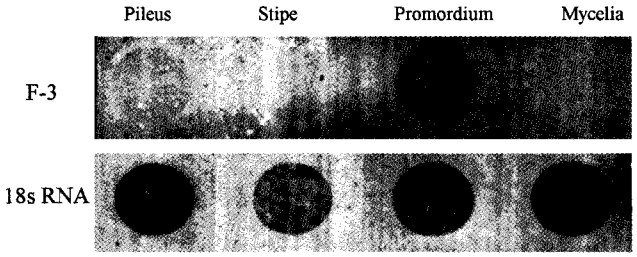

Fig. 5. Northern Hybridization of the unfractionated RNA of fragment-3 immobilized by the slot blotting.

preparations of the three fungal tissues, excluding the tissue of pileus, were selected for the analyses of DNA sequence. In other words, the possible genes related to the morphogenesis of basidiocarp should be existed in the fungal tissues of mycelia, primordium and stipe (Table 2).

All fragments analyzed by DNA sequence (Table 3) were searched to be matched with other DNA sequences of gene provided from BlastX (http://www.ncbi.nlm.nih.gov). However, the 15 to 20 bps, the partial region, of fragment were completely matched with those of several genes showed in Table 4. The fragments selected from this work, especially A+T rich DNA sequence in F3-1, was considered to be some gene of Mitochondrion of Yeast (gb|U69572.1|CPU69572 Mitochondrion *Culex pipiens* A+T rich or gb|U41277.1|CELC06E4 *Caenorhabditis elegans*), and speculated to be related to respiration during morphogenesis (Kondoh *et al.*, 1995; Ng *et al.*, 2000; Zhang *et al.*, 1998). The information related to F-18 fragment was revealed to be the gene of Human DNA sequence (emb|AL121776.19|HSJ1050K3 Human DNA sequence) and to be related to their structural gene having unknown functions. The analyses of the protein for the DNA sequences of the fragment selected were not conducted because finding the genes related to morphogenesis of basidiocarp were aimed by using the methology of DDRT-PCR. The four fragments selected in this work will be analyzed further by confirmation of expression pattern, cloning and/or the loss of function.

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Table 4. Sequence similarity of cloned genes

	Code number	The organisms and some descriptions	Homology sequences
F3-1	gb|U69572.1|CPU69572	Mitochondrion Culex pipiens A+T rich m	97→119: 636 atatattattaaatttatatatt 658
	gb|U41277.1|CELC06E4	*Caenorhabditis elegans* cosmid C06E4	99→119: 15414 atattattaaatttatatatt 15394
	gb|AE003442.1|AE003442	*Drosophila melanogaster* genomic scaf	97→116: 167763 atatattattaaatttatat 167782
	gb|AC004845.2|AC004845	*Homo sapiens* clone RP4-635O5, comple	97→120: 50459 atatattatttaatttatatattc 5048
F14-1	gb|AE003528.1|AE00319528	*Drosophila melanogaster* genomic	75→95: 281829 gcaatatttatgcattaagta 281849
	gb|AC006075.1|AC006075	*Homo sapiens* chromosome 16	108→128: 155819 atgtgcttgaaacaagagaaa 155799
	gb|U73649.1|U73649	Human Chromosome 11 Cosmid	103→123: 21689 agcatatgtgcttgaaacaag 21669
	gb|U61947.1|CELC06G3	*Caenorhabditis elegans* cosmid	25→43: 1920 aggagtaatttattgacaa 1938
F14-2	gb|J05161.1|LUMHBC	Earthworm (*L.terrestris*) extracellular	50→74: 1111 atgactatgacgacgacgacgatga 1135
	emb|AJ249549.1|EMU249549	Echinococcus multilocularis	12→39: 2295 ggatgaaggggaattcgatgaggatgag 23
	ref|NC_001139.1	*Saccharomyces cerevisiae* chromosome VII	50→74: 70202 atgacgatgacgacgacgacgatga 70226
	emb|Z72749.1|SCYGL227W	*S. cerevisiae* chromosome VII	50→74: 872 atgacgatgacgacgacgacgatga 896
F 18	emb|AL365234.1|ATT30N20	*Arabidopsis thaliana* DNA chromosome	68→87: 16388 tatacacttatgtcaaatct 16407
	emb|AL121776. |HSJ1050K3	Human DNA sequence	170→189: 74436 aacaggtggatgaaaagccg 74455
	gb|AC009541.16|AC009541	Human Chromosome 7 clone	147→165: 80344 ggaatctcagtctacaaaa 80362
	gb|AC002352.1|AC002352	*Homo sapiens* 12q24 PAC P256D10	129→147: 6002 tgcacctttgaaggatttg 6020

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