In situ RNA-RNA Hybridization: A Useful Method For Analysis of the Distribution of Transcripts of Various Genes in *Lentinula edodes* Fruiting Bodies

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The in situ RNA-RNA hybridization was renewed understanding that it is a useful method for analysis of the distribution of transcripts of various genes in fruiting bodies of Lentinula edodes. By using this method, we obtained the following results. Large amounts of the transcripts of ribonucleotide reductase small subunit gene (Le.rnr2) and UMP-CMP kinase gene (uck1), which is a target of developmental regulator PRIB, are present in both hymenium and outer region of trama in the hymenophore (gill tissue). The hymenium is the part for production of basidiospores and the outer region of trama is the region branching out into subhymenium (on the top of which hymenium is formed). The Le.ras transcript is present mostly in outer region of trama and in trama cells, while the transcript of trimeric G-protein αsubunit gene (Le.ga1) is mostly in hymenium. The transcript of *mfbC* gene, which is the target of PRIB and probably encodes the protein interacting with a putative translation initiation factor 5A (eIF5A), is present in outer region of trama. The transcript of *hyd1* (hydrophobin 1 gene), whose expressed product is considered to be involved in the formation in the extracellular matrix of lined air channels with a hydrophobic membrane, is present everywhere in the mycelial tissues of developing fruiting bodies except for the top parts of pileus (cap) and for prehymenophore. The region surrounding prehymenophore contains a high level of the transcript. These results suggest that *Le.rnr2* and *uck1* genes play a role mainly in the nucleotide biosynthesis essential for production of basidiospores and for divergence of trama cells into subhymenium cells. The *Le.ras* and *mfbC* play a role in divergence of mycelial cells and the *Le.ga1* plays a role in spore-production. The hydrophobin-mediated air channels may be formed almost all the parts of developing fruiting bodies.

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
© 2005 Universidad Pública de Navarra, Spain. ISBN 84-9769-107-5

1. Introduction

To elucidate the molecular mechanism of the fruiting-body formation of homobasidiomycete *Lentinula edodes*, we previously isolated various genes that regulate development and analyzed their properties and functions using genetical, biochemical and cell-biological methods. To investigate the transcriptional expression of isolated genes during formation of fruiting body and in parts of fruiting body, usually total RNA was isolated from the corresponding mycelial cells and analyzed by Northern blotting. Here we present the data demonstrating that in situ RNA-RNA hybridization is a useful method to investigate the details of distribution of transcripts of isolated genes in parts of mature fruiting body such as hymenophore and stipe and in immature small fruiting body.

2. Method: In situ RNA-RNA hybridization

Parts of mature fruiting bodies and the whole immature small fruiting bodies were fixed with 4% paraformaldehyde in PBS at 4 °C for 4 h and they were cut into 10-μm ultrathin longitudinal cryosections according to the method of Bochenek and Hirsch (1990). The cDNA fragments encoding the conserved regions of proteins were cloned into the *Eco*RI and *Hin*dIII sites of pSPT18 vector (Roche Diagnostics) and then, sense and antisense RNA probes were prepared by *in vitro* transcription with digoxigenine-UTP (Roche Diagnostics). Hybridization work and immunological detection of the hybridized probe were as described previously (Kaneko and Shishido 2001).

3. Results

3.1. *Ribonucleotide reductase small subunit gene* (Le.rnr2) *and UMP-CMP kinase gene* (uck1)

In the nucleotide metabolism, UDP, CDP, ADP and GDP are reduced to

the corresponding deoxyribonucleoside diphosphates (dNDP) to serve as precursors for syntheses of dNTPs. These reductions are catalyzed by ribonucleotide reductase (RNR) which consists of a heterodimer (α2ß2) that contains two non-identical homodimers: α2 is large subunit and ß2 is small subunit (Mathews et al. 1987). RNR plays a regulatory role in maintaining a balanced pool of all four deoxyribonucleoties (Reichard 1988). The small subunit gene (*Le.rnr2*) was cloned by Kaneko and Shishido (2001) from *L. edodes*. UMP-CMP kinase gene (*uck1*) of *L. edodes* (Kaneko et al. 1998) is a target of developmental regulator PRIB (565 amino acids (aa)) protein with a "Zn(II)2Cys6 zinc cluster" DNA-binding motif (Endo et al. 1994). The recombinant UMP-CMP kinase protein catalyzes the phosphoryl transfer from ATP to UMP and CMP efficiently and also to AMP and dCMP with lower efficiencies (Kaneko et al. 1998).

A Northern-blot analysis was carried out to investigate the expression of the *Le.rnr2* and *uck1* genes during fruiting-body formation of *L. edodes*. The intensity of hybridization bands gradually increased in proportion to enlargement/growing of fruiting bodies and the most intense signals were detected in the fruiting-body maturation stages (Kaneko et al. 1998, Kaneko and Shishido 2001). The *Le.rnr2* and *uck1* genes were shown to be actively transcribed in hymenophore of mature fruiting body (Kaneko et al. 1998, Kaneko and Shishido 2001).

The hymenophore is a complicated gill tissue consisting of trama, subhymenium and hymenium on which a large number of basidia and basidiospores are formed (Fig. 1). The trama cells diverge to form subhymeni-

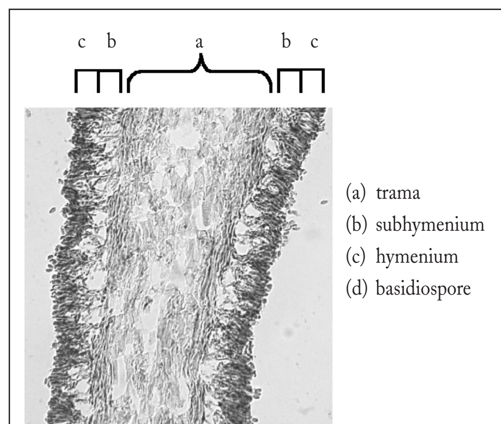


Figure 1. Hymenophore (gill tissue) of mature fruiting body of *L.edodes*

um on the top of which hymenium is formed.

In situ RNA-RNA hybridization was carried out to investigate the expression the *Le.rnr2* and *uck1* genes in the parts of hymenophore of fruiting body. The *Le.rnr2* and *uck1* antisense strand probes showed a specific distribution of the transcripts in the hymenophores of mature fruiting body (Fig. 2A and 2C), whereas their sense strand probes gave no signal (Fig. 2B and 2D). The intense signals of the *Le.rnr2* and *uck1* transcripts were detected in both the hymenium and the outer region of trama (the region branching out into subhymenium). The majority of trama cells and subhymenium cells gave weaker signals. These results imply that *Le.rnr2* and *uck1* genes play a role mainly in the nucleotide biosynthesis essential both for production of basidiospores and for divergence of trama cells into subhymenium cells in the hymenophore. During both the production of basidiospores and the divergence of mycelial cells in hymenophore biosyntheses of nucleic acids, carbohydrates, lipids etc. must be significantly active. Deoxyribonucleoside diphosphates and ribonucleoside diphosphates synthesized by ribonucleotide reductase or ribonucleoside monophosphate kinase all are required for syntheses of these biomolecules. Of course both genes are considered to be also involved in the nucleotide biosynthesis essential for growth of mycelial cells in other part(s) of fruiting body.

3.2. *Small monomeric Ras protein gene* (Le.ras) *and heterotrimeric G-protein a-subunit gene* (Le.ga1)

A Northern-blot analysis was carried out to investigate the expression of the *Le.ga1* gene during fruiting-body formation of *L. edodes*. Previous our study showed that the *Le.ras* is transcribed at similar levels in the course of fruiting-body formation (Hori et al. 1991). The *Le.ga1* is most actively transcribed in the fruiting body of maturation stage and is also transcribed in the preprimordial vegetative mycelia (Tanaka et al. 2005). Levels of the transcripts of *Le.ras* and *Le.ga1* were analyzed in parts of fruiting body of *L. edodes*. The hymenophore and stipe of mature fruiting body contained markedly higher levels of the transcripts of both genes when compared with the hymenophore-depleted pileus (Tanaka et al. 2005).

To investigate the expression of the *Le.ras* and *Le.ga1* genes in parts of the

hymenophore and stipe, in situ RNA-RNA hybridization was done. An intense signal of the *Le.ras* transcript was detected especially in the outer region of trama and relatively weak signal was observed in the trama cells (Fig. 2E). Differently from the case of hymenophore, the *Le.ras* transcript is pres-

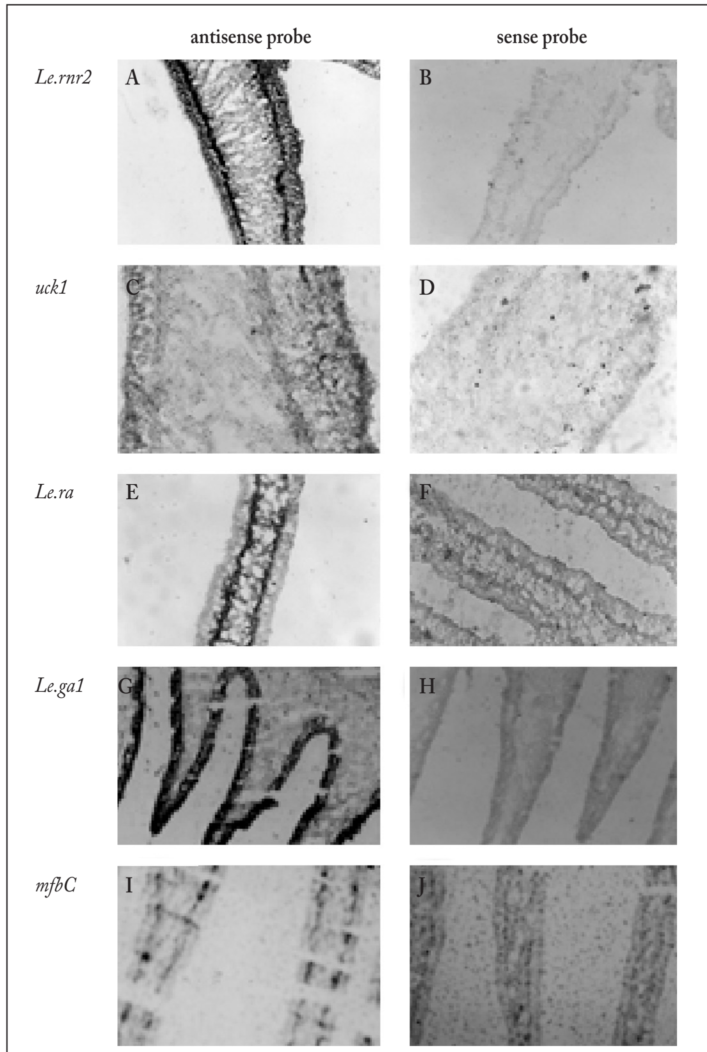


Figure 2. Expression of various genes in hymenophore of *L.edodes*

ent diffusibly in the stipe (data not shown). On the other hand, the *Le.ga1* transcript is present mostly in hymenium and subhymenium (Fig. 2G). The distribution pattern of the *Le.ga1* transcript in stipe was similar to that of *Le.ras* (data not shown). These results seem to suggest the possibility that *Le.ga1* is involved in production of basidiospores and *Le.ras* gene is involved in formation of inner part of hymenophore and in divergence of trama cells into subhymenium cells.

3.3. *Developmentally regulated* mfbC *gene*

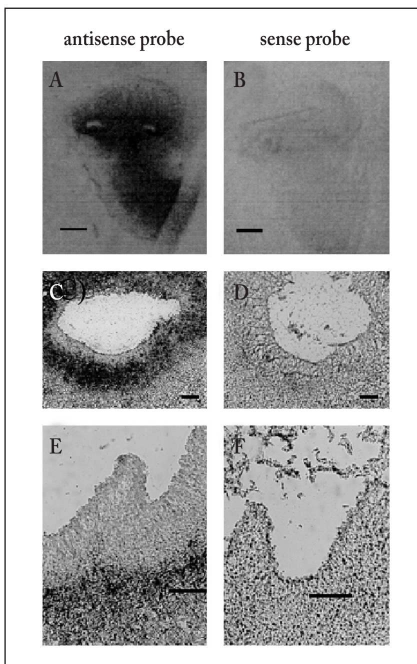
mfbC gene was cloned by Miyazaki et al. (2004) as a target of developmental regulator PRIB protein. As mentioned above, the *uck1* is also target of PRIB. The *mfbC* is a homologue of *S. cerevisiae YJR070C* /Lia (Thompson et al. 2003) and probably encodes the protein interacting with a putative translation initiation factor 5A (eIF5A), which is essential for cell viability and evolutionarily conserved (Thompson et al. 2003). Although the function of eIF5A is still obscure, there is the possibility that this factor might have a role in the translation of a specific subset of mRNAs.

Northern-blot analysis showed that all parts of the fruiting body contain *mfbC* transcript. But hymenophores and stipe contained higher levels of the transcript than the hymenophore-depleted pileus (Miyazaki et al. 2004). In situ RNA-RNA hybridization was carried out to investigate the expression of the *mfbC* gene in the parts of hymenophore, stipe, and hymenophore-depleted pileus (Miyazaki et al. 2004). Although the *mfbC* transcript appeared to distribute diffusively in the stipe and hymenophore-depleted pileus, this transcript showed a region-specific distribution in hymenophore (gill tissue). High levels of the *mfbC* transcript were detected in the outer region of the trama, the region branching out into the subhymenium (Fig. 2I). These results suggest that the *mfbC* gene is involved in the divergence of trama cells into subhymenium cells, but not in the production of basidiospores. To clarify the physiological function of the *mfbC* gene, however, more detailed ge-

netical and biochemical approaches are necessary.

3.4. *Hydrophobin 1 gene* (Le.hyd1)

Hydrophobins are moderately hydrophobic small proteins (100-150 amino acid residues) containing eight cysteine residues in a conserved pattern (Wessels 2000). These compounds appear to be unique to mycelial fungi such as basidiomycetes and ascomycetes, where they probably act in morphogenesis and pathogenesis (Wessels 2000). Three hydrophobin genes *SC1, SC4, and SC6* of *Schizophyllum commune* are expressed in dikaryons only, at the time of fruiting-body formation (Van Wetter 2000). Immunoelectron microscopy showed that SC4, the most abundant hydrophobin of the three



gene products, is secreted into the mucilage that surrounds hyphae of the plectenchyma (fungal tissues) of the fruiting bodies, lining air channels within them with a hydrophobic membrane (Van Wetter 2000).

Figure 3. Distribution of the *Le.hyd1* transcript in immature small fruiting bodies of L.edodes (A and B) and distribution of the le.hyd1 transcript in mycelial tissues around the prehymenophore of immature fruiting bodies (C-F). Bar, 200 μm

We isolated two hydrophobin genes, *Le.hyd1* and *Le.hyd2*, from *L. edodes* dikaryotic strain (Nishizawa et al. 2002). Northern blotting showed that immature small fruiting bodies that had just developed from primordia (pileus and stipe do not yet develop) contained the highest level of *Le.hyd1* transcript. Enlarged immature fruiting bodies also contained high levels of the transcript, but vegetatively growing mycelia or primordia and mature fruiting bodies contained no or a little *Le.hyd1* transcript. In the case of *Le.hyd2*, its transcript level was high in dikaryotic vegetative mycelial tissues.

Results of in situ RNA-RNA hybridization showed the presence of the *Le.hyd1* transcripts everywhere in the mycelial tissues of developing fruiting bodies except for the top parts of the pileus (cap) and for the prehymenophore (Fig. 3A). A high level of the transcript was detected in the parts surrounding the prehymenophore (Fig. 3C and E). As mentioned earlier, hydrophobins seem to be involved in the formation in the extracellular matrix of lined air channels with a hydrophobic membrane. These channels may help to provide gas exchange during respiration in mycelial tissues of developing fruiting bodies. Our results suggested that hydrophobin-mediated air channels may be formed all over the mycelial tissues of developing fruiting bodies except for the top parts of the pileus (cap) and for the prehymenophore. In particular, the parts surrounding the prehymenophore may come to have a large number of air channels.

4. Acknowledgements

This work was supported by a Grant-in Aid for Scientific Research (no. 13660084) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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