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Molecular cloning of developmentally specific genes by representational difference analysis during the fruiting body formation in the basidiomycete *Lentinula edodes*

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Abstract

To understand molecular mechanisms of the fruiting body development in basidiomycetes, we attempted to isolate developmentally regulated genes expressed specifically during the fruiting body formation of *Lentinula edodes* (Shiitake-mushroom). cDNA representational difference analysis (cDNA-RDA) between vegetatively growing mycelium and two developmental substages, primordium and mature fruiting body, resulted in an isolation of 105 individual genes (51 in primordium and 54 in mature fruiting body, respectively). A search of homology with the protein databases and two basidiomycetous genomes in *Phanerochaete chrysosporium* and *Coprinopsis cinerea* revealed that the obtained genes encoded various proteins similar to those involved in general metabolism, cell structure, signal transduction, and responses to stress; in addition, there were apparently several metabolic pathways and signal transduction cascades that could be involved in the fruiting body development. The expression products of several genes revealed no significant homologies to those in the databases, implying that those genes are unique in *L. edodes* and the encoding products may possess possible functions in the course of fruiting body development. RT-PCR analyses revealed that 20 candidates of the obtained genes were specifically or abundantly transcribed in the course of the fruiting body formation, suggesting that the obtained genes in this work play roles in fruiting body development in *L. edodes*.

Keywords: Basidiomycete; *Lentinula edodes*; Fruiting body development; Fruiting body formation; Gene cloning; Representational difference analysis

1. Introduction

Basidiomycetes and ascomycetes are "higher fungi" than other microorganisms in their developmental complexities. For example, basidiomycetous mushrooms commonly develop a fruiting body as a large characteristic organ that produces many basidiospores. In the basidiomycete *Coprinopsis cinerea*, developmental processes including fruiting body formation and sporulation mechanisms essential for sexual reproduction have been briefly observed (Kües, 2000), but details of molecular mechanisms of fruiting body development in basidiomycetous mushrooms are not well known.

The mycelial development of the basidiomycetous mushroom *Lentinula edodes* (Shiitake-mushroom), which is one of the most popular cultivated mushrooms in the world (Chang and Miles, 1991), is as follows. An aggregation of vegetatively growing mycelia on sawdust-rice bran medium results in the formation of thick film-like mycelia (preprimordial mycelia) (Takagi et al., 1988). Primordia that have formed on the preprimordial mycelia grow into immature fruiting bodies and progressively into mature fruiting bodies (Kajiwara et al., 1992). In recent years, several genes, proteins, and biochemical compounds involved in the fruiting of *L. edodes* have been reported. A cyclic adenosine monophosphate

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(cAMP), a well-known second messenger in various organisms, is closely related in the onset of fruiting body development and there may be a relationship between intracellular cAMP levels and expression of cAMP-dependent genes in *L. edodes* (Hori et al., 1991). Lectin, an extracellular matrix, seems to be involved in the formation of hyphal aggregates of brown mycelial film in *L. edodes*, which is a preceding state of primordial formation (Tsivileva et al., 2001). The *priB* transcript is abundant in primordia of *L. edodes* (Endo et al., 1994) and random binding-site analysis suggests the expression product of *priB* binds 16-bp consensus DNA sequence (Miyazaki et al., 1997). The *mfbC*, a target gene regulated by the expression product of *priB*, is transcribed almost exclusively in mature fruiting bodies of *L. edodes* (Miyazaki et al., 2004b). The *Le.cdc5* gene, a homolog of *Schizosaccharomyces pombe cdc5*$^{+}$, is most actively transcribed in primordia and small immature fruiting bodies of *L. edodes* and Le.CDC5 peptide binds to a 7-bp DNA sequence with the consensus sequence of 5′GCAATGT3′ (Miyazaki et al., 2004a). The *mfbAc* transcript in *L. edodes* is abundant in the pileus but not in the gill tissue or the stipe (Kondoh et al., 1995). The *uck1*, which encodes an UMP-CMP kinase in *L. edodes*, is actively transcribed in the gill tissue of the mature fruit body where basidiospores are formed (Kaneko et al., 1998). Several hydrophobins found in *L. edodes* are probably responsible for the hydrophobicity of fruiting body surface and formation of fruit bodies (van Wetter et al., 2000) and their homologs also exist in other basidiomycetous mushrooms, such as *Schizophyllum commune* and *Agaricus bisporus* (Dons et al., 1984; Lugones et al., 1996; Ng et al., 2000). In situ RNA–RNA hybridization showed that the *L. edodes* hydrophobin 1 gene transcripts exist in the mycelial tissues of developing fruiting bodies (Nishizawa et al., 2002). A study of the laccase production in various developmental stages of *L. edodes* suggested that it plays a role in the morphogenesis of the mushroom (Zhao and Kwan, 1999).

Still, current information about the biological processes of the fruiting body initiation and development in the basidiomycetes is limited (Kües and Liu, 2000). So far, there has been no conclusive evidence of key genes to promote the fruiting development in basidiomycetous mushrooms, including *L. edodes*. To understand developmental mechanisms of fruiting body formation of the basidiomycete in detail, it is important to have much more information about the function of genes in the fruiting body development. Recently, several attempts to identify and classify genes in several basidiomycetes have been reported. A report in *A. bisporus* revealed that single-pass sequencing identified 477 expressed sequence tags (ESTs) including 466 not previously described in the database (Ospina-Girald et al., 2000). Using single-pass sequencing of cDNA clones in *Pleurotus ostreatus*, 41% of the liquid-cultured mycelia ESTs and 50% of the fruit body ESTs showed significant similarity to protein sequences described in the databases (Lee et al., 2002). For *Ustilago maydis*, which grows in *Zea mays* and elicits the formation of black teliospores within tumors, analyses of 2871 ESTs are assembled into 1293 contiguous sequences (Sacadura and Saville, 2003) and functional categorization and comparative analyses of 3074 assembled contiguous sequences from 7455 ESTs were provided (Nugent et al., 2004). In the basidiomycete *Phanerochaete chrysosporium*, which is a white rot fungus showing no morphological changes to an obvious fruiting body such as *L. edodes*, a whole genome sequencing project has recently been accomplished (Martinez et al., 2004). The genomic sequence of *C. cinerea*, a basidiomycete forming the fruiting body, has been analyzed and the data have been recently released by the Broad Institute. These data from genomic researches on these basidiomycetes have given us useful information for obtaining interesting genes in basidiomycetes, but they are insufficient for identifying the specific genes responsible for the molecular mechanisms involved in the fruiting body development.

In recent years, several experiments to isolate groups of genes expressed in the fruiting body development of basidiomycetous mushrooms have been also reported. RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) was used to identify differentially expressed genes in fruit body development in *L. edodes* and 13 RAP fragments out of 33 obtained fragments showed high sequence similarity to known gene products (Leung et al., 2000). Using differential screening in *L. edodes*, six clones were identified as fruit-body-specific genes (Hirano et al., 2004). A modified differential display to analyze gene expression in the ectomychorrizal fungus *Tuber borchii* resulted in the identification of 25 amplicons with apparent differential expression during fruit body development (Zeppa et al., 2002). However, a previous data on the basidiomycete *S. commune* revealed that the entire poly(A)-containing RNA complexity represented the existence of 10,000 different RNA sequences isolated from fruiting and non-fruiting mycelia, 90% of which were identical (Zantinge et al., 1979). This result suggests that at least a few percents (corresponding to a few hundred) of all genes on the basidiomycetous chromosome are differentially transcribed between the fruiting body and normally growing mycelia.

Thus, to further examine the specific genes expressed within the fruiting body development of the basidiomycetous mushroom and to better understand the currently unclear mechanism of the fruiting body formation in detail, we used cDNA representational difference analysis (cDNA-RDA) (Hubank and Schatz, 1994), which is a powerful application of subtractive hybridization and is believed to reflect a large number of relevant gene transcripts. Here we describe how 105 different genes containing putatively novel transcripts were successfully