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Cloning of a Gene Specifically Expressed During Early Stage of Fruiting Body Formation in *Flammulina velutipes*

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팽이버섯의 자실체형성 초기과정에서 특이적으로 발현하는 유전자의 클로닝

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ABSTRACT: A cDNA library was constructed using mRNA from the cells of 7-day-old cultures of *Flammulina velutipes* after induction of fruiting treatment. A cDNA clone, FVFD16 (*Flammulina velutipes* fruiting body differentiation), was selected by differential screening. The expression property of the FVFD16 gene was examined by Northern blot analysis. FVFD16 represents mRNA that is specifically expressed during differentiation of fruit bodies. The conspicuous accumulation of the FVFD16 mRNA was detected in 4-day-old and 1-day-old cultures. The nucleotide sequence of the FVFD16 gene was determined and the mRNA contained an open reading frame that encoded a putative protein of 128 amino acid residues (13.5 kDa).

KEYWORDS: *Flammulina velutipes*, cDNA Library, Differential Screening, Fruiting Body Differentiation

The differentiation of fruiting bodies from vegetative mycelia and its regulation have been the subject of considerable interest to those who study basidiomycetes (Azuma *et al.*, 1996).

Formation of fruiting bodies is directly and indirectly correlated with exposure to various environmental factors and time, with the accumulation of several specific mRNAs (Ruiters and Wessels, 1989). In basidoimycetes, the genetics of formation of fruiting bodies have been well studied in *Schizophyllum commune* and *Lentinus edodes* (Kajiwara *et al.*, 1992; Kondoh and Shishido, 1994; Ruiters and Wessels, 1986; Schuren and Wessels, 1990). At fruiting, a number of novel abundantly expressed mRNAs that account for 5% of the RNA in *S. commune* is produced (Dons *et al.*, 1984). In particular, genes for hydrophobins *Sc1, Sc3* and *Sc4* are expressed at high levels when fruiting bodies are formed (Hoge *et al.*, 1982; Mulder and Wessels, 1986; Schuren and Wessels, 1990; Schuren *et al.*, 1993; Wessels *et al.*, 1987; Wessels, 1992). In *Lentinus edodes*, expression of the *priA* and *priBc* genes is developmental regulated during formation of fruiting bodies (Endo *et al.*, 1994; Kajiwara *et al.*, 1992; Kondoh and Shishido, 1994).

In our previous study of the basidiomycete *Flammulina velutipes* (Curt.: Fr.) Sing., we showed that the number of total and stage-specific polypeptides increased significantly in 5- to 7-day-old cultures that did not exhibit any obvious morphological differences from vegetatively growing mycelia (Miura *et al.*, 1994). Moreover, the morphological changes of hyphae during the development of fruiting bodies were investigated under the same conditions by scanning electron microscopy (SEM) (Azuma *et al.*, 1996). Several genes expressed specifically during the formation of fruiting bodies have been isolated by differential screening. The mRNA transcript of the *FDS* gene was detected in 4- to 21-day-old cultures and might be related to differentiation of fruiting bodies. The structural features of the gene and its flanking sequences in the genome were characterized (Azuma *et al.*, 1996).

We propose here that the FVFD16 (*Flammulina velutipes* fruiting body differentiation) cDNA we cloned is associated with differentiation of fruiting bodies prior to the first detectable morphological changes.

Materials and Methods

Strain and culture condition

The *Flammulina velutipes* strain used in this work was obtained from the Hokkaido Forest Products Research Institute. An agar disk, 5 mm in diameter, was cut from the

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culture that had been grown on potato dextrose agar medium at 25°C in the dark. This was placed in a 500 m*l* flask containing 300 m*l* of liquid medium [20 g/*l* soluble starch, 20 g/*l* sucrose, 1.5 g/*l* polypetone, 3 g/*l* yeast extract, 10 mM potassium phosphate (pH 5.0), 50 mg/*l* $MgSO_4 \cdot 7H_2O$, 10 mg/*l* $MnSO_4 \cdot 4H_2O$, 4 mg/*l* $ZnCl_2$, 1 mg/*l* $CuSO_4 \cdot 5H_2O$, 50 mg/*l* $CaCl_2 \cdot 2H_2O$] and cultured with shaking at 25°C for 10d in the dark. Ten ml of this culture was placed in a 200 m*l* glass vessel containing a sawdust and wheat bran (4 : 1) medium and cultured at 25°C and a relative humidity of 70% for 21d in the dark, then transferred to a room that was under continuous illumination by a fluorescent lamp and at 19°C to form the fruiting body (fruiting treatment).

Preparation of RNA

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method with some modifications (Azuma *et al.*, 1996). The harvested material immediately was frozen in liquid nitrogen and stored at -80°C. Frozen mycelia were homogenized in Solution-D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium N-lauroyl sarcosinate, 0.1 M mercaptoethanol) for 5 min. And then 2 M sodium acetate (0.1, v/v), water-saturated phenol (1, v/v), chloroform/isopropyl alcohol (0.2, v/v) were added to the homogenate and mixed. After centrifugation at 10,000×g at 4°C for 20 min, the aqueous phase was treated with isopropyl alcohol to form a precipitation. The precipitated RNA was dissolved in TE-HPRI [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 units/m*l* human placenta RNase inhibitor]. Three volumes of 4 M sodium acetate (pH 7.0) were added to the RNA solution and allowed to settle at -20°C for 1h. After centrifugation at 10,000×g at 4°C for 20 min, the precipitate was washed with 70% ethanol and dissolved in TE-HPRI. Poly(A)$^+$RNA was isolated by chromatography on oligo (dT)- cellulose.

Differential screening

The nylon-membranes were prepared from the cDNA library and were screened with single stranded ^{32}P-labeled cDNA probes synthesized by using poly(A)$^+$RNA isolated from 0- and 7-day-old cultures. The nylon membranes were prehybrdized for 1 h at 42°C in a solution containing 6x SSC [1x SSC is 0.15 M NaCl and 15 mM Na-citrate, (pH 7.0), 0.1% SDS], 5x Denhardt's medium [1x Denhardt's is 0.02% polyvinypyrrolidone (PVP), 0.02% Ficoll, 0.02% bovine serum albumin (BSA)], 100 μg/m*l* of denatured herring sperm DNA, and 50% formamide. Hybridization was conducted at 42°C for 16 h in the presence of single stranded ^{32}P-labeled cDNA probes prepared from poly(A)$^+$ RNA of 0-day-old cultures or 7-day-old cultures. Filters were washed with 2x SSC containing 0.1% SDS at room temperature for 5 min and 30 min, and then three times at 68°C for 30 min. Filters were exposed to X-ray film (Fuji RX) for 24 h with an intensifying screen at -80°C.

Northern hybridization

Total RNA of 10 μg was subjected to electrophoresis in 1% agarose gel containing formaldehyde and blotted onto a Hybond-N$^+$ filter (Amersham) in 20x SSPE [360 mM NaCl, 20 mM NaH_2PO_4 (pH 7.4), 2 mM EDTA (pH 7.4)]. The prehybridization, hybridization and washing were performed by the same methods as described for the differential screening. Radioactivity was recorded on the imaging plate of a Bioimaging Analyzer (Fujix BAS2000; Fuji Photo Film, Tokyo) with an exposure time of 4 h. The intensities of the signal bands were quantitated with an analyzer system.

DNA sequence analysis

cDNA inserts in λgt10 were subcloned to pBluescript II KS(+). The nucleotide sequence was determined by the dideoxy sequencing method (Sanger and Coulson, 1977). The DNA and protein sequences were analyzed using GENETYX soft ware (Software Development Co., Ltd., Tokyo) and software on the network, such as tfasta, blastp, tblastn and PSORT.

Results and Discussion

A cDNA library was prepared from mRNA from a culture of *Flammulina velutipes* 7 days after the start of the treatment for induction of fruiting (Azuma *et al.*, 1996).

Differential screening was performed by plaque hybridization with the cDNA library and single-stranded ^{32}P-labeled cDNA probes that have been synthesized from poly(A)$^+$ RNA isolated from cultures prior to induction of fruiting and 7-day-old cultures.

One cDNA clone, FVFD16 (*Flammulina velutipes* fruiting body differentiation), was detected as the cDNA that corresponded to an mRNA that was abundantly expressed during differentiation of fruiting bodies.

Figure 1 shows the results of the Northern blot analysis. The FVFD16 transcript was abundant in 1-day-old and 4-day-old cultures. It was detectable in 7-, 10- and 14-day-old cultures, but absent from fresh (harvested before treatment to induce fruiting) and 21-day-old cultures (with mature fruiting bodies). To endure equal loading and transfer of RNA preparations, the Northern-blots were hybridized with a control probe (C probe) that was isolated as a constantly expressed gene in differential screening (Azuma *et al.*, 1996).

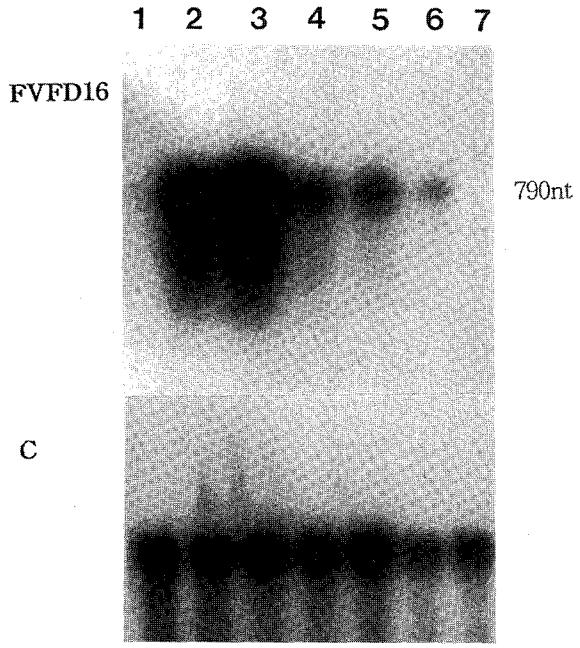

Fig. 1. Autoradiogram of a Northern blot after hybridization with a probe for the FVFD16 gene. Each lane contained 10 μg of RNA. RNA was isolated from the 0 day (lane 1), 1 day (lane 2), 4 day (lane 3), 7 day (lane 4), 10 day (lane 5), 14 day (lane 6) and 21 day (lane 7) cultures after induction of fruiting. The 0 day cultures was harvested before induction. The RNA samples were fractionated on 1% agarose gel, transferred to a nylon filter and allowed to hybridize with the ^{32}P-labeled FVFD16 cDNA probe, and control cDNA probe (C) is shown as a loading control. The size of FVFD16 transcript is on the right (nucleotide: nt).

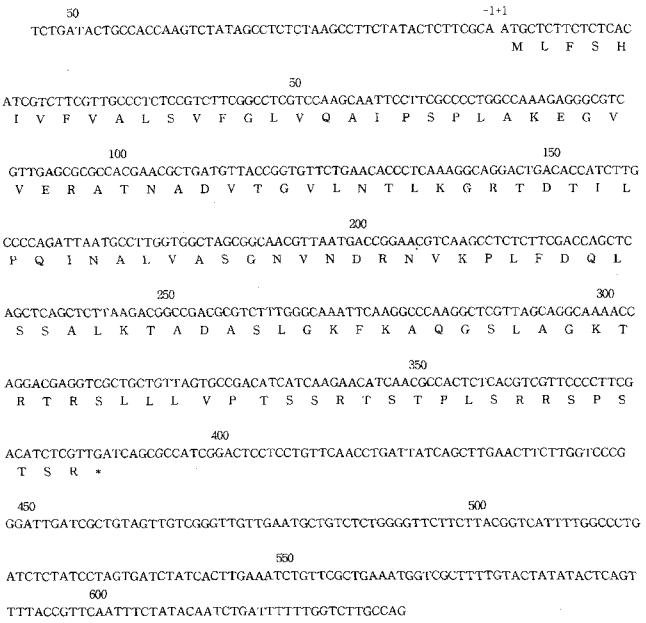

Fig. 2. Nucleotide sequence of the FVFD16 cDNA and the deduced amino aicd sequence. The putative site of initiation of translation was taken as position 1. The termination codon TGA is marked with an asterisk*. The NH_2-terminal region is presumed 19 amino acid residues from methionine.

Thus, expression of the FVFD16 gene first increased and then decreased with time after induction of fruiting. The FVFD16 mRNA was not detectable before induction of fruiting or in mature fruiting bodies. The concentrations of other mRNAs also appear to change significantly with time and under different cultivation conditions (Dons *et al.*, 1984; Hoge *et al.*, 1982; Ruiters and Wessels, 1989; Wessels *et al.*, 1992). In view of the considerable increase in the level of the FVFD16 mRNA during initiation of fruiting bodies, we suggest that the expression of the FVFD16 gene is related to the differentiation of fruiting bodies.

The cDNA insert of FVFD16 was 688 bp long and the corresponding mRNA was determined by Northern bloting analysis to be approximately 790 nucleotides (nt) long, including a poly(A)$^+$ tail of about 100 nt. The mRNA contained an open reading frame that encoded a putative protein of 128 amino acid residues (13.5 kDa; Fig. 2).

Comparison of the sequence of FVFD16 with nucleotide sequences in the EMBL and GenBank databases did not reveal any significant homology with known genes or proteins. However, analysis by the computer program, PSORT, predicted that the FVFD16 gene contains a putative amino-terminal region. A hydropathy plots indicated that the putative FVFD16 protein has some hydrophobic segments characteristic of integral membrane proteins. Thus, the product of the FVFD16 gene is likely to be a secretory protein or an integral membrane proteins related to the differentiation of fruiting bodies.

적 요

팽이버섯의 자실체 분화 과정에서 특이적으로 발현하는 유전자 분리를 위한 cDNA library는 발이처리 후 7일째 배양한 균사체의 mRNA에 의해 만들어졌다. cDNA 클론 FVFD16(*Flammulina velutipes* fruiting body differentiation)은 자실체 분화 과정에서 특이적으로 발현되는 클론으로 differential screening에 의해 선발되었다. Northern 분석에 의해 FVFD16의 발현 특성을 관찰한 결과, 1일과 4일째의 균사체에서 현저한 발현량을 나타내었다. FVFD16의 염기 서열을 검색한 결과, FVFD16의 mRNA는 open reading frame을 포함한 128의 아미노산 잔기(13.5kDa)를 가진 단백질로 추정되었다.

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