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Molecular and cellular biological studies on two nucleotide biosynthesis genes and their expression products of the basidiomycete *Lentinus edodes*

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1999

Contents

CHAPTER 1

General Introduction

Basidiomycetes are unique microorganisms with dramatic morphological differentiation from vegetative mycelia into huge fruiting-bodies in which a large number of basidiospores are produced (see Fig.1-1). Basidiospores produced in/on basidia of fruiting body germinate to form monocaryotic hyphae. Compatible monocaryotic hyphae mate to be fertile dicaryotic hypha which has clamp connections. In the dicaryotic hypha, the nucleus originated from each compatible monocaryotic hypha does not fuse immediately after the mating. Nuclear fusion occurs only in basidia of fruiting body. Under the conditions of nutrient starvation, low temperature, blue-light irradiation, etc., vegetative growth converts to sexual reproductive phase. Primordium is formed on the highly aggregated mycelia (complicated structure of hyphae) and it develops gradually to mature fruiting body consisting of stipe, pileus, and hymenophores (gill tissues) on which basidia are formed. In basidium, chromosomes of each nucleus are replicated and then fused. Meiosis occurs and four nuclei are produced. The four nuclei are divided into four prespore cells and duplicated in them. One set of nuclei moves back to the basidium and the other remains in the prespore. The prespore maturates to be basidiospore.

To elucidate the molecular mechanism of fruiting-body formation of the basidiomycete, our group have isolated various developmentally regulated genes from the most popular basidiomycete *Lentinus edodes* (*Shiitake*) (Shishido, 1994). The *priB* cDNA (*priBc*), one of them, was isolated from a cDNA library of primordia of *L. edodes* (Endo et al., 1994). As was expected, the *priB* gene was most actively transcribed in primordia, and immature and mature fruiting-bodies also contained lower levels of the *priB* transcript (Fig.1-2). The deduced PRIB protein (64 kDa, 565 amino acid (aa)) contained a 'Zn(II)2Cys6 zinc cluster' DNA-binding motif observed in *Saccharomyces cerevisiae* GAL4, *Neurospora crassa* QA1F and other yeast and fungal transcription factors (Vallee et al., 1991; Kraulis et al., 1992). The PRIB protein produced in *Escherichia coli* using the bacteriophage T7 expression system was found to bind to the DNA fragment containing the upstream region

primordia

immature
fruiting body

formation of
basidium
(n+n)

fruiting body
formation

mature fruiting body

replication
of DNA
(2n+2n)

nuclear fusion
(4n)

growth of
dicaryon (n+n)

meiosis
(4Xn)

dicaryotization and
fugion of hypha

sporulation of
basidiospore (n)

growth of
monocaryon (n)

germination of
basidiospore (n)
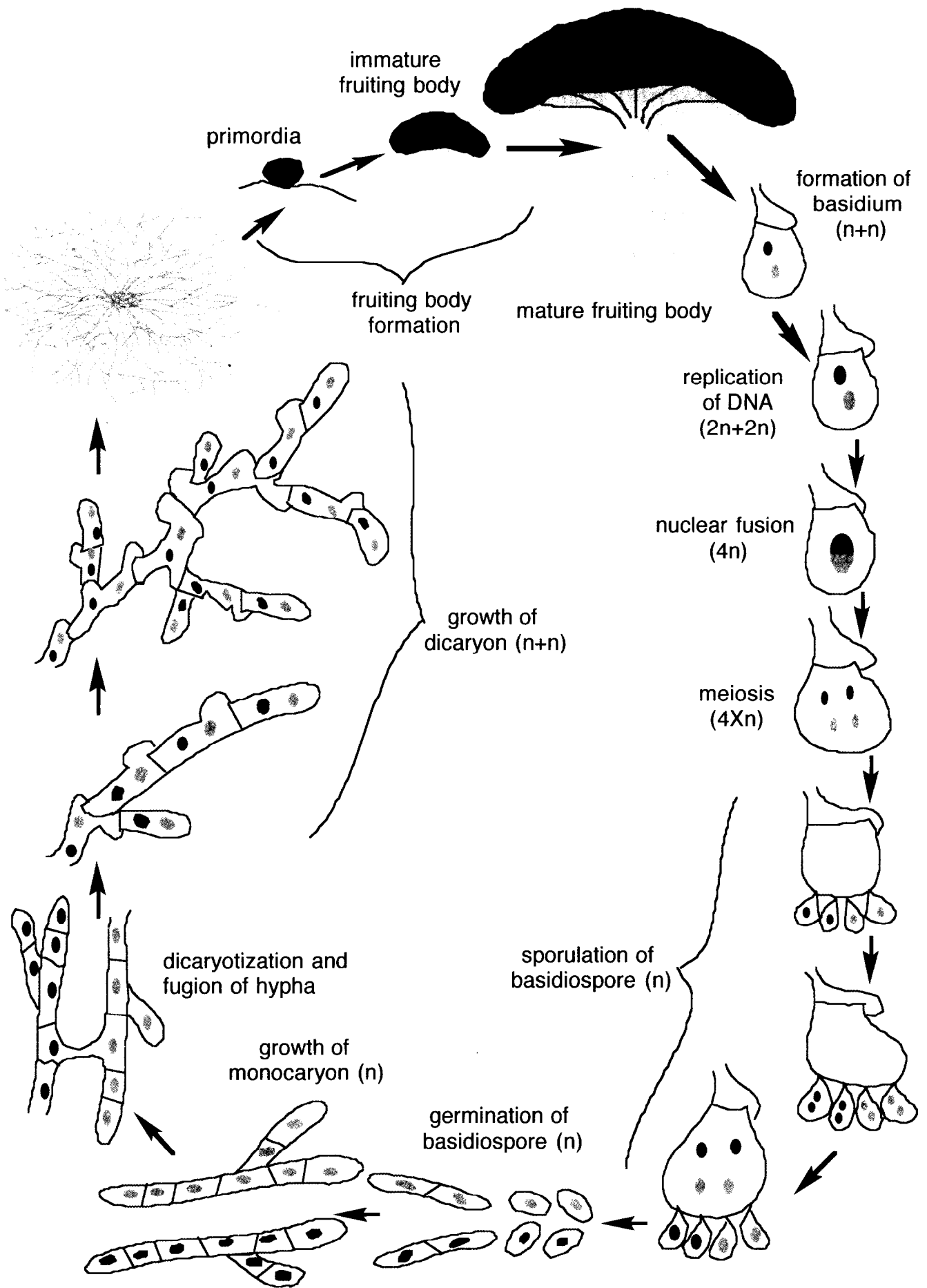
Fig. 1-1　The life cycle of the basidiomycete

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of *priB* gene (Endo et al., 1994). Our recent data (Miyazaki et al., 1997) revealed that the consensus sequence of binding of PRIB is 16 bp 5'GGGGGGGGACAGGANCC3'. Four sequences similar to this were actually found in the upstream region of *priB* gene. The *N. crassa* QA1F has been reported to be an activator protein required for the quinic-acid induction of transcription in the *qa* gene cluster which comprises a contiguous set of five genes that control quinate-shikimate utilization (Baum et al., 1987).

The analysis of the 5' upstream region of *priB* gene revealed an isolation of the novel gene designated *Le.cdc5* encoding 842 amino acid, which was also actively transcribed in primordia (Fig.1-2). The deduced Le.CDC5 protein contained the N-terminal amino acid sequence similar to those of *Schizosaccharomyces pombe* cdc5⁺gene product (*pombe* Cdc5) and other proteins related to *pombe* Cdc5 (named PCDC5RPs). This protein produced in *Escherichia coli* using the glutathione S-transferase gene fusion system was found to bind to 7 bp consensus sequence such as 5'GCAATGT3' or 5'ACATTGC3' (Miyazaki, 1999).

These findings, taken together, led the author to analyze the nucleotide sequences of the 3' flanking region of *priB* gene, resulting in the isolation of a *S. cerevisiae URA6* gene homologue encoding UMP kinase. The promoter region of *URA6* gene homologue contained two sequence of binding of PRIB. The deduced amino acid sequence of the *URA6* homologue gene (named *uck1* gene) product shows a significant homology to those of *S. cerevisiae* UMP kinase, *Dictyostelium discoideum* (slime mold) UMP-CMP kinase, pig UMP-CMP kinase and many AMP kinase genes. These nucleoside monophosphate (NMP) kinases catalyze the transfer of a phosphate group from ATP to various nucleoside monophosphate (NMP) (Anderson, 1973; Fig.1-3). The NMP kinase family is divided into subgroups consisting of UMP (or UMP/CMP) kinases (UCK), adenylate kinases (AK), guanylate kinases, and thymidylate kinases, on the basis of the differences in substrate specificity for NMP as a phosphoryl acceptor. The author showed in the chapter 2 that the *uck1* product phospholylates UMP, CMP, AMP, etc. Northern-blot analysis revealed that the
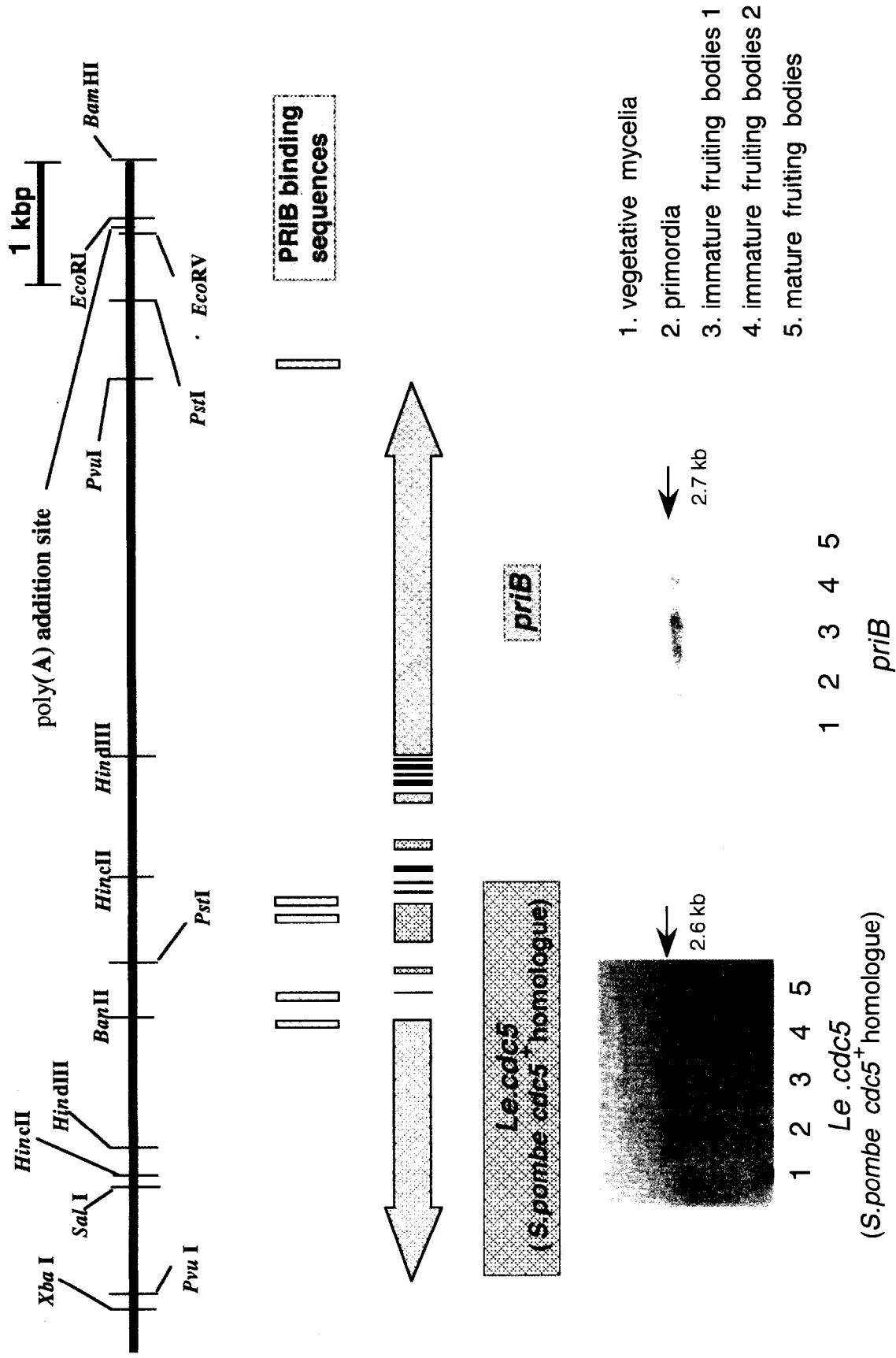
Fig. 1-2 Arrangement of *priB* and *Le.cdc5* (*S.pombe cdc5* [+]homologue) on chromosome of *L.edodes*

1. vegetative mycelia
2. primordia
3. immature fruiting bodies 1
4. immature fruiting bodies 2
5. mature fruiting bodies

poly(A) addition site

PRIB binding sequences

priB

Le.cdc5
(*S.pombe cdc5* [+]homologue)

2.7 kb

2.6 kb

1 2 3 4 5
priB

1 2 3 4 5
Le.cdc5
(*S.pombe cdc5* [+]homologue)

1 kbp

Xba I
Sal I
*Hinc*II
*Hind*III
Pvu I
*Bgl*II
*Hinc*II
*Pst*I
*Hind*III
*Pvu*I
*Pst*I · *Eco*RV
*Eco*RI
*Bam*HI

5
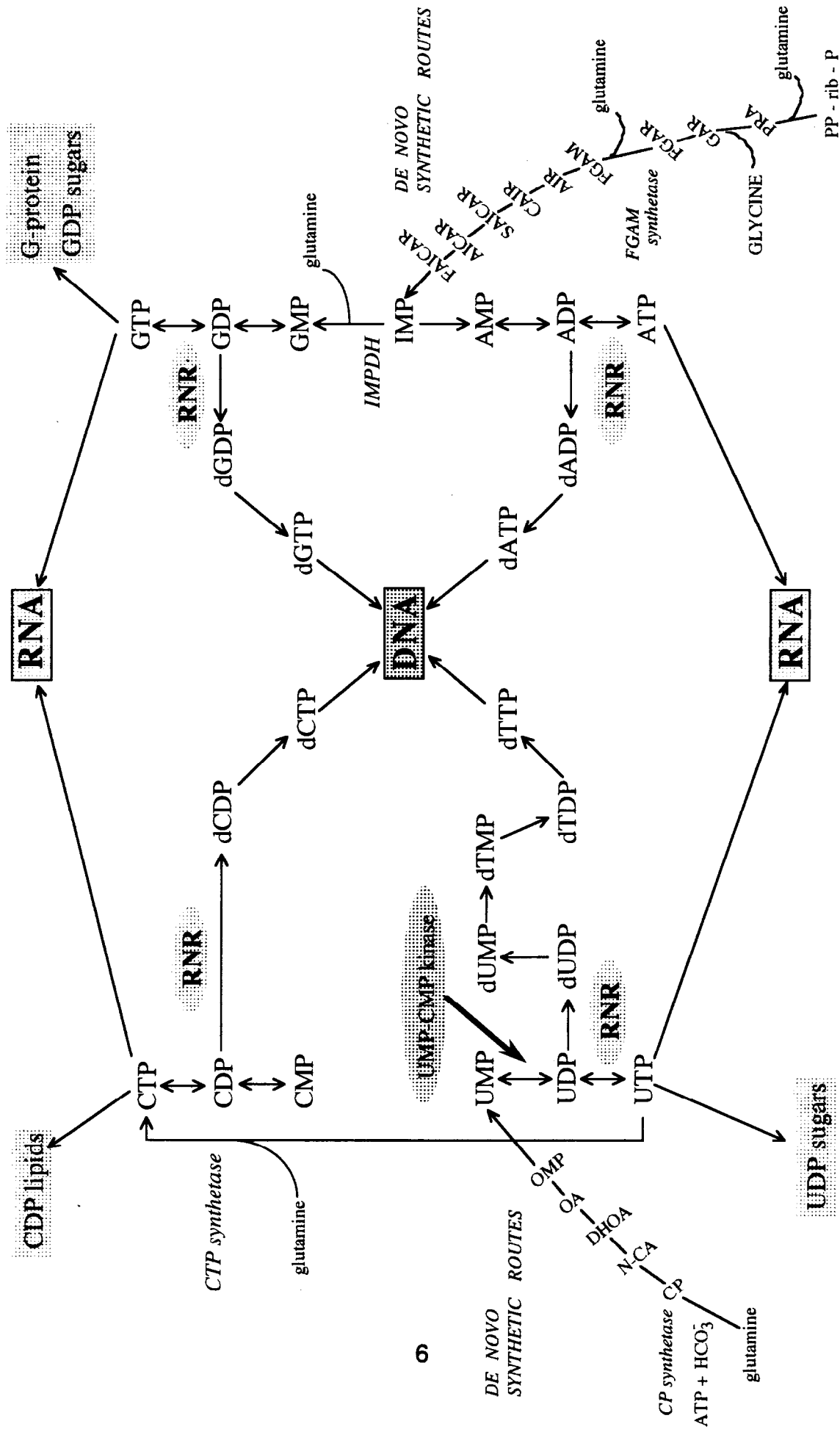
Fig. 1-3 Pathway for purine and pyrimidine metabolism

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uck1 gene is actively transcribed in hymenophores (gill tissues) of mature fruiting body of *L. edodes*. This implies that *uck1* product (UMP-CMP kinase) which synthesizes UDP, CDP and ADP play a role in the hymenophores in which basidiospores are formed.

In the nucleotide metabolism, UDP, CDP and ADP were reduced to the corresponding deoxyribonucleoside diphosphates (dNDP) to serve as precursors for syntheses of dNTP (see Fig.1-3). These reductions are catalyzed by identical Ribonucleotide reductase (RNR) in eucaryotes. RNR also reduces GDP to dGDP and plays a regulatory role in maintaining a balanced pool of all four deoxyribonucleotides (Reichard, 1988; see Fig.1-4). Therefore its activity is cell-cycle dependent and is highly transcriptionally regulated (Elledge et al., 1992; Tuggle et al., 1990; Björklund et al., 1990). To study the biosynthesis of deoxyribonucleotide during fruiting-body formation, the author attempted to isolate the RNR genes encoding RNR large (R1) and small (R2) subunits, resulting in the isolation of two cDNA fragments (*Le.rnrB1* and *Le.rnrB2*) encoding small subunit (R2) from mRNAs in mature fruiting bodies of *L. edodes* using RT-PCR technique. Furthermore the author isolated *Le.rnrB1* cDNA containing the entire ORF. Northern-blot analysis showed that the transcripts of the *Le.rnrB1* and *Le.rnrB2* genes were most abundant in the hymenophores (gill tissues) of mature fruiting bodies, in analogous to *uck1* gene. The hymenophore in the mature fruiting body is complicated gill tissue on which a large number of basidia are formed. The author carried out *in situ* RNA hybridization to localize the part(s) of hymenophore at where these nucleotide biosynthesis genes are actively transcribed. In this study the author describes the experimental results of the two sorts of nucleotide biosynthesis gene. These results are considered to give valuable informations for the molecular mechanism of basidiospore formation of *L. edodes*.

General activity site
ATP dATP

General activity site
ATP dATP

R1
(α)

R1
(α)

Substrate specificity site
ATP dATP
dTTP dGTP

Substrate specificity site
ATP dATP
dTTP dGTP

Substrate-binding site SH
NDP

SH Substrate-binding site
NDP

Free radical

Free radical

Fe^{3+} Fe^{3+}

Fe^{3+}

Fe^{3+}

Fe^{3+}

R2
(β)

R2
(β)
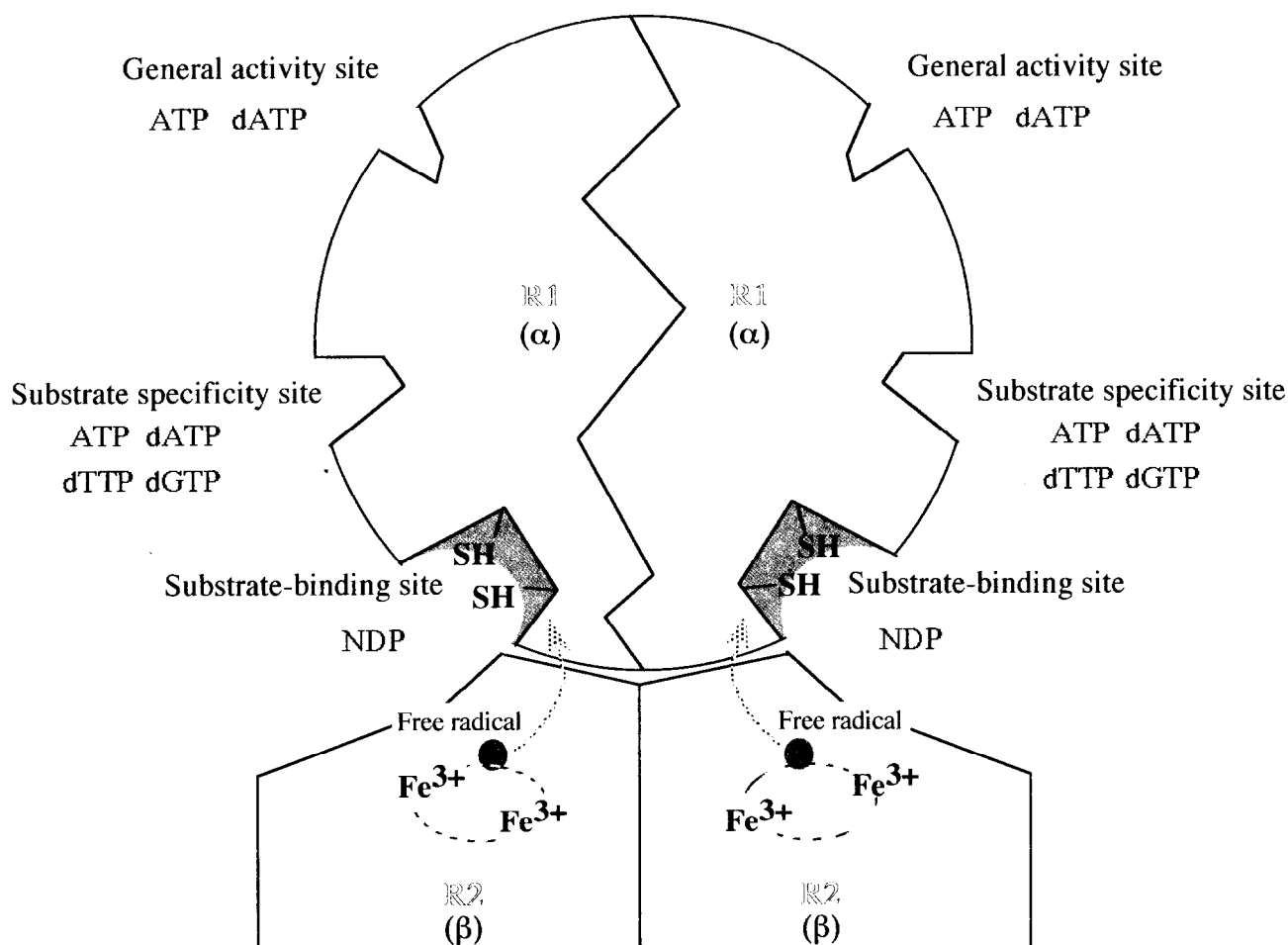
Fig. 1-4   Model of ribonucleotide reductase (RNR)

RNRs in eucaryotes consists of a heterodimer ($\alpha_2\beta_2$) that contained two nonidentical homodimers (Mathews et al., 1987), the large subunit (α or R1) and the small subunit (β or R2), each of which is inactive by itself. Each monomer (90-kDa) of the R1 dimer contains one substrate-binding site with redoxactive thiols and two separate allosterically regulated binding sites for dNTPs. Each monomer (45-kDa) of the R2 dimer contains a tyrosyl free radical that is essential for catalytic activety. This tyrosyl free radical is generated and maintained by an oxygen-linked binuclear ferric iron center buried insidethe protein (Petersson et al., 1980). RNR is inhibited by high concentrations of dATP (Reichard 1993; Brown et al.,1969). In contrast, the enzyme needs ATP to be active. Thus, the relative concentrations of ATP and dATP regulate the activity of the enzyme. RNR is further regulated in a unique way by a second class of allosteric activators which influence the substrate specificity of the enzyme so as to obtain a balanced supply of deoxyribonucleotides for DNA synthesis. The substrate specificity is shifted from pyrimidine reduction to the reduction of GDP by binding of dTTP at the specificity site, and to ADP reduction by the binding of dGTP. This series of events has been described as a sequential transformation of the substrate specificity. The binding of nucleotide effectors takes place at two different sites (Reichard 1993; Brown et al.,1969). ATP and dATP bind to the general activity site, while ATP, dATP, dTTP and dGTP bind at the substrate specificity site.

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CHAPTER 2

Cloning, sequence analysis and expression of the basidiomycete *Lentinus edodes* gene encoding UMP-CMP kinase, the homologue of *Saccharomyces cerevisiae URA6* gene

2-1. Introduction

The *priB* gene isolated from a cDNA library of primordia of *L. edodes,* was most actively transcribed in primordia, and immature and mature fruiting bodies also contained lower levels of this transcript. Its product, PRIB binds to 16 bp consensus sequence and is supposed to play a role of fruiting-body formation of *L. edodes.* By genomic binding selection method, Miyazaki isolated two genomic DNA fragments contained 16 bp PRIB-binding site and the gene transcribed actively in the mature fruiting body. One of each encoded the protein highly homologous to *S. cerevisiae* YJ40 protein, of which function was not yet known (Miyazaki, 1999). As described in the chapter 1, four sequences similar to the 16 bp consensus sequence binding of PRIB were found in the upstream region of *priB* gene and within *Le.cdc5* gene encoding DNA binding protein related to *pombe* Cdc5, while it had been shown that 3'-noncoding region of *priB* also contained one of binding sequence of PRIB. The author analyzed genomic DNA region downstream from the poly(A)-addition site of *priB*, resulting in the presence of two sequences similar to the 16 bp consensus sequence binding of PRIB and the isolation of *S. cerevisiae* URA6 gene homologue encoding UMP kinase.

2-2. Materials and methods

2-2.1. Cloning of genomic DNA region downstream from the poly(A)-addition site of priB

A genomic DNA library was prepared by insertion of size-fractionated *Eco*RI- or *Hind*III-digest of *L. edodes* FMC2 (Katayose et al., 1990) genomic DNA, into *Eco*RI- or *Hind*III-cleaved and dephosphorylated Charomid 9-36 vector (Saito et al., 1986), followed by transformation of *E. coli* DH1 with the

packaging mixture (Sambrook et al., 1989). These libraries were screened by using ^{32}P-labeled probes (Feinberg et al., 1983) of the 400 bp EcoRI - BamHI fragment (for the EcoRI-derived library) and the 450 bp PstI - EcoRI fragment (for the HindIII-derived library) (see Fig. 2-1). Previous published conditions (Hori et al., 1991) were used for the hybridization work (Hori et al., 1991). The genomic DNA clones were restriction mapped and the restriction fragments were analyzed by direct DNA sequencing after subcloning into pUC19. Sequencing was carried out by the dideoxy chain-termination method of Sanger et al. (1977).

2-2.2. Gel mobility-shift assay

Restriction DNA fragments were end-labeled with [α-^{32}P]-dCTP and Klenow fragment. About 5 fmol each of ^{32}P-labeled DNA fragments were incubated with approx. 20 fmol or 60 fmol of PRIB in 50 μl of the binding buffer (25 mM HEPES-KOH (pH 7.9), 50 mM KCl, 5 mM MgCl$_2$, 0.1 mM ZnCl$_2$, 1 mM EDTA (pH 8.0), 300 mg/ml BSA) at room temperature for 15 min according to the method of Miyazaki et al. (1997 and 1999). The reaction mixtures were electrophoresed in 4 % native polyacrylamide gels using 0.5×TBE buffer. The gels were dried and autoradiographed.

2-2.3. Screening of cDNA library

The cDNAs synthesized using poly(A)$^+$ RNAs which were actively expressed in mature fruiting bodies of L. edodes FMC2 was ligated to EcoRI/NotI adaptor after filling up the ends and then, inserted into EcoRI-cut and dephosphorylated λgt10 vector (Huynh et al., 1985) basically according to the procedures previously described (Hori et al., 1991). This mature fruiting-body cDNA library was screened by plaque hybridization using the ^{32}P-labeled

500-bp genomic *Eco*RI - *Eco*RV fragment (see Fig. 2-1) under the conditions previously reported (Hori et al., 1991). Two positive cDNA clones were obtained; one clone contained a DNA insert of almost the expected 0.7 kb and the other contained the 0.6 kb insert, being a deletion derivative of the 0.7 kb insert. The restriction fragments were subcloned into pUC19 and sequenced.

2-2.4. Construction of L. edodes *UMP-CMP kinase expression plasmid and production in* E. coli *of the protein*

The cDNA fragment (nucleotide +61 to +773) bearing the *L. edodes* UMP-CMP kinase-coding sequence without N-terminal 20-amino acid-coding sequence and the 3' untranslated sequence (see Fig. 2-2) was inserted into the *Eco*RI site of the glutathione S-transferase (GST) fusion vector pGEX-2TK (Smith et al., 1988). The resulting recombinant plasmid, named pGEX-2TK-UCK was introduced into *E. coli* BL21. Cultivation of the transformant in 2×YT medium (Yeast extract 1.0%, Tryptone 1.6%, NaCl 0.5%, pH 7.0) at 28°C for 3 h, induction of the culture by 1 mM isopropyl-ß-D-thiogalactoside (IPTG) for 3 h, preparation of *E. coli* protein extracts, and purification of the UMP-CMP kinase at 28°C all were performed according to the procedures of GST Gene Fusion System protocol supplied by Pharmacia Biotech. The GST-(UMP-CMP kinase) fusion protein was purified by a batch method using Glutathione-Sepharose 4B and cleaved at its fusion junction with Thrombin whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX-2TK plasmid (see Fig. 2-4). The Thrombin-digest was applied to Glutathione-Sepharose 4B and the UMP-CMP kinase protein was purified as an unadsorbable protein. The protein samples (approx. 5 - 10 µg) were resolved by 0.1 % SDS-12.5 % PAGE and subsequently stained with Coomassie brilliant blue.

2-2.5. Nucleoside monophosphate kinase assay

Kinase activity toward various nucleoside monophosphates was measured by the following two methods. To the assay mixture containing 75 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2.5 mM $[\gamma\text{-}^{32}P]$-labeled ATP, and 2.5 mM nucleoside monophosphate, 25 ng of the purified *L. edodes* UMP-CMP kinase was added and incubated at 37°C for 30 min. The reaction mixtures were spotted onto polyethyleneimine-cellulose thin-layer sheets and developed by ascending chromatography in a solvent of 0.5 M ammonium formate titrated to pH 3.4 with formic acid according to the method of Choi et al. (1989).

The other assay mixture contained 87 mM triethanolamine-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 5 units of pyruvate kinase, 20 units of lactate dehydrogenase, 1 mM ATP, and 0.05 - 0.5 mM nucleoside monophosphate according to the method reported by Okajima et al. (1995). After pre-equilibration at 25°C for about 2 min, 10 ng of the purified *L. edodes* UMP-CMP kinase was added and incubated at 25°C for 1 min. Kinase activity was determined by monitoring the decrease of the absorbance at 340 nm (Okajima et al., 1995).

2-2.6. Northern-blot analysis

Total *L. edodes* cellular RNA was isolated from the preprimordial mycelia, primordia, immature fruiting bodies (fruiting-body development stage I, II and III), mature fruiting bodies (fruiting-body maturation stage I, II and III), hymenophores-depleted pileus, stipe, and hymenophores (gill tissues) according to the method of Han et al., (1987). The RNA samples (25 µg each) were size-fractionated on 2.2 M formaldehyde -1.2 % agarose gels (Lehrach et al., 1977), transferred to a nylon filter (Reed et al., 1985), hybridized with ^{32}P-labeled probes of *L. edodes* genomic restriction fragments and cDNA under stringent conditions according to the method of (Hori et al., 1991) and

autoradiographed.

2-3. Results

2-3.1. PRIB protein binds to the approx. 1.0 kb 3' downstream region from the poly(A)-addition site of priB *gene*

The author cloned the 2 kb *Eco*RI - *Eco*RI and 6.5 kb *Hin*dIII - *Hin*dIII fragments covering the 7 kb downstream region from the poly(A)-addition site of *priB* gene and constructed the restriction maps (see Fig. 2-1). Sequence analysis suggested that there exist two 16 bp sequences of 5'GTGGGGAGTAAGATCC3' and 5'GTGAGGAAATGAAGCC3' homologous to the PRIB-binding consensus sequence 16 bp 5'GGGGG GGACAGGANCC3' (Miyazaki et al., 1997) at 852 and 1074 nucleotide 3' downstream from the poly(A)-addition site of *priB* gene (see Fig. 2-1). To examine whether PRIB actually binds to the DNA fragments containing the two 16 bp sequences, a gel mobility-shift assay using native polyacrylamide gels was done for the following three ^{32}P-end-labeled restriction fragments, i.e., 228 bp *Eco*RV - *Xba*I fragment (Probe A) and 340 bp *Xba*I - *Pst*I fragment (Probe B) both containing either one of the 16 bp sequences and 127 bp *Hin*dIII - *Eco*RV fragment (Probe C) containing no 16 bp sequence (Fig. 2-2A). As shown in Fig. 2-2B, PRIB was found to bind to the ^{32}P-labeled probe A (panel B1) and probe B (panel B2), but not to probe C (panel B3). Binding of PRIB to the ^{32}P-labeled probe A (and Probe B) was inhibited by cold competitor probe A (and Probe B), but not by probe C (panels B1 and B2). These results indicate the binding of PRIB to the approx. 1.0 kb downstream flanking region of *priB* gene, and the possibility that a gene regulated by PRIB exists in the further downstream region of *priB* gene.

Fig. 2-1 Arrangement of *priB*, *Le.cdc5* (S.pombe cdc5⁺ homologue), and *uck1* (S.cerevisiae URA6 homologue) on chromosome of *L.edodes*

Restriction map of the region containing *Le.cdc5*, *priB* and *uck1* genes and their flanking regions on the chromosome of *L. edodes*. All genes are represented by their coding regions. Seven vertical striped boxes show putative PRIB-binding sites. The cloned two fragments of 2-kb *Eco*RI - *Eco*RI fragment (clone 1) and 6.5-kb *Hind*III - *Hind*III fragment (clone 2) covering the 7-kb downstream region from the poly(A)-addition site of *priB* gene are shown. Probes used for the cloning of genomic and complementary DNAs are also shown.

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(A)

poly(A)-addition site of
priB

HindIII   EcoRV   XbaI   PstI   *uck1*

poly(A)-addition site of
priB

Probe

C

A

B

(B1)

PRIB	(fmol)	–	20	60	60	60	–
competitor A	(pmol)	–	–	–	0.5	2.5	–
competitor C	(pmol)	–	–	–	–	–	2.5

1 2 3 4 5 6

Probe A

(B2)

PRIB	(fmol)	–	20	60	60	60	60
competitor B	(pmol)	–	–	–	0.5	2.5	–
competitor C	(pmol)	–	–	–	–	–	2.5

1 2 3 4 5 6

Probe B

(B3)

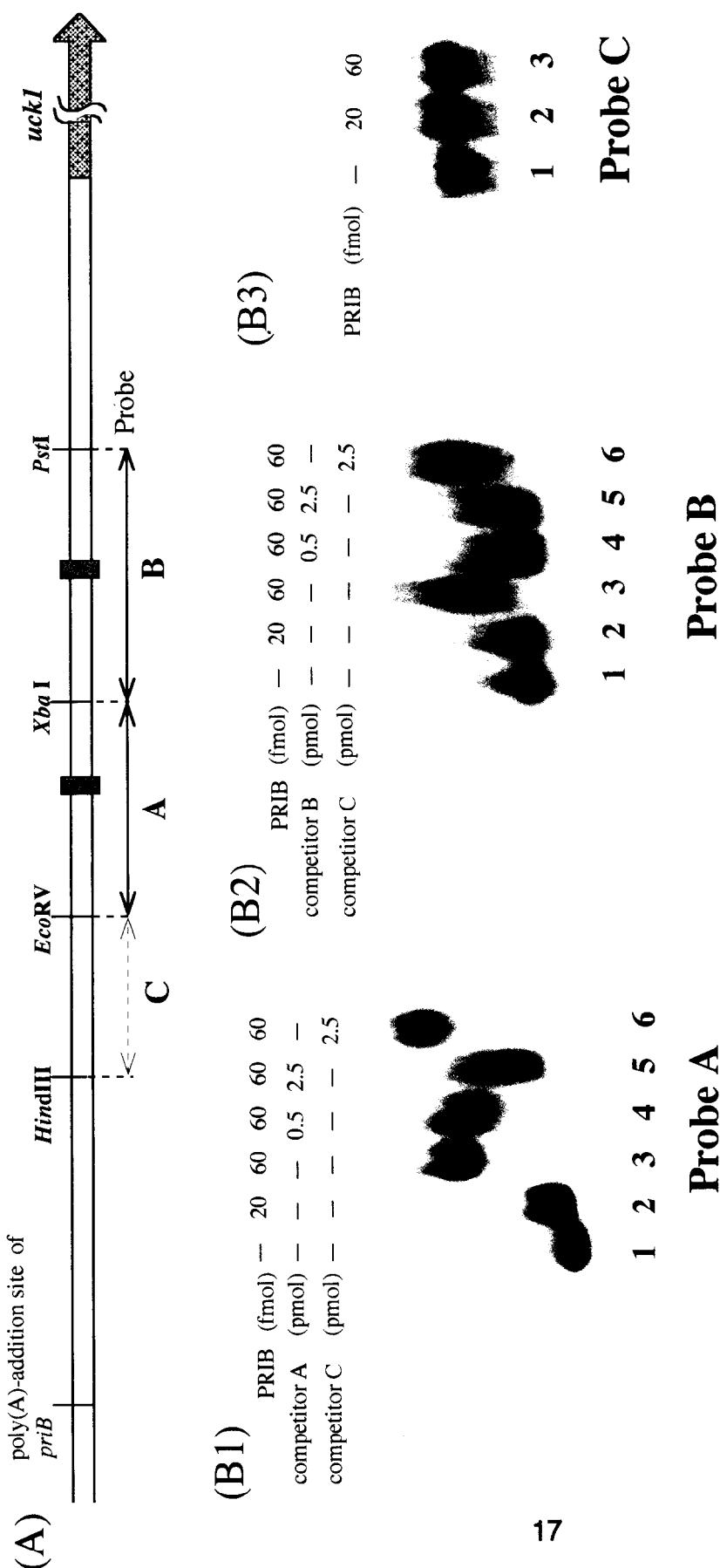| PRIB	(fmol)	–	20	60

1 2 3

Probe C

Fig.2-2.   Binding of PRIB to the downstream region from the poly(A)-addition site of *priB* gene.

(A) Structural features of the downstream region from the poly(A)-addition site of *priB* gene. Shaded boxes within the 228 bp *EcoRV* - *XbaI* fragment (Probe A) and 340 bp *XbaI* - *PstI* fragment (Probe B) indicate the 16 bp sequences of 5'GTGGGGAGT AAGATCC3' and 5'GTGAGGAAATGAAGCC3' respectively, which exist 852 and 1074 nucleotide 3' downstream from the poly(A)-addition site of *priB* gene and homologous to the PRIB-binding consensus 16 bp sequence 5'GGGGGGGACAGGANCC3'. (B1), (B2) and (B3) Gel mobility-shift assay in native polyacrylamide gels. The assay was done for the following three 32P-end-labeled restriction fragments, i.e., 228 bp *EcoRV* - *XbaI* fragment (Probe A) (B1) and 340 bp *XbaI* - *PstI* fragment (Probe B) (B2), and 127 bp *HindIII* - *EcoRV* fragment without the 16 bp sequences (Probe C) (B3). (B1, B2) Lane 1, without PRIB ; lane 2, 20 fmol of PRIB ; lanes 3 - 6, 60 fmol of PRIB. Five fmol of 32P-end-labeled Probes A (panel B1) or B (panel B2) was used in all lanes. Cold DNA fragment A (panel B1) or B (panel B2) was used 0.5 pmol in lane 4 and 2.5 pmol in lane 5. Cold DNA fragment C was used 2.5 pmol in lane 6 of panels B1 and B2. (B3) Lane 1, without PRIB ; lane 2, 20 fmol of PRIB ; lane 3, 60 fmol of PRIB. Five fmol of 32P-end-labeled Probe C was used in all lanes.

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2-3.2. Cloning and nucleotide sequence analysis of uck1 *gene from* Lentinus edodes

Northern-blot analysis using each of the subcloned fragments (2 kb *Eco*RI - *Eco*RI and 6.5 kb *Hind*III - *Hind*III fragments) as a probe suggested the presence of a gene, which is actively transcribed at the stage of mature fruiting bodies, approx. 2.0 kb downstream from the poly(A)-addition site of *priB* (Fig. 2-1). By using the probe of 500 bp *Eco*RI - *Eco*RV fragment which gave the hybridization signal (see Fig. 2-1), the author isolated a corresponding 0.7 kb cDNA clone from a mature fruiting-body cDNA library of *L. edodes*. The cloned cDNA, designated *uck1*c, consisted of 713 bp encoding 207 amino acid with a remarkable homology to the amino acid sequence of UMP kinase (204 amino acid) encoded by *S. cerevisiae* URA6 gene (Liljelund et al., 1989) (see Figs. 2-3 and 2-4). The approx. 3 kb genomic sequence was determined and compared with the *uck1* cDNA sequence. It was found that the coding sequence of *uck1* gene is interrupted by four small introns and *uck1*c lacks the 60 bp sequence encoding the N-terminal 20 amino acid (Fig. 2-3).

The 360 nucleotide sequence preceding the translation start codon of *uck1* contained TATTTA and CCAAT boxes (Breathnach et al., 1981) and a CT-motif (Gurr et al., 1988). A putative poly(A)-addition signal (AATCAAA) was found 59 nucleotide downstream from the translation stop codon, however, the poly(A)-addition site was not determined because *uck1*c lacked 3' poly(A) tail.

2-3.3. Comparison of the amino acid sequences of uck1 *gene product (UMP-CMP kinase) and various nucleoside monophosphate kinase proteins*

To determine the relationship between the *uck1* and other nucleoside monophosphate kinase genes, their derived amino acid sequences were compared (Fig. 2-4). *S. cerevisiae* URA6 gene product (UMP kinase), *Dictyostelium discoideum* (slime mold) UMP-CMP kinase, pig UMP-CMP

```
-360  AGGGGCACCGGCATTGTGTGACAGGCGACGAGGACATATATGTCCGTCAGTTGCTTTTATGTGCTCGGCTTGTGAGCAGAAGTTGGTTTT
      TATA box
-240  ATTTATTTAATCTGCAGCTAATTCAATGTATCAATGTATGGTGATTGTTTGGTCTTGATCAGACAGCACAAACCTCCAAACCCTTTCTGCTTCTGTCTCCGGTCTTTCTTTGGGCTCC
      CCAAT box                                                  CT motif
-120  TTTGTCCTGTTTTATTCATTAGCAAAATACCGCGAAAAACCAATCCAGCTTCCGAGCTTCCTGACAACTTGATCATCCTCTTGTACCTCCTTCCTGTCCTGTGATAACTAAAACCTAA

   1  ATGCCTACTATCATCGACAAATTGTCTGATGCTCTCCATCTTCACTCCCACCAGAGCACTTTCGACTCAAAAGTAACGGTTATCTTTGTCTTTGGTGGTCCTGGTGCAG gtatgtagcc
      M   P   T   I   I   D   K   L   S   D   A   L   H   L   H   S   H   Q   S   T   F   D   S   K   V   T   V   I   F   V   L   G   G   P   G   A     36
                                                                                                                       mononucleotide-

 120  atccagccatcttcgatccgtccatttttaattcaatatttcag GCAAAGGACCCAGGTGCGCGCCTCGTTGAAGACTTTAGCTTCTCCATCTTTCAG gtgcgttttgttttttg
                                                    Q   C   A   R   L   V   E   D   F   S   F   S   H   L   S
                                                                                                                                                55
                                                G   K   G   T
                                               binding motif

 239  cttataccatagagtgaattcggagcttgcag CTGGAGACCTCCTCCGTGCAGAACAGCACCGCGAAGGTTCTGAATACGGCCAGCTCATTCAAAACCTGTATCAAGGAAGGCTCTATAG
                                       A   G   D   L   L   R   A   E   Q   H   R   E   G   S   E   Y   G   Q   L   T   C   I   K   E   G   S   I     84
                                                                            NMP binding domain

 358  TGCCAATGGAAGTTACCGTTAAACTCCTTGAGAACGCCAATGACCGCCACTCTGGCGAACGAAGATCTGGAGAGGGATGGACTGGACGGTCAAGGTCGCTTCCTTATCGATGGGTTCCTC
      V   P   M   E   V   T   V   K   L   L   E   N   A   M   T   A   T   L   A   E   R   R   S   G   E   G   W   T   D   G   Q   G   R   F   L   I   D   G   F   P     124

 478  GAAAAATGACCAGCAGAGAAGTTGAACACGACGTTGAAAAAGCAAGCTCGGTTTGTCTTTTCTACCACCCAGAGGTCATGCTTGATCGTTCTTCTTGAACGAGCAAAACTAGCG
      R   K   M   D   Q   A   E   K   F   F   E   H   D   V   G   K   A   T   A   V   L   F   F   S   T   T   Q   E   V   M   L   D   R   L   L   E   R   G   K   T   S     164
                                                                                                                                                  LID domain

 598  GGAGGGAGGACGACCAACGTTGAGAGTATCAAGAAAACGCTTCA gtaagtacctcttcgtcctatgagattccttgggtacctacattaccatag ATACGTACAAGGAACAGACCATGCC
      G   R   E   D   D   N   V   E   S   I   K   K   R   F                                                    N   T   Y   K   E   Q   T   M   P     187

 717  GGTCATTGAACACTATGAGAAAACTCGGAAAAGTGATAGAG gtgtgtgatgtgttaccaacattccactcggtgactatgctcatcctgttag ATTGATAGTTCTGTCTCTCATTGAAG
      V   I   E   H   Y   E   K   L   G   K   V   I   E                                                  I   D   S   S   V   S   I   E     208

 836  AGGTACACCAGAAAACCAGGTCTGCTGTAGCTAAACTTCTTTCTGGCTCTACCGCCTAAAGCTACTGTAATTACTGGATTCATTGATATCAAGGAGGACCATACAAGGAATACATTAAA
      E   V   H   Q   K   T   R   S   A   V   A   K   L   L   S   G   G   S   T   A   *     227

 956  TCAAAAATATTTATCCATGGACCATGAATCCCGC
```

Fig. 2-3 The nucleotide sequences of the *L. edodes uck1* gene and deduced amino acid sequences.

The presence of introns in lower-case letters was deduced from the comparison of the cDNA sequence. The nucleotide sequence coordinates and amino acid sequence coordinates are presented on the left-hand side and right-hand side, respectively. The start codon is assigned the +1 coordinate. The amino acid residues responsible for the bindings of phosphoryl donor ATP and phosphoryl acceptor nucleoside monophosphates and those responsible for the transfer of phosphate group from ATP to nucleoside monophosphates are shaded (refer to the legend of Fig. 2-4). The conserved mononucleotide-binding motif observed in many ATP-binding proteins (Möller and Amons, 1985) and NMP binding and LID domains reported for AMP kinases or for AMP kinase-related proteins (Schulz et al., 1990; Müller-Dieckmann and Schulz, 1995) are boxed. The TATTTA and CCAAT boxes and CT-motif are boxed. Consensus sequences for splicing are underlined. Dotted line and horizontal arrows in 3' untranslated region indicate a putative poly(A)-addition signal and a direct repeat. The sequence data reported in this paper will appear in the DDBJ/EMBL/Gen Bank nucleotide sequence databases under accession NO. AB005742.
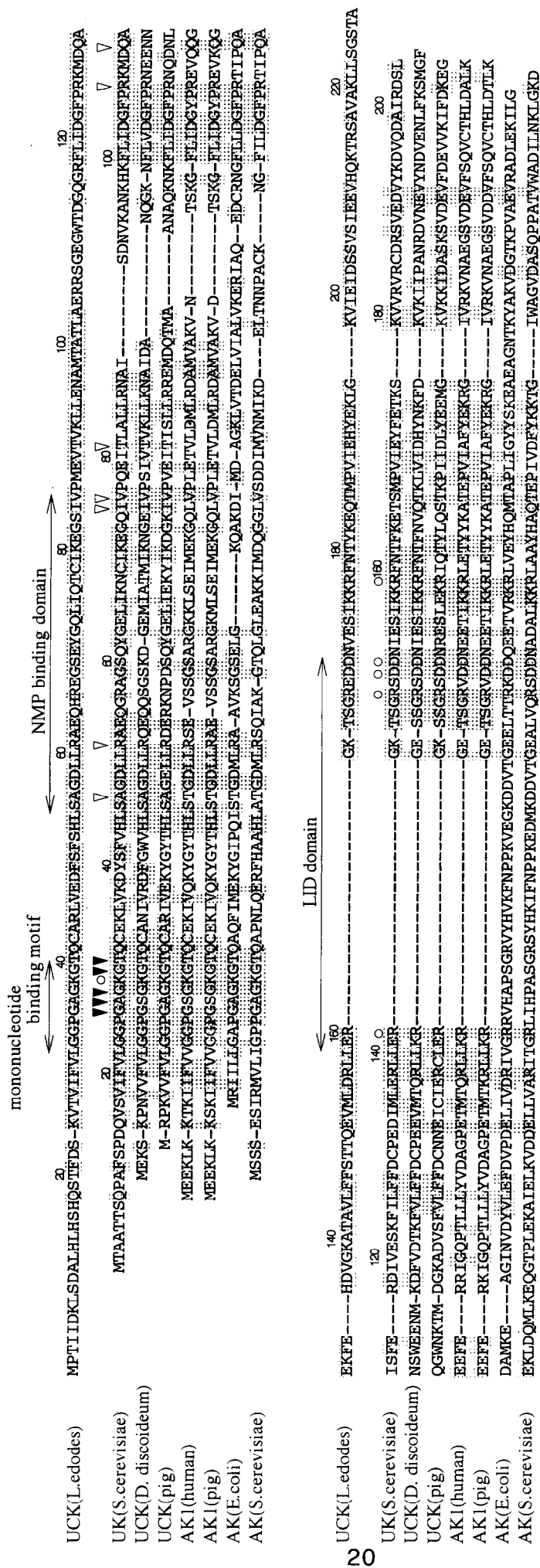
mononucleotide
binding motif   NMP binding domain

```
UCK(L.edodes)      MPTIIDKLSDALHLHSHQSTFDS-KVTVIFVLGGPGAGKGTQCARLVEDESFSHISAGDLLRAEQHREGSEVGQLIQTCIKEGSIVPMEVTVKLLENAMTATLAERRSGEGWTDGQGRFLIDGFPRKMDQA

UK(S.cerevisiae)   MTAATTSQPAFSPDQVSVIFVLGGPGAGKGTQCEKLVKDYSFVHLSAGDLLRAEQGRAGSQYGELIKNCIKEGQIVPQEITLALLRNAI------SDNVKANKHKFLIDGFPRKMDQA
UCK(D. discoideum) MEKS-KPNVFVLGGPGSGKGTQCANIVRDFGWVHLSAGDLIRQEQQSGSKD-GEMIAIMIKNGEIVPSIVTVKLLKNAIDA------NQGk-NFLVDGFPRNEENN
UCK(pig)           M-RPKVFVLGGPGAGKGTQCARIVEKYGYTHLSAGELLRDERKNPDSQYGELIEKYIKDGKIVPVEITISLLRREMDQTMA------ANAQKNKFLFDGFPRNQDNL
AK1(human)         MEEKLK-KTKIIFVVGGPGSGKGTQCEKIVQKYGYTHLSTGDILRSE-VSSGSARGKKLSEIMEKGQLVPLETVLDMLRDAMVAKV-N------TSKG-FLIDGYPREVQQG
AK1(pig)           MEEKLK-KSKIIFVVGGPGSGKGTQCEKIVQKYGYTHLSTGDILRAE-VSSGSARGKMLSEIMEKGQLVPLETVLDMLRDAMVAKV-D------TSKG-FLIDGYPREVKQG
AK(E.coli)         MRIILLGAPGAGKGTQAQFIMEKYGIPQISTGDMLRA-AVKSGSELG------KQAKDI-MD-AGKLVTDELVIALVKERIAQ--EDCRNGFLLDGFPRTIPQA
AK(S.cerevisiae)   MSSS-ESIRMVLIGPPGAGKGTQAPNLQERFHAAHLATGDMLRSQIAK-GTQLGLEAKKIMDQGLVSDDIMVNMIKD----NG-FIIDGFPRTIPQA
```

LID domain

```
UCK(L.edodes)      EKFE------HDVGKATAVLFFSTTQEVMLDRLLER------GK-TSGREDDNVFSIKKRFNTYKEQTMPVIEHYEKLG------KVIEIDSSVSIEEVHQKTRSAVAKLLSGSTA

UK(S.cerevisiae)   ISFE----RDIVESKFILFFDCPEDIMLERLLER------GK-TSGRSDDNIESIKKRFNTFKETSMPVIEYFETKS------KVVRVRCDRSVEDYKDVQDAIRDSL
UCK(D. discoideum) NSWEENM-KDFVDTKFVLFFDCPEEVMTQRLLKR------GE-SSGRSDDNIBSIKKRFNTFNVQTKLVIDHYNKFD------KVKIIPANRDVNEYNDVENLFKSMGF
UCK(pig)           QGWNKTM-DGKADVSFVLFFDCNNEICIERCIER------GK-SSGKSDDNRBSLEKRIQTYLQSTKPIIDLYEEMG------KVKKIDASKSVDFVFDEVVKIFDKEG
AK1(human)         EEFE------RRIGQPTLLEYVDAGPETMTQRLLKR------GE-TSGRVDDNEETIKKRLETYYKATEPVIAFYEKRG------IVRKVNAEGSVDFVFSQVCTHLDALK
AK1(pig)           EEFE------RKIGQPTLLEYVDAGPETMTKRLLKR------GE-TSGRVDDNEETIKKRLETYYKATEPVIAFYEKRG------IVRKVNAEGSVDDVFSQVCTHLDTLK
AK(E.coli)         DAMKE----AGINVDYVLEFDVPDELIVDRIVGRRVHAPSGRVYHVKFNPPKVEGKDDVTGEELTTRKDDQEETVRKRLVEYHQMTAPLIGYSKEAEAGNTKYAKVDGTKPVAEYRADLEKILG
AK(S.cerevisiae)   EKLDQMLKEQGTPLEKAIELKVDDELLVARITGRLLHPASGRSYHKIFNPPKEDMKDDVTGEALVQRSDDNADALKKRLAAYHAQTEPIVDFYKKTG------IWAGVDASQPPATYWADILNKLGKD
```

20

Fig.2-4 Comparison of the amino acid sequences of L. edodes UMP-CMP kinase and the other nucleoside monophosphate kinases

The amino acid sequences of L. edodes UMP-CMP kinase, S. cerevisiae UMP kinase (Liljelund et al., 1989), D. discoideum UMP-CMP kinase (Wiesmüller et al., 1990), pig UMP-CMP kinase (Okajima et al., 1995), human AMP kinase (Matsuura et al., 1989), pig AMP kinase1 (Heil et al., 1974), E. coli AMP kinase (Brune et al., 1985), S. cerevisiae AMP kinase (Magdolen et al., 1987) are shown. The amino acid sequences were aligned to optimize matches. The numbers of the positions of amino acid residues relative to the start codon are given for L. edodes UMP-CMP kinase and S. cerevisiae UMP kinase. The amino acid residues identical to the L. edodes UMP-CMP kinase are shaded. The mononucleotide-binding motif, NMP binding and LID domains are indicated. Gaps of amino acid residues are shown by a dash. The amino acid residues responsible for the bindings of ATP (closed arrowheads) and nucleoside monophosphates (open arrowheads) and those responsible for the transfer of phosphate group from ATP to nucleoside monophosphates (open circles) all identified in S. cerevisiae UMP kinase (Müller-Dieckmann and Schulz, 1995) are shown.

kinase, human AMP kinase 1, pig AMP kinase 1, *E. coli* AMP kinase, and *S. cerevisiae* AMP kinase proteins consist of 204, 194, 196, 194, 194, 214, and 222 amino acid, respectively. Among these kinase proteins, the *S. cerevisiae* UMP kinase was most homologous to the *L. edodes uck1* gene product, showing an overall identity of 51.1% (51.1% homology). *D. discoideum* UMP-CMP kinase and pig UMP-CMP kinase showed 42.7% and 41.0% homology to the *L. edodes uck1* gene product. Human AMP kinase 1, pig AMP kinase 1, *E. coli* AMP kinase, and *S. cerevisiae* AMP kinase showed 37.0%, 37.0%, 26.4%, and 24.7% homology, respectively, to the *L. edodes uck1* gene product. The *L. edodes uck1* gene product contained the conserved mononucleotide (phosphoryl donor ATP)-binding glycin-rich region (amino acid residues 32-40) (Möller et al., 1985) observed in many ATP-binding proteins such as nucleoside monophosphate kinase, F_1-ATPase and *ras* p21 protein, the NMP binding domain and LID domain that closes over the substrate upon binding (Scheffzek et al., 1996) similar to the other UMP-CMP kinase. Recently, Müller-Dieckmann et al reported each residues relation with enzyme activity of *S. cerevisiae* UMP kinase by X-ray crystallographic studies. The *L. edodes uck1* gene product also contained the all amino acid residues of positions 56, 61, 84, 85, 90, 125 and 129 responsible for the binding of phosphoryl acceptor, i.e., nucleoside monophosphate identified in the *S. cerevisiae* UMP kinase (Müller-Dieckmann et al., 1995), and the all amino acid residues of positions 38, 160, 166, 168, 169 and 177 responsible for the transfer of phosphate group from ATP to the phosphoryl acceptor identified in the *S. cerevisiae* UMP kinase (Müller-Dieckmann and Schulz, 1995).

The AMP kinases from vertebrates have been reported to be separated into three isoforms, AK1, AK2 and AK3, based on structure, subcellular localization and substrate specificity (Fukami-Kobayashi, 1996). AK1 is the short type with the amino acid sequence being 27 residues shorter than sequences of the long types, AK2 and AK3. AK1 is present in the cytosol, AK2 in the mitochondrial intermembrane and cytosol and AK3 in the mitochondrial matrix. AK3 utilizes GTP more efficiently than ATP. The long and short type

AKs differ in the LID domain. As shown in Fig.2-4, the *L. edodes uck1* gene product was shown to be the short type as like all known UMP-CMP kinases. Substrate specificity for UMP/CMP can be acquired by a single replacement of amino acid in chicken AK1 (Okajima et al., 1993). These, taken together, seem to imply the localization of *L. edodes uck1* gene product in cytosol. To clarify this, a detailed analysis is necessary.
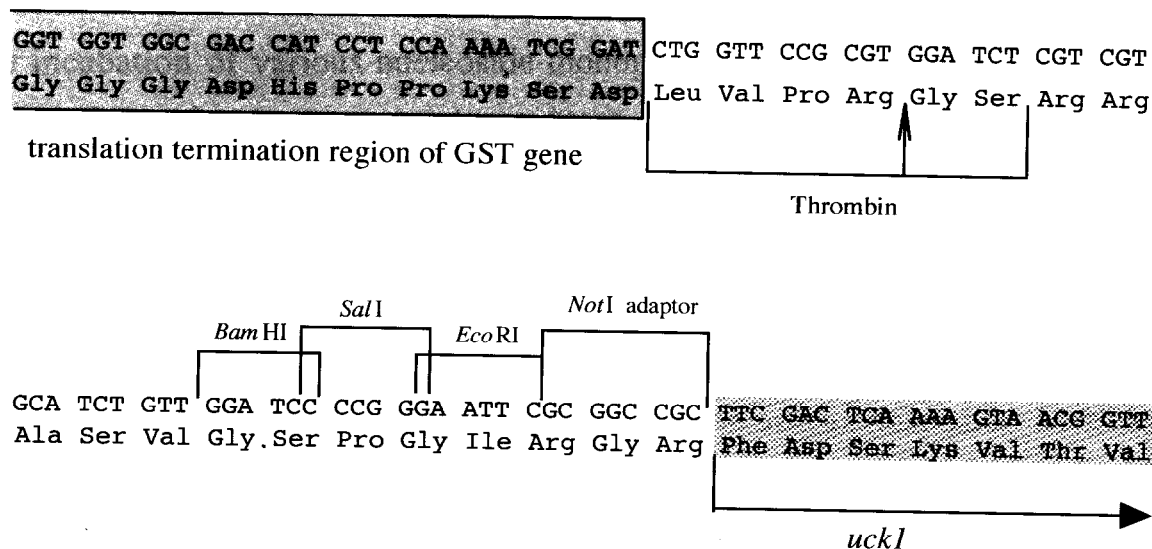
2-3.4. Production of the L. edodes uck1 gene product (UMP-CMP kinase) in E. coli

To confirm the kinase activity of the *L. edodes uck1* gene product, the author attempted to produce it in *E. coli*. The *uck1* cDNA was found to lack 5'-terminal 60 nucleotide sequence, but its coding N-terminal 20 amino acid are not conserved among various nucleoside monophosphate kinase proteins (see Fig. 2-4) and they seem unlikely to be essential for an enzymatic function of the kinase. So the author decided to use this cDNA for experiments. A large amount of the *uck1* gene product was successfully produced in BL21 (Studier et al., 1986) using the glutathione S-transferase (GST) fusion vector pGEX-2TK (Smith and Johnson, 1988) (Fig. 2-5). The GST-*uck1* gene product fusion protein was purified to homogeneity by a batch method using Glutathione-Sepharose 4B. The fusion protein was cleaved with Thrombin at its fusion junction and applied to Glutathione-Sepharose 4B. The *uck1* gene product (25 kDa) was purified as an unadsorbable protein (Fig. 2-5).

2-3.5. Nucleoside monophosphate kinase activity of the purified L. edodes uck1 gene product (UMP-CMP kinase)

The purified *L. edodes uck1* gene product was examined for the ability of transfer of the phosphate group from $[\gamma\text{-}^{32}P]$ ATP to nucleoside monophosphate yielding the ^{32}P-labeled nucleoside diphosphate.
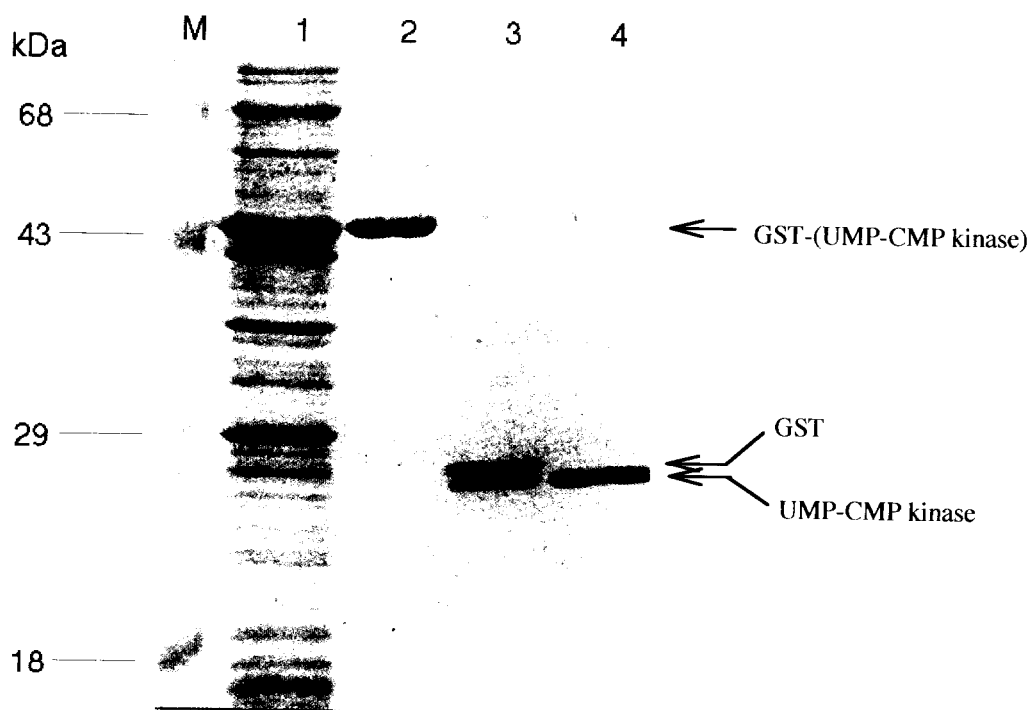
(a)

| GGT GGC GGC GAC CAT CCT CCA AAA TCG GAT | CTG GTT CCG CGT GGA TCT CGT CGT |
| Gly Gly Gly Asp His Pro Pro Lys Ser Asp | Leu Val Pro Arg Gly Ser Arg Arg |

translation termination region of GST gene

Thrombin

                    Sal I   NotI adaptor
        Bam HI   EcoRI

GCA TCT GTT GGA TCC CCG GGA ATT CGC GGC CGC TTC GAC TCA AAA GTA ACG GTT
Ala Ser Val Gly Ser Pro Gly Ile Arg Gly Arg Phe Asp Ser Lys Val Thr Val

uck1

(b)

Fig.2-5 Production of the *L. edodes uck1* gene product (UMP-CMP kinase) in *E. coli* by the GST-fusion/expression vector pGEX-2TK.

The cDNA fragment bearing the *L. edodes* UMP-CMP kinase-coding sequence without N-terminal 20-amino acid-coding sequence was inserted into the *Eco*RI site of pGEX-2TK. (a) The nucleotide and deduced amino acid sequences at the fusion junction between the translation termination region of *Schistosoma japonicum* glutathione S-transferase (GST) gene and *L. edodes uck1* gene. (b) SDS-PAGE of protein samples. Lanes 1, protein extract from the *E. coli* BL21 carrying plasmid pGEX-2TK-UCK ; 2, GST-(UMP-CMP kinase) fusion protein ; 3, Thrombin-digest of the fusion protein ; 4, purified UMP-CMP kinase. Markers are shown in lane M.

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After incubation of various nucleoside monophosphates with the purified *uckl* gene product, the reaction mixtures were spotted onto polyethyleneimine-cellulose thin-layer chromatography plates to reveal the formation of the ^{32}P-labeled diphosphates. As shown in Fig. 2-6, the *uckl* gene product actually showed nucleoside monophosphate kinase activity. UMP, CMP, and AMP were found to be effective phosphate acceptors, while dCMP showed a weak phosphate-acceptor activity. The other nucleoside monophosphates such as GMP, dTMP, dAMP, and dGMP were not used as a phosphate acceptor.

Kinetic studies were carried out to evaluate the relative substrate activities of UMP, CMP, AMP and dCMP for the *uckl* gene product according to the method reported by Okajima et al. (1995). The relative phosphoryl acceptor activities of the four substrates were determined under the condition at which the concentration of phosphoryl donor ATP was kept constant at 1 mM. The results are shown in Table 1. The *uckl* gene product showed markedly higher activities toward UMP and CMP than AMP. The activity toward dCMP was very low. These revealed the *uckl* gene product to be UMP-CMP kinase.

2-3.6. Expression in L. edodes *of the* L. edodes uckl *gene*

Northern-blot analysis was done to investigate expression of the *L. edodes uckl* gene during mycelial development of *L. edodes*. Total cellular RNA was isolated from preprimordial mycelia, primordia, immature fruiting bodies and mature fruiting bodies. The stages of immature fruiting bodies and mature fruiting bodies were divided into three substages. The former was fruiting-body development stage I, II and III and the latter was fruiting-body maturation stage I, II and III (Fig.2-7). The RNA samples were subjected to the blot analysis using probe of the *uckl* cDNA or cDNA (1.2 kb) of *L. edodes ras* which has been shown to be transcribed at similar levels during mycelial development in fruiting-body formation of the fungus in our previous paper (Hori et al., 1991; Kajiwara et al., 1992) (Fig. 2-8 (a)).
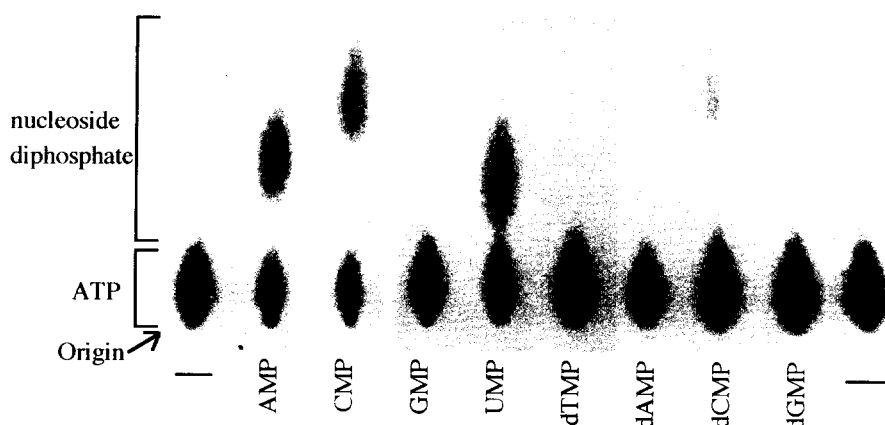
Fig. 2-6

Enzymatic activities of the purified *L. edodes* UMP-CMP kinase using various nucleoside monophosphates as the substrate.

The activity was assayed based on the transfer of $[\gamma\text{-}^{32}P]$ of ATP to the substrate according to the method described in Materials and methods. The nucleoside monophosphates used are marked on bottom of the panel, and the bars on bottom (-) indicate the $[\gamma\text{-}^{32}P]$ ATP only as controls. The origin and migration of the nucleoside diphosphate and ATP points are marked on the left.

Substrate	Relative activity (%) [a]
UMP	100
CMP	95.9
AMP	40.9
dCMP	13.1

Table 1 Phosphoryl acceptor specificity of *L. edodes* UMP-CMP kinase.

[a] The enzyme was assayed at 25°C in 87 mM triethanolamine-HCl (pH 7.5) containing 10 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM nucleoside monophosphate indicated, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 20 units of pyruvate kinase, and 20 units of lactate dehydrogenase. The activity of the enzyme with the MgATP-UMP pair was taken as 100.
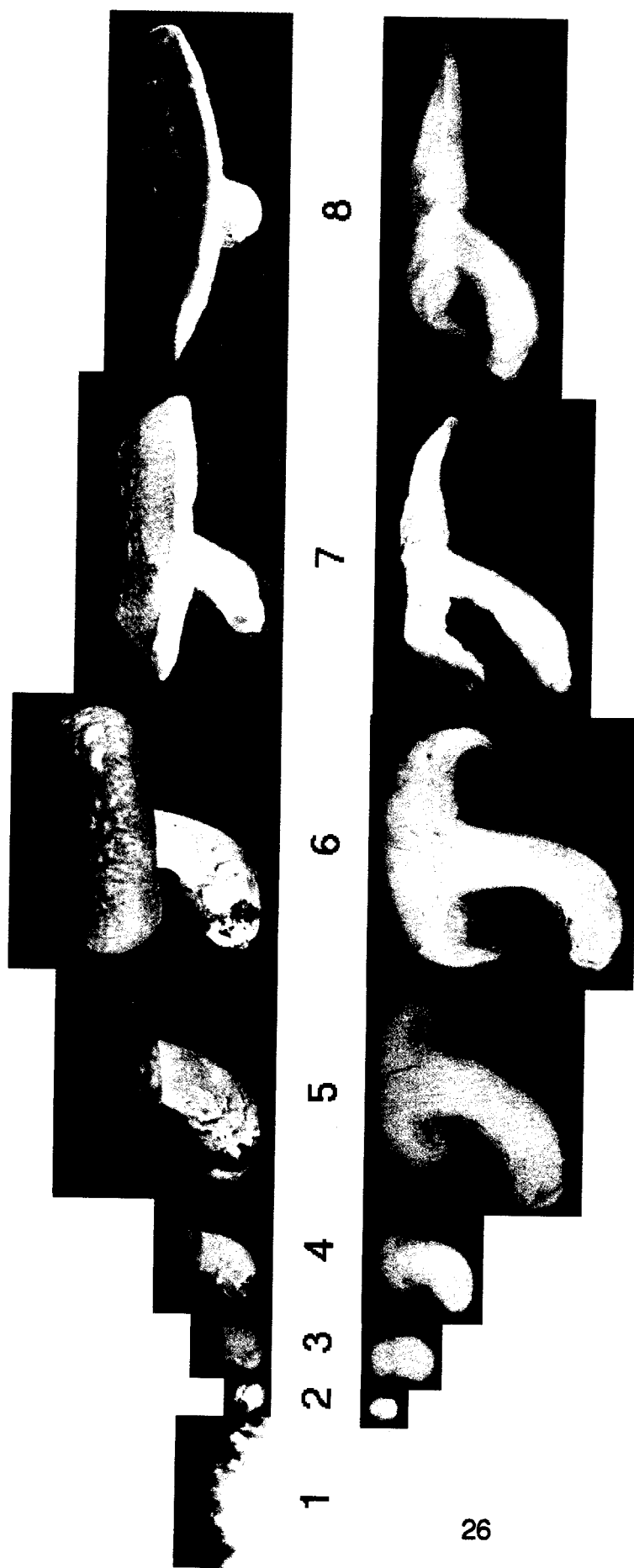
25

1. preprimordial mycelia

2. primordia

3. fruiting-body development stage I

4. fruiting-body development stage II

5. fruiting-body development stage III

6. fruiting-body maturation stage I

7. fruiting-body maturation stage II

8. fruiting-body maturation stage III

Fig. 2-7 Development of the fruiting body of the basidiomycete *Lentinus edodes*

26

(a)

(b)

1. preprimordial mycelia
2. primordia
3. fruiting-body development stage I
4. fruiting-body development stage II
5. fruiting-body development stage III

6. fruiting-body maturation stage I
7. fruiting-body maturation stage II
8. fruiting-body maturation stage III

1. hymenophore-depleted pileus
2. stipe
3. hymenophore
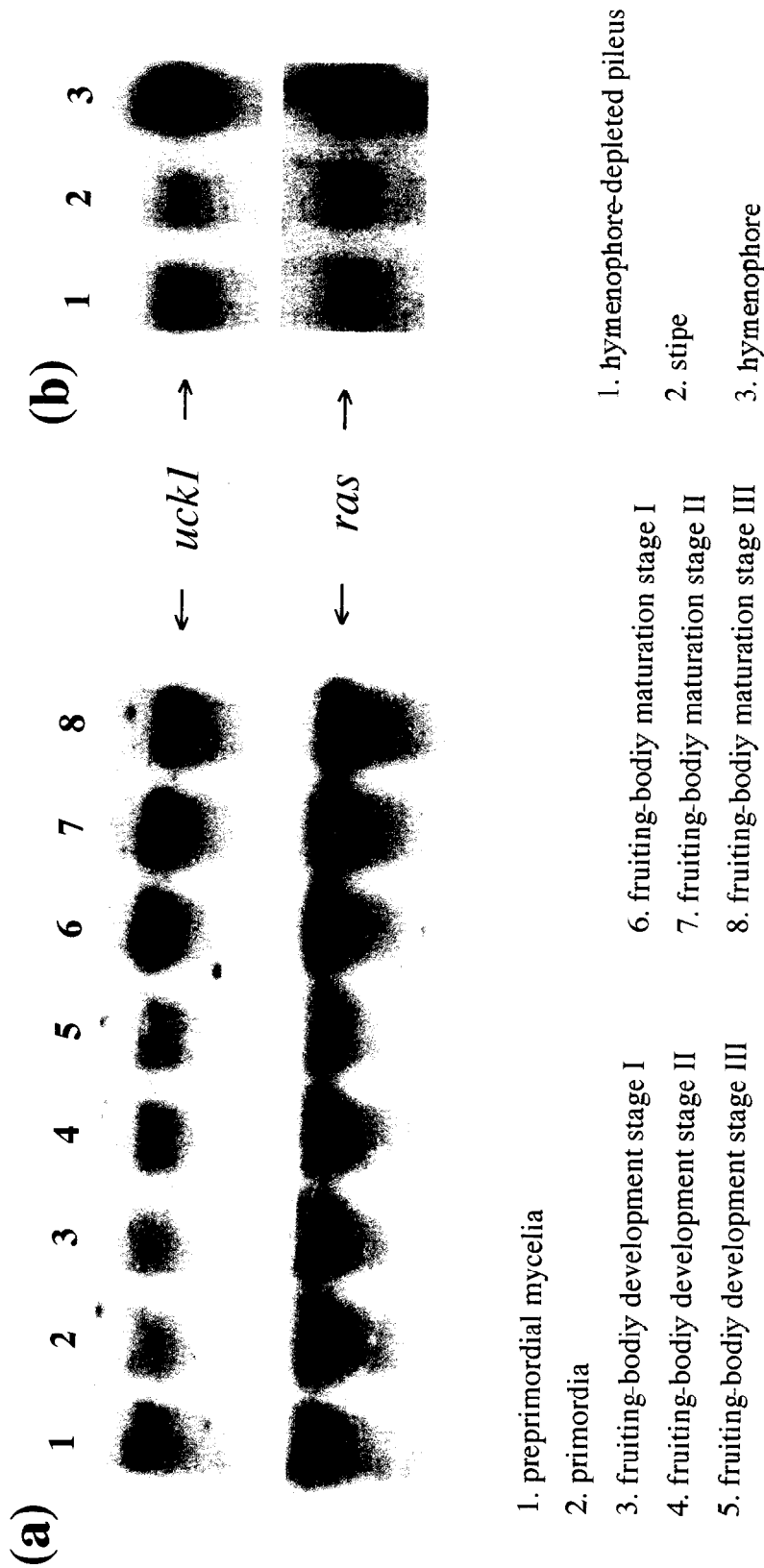
Fig.2-8 Transcriptional expression of the *uck1* gene of *L. edodes* during development of the fruiting body (a) and in parts of the fruiting body (b).

(a) Total cellular RNA samples (25 μg each) isolated from the preprimordial mycelia (lane 1), primordia (lane 2), fruiting-body development stage I, II and III (lane 3 - 5) and fruiting-body maturation stage I, II and III (lane 6 - 8) were analyzed by Northern-blot hybridization using ^{32}P-labeled cDNA probes of *uck1* gene and *L. edodes ras*.
(b) Total cellular RNA samples (25 μg each) isolated from hymenophore-depleted pileus (lane 1), stipe (lane 2), and hymenophore (lane 3) were analyzed by Northern-blot hybridization using ^{32}P-labeled cDNA probes of *uck1* gene and *L. edodes ras*.

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As was expected, a distinct signals of approx. 1 kb (*uckl* gene band) were detected in all RNA blots. The most intense signals were detected in the fruiting-body maturation stage I, II and III (lanes 6, 7 and 8). Although preprimordial mycelia also contained a considerable amount of the transcript, week signals were detected in the primordia and fruiting-body development stage I, II and III (lanes 1 to 5). The 1.2-kb *ras* signals were detected in all RNA blots and their intensities were similar (lanes 1 to 8), ensuring an equal loading and transfer of RNA preparations. These results suggest that the *uckl* gene may play a role especially in mature fruiting bodies during fruiting-body formation.

Levels of the transcript of the *uckl* gene were analyzed in hymenophores (gill tissues) (lane 3), hymenophores-depleted pileus (lane 1) and stipe (lane 2) of the fruiting bodies using probe of the *uckl* cDNA and *L. edodes ras* cDNA (Fig. 2-8 (b)). The hymenophores contained markedly higher level of the transcript than hymenophores-depleted pileus. The stipe contained lower level of this transcript. Complete removal of the hymenophores from the pileus was difficult, so the signal given by the hymenophores-depleted pileus may include the signal derived from the contaminated hymenophores.

2-4. Discussion

The *uckl* product produced in *E. coli* utilized UMP, CMP, AMP and dCMP as phosphate acceptors with relative reaction rates of 100%, 95.9%, 40.9% and 13.1%, respectively. The relative reaction rates to the nucleoside monophosphates of other UMP-CMP kinase have been reported as follows, CMP (100%), UMP (87.7%), AMP (12.3%) and TMP (1.75%) in *D. discoidium* (Wiesmüller et al., 1990), and UMP (100%), CMP (26%), AMP (4%) and dAMP (0.46%) in pig (Okajima et al., 1995). Recently, UMP-CMP kinase genes were isolated from *Arabidopsis thaliana* and human (Zhou et al.,

1998; Rompay et al., 1999). The *A thaliana* enzyme efficiently utilizes CMP (100%) and UMP (90%) as phosphate acceptors, but inefficiently use AMP (0.5%). The human enzyme efficiently utilize CMP (100%) and UMP (95%), but inefficiently use dCMP (12%), dAMP (5%), AMP (3.5%) and dUMP (3%). *S. cerevisiae* UMP kinase which shows the highest homology to *uck1* product, has been reported to show the relative activity of about 30% for AMP and 10% for CMP compared with UMP (Müller-Dieckmann, 1990). Differently from those of AMP kinase and GMP kinase, the substrate specificity of UMP-CMP kinase and UMP kinase is not regulated strictly. Indeed, *URA6* gene encoding *S. cerevisiae* UMP kinase has been isolated as a multicopy suppressor of *S. cerevisiae* deficient in AMP kinase (Schricker et al., 1992). This implies that *S. cerevisiae* UMP kinase may compensate the deficiency of AMP kinase. The author showed that *L. edodes* UMP-CMP kinase also utilized AMP as a phosphate acceptor.

Northern-blot analysis showed that the transcript of *uck1* was remarkable abundant in hymenophores (gill tissues) of mature fruiting-body of *L.edodes*. These indicated that UMP-CMP kinase may play a role mainly in hymenophores (gill tissues) in which basidiospores are produced. The following is considered from the experimental results. During production of basidiospores, biosynthesis of nucleic acids, carbohydrates, lipids etc. and generation of energy source should be active. UDP, CDP and ADP synthesized by the UMP-CMP kinase serve as precursors for syntheses of UTP, CTP and ATP all are substrates for RNA polymerases. UTP and CTP are also involved in the generation of high-energy intermediates, such as UDP-glucose and UDP-galactose in carbohydrate synthesis and CDP-acylglycerol in lipid synthesis. They should require ATP-related active energy metabolism.

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CHAPTER 3

Cloning and sequence analysis of the cDNA genes encoding ribonucleotide reductase small subunits from the basidiomycete *Lentinus edodes*

3-1. Introduction

As described in the chapter 2, *uck1* present in the 3'-flanking region of *priB* was actively transcribed in the hymenophores (gill tissues) of mature fruiting body. This implies that UDP, CDP and ADP are synthesized actively in the hymenophores in which basidiospores are formed.

The author attempt to isolate the RNR genes encoding an enzyme which catalyzes the reductions from UDP, CDP, ADP and GDP to dUDP, dCDP, dADP and dGDP, and play a key role in the pathway of DNA synthesis. RNR from eucaryotes consists of a heterodimer $(\alpha_2\beta_2)$ that contained two nonidentical homodimers (Mathews et al., 1987); α (or R1) is large subunit and β (or R2) is small subunit (see Fig.1-4). The PCR experiments using mRNAs prepared from the hymenophores of *L. edodes* and genomic DNA of the fungus resulted in the isolation of two cDNA fragments encoding small subunit (R2). By using the cloned cDNA fragment as a probe, cDNA containing the entire ORF was isolated from the cDNA library of mature fruiting body of *L. edodes*.

3-2. Materials and Methods

3-2.1. Cloning of two cDNA fragment and genomic DNA fragment encoding ribonucleotide reductase small subunit using for polymerase chain reaction (PCR)

The genomic DNA isolated from vegetative cells of *L. edodes* FMC2, and the purified mRNAs (poly(A)$^+$ RNAs) isolated from hymenophores of mature fruiting-body of *L. edodes* FMC2 were subjected to PCR. Each two sets of degenerate primer DNAs which correspond to conserved amino acids in known eukaryotic RNR large (R1) and small (R2) subunits, were designed for

reaction as follows; large subunit (primer 1: 5'-GCACCAATGCCWACYGC YTCAAC-3' and primer 2: 5'-TACATHCCRGTYTTMARACC-3') and small subunit (primer 1: 5'-GAAGGTRTYTTYTTYTCHGG-3' and primer 2: 5'-AACCAACARWCKRTCAGCRACRAA-3'). Some genomic and cDNA fragments encoding RNR small subunit were acquired by KOD dush (TOYOBO) in that condition of 45 cycles in which each cycle was 30 sec at 94°C, 5 sec at 47 °C, and 30 sec at 74 °C. These amplified fragments were inserted into *Xcm*I site of p123T and sequenced. On the other hand, we could not obtain any genomic and cDNA fragments encoding RNR large subunit.

3-2.2. Genomic Southern-blot analysis

The genomic DNA isolated from vegetative monocaryotic mycelia of *L. edodes* FMC2, were digested by *Bam*HI, *Eco*RI and *Hin*dIII. Each digested genomic DNA (30 μg) were subjected to Southern-blot analysis using ^{32}P-labeled probes of cDNA fragment of *Le.rnrB1* and *Le.rnrB2* according to the method previously reported (Southern, 1975).

3-2.3. Screening of cDNA library

This was done as described in the chapter 2

3-2.4. Northern-blot analysis

This was done as described in the chapter 2

3-3. Results and Discussion

3-3.1. Cloning and nucleotide sequence analysis of Le. rnrB1 cDNA encoding ribonucleotide reductase small subunit B1 from L. edodes

The author designed two sets of the degenerated PCR primers for amplification of *L. edodes* DNA fragments which encode conserved amino acid sequences of large and small subunits of ribonucleotide reductase (RNR) among *S. cerevisiae*, *S. pombe*, mouse and *Arabidopsis thaliana* (Elledge et al., 1987; Sarabia et al., 1993; Thelander et al., 1986; Philipps G., 1995). Mixture of mRNAs prepared from the hymenophores of *L. edodes* and genomic DNA of the fungus were used for the PCR experiments. Although genomic DNA and cDNA fragments encoding RNR large subunit were not obtained, two cDNA fragments (306 bp each) and one genomic DNA fragment(368 bp) encoding RNR small subunit(s) were isolated (Fig.3-1). Nucleotide sequence analysis suggested that two cDNA fragments encode the amino acid sequences most homologous to that of *S. pombe* RNR small subunit, showing an overall identity of 77.5% and 74.5% respectively (Fig. 3-4). The genomic DNA fragment was shown to encode the amino acid sequence identical to one of them, which is interrupted by a small intron (Fig. 3-1). Two cDNA fragments were very homologous each other. Their nucleotide and deduced amino acid sequences showed 72.9% and 80.4% identity respectively. Southern-blot analysis for the genomic DNA prepared from monocaryotic mycelia of *L. edodes* suggested that these cDNA fragments were derived from different two genes (Fig.3-2). As described later, Northern-blot analysis showed that one gene, designated *Le.rnrB1*, is actively transcribed during fruiting-body formation but the other gene, designated *Le.rnrB2*, is poorly transcribed (Fig.3-5).

The author attempted to isolate *Le.rnrB1* and *Le.rnrB2* cDNAs containing the entire ORF of RNR small subunits from cDNA library of fruiting body of

(a) *Le.rnrB1* cDNA fragment encoding RNR small subunit 1 (isolated by RT-PCR)

```
                GAAGGTGTTTTTTTTTTTCGG
1   GAAGGTGTTGTTTTTTTTCCGGCTCCTTCCGCGTCCATCTTTGGATGAAGAAGCGTGATTAATGCCTGGCTTCTCAAACGAGCTCATCAGTCGTGATGAGGGTATGCATACC
    E  G  V  F [   ] S  G  S [ A ] S  I  F  W  M  K  K  R  G  L  M  P  G  L  A  F  S  N  E  L [   ] S  R  D [E] G  M [H] T   39
118 GACTTCGCGTGCCTTCTCTTCAGCCACCTCCGTCGTTCATCCTGACACCGTTGAACGTATTATCAAAGAAGCAGTCGCTGTTGAGCAAGAGTTCTTGACTGACGCGCTTCCT
    D  F  A  C  L  L  F  S  H  L  R  R  R  P  H  P  D  T  V  E  R  I  I  K  E  A  V  A  I  E  Q  E  F  L  T  D  A  L  P   78
235 GTAAGCTTGATCGGCATGAACGCGACATTGATGTGTCAATATATCAGTTTGTTGCTGACCGACTGTTGGTT
    V  S  L  I  G  M  N  A  T  L  M  C  Q  Y  I  E  F  V  A  D  R  L  L  V
                    DTTYGTYGCTGAYMGNYTGYTGGTT                                      102
```

(b) *Le.rnrB2* cDNA fragment encoding RNR small subunit 2 (isolated by RT-PCR)

```
                GAAGGTRTTRTTTTTTTCKGG
1   GAAGGTGTGTTTTTTTTTCCGGGTCGTTCGCTTCCATTTCTGGTTAAAGAAGCGCGGCCTGGCCTAACTTATTCAAACGAGCTCATCAGCCGAGATGAAGGTTTACATACC
    E  G  V  F [   ] S  G  S [ A ] S  I  F  W  L  K  K  R  G  L  M  P  G  L  T  Y  S  N  E  L [   ] S  R  D  E [G] L [H] T   39
118 GATTTCGCGTGCTCCTTACCACCCATCTAAAGCGCCCGCCAACGGGTATCATCAAGAAAATTATAACGGAAGCGGTTGCTATCGAACAAGATTTTTGACTGATGCCCTTCCT
    D  F  A  C  L  L  T  T  H  L  K  R  P  A  Q  P  G  I  I  K  K  I  I  T  E  A  V  A  I  E  Q  D  F  L  T  D  A  L  P   78
235 GTGTCACTCATAGGCATGAATGCAAGTTTGATGCGGCAATACATTCAATTCGTTCGTGCTGATAGACTGTTGGTT
    V  S  L  I  G  M  N  A  S  L  M  R  Q  Y  I  Q  F  V  A  D  R  L  L  V
                    HTGTTRGCTGAYMGNYTGYTGGTT                                          102
```

(c) *Le.rnrB2* genomic DNA fragment encoding RNR small subunit 2 (isolated by genomic PCR)

```
                GAAGGTRTTRTTTTTTTCHGG
1   GAAGGTGTTTTTTTTTCCGGGTCGTTCGCTTCCATTTCTGGTTAAAGAAGCGCGGCCTGGCCTAACTTATTCAAACGAGCTCATCAGCCGAGATGAAGGTTTACATACC
    E  G  V  F [   ] S  G  S [ A ] S  I  F  W  L  K  K  R  G  L  M  P  G  L  T  Y  S  N  E  L [   ] S  R  D  E [G] L [H] T   39
118 GATTTCGCGTGCTCCTTACCACCCATCTAAAGCGCCCCGCCAACGGGTATCATCAAGAAAATTATAACGGAAGCGGTTGCTATCGAACAAGATTTTTTGACTGgtacgtttcat
    D  F  A  C  L  L  T  T  H  L  K  R  P  A  Q  P  G  I  I  K  K  I  I  T  E  A  V  A  I  E  Q  D  F  L  T                 74
235 cccggtttccacactgaaatccagctgtgttcacaacgtcgttgtattgtagATGCCCTTCCTGTCACTCATAGGCATGAATGCAAGTTGATGCGGCAATACATTCAATTCGTCG
                                                         D  A  L  P  V  S  L  I  G  M  N  A  S  L  M  R  Q  Y  I  Q  F  V  96
352 CTGATAGACTGTTGGTT
    A  D  R  L  L  V
        CTCAYAGNYTGYTGGTT                                                         102
```

Fig.3-1

The nucleotide sequences and deduced amino acid sequences of the *Le.rnrB1* cDNA fragment (a), and *Le.rnrB2* cDNA fragment (b), and *Le.rnrB2* genomic DNA fragment (c) isolated by RT-PCR or genomic PCR.

The nucleotide sequence coordinates and amino acid sequence coordinates are presented on the left-hand side and right-hand side, respectively. The first nucleotide of each fragment is assigned the +1 coordinate. The degenerated primers for PCR are shaded. The presence of introns in lower-case letters (c) was deduced from the comparison with the *Le.rnrB2* cDNA fragment. Consensus sequences for splicing are underlined. The residues for iron ligand (Glu 1, 35; His 38) are boxed. Amino acids essential for the tyrosyl radical and its environment (Phe 5, 9; Ile 31) are shaded. (refer to the legend of Fig. 3–4).

(a)

Le.rnrB1 GAACGCTGTTTTTTTCCGGCGCTCCTTCCGTCCATCTTTGGATGAAGAAGCGTTGATTAATGCCGTCTTGGCGGTTCTCAACCAG
Le.rnrB2 GAACGCTGTTTTTTTCCGGAGGTCGCTTCGTTCCATTTCTGGTTAAACACAGGCCCCTGATGCCCCTAACTTATTCAAACGAG

Le.rnrB1 CTCATTCAGTCGTCGATGAGGGCATACCGACTTCGGCCTTCTCTTCAGCCACTCCGTCGTCGTCCTCATCCTGACACCGTT
Le.rnrB2 CTCATTCAGGCCGAGATCGAAGGTTTACATACCACCGATTTTGCGTTGTCTTCCTTACCACCCATCTAAAGGCCCCGCCAACCGGGTATCATC

Le.rnrB1 GAACGTATTATCAAGTAGCAGCGCCTATCGAGCAAGAGTTCTTGACTGACGCGCTTGATCGGGATGAACGGCGACA
Le.rnrB2 AAGAAAATTATAACGGAAGCGGTTGCTATCGAACAAGATTTTTGACTGAAGCTTGTCACTCATAGCCATGAATGCCAAGT

Le.rnrB1 TTTGATCTGTCAATATATCGAGTTTGTGCTTGACCGACTGTTTGGTT
Le.rnrB2 TTTGATGCGGCAATTACAATTCATTCGGTCGGTGATAGAGCTGTTTGGTT

(b)

Le.rnrB1

BamHI digestion
EcoRI digestion
HindIII digestion

→ 8.3kb
→ 6.1kb
→ 4.8kb

Le.rnrB2

BamHI digestion
EcoRI digestion
HindIII digestion

→ 6.4kb
→ 4.3kb
→ 2.5kb

Fig.3-2 **Comparison of the nucleotide sequences between Le.rnrB1 and Le.rnrB2 cDNA fragments and genomic Southern-blot analysis using the probes of Le.rnrB1 and Le.rnrB2 cDNA fragments**

(a) The nucleotide sequences of Le.rnrB1 and Le.rnrB2 cDNA fragment isolated by RT-PCR are shown. Each identical nucleotides are shaded. (b) The genomic DNA isolated from vegetative monocaryotic mycelia of L. edodes FMC2, were digested by BamHI, EcoRI and HindIII. Each digested genomic DNA (30 μg) were subjected to Southern-blot analysis using ^{32}P-labeled probes of cDNA fragment of Le.rnrB1 and Le.rnrB2

L. edodes, obtaining an approx. 1.5-kbp cDNA fragment. Nucleotide sequence analysis revealed that the 1.5 kb fragment consists of 1461 bp and contained 1254 bp ORF (from nucleotide 37 to 1293 in Fig.3-3) encoding 418 amino acid protein with a predicted molecular mass of 48 kDa. The ORF product was found to be RNR small subunit and it's deduced amino acid sequence was most homologous to that of RNR small subunit of *S. pombe* *suc22* (Sarabia et al., 1993). Here the author designated the 1461 bp cDNA as *Le.rnrB1* cDNA. The author couldn't isolate *Le.rnrB2* cDNA. The level of transcription of *Le.rnrB2* is very low, therefore isolation of its cDNA must be so not easy.

3-3.2. Comparison of the amino acid sequences of Le.RNRB1 *and various RNR small subunit proteins*

To determine the relationship between *Le.rnrB1* and other RNR small subunit (R2) genes, their derived amino acid sequences were compared (Fig.3-4). The R2 gene product of *S pombe* ($suc22^+$), mouse, human, *D. discoideum* (*rnrB*), *S. cerevisiae* (*RNR2* and *RNR4*) and *A. thaliana* (RNR2) consist of 391, 390, 389, 338, 399, 345 and 340 amino acids, respectively. Among these proteins, the *S. pombe* R2 subunit was most homologous to the *Le.RNRB1*, showing an overall identity of 71.9% (71.9% homology). The R2 subunit of mouse, human, *D. discoideum* , *S. cerevisiae* (*RNR2*) and *A. thaliana* showed 71.1%, 71.0%, 66.8%, 63.2% and 62.0% homology, respectively. The *Le.RNRB1* contained the essential residues for the catalytic activity and the structure of the enzyme, the iron ligands residues (Asp 161; Glu 192, 255, 289; His 195, 292), the tyrosyl radical and environment (Tyr 199; Phe 259, 263; Ile 285), and the radicals involved in binding to the R1 subunit (Trp 124, Glu 128, Asp 134, Arg 388, Tyr 392) (Kauppi et al., 1996). These conserved amino acid sequence implied that the *Le.rnrB1* gene product behaved as ribonucleotide reductase small subunit in *L. edodes*.

Fig.3-3 The nucleotide sequences of the *Le.rnrB1* cDNA and deduced amino acid sequences.

The nucleotide sequence coordinates and amino acid sequence coordinates are presented on the left-hand side and right-hand side, respectively. The start codon is assigned the +1 coordinate. The nucleotide sequences (+763 to +1068) of the *Le.rnrB1* cDNA fragment isolated by RT-PCR are boxed. The residues for iron ligand (Asp 161; Glu 192, 255, 289; His 195, 292) are boxed. Amino acids essential for the tyrosyl radical and its environment (Tyr 199; Phe 259, 263; Ile 285) are shaded. Amino acids essential for the radicals involved in binding to the large subunit (R1) (Trp 124, Glu 128, Asp 134, Arg 388, Tyr 392) are circled. These amino acids are essential for the structure or the enzyme activity (refer to the legend of Fig. 3-4).
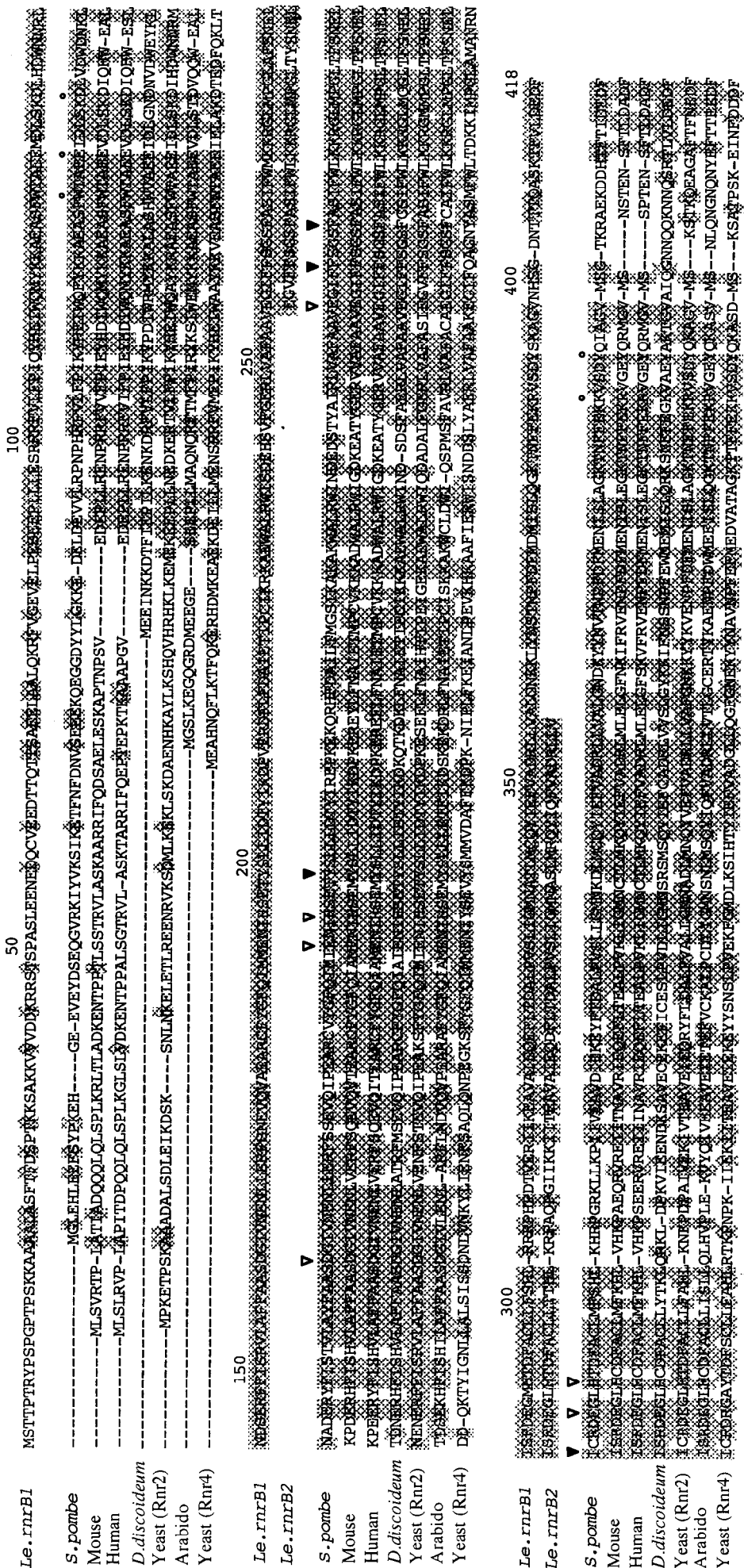
Fig.3-4 Comparison of the amino acid sequences of *L. edodes* RNR small subunit(s) and the other RNR small subunits.

The amino acid sequences of *Le.rnrB1* gene product, partial product derived from *Le.rnrB2* cDNA fragment, *S pombe* RNR2 (Fernandez Sarabia et al., 1993), mouse M2 subunit (Thelander et al 1986), human M2 subunit (Pavloff et al., 1992), *D. discoideum* RNRB (Tsang et al., 1996), *S. cerevisiae* Rnr2 (Elledge et al., 1987) and Rnr4 (Huang et al., 1997) and *A. Thaliana* RNR2 (Philipps et al., 1995) are shown. The amino acid sequences were aligned to optimize matches. The numbers of the positions of amino acid residues relative to the start codon are given for *Le.rnrB1* gene product. The amino acid residues identical to the *Le.rnrB1* gene product are shaded. Gaps of amino acid residues are shown by a dash. The amino acid residues responsible for iron ligand (open arrowheads) and the tyrosyl radical and its environment (closed arrowheads) and the radicals involved in binding to the large subunit (R1) (open circles) are shown. These amino acids are essential for the structure or the enzyme activity (Kauppi et al., 1996).

3-3.3. Expression in L. edodes *of the* Le.rnrB1 (*and* Le. rnrB2) *genes*

A Northern-blot analysis was carried out to investigate the expression of the *Le.rnrB1* (and *Le.rnrB2*) gene(s) during mycelial development of *L. edodes*. Total cellular RNA was isolated from preprimordial mycelia, primordia, immature fruiting bodies, and mature fruiting bodies as described in the chapter 2. The RNA samples were subjected to the blot analysis using cDNA probe of *Le.rnrB1; Le.rnrB2* (fragment) or *Le.ras* (Fig.3-5a). In the case of *Le.rnrB1* cDNA probe, a distinct signal of approx. 1.5 kb (*Le.rnrB1* gene band) was detected in all RNA blots (lanes 7 and 8), only except for the RNA blot of preprimordial mycelia (lane 1). The intensity of the band gradually increased in proportion as fruiting bodies grew (lanes 2 to 8), and the most · intense signals were detected in the fruiting-body maturation stages II and III (lanes 7 and 8). In the case of *Le.rnrB2* probe, a very weak signal of approx. 1.5 kb (*Le.rnrB2* gene band), were detected. Transcriptional expression pattern of *Le.rnrB2* during fruiting-body formation was similar to that of *Le.rnrB1*. In the case of the cDNA probe of *Le.ras*, which has been shown to be transcribed at similar levels during mycelial development in fruiting-body formation, the 1.2-kb *ras* signals were detected in all RNA blots, and their intensities were similar (lanes 1 to 8), ensuring an equal loading and transfer of RNA preparations.

Levels of the transcripts of the *Le.rnrB1* (and *Le.rnrB2*) gene(s) were analyzed in hymenophores (gill tissues) (lane 3), hymenophores-depleted pileus (lane 1) and stipe (lane 2) of the fruiting-body maturation stage II using cDNA probes of the *Le.rnrB1, Le.rnrB2* or *Le.ras* (Fig.3-7b). The hymenophores contained a markedly higher level of the *Le.rnrB1* transcript than the hymenophores-depleted pileus, and the signal was given by the hymenophores-depleted pileus may include the signal derived from the contaminated hymenophores. The stipe did not contain detectable amount of the transcript (lane2). These results indicated that *Le.rnrB1* was actively transcribed in the hymenophores of the fruiting-body maturation stage.

(a)

Le.rnrB1 →

Le.rnrB2 →

← ras

1. preprimordial mycelia
2. primordia
3. fruiting-bodiy development stage I
4. fruiting-bodiy development stage II
5. fruiting-bodiy development stage III
6. fruiting-bodiy maturation stage I
7. fruiting-bodiy maturation stage II
8. fruiting-bodiy maturation stage III

(b)

Le.rnrB1 →

Le.rnrB2 →

← ras

1. hymenophore-depleted pileus
2. stipe
3. hymenophore
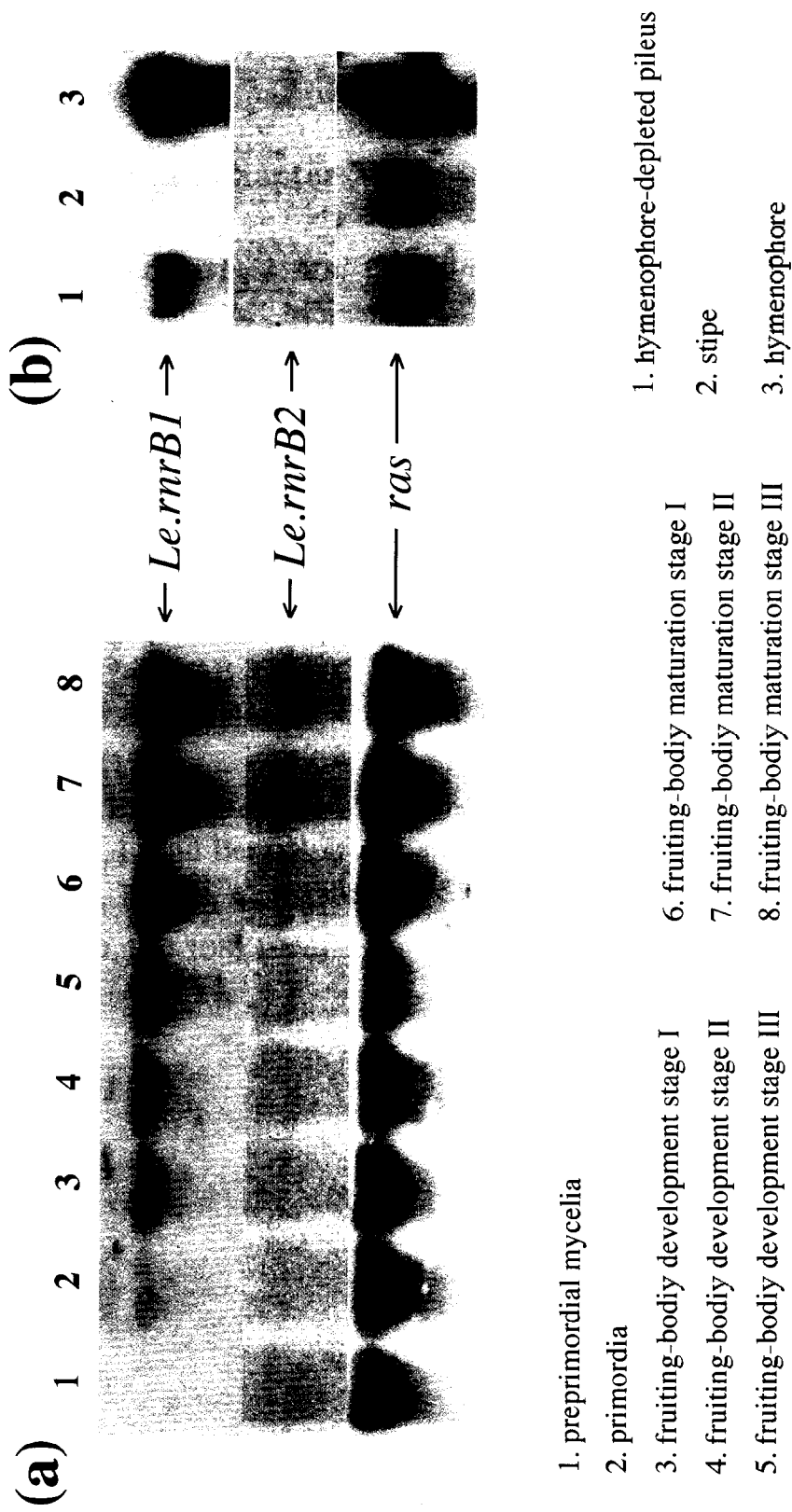
Fig.3-5
Transcriptional expression of the Le.rnrB1 and Le.rnrB2 genes during development of the fruiting body (a) and in parts of the fruiting body (b) of L. edodes

(a) Total cellular RNA samples (25 μg each) isolated from the preprimordial mycelia (lane 1), primordia (lane 2), fruiting-body development stage I, II and III (lane 3 - 5) and fruiting-body maturation stage I, II and III (lane 6 - 8) were analyzed by Northern-blot hybridization using ^{32}P-labeled cDNA probes of Le.rnrB1, Le.rnrB2 and L. edodes ras. (b) Total cellular RNA samples (25 μg each) isolated from hymenophore-depleted pileus (lane 1), stipe (lane 2), and hymenophore (lane 3) were analyzed by Northern-blot hybridization using ^{32}P-labeled cDNA probes of Le.rnrB1, Le.rnrB2 and L. edodes ras.

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The author also detected signals of *Le.rnrB2* similar to the transcript pattern of *Le.rnrB1*, although it was considerably weaker than that of *Le.rnrB1*.

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CHAPTER 4

Expression of *uck1* and *Le.rnrB1* in hymenophore mature fruiting body and fruiting body of develop stage

4-1 Introduction

As described in the chapters 2 and 3, Northern-blot analyses showed that two nucleotide biosynthesis genes, *uckl* and *Le.rnrB*, were actively transcribed in hymenophores of the mature fruiting body of *L. edodes*. The hymenophore is complicated gill tissue consisting of trama, subhymenium and hymenium on which a large number of basidia are formed (see Fig.4-1). As described in the chapter 1, nucleotide metabolism must be active in the basidia, because fusion of two nuclei, meiosis, replication, etc. occur to produce basidiospores. These led the author to analyze the expression of *uckl* and *Le.rnrB1* in parts of the hymenophores of both mature fruiting body and immature fruiting body by using *in situ* hybridization technique. In the experiments, the expression of *Le.ras* gene was also examined.

4-2. Materials and Methods

4-2.1. Preparation of the longitudinal sections of fruiting-body maturation and development stage of L. *edodes— fixation and sectioning*

The author applied different fixation method for *in situ* RNA hybridization to fruiting-body maturation stage II and development stage II, according to Koji (1998), Fukuda et al. (1997), Bochenek et al. (1990) and Jackson (1991). The suitable ultrathin cryosection and the distinct hybridization signals were obtained with 4% paraformaldehyde in phosphate-buffered saline (PBS) (137mM NaCl, 2.68mM KCl, 10mM NaH_2PO_4, 1.76mM KH_2PO_4), pH 7.4 (4 h at 4°C). After fixation, the tissues were transferred to 0.5M Mannitol/0.02% DEPC solution (30 min×2, 4°C) in order to remove fix solution. Subsequently they were embedded in OCT Compound (Tissue-Tek) and frozen in liquid nitrogen or on dry ice. Using a cryostat (kept at -18°C), 8- to 10-μm ultrathin
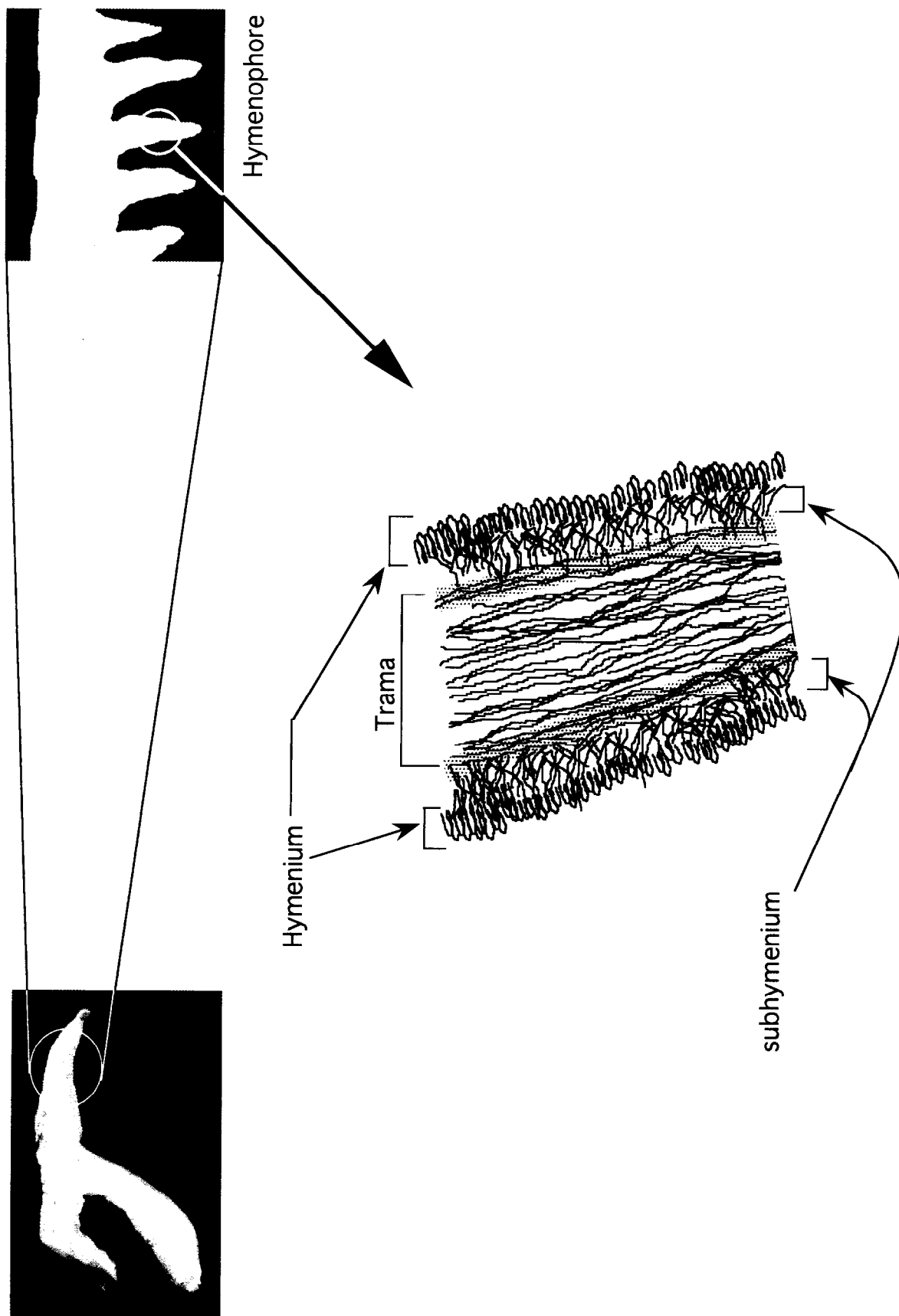
Fig. 4-1   Hymenophores of mature fruiting body of *L. edodes*

Colored zones are the border between the trama and subhymenium

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sections were cut and attached to silanized slides (DAKO). The slides were dried up in a slide box at 45°C overnight and were kept at -80°C until they ready to be hybridized. At that time, the slides were gradually warmed to room temperature.

4-2.2. The synthesis of RNA probe

Each RNA probes were prepared by *in vitro* transcription with digoxigenin-UTP (Roche Diagnostics GmbH) according to the manufacturer's instructions and Nomura et al. (1994). The *uck1* cDNA fragment (nucleotide +167 to +713) and the *Le.ras* cDNA fragment (nucleotide +241 to +1000) without 5' region encoding mononucleotide-binding glycin-rich region, were inserted into *Hinc*II site of pSPT18. The *Le.rnrB1* cDNA fragment (nucleotide +296 to +999) was cloned in pSPT18 between the sites *Hind*III and *Eco*RI. The resulting plasmids were called pSPT18-*uck*, pSPT18-*ras* and pSPT18-*rnrB1*. Five µg of pSPT18-*uck* and pSPT18-*ras* linearized by *Hind*III were used as a template for the synthesis of antisense digoxigenin-UTP-labelled RNA probes by T7 RNA polymerase (Roche Diagnostics GmbH), while 5 µg of pSPT18-*rnrB1* linearized by *Eco*RI was also used as a template for the synthesis of antisense RNA probes by SP6 RNA polymerase (Roche Diagnostics GmbH). For the synthesis of the sense probes, pSPT18-*uck* and pSPT18-*ras* were linearized by *Eco*RI, and pSPT18-*rnrB1* was linearized by *Hind*III, and each sense probes were synthesized in the presence of SP6 RNA polymerase (pSPT18-*uck* and pSPT18-*ras*) and T7 RNA polymerase (pSPT18-*rnrB1*) (Roche Diagnostics GmbH). Each RNA probes were hydrolyzed for 10 min (pSPT18-*uck*) and for 12 min (pSPT18-*ras* and pSPT18-*rnrB1*) at 60°C in the presence of carbonate buffer (60mM Na_2CO_3 and 40mM $NaHCO_3$), pH 10.2. An equal volume of 0.2 M sodium acetate, pH 6.0, was used to stop the hydrolysis reaction. The samples were incubated with DNaseI (RNAase free) (0.2 U/ml) for 10 min at 37°C and the RNA probes precipitated with 400 mM LiCl and 3 vols of ethanol. Each RNA pellets were solubilized in 100 µl H_2O

DEPC.

4-2.3. The treatment of each longitudinal sections before hybridization

Before hybridization, each ultrathin longitudinal sections on slides were immersed in 0.2 M HCl for 20 min and in 0.2% Triton X-100/PBS for 10 min at room temperature, washing in H_2O each. Subsequently they were rinsed with protenase K buffer (100 mM Tris-HCl, pH 7.5 and 50 mM EDTA), and treated with 1 µg/ml proteinase K in protenase K buffer for 15 min at 37°C. After brief washing in H_2O, their slides were rinsed in 0.1 M triethanolamine, pH 8.0, for 5 min and acetylated in 0.25% acetic anhydride in 0.1 mM triethanolamine, pH 8.0, for 20 min at room temperature. After rinsed in H_2O, they were dehydrated in a series of increasing ethanol concentrations (30%, 50%, 75%, 95%, 99.5%) for 5 min each and then air dried.

4-2.4. Hybridization and Post-Hybridization

Each RNA probes were heat denatured at 80°C for 10min and diluted to final 1µg/ml in hybridization buffer (50% formamide, 300mM NaCl, 10mM Tris-HCl (pH7.4), 1mM EDTA, 1×Denhart, 0.25% SDS, 10% dextran sulfate, 100µg salmon sperm and 80U/ml RNase inhibitor). Hybridization was done at 47°C overnight according to Koji (1998), Fukuda et al. (1997), Bochenek et al. (1990) and Jackson (1991). Then the slides were washed twice in 2×SSC, 50% formamide at 47-50°C for 30min, followed by two washings by 2×SSC at 47-50°C for 30min and treated with 20µg/ml RNase A in this buffer at 37°C for 30min. The slides were then washed again in 2×SSC for 1hr and finally washed twice in 0.1×SSC, each at 47-50°C for 30min. Subsequently they were dehydrated in a series of increasing ethanol concentrations (30%, 50%, 75%, 95%, 99.5%) for 5 min each, and immersed twice in acetone for 5 min at room temperature and then air dried.

4-2.5. Immunological Reaction

Immunological detection of each hybridized probe was carried out as described in the Roche Diagnostics GmbH digoxigenin-nucleic acid detection kit with some modifications. The slides were incubated at room temperature for 2hr in blocking reagent (Buffer 2) (Roche Diagnostics Gmb). These were followed by an overnight incubation in dilute antibody-conjugate (1 in 1000) in Buffer 2 (Roche Diagnostics Gmb), and twice washes of 15 min each in Washing buffer (Roche Diagnostics Gmb) without antibody. After the slides were briefly washed in Buffer 3 (Roche Diagnostics Gmb), they incubated from 5-6 hr to overnight in color-substrate solution (NBT/BCIP solution) (Roche Diagnostics Gmb). The color reaction was stopped with 10 mM Tris, 1 mM EDTA (pH 8.0) (Ohtani et al., 1996).

4-3. Results

4-3.1. Detection of total RNA molecules in the mycelial cells of stipe, pileus and hymenophores of L. edodes in fruiting body

Longitudinal ultrathin section of stipe, pileus and hymenophores (gill tissues) of fruiting-body maturation stage II of *L. edodes* were prepared and RNA molecules present in them were fixed and stained shocking pink with the methylgreen pyronin stain solution, by which DNAs were stained blue. As shown in panels A and B of Fig.4-2, the stipe cells were relatively long and thick, and run parallel. RNAs, which are stained shocking pink, were detected in all parts of the stipe cells. The pileus cells (panel C of Fig.4-2), which were thin and reticular, were stained pink in all parts. The mycelial cells of subhymenium enclose the trama and diverge to the outside. They were covered with the hymenium, the cells of which were small and crowded, and included
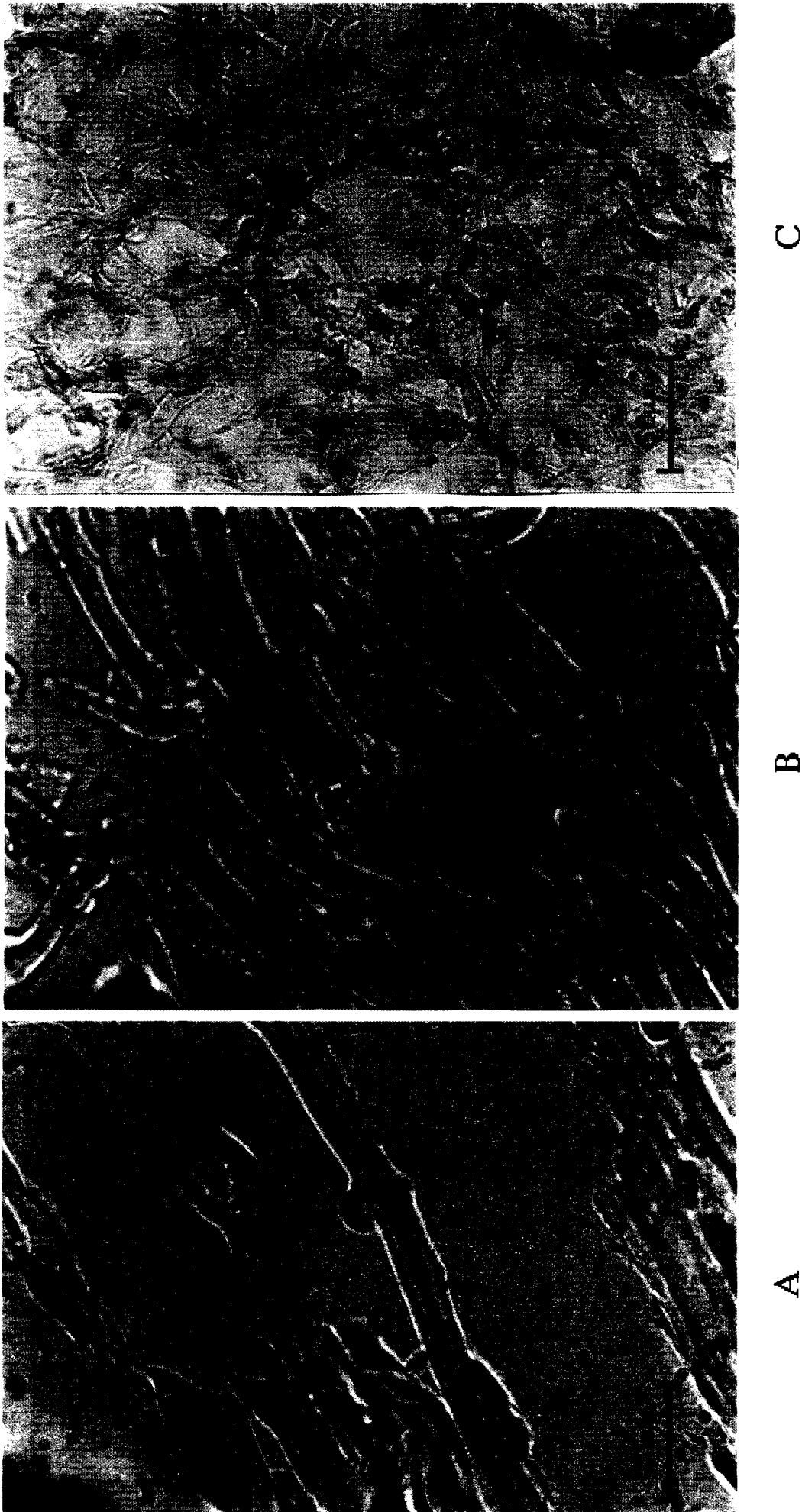
Fig.4-2 Detection of total RNA molecules in the mycelial cells of stipe, pileus and hymenophore of fruiting body of *L. edodes*

Longitudinal ultrathin section of stipe, pileus and hymenophore (gill tissue) of fruiting-body maturation stage II of *L. edodes* were prepared and RNA molecules present in them were fixed and stained shocking pink with the methylgreen pyronin stain solution, by which DNAs were stained blue. Panels *A* and *B* show stipe cells: (Magnification, ×1000). Panel C shows pileus cells: (Magnification, ×400).
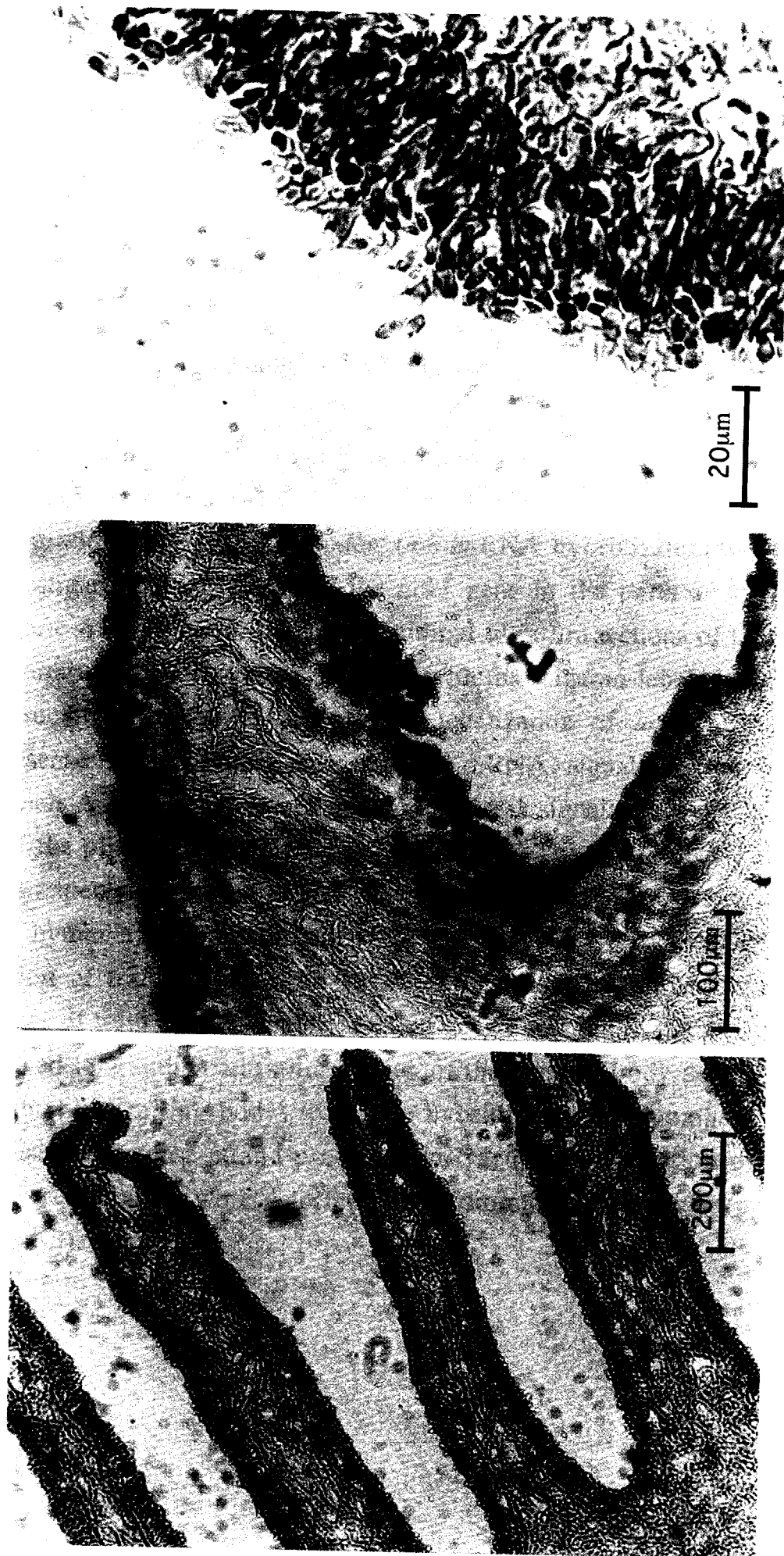
50

Fig.4-2 Detection of total RNA molecules in the mycelial cells of stipe, pileus and hymenophore of fruiting body of *L. edodes*

Panels D, E and F show hymenophore cells.
Panels D: (Magnification, ×100), E: (Magnification, ×200) and Panel F: (Magnification, ×1000).

many basidia and basidiospores as shown in panel F of Fig.4-2. RNAs were detected abundantly in the hymenium cells and the outer region of trama (the border between the trama and the subhymenium) also contained higher amounts of RNAs than the central region of the trama (panels D and E Fig.4-2).

4-3.2. Expression of uck1 gene in the hymenophores of L. edodes

As describe in the chapter 2, Northern-blot analysis showed that the *uck1* is actively transcribed in the hymenophores (gill tissues) of fruiting-body maturation stage II of *L. edodes*. *In situ* RNA hybridization was carried out to investigate the expression of the *uck1* gene in the parts of hymenophores of mature fruiting body. Fixed longitudinal ultrathin sections of the fruiting-body maturation stage II were hybridized with digoxigenin-labeled *uck1* antisense or sense strand probe to analyze the localization of *uck1* mRNA. The *uck1* antisense strand probe gave distinct mRNA signals in the hymenophores (panels A, and C and D of Fig.4-3) and weak signals were detected in the stipe and the pileus (panel, E and G of Fig.4-3). The *uck1* sense strand probe, as was expected, gave no signal. The intense signals were detected especially in the hymenia of hymenophore and weak signals were detected in the outer region of trama (the border between the trama and the subhymenium). The center of trama and the subhymenium gave a negligible signal.

Fixed longitudinal ultrathin section of the fruiting-body development stage II (immature fruiting body) were also hybridized with digoxigenin-labeled *uck1* antisense or sense strand probe to analyzed the localization of *uck1* mRNA. The weak signals were detected only in immature hymenium (panels H to J of Fig.4-3).
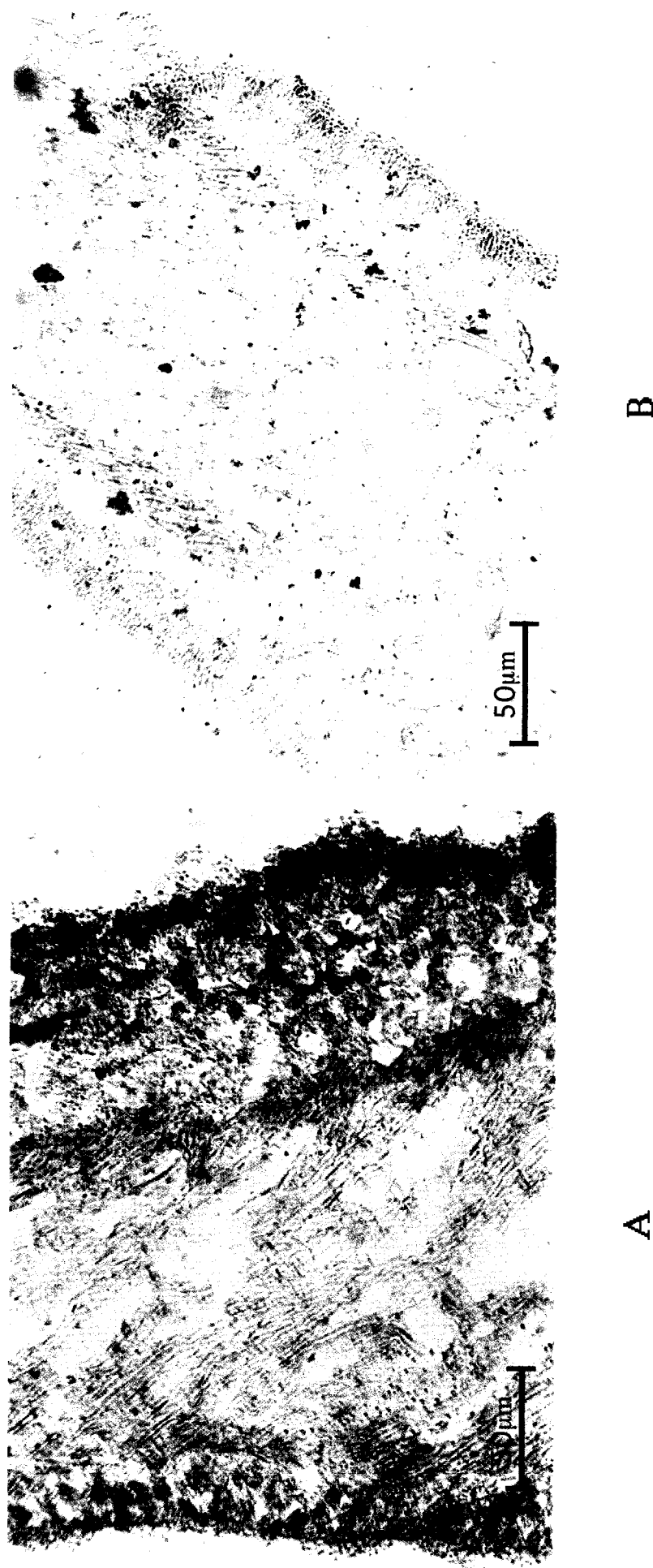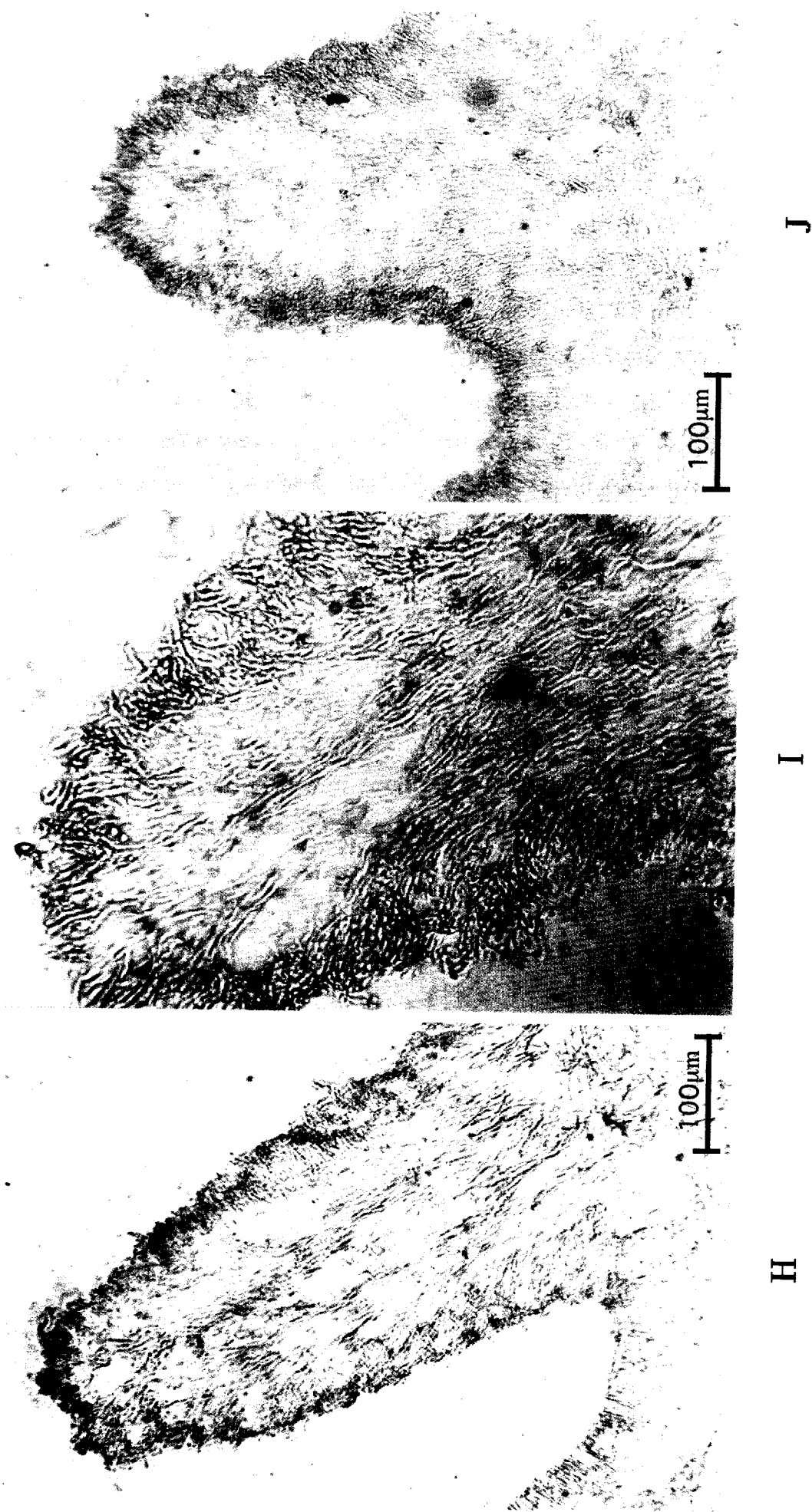
Fig.4-3   Expression of *uck1* gene in the hymenophore of fruiting body of *L. edodes*

Fixed longitudinal ultrathin sections of the fruiting-body maturation stage II were hybridized with digoxigenin-labeled *uck1* antisense or sense strand probe to analyze the localization of *uck1* mRNA. Panels A, C and D show hymenophore cells hybridized with *uck1* antisense strand probe. Panel B shows hymenophore cells hybridized with *uck1* sense strand probe. Panels A and B: (Magnification, ×400)

Fig.4-3 Expression of *uck1* gene in the hymenophore of fruiting body of *L. edodes*
Panel C: (Magnification, ×1000) ;   Panel D: (Magnification, ×100)

Fig.4-3 Expression of *uck1* gene in the stipe and pileus cells of fruiting body of *L. edodes*

Panels E and G show stipe cells and pileus cells each with *uck1* antisense strand probe. Panel F shows stipe cells hybridized with *uck1* sense strand probe. (Magnification, ×1000)
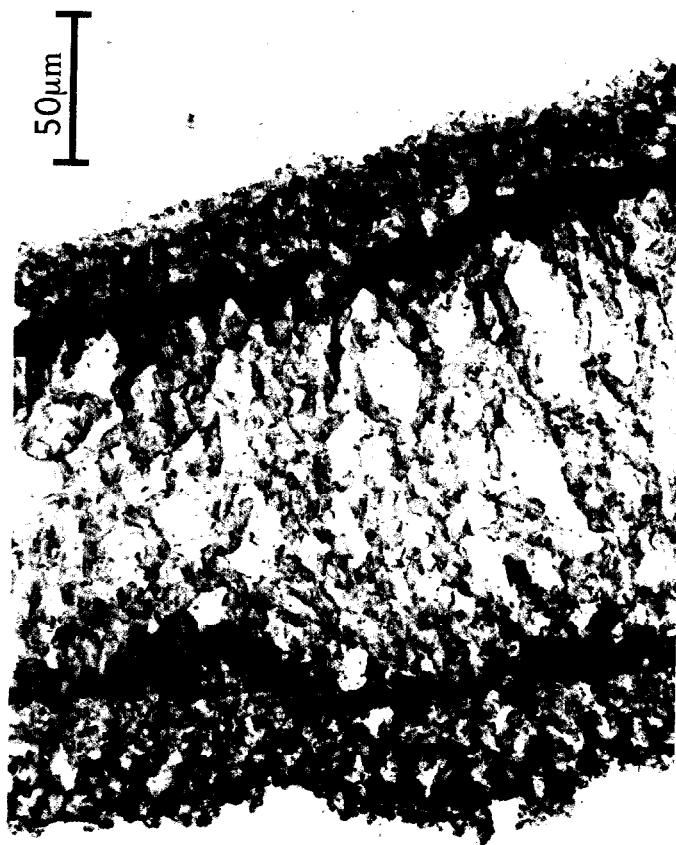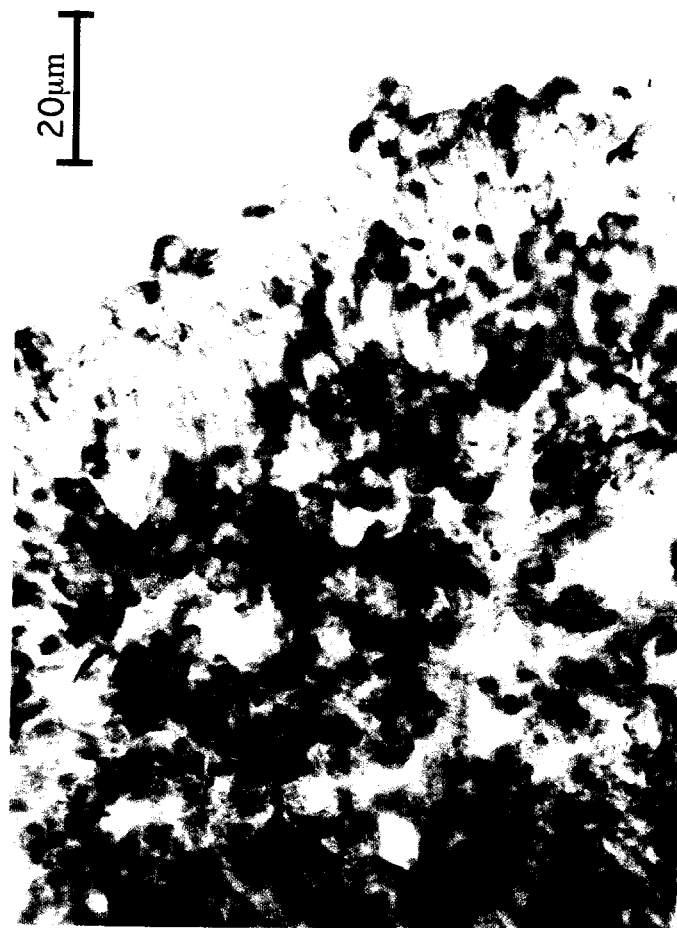
Fig.4-3 Expression of *uck1* gene in the hymenophore of immature fruiting body of *L. edodes*

Fixed longitudinal ultrathin section of the fruiting-body development stage II (immature fruiting body) were hybridized with digoxigenin-labeled *uck1* antisense strand probe. Panels H and I show hymenophore cells hybridized with *uck1* antisense strand probe. Panel J shows hymenophore cells hybridized with *uck1* sense strand probe. Panels H and J: (Magnification, x200): Panel I: (Magnification, x400)
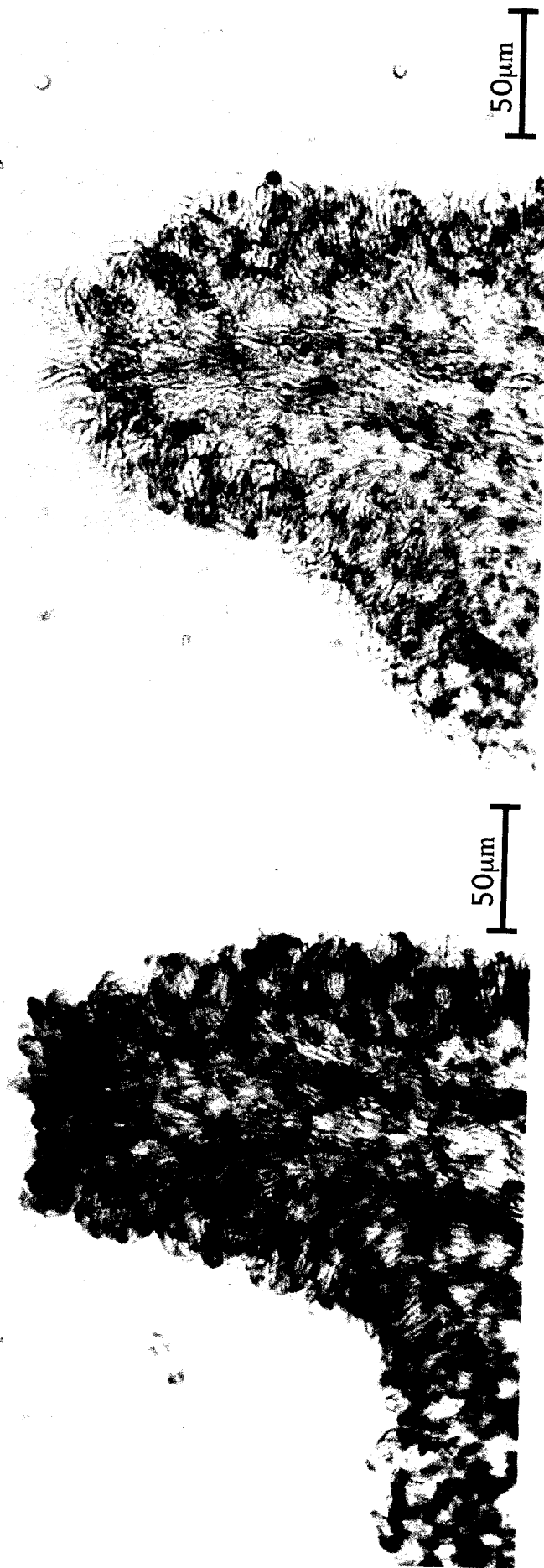
4-3.3 Expression of Le.rnrB1 *gene in the hymenophores of* L. edodes

As describe in the chapter 3, Northern-blot analysis showed that the *Le.rnrB1* were actively transcribed in the hymenophores (gill tissues) of fruiting-body maturation stage II of *L. edodes* in analogous to *uck1*. *In situ* RNA hybridization was carried out to investigate the expression the *Le.rnrB1* gene in the parts of hymenophores of mature fruiting-body. Fixed longitudinal ultrathin sections of the fruiting-body maturation stage II were hybridized with digoxigenin-labeled *Le.rnrB1* antisense or sense strand probe to analyze the localization of *Le.rnrB1* mRNA. The *Le.rnrB1* antisense strand probe gave distinct mRNA signals in the hymenophores (panels A, and C and D of Fig.4-4) and weak signals were detected in the pileus, whereas the stipe gave a negligible weak signals. The *Le.rnrB1* sense strand probe, as was expected, gave no signal. The intense signals were detected especially in the hymenia and the outer region of trama (the border between the trama and the subhymenium). The center of trama and subhymenium gave weak signals.

Fixed longitudinal ultrathin section of the fruiting-body development stage II (immature fruiting body) were also hybridized with digoxigenin-labeled *LernrB1* antisense or sense strand probes to analyzed the localization of *Le.rnrB1* mRNA. The weak signals were detected widely from center of trama to hymenium in immature hymenopore (panel E and F of Fig.4-4).

Fig.4-4 **Expression of *Le.rnrB1* gene in the hymenophore of fruiting body of *L. edodes***

Fixed longitudinal ultrathin sections of the fruiting-body maturation stage II were hybridized with digoxigenin-labeled *Le.rnrB1* antisense or sense strand probe to analyze the localization of *Le.rnrB1* mRNA. Panel A, C and D show hymenophore cells hybridized with *Le.rnrB1* antisense strand probe. Panel B shows hymenophore cells hybridized with *Le.rnrB1* sense strand probe. Panel A and B: (Magnification, ×100) .

Fig.4-4 Expression of *Le.rnrB1* gene in the hymenophore of fruiting body of *L. edodes*

Panel C: (Magnification, ×400); Panel D: (Magnification, ×1000)

Fig.4-4 Expression of *Le.rnrB1* gene in the hymenophore of immature fruiting body of *L. edodes*

Fixed longitudinal ultrathin section of the fruiting-body development stage II (immature fruiting body) were also hybridized with digoxigenin-labeled *Le.rnrB1* antisense strand probe. Panel E shows hymenophore cells hybridized with *Le.rnrB1* antisense strand probe. Panel F shows hymenophore cells hybridized with *Le.rnrB1* sense strand probe. (Magnification, ×400)

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4-3.4 Expression of ras *gene in the hymenophores of* L. edodes

In the chapters 2 and 3, *L. edodes ras* gene was used as control probe for Northern-blot analysis. *In situ* RNA hybridization was also carried out to investigate the expression of *Le.ras* gene in the parts of hymenophores of mature fruiting body. Fixed longitudinal ultrathin sections of the fruiting-body maturation stage II were hybridized with digoxigenin-labeled *Le.ras* antisense or sense strand probe to analyze the localization of *Le.ras* mRNA. The only intense signals were detected especially in the outer region of trama (the border between the trama and the subhymenium)(panels A to D of Fig.4-5).

Fixed longitudinal ultrathin section of the fruiting-body development stage II (immature fruiting body) were also hybridized with digoxigenin-labeled *Le.ras* antisense or sense strand probe. The distinct signals were also detected in the outer region of trama, of which width seems to be relatively narrow when compared with that of mature fruiting body (fruiting-body maturation stage II) (panels G to I of Fig.4-5).

4-4. Discussion

In situ RNA hybridization of the mature fruiting body of *L. edodes* showed that two nucleotide biosynthesis genes, *uck1* and *Le.rnrB1* were actively transcribed in the hymenium cells which develops into basidia. The hymenium was formed from the subhymenium during the fruiting-body development stages. First the hymenium consists of long elliptic cells such as subhymenium and each cells contain two nuclei. In the fruiting-body maturation stage, the hymenium cells change into truncheon-shaped cells (basidia) from which four horned sterigmas arise. During development of sterigmas into basidiospores, fusion of nuclei and meiosis occur and then, four nuclei migrate from basidia to enlarged sterigmas prespores and duplicate in them. One set of (four) nuclei moves back to the basidium and the other remains in the prespore.
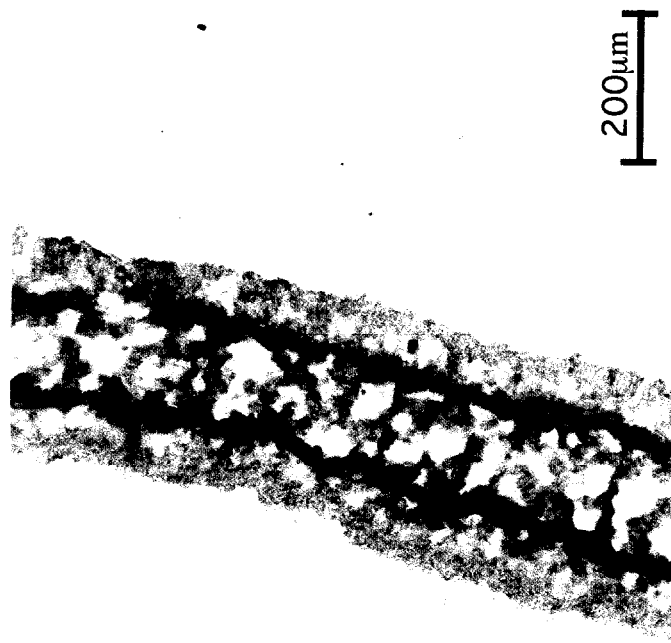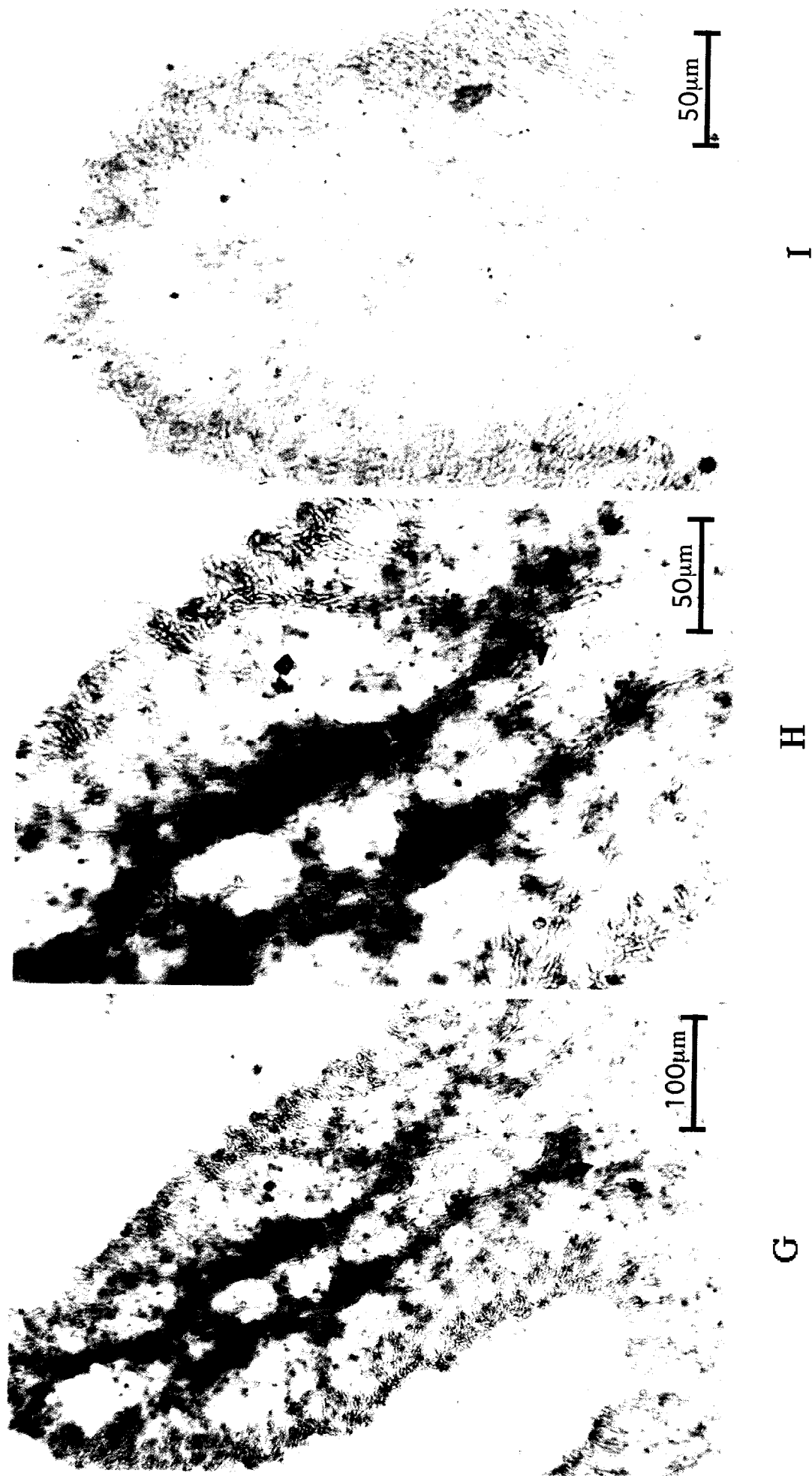
Fig.4-5   Expression of *Le.ras* gene in the hymenophore of fruiting body of *L. edodes*

Fixed longitudinal ultrathin sections of the fruiting-body maturation stage II were hybridized with digoxigenin-labeled *Le.ras* antisense or sense strand probe to analyze the localization of *Le.ras* mRNA. Panels A, C and D show hymenophore cells hybridized with *Le.ras* antisense strand probe. Panel B shows hymenophore cells hybridized with *Le.ras* sense strand probe. Panels A and B: (Magnification, ×100)

Fig.4-5 **Expression of *Le.ras* gene in the hymenophore of fruiting body of *L. edodes***

Panel C: (Magnification, ×400); Panel D: (Magnification, ×1000)

C　　　　　　　　　　　　　　D

F

E

Fig.4-5   Expression of *Le.ras* gene in the stipe cells of fruiting body of *L. edodes*

Panel E and F show stipe cells, Panel E with *Le.ras* antisense strand probe, Panel F with *Le.ras* sense strand probe. (Magnification, ×1000)

Fig.4-5 **Expression of *Le.ras* gene in the hymenophores of immature fruiting body of *L. edodes***

Fixed longitudinal ultrathin section of the fruiting-body development stage II (immature fruiting body) were also hybridized with digoxigenin-labeled *Le.ras* antisense strand probe. Panels G and H show hymenophore cells hybridized with *Le.ras* antisense strand probe. Panel I shows hymenophore cells hybridized with *Le.ras* sense strand probe.
Panel G: (Magnification, x200); Panels H and I: (Magnification, x400)

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The prespores were maturated and released (Nakai, 1992). In mature fruiting body and fruiting body of the development stage, the transcripts of the *uck1* and *Le.rnrB1* detected in the whole region of hymenium, suggesting that *uck1* and *Le.rnrB1* genes play a role in the nucleotide biosynthesis essential for production of basidiospores. During the production of basidiospores, the biosyntheses of nucleic acids, carbohydrates, lipids etc. must be active. Ribonucleoside and deoxyribonucleoside diphosphates synthesized by *uck1* gene product (UMP-CMP kinase) and *Le.rnrB1* gene product (Ribonucleoside diphosphate reductase (RNR) small subunit) all are required by the biosyntheses of the biomolecules.

Intense signal of *Le.rnrB1* transcript was detected in the outer region of trama (the border between the trama and the subhymenium). In the case of *uck1* transcript, only weak signal was detected in it. Of interest was the fact that the *Le.ras* gave intense signal of its transcript exclusively in the outer region of the trama of hymenophore of fruiting body. This region is the parts divergent from trama cells which run in parallel to subhymenium cells (see Fig.4-1). The actively transcription of *Le.rnrB1* and *Le.ras* in the divergent parts suggests the possibility that these genes play a role for the divergence of trama cells in hymenophore.

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CHAPTER 5

Conclusions

In this study, the author isolated two nucleotide biosynthesis genes from the basidiomycete *L. edodes*. The *uck1* gene is present in the 3'-flanking region of *priB* and encodes UMP-CMP kinase of which recombinant protein produced in *E. coli* catalyzed the phosphoryl transfer from ATP to UMP and CMP efficiently and also to AMP and dCMP with lower efficiencies. The reduction of ribonucleoside diphosphates produced by UMP-CMP kinase, etc. to deoxyribonucleoside diphosphates are catalyzed by ribonucleotide reductase (RNR). The author also isolated the *Le.rnrB1* gene encoding RNR small subunit (R2). Northern-blot analysis showed that these genes were most actively transcribed in hymenophore of mature fruiting bodies during fruiting-body formation.

The *in situ* hybridization analysis showed the presence of the transcripts of these genes in hymenium and the outer regions of trama (the border between the trama and the subhymenium) in hymenophore. Hymenium contains many basidia in which fusion of two nuclei, meiosis, replication, etc. essential for production of basidiospores occurs. The outer regions of trama contain the cells divergent from trama to the subhymenium. These imply that two nucleotide biosynthesis genes (*uck1* and *Le.rnrB1*) play a role in the nucleotide biosynthesis essential for production of basidiospores and for the divergence of trama cells to subhymenium cells in hymenophore.

In this study, I could not study the localization of the expression products of *uck1* and *Le.rnrB1* in hymenophore of fruiting body. However the author belives that this study gives valuable informations for understanding the molecular mechanism of basidiospore formation of *L. edodes*.

Molecular and cellular biological studies on two nucleotide biosynthesis genes and their expression products of the basidiomycete *Lentinus edodes*

List of papers

Papers for Dissertation:

S. Kaneko, Y. Miyazaki, T. Yasuda, and K. Shishido. Cloning, sequence analysis and expression of the basidiomycete *Lentinus edodes* gene *uck1*, encoding UMP-CMP kinase, the homologue of *Saccharomyces cerevisiae URA6* gene., *Gene*, **211**, p.259-266 (1998).

S. Kaneko, K. Shishido. Cloning and sequence analysis of the cDNA genes encoding ribonucleotide reductase small subunits from the basidiomycete *Lentinus edodes*., submitted.

Other Papers:

K. Shishido, Y. Miyazaki, T. Yasuda. and S. Kaneko. Molecular mechanism of fruiting body formation in the basidiomycete *Lentinus edodes*., *Proceedings of Progress in Mycological Sciences in Japan and the United Kingdom, Sixth International Symposium of the Mycological Society of Japan* (*ISMSJ-6, 1998*) (Nov. 26-27, 1998, Chiba, Japan), pp.5-10 (1998).

K. Shishido, Y. Miyazaki, T. Yasuda. and S. Kaneko. Structure and function of fruiting genes in *Lentinus edodes*., *Abstract of Sixth International Mycological Congress IMC6* (August 23-28, 1998, Jerusalem, Israel), p. 54 (1998).

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