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Molecular Biology and Molecular Breeding of Basidiomycete Fungi

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Fruiting-body formation of basidiomycete fungi is one of the most typical and attractive morphological differentiation observed in microorganisms. White-rot basidiomycete fungi are known to be the only organisms which can degrade lignin, an intractable aromatic polymer, and they also degrade aromatic pollutants such as pentachlorophenol and dioxins. We have studied on the molecular mechanism of fruiting-body formation of *Lentinula edodes* (Shiitake) as a molecular biological basic research and have molecular-genetically produced the basidiomycete fungal strains useful for recycle/reuse of waste plant biomass and degradation of aromatic pollutants as a molecular breeding application research.

I. Molecular biology of *L. edodes* : Developmental regulator PRIB and its target genes

To elucidate the molecular mechanism of fruiting-body formation of *L. edodes,* we have isolated various developmentally regulated genes, one of which, *priB* was most abundantly transcribed in primordia and at early stages of fruiting-body formation [1].

Mature fruiting bodies also contained this transcript. The deduced PRIB protein (565 amino acid residues) contained two characteristic DNA-binding motifs, a Zn(II)2Cys6 zinc-cluster motif [2] and a bZIP motif (consisting of basic amino acid residues and leucine zipper) [3]. The PRIB protein possessed two highly basic amino acid sequences (amino acids 182 – 186 and 354 – 362) as possible nuclear localization signals [4] and a putative phosphorylation sequence (Arg-Arg-Arg-Asp-Ser) which perfectly matches with the consensus phosphorylation sequence (Arg/Lys-Arg/Lys-Xaa (any amino acids)-Ser/Thr) by cAMP-dependent protein kinase (A kinase) [5]. The yeast two-hybrid analysis showed that the PRIB fragment containing the bZIP forms homodimer [6].

To analyze the sequences of PRIB binding, random bindng-site selection analysis [7] was done using a pool of random 24 bp oligonucleotides with 13 bp PCR primer sites at each end. The oligonucleotides (50 bp) selected for PRIB binding were cloned into pUC19. A total of 303 cloned DNA fragments were picked randomly and sequenced. The PRIB binding sites could be grouped into 25 individual sequences, suggesting a consensus sequence of 16 bp, 5'-$G_{20}G_{22}G_{23}G_{24}G_{22}G_{19}G_{19}A_{15}C_{22}A_{23}G_{19}G_{22}A_{18}NC_{13}C_{17}$-3' (the numerals on the right of the bases indicate how many bases out of 25 in the cloned binding sites are identical in the consensus sequence)[8]. The upstream region of *priB* contained four 16 bp sequences similar to the consensus sequence of PRIB binding [6]. The *L. edodes* UMP-CMP kinase gene, *uck1*, that is

located downstream of *priB*, contained two 16 bp consensus-like sequences in its upstream region [9]. These imply the possibility that *priB* and *uck1* genes are the targets of PRIB protein.

To examine whether *priB* and *uck1* are selected as the target genes of PRIB and to test the possibility of the presence of other target gene(s) of PRIB, we carried out genomic binding-site (GBS) cloning experiment [10] of the *Hin*dIII-digests of *L. edodes* DNA by using the N-terminal fragment of PRIB containing the zinc-cluster and bZIP motifs. The experiment selected three *Hin*dIII fragments each of which bears *priB*, *uck1*, or a new gene (named *mfbC*). The deduced 330 amino acid sequence of the *mfbC* gene product (MFBC) was found to be highly homologous to the 325 amino acid sequence of *Saccharomyces cerevisiae YJR070C/Lia1* gene product (YJR070C/Lia1) which is the protein interacting with the putative translation initiation factor 5A (eIF5A) [11]. The evidence that *priB* gene is the target of PRIB suggests an autoregulation of transcription of *priB* gene. Just downstream of *priB* gene there exists *uck1* gene [9]. It is interesting to note that the gene of *Neurospora crassa* QA1F protein with a Zn(II)Cys6 zinc-cluster DNA-binding motif and the target genes of QA1F are also clustered on the chromosome [12], even though the location of *mfbC* on *L. edodes* chromosome remains to be determined.

A Northern blotting was carried out to investigate the expression of the *mfbC* gene in the course of fruiting-body formation of *L. edodes*. Total cellular RNA was isolated from preprimordial aggregated mycelia, primordia, immature fruiting bodies, and mature fruiting bodies. The stages of immature fruiting bodies were divided into fruiting-body developmental stages I, II, and III as before [13]. The RNA samples were subjected to northern blotting using the ^{32}P-labeled *mfbC*c as a probe. Almost only mature fruiting bodies contained a large amount of *mfbC* transcript. So the *mfbC* gene, the homologue of *S. cerevisiae YJR070C/Lia1* was considered to play a role at the final stage of fruiting-body formation of *L. edodes*, possively regulating the translation of mRNAs produced in mature fruiting bodies. Levels of the transcript of *mfbC* gene were analyzed in hymenophores, hymenophores-depleted pileus and stipe of mature fruiting bodies using the probe of *mfbC*c. All parts of the fruiting body were found to contain *mfbC* transcript. But hymenophores and stipe contained higher levels of the transcript than the hymenophores-depleted pileus.

In situ RNA-RNA hybridization was carried out to investigate the expression of the *mfbC* gene in the parts of hymenophore, stipe and hymenophores-depleted pileus. The hymenophore is a complicated gill tissue consisting of trama, subhymenium, and hymeniun on which a large number of basidia and basidiospores are formed. The trama cells diverge to form subhymenium on the top of which hymenium is formed. Fixed longitudinal ultrathin sections of the hymenophores were hybridized with digoxigenin-labeled *mfbC*c antisense or sense strand probe. The *mfbC*c antisense strand probe gave an intense signal in the outer region of trama (the region branching out into subhymenium). The same experiment was done also for the fixed longitudinal ultrathin sections of the stipe and hymenophores-depleted pileus. However a discrete hybridization signal was not observed, suggesting a diffusible distribution of the *mfbC* transcript in them. Our previous study with the *uck1* gene [9, 13] showed that a markedly large amount of the *uck1* transcript is present in both hymenium and outer region of trama in the hymenophore. These results suggest that *mfbC* gene is involved in divergence of trama cells into subhymenium cells, but not in production of basidiospores. On the other hand, *uck1* gene may play a role in the nucleotide biosynthesis essential both for production of basidiospores and for divergence of trama cells.

To clarify the physiological function of *mfbC* gene, however, more detailed genetical and biochemical approaches are necessary.

Ⅱ. Molecular breeding of Basidiomycete fungal strains

Ⅱ-1. Production of the *Coprinus cinereus* strains with high lignin- and xylan-degrading activities and their use for an efficient isolation of cellulose from rice straw

Plant biomass *i.e.*, lignocellulose, is the most abundant renewable organic resource on earth. Lignocellulosic residues themselves are not high-value materials. However, if we can develop biological methods for the isolation of cellulose, which is convertible into ethanol by fermentation, from waste plant biomass, it is meaningful and remarkable from the point of view of obtaining a source of energy. In order to isolate celllulose from plant biomass, of which major components are cellulose, hemicellulose, and lignin, it is required to eliminate lignin and hydrolyze hemicellulose. As described in introduction white-rot basidiomycete fungi degrade lignin. They can also hydrolyze hemicelluloses such as a xylan. However the productions of lignin-degrading enzymes such as manganese (II) peroxidase (MnP) and lignin peroxidase (LiP) and xylan-hydrolyzing enzymes such as endo-(1,4)-β-xylanase are all inducible. Therefore the constitutive and high level producution of these enzymes in basidiomycete fungi is important for efficient use of lignocellulosic residues.

i) Molecular breeding of the *Coprinus cinereus* strains with high lignin-decolorization and –degradation activities using novel heterologous protein expression vectors

Two chromosome-integrating vectors, pLC1 and pLC2, were used. The former is the pUC19-based vector carrying the *L. edodes ras* gene promoter and *priA* gene terminator, and the latter is the pBR322-based vector carrying the basal promoter and the terminator of the *priA* gene [14]. The MnP cDNA (*mnpc*) derived from *Pleurotus ostreatus* was fused between the promoter and terminator of pLC1 and pLC2, yielding the recombinant plasmids pLC1-mnp and pLC2-mnp. These plasmids were introduced into protoplasts of the monokaryotic *C. cinereus trp1* strain with the *C. cinereus TRP1*-containing plasmid pCc1001 by co-transformation. Two Trp^+ transformants for each plasmid, showing clearly higher lignin-decolorization activities, were obtained through introduction of pLC1-mnp and pLC2-mnp. Southern-blot analysis revealed that the four transformants all possess *mnpc* sequence on their chromosomes. One Trp^+ MnP^+ transformant (named CcTF2-7(Po.MnP)), which was derived from the introduction of pLC2-mnp and carried the highest copies (approx. 10) of *mnpc*, showed remarkably high lignin-decolorization and -degradation activities; at the time of cultivation when only 35% – 40% of the lignin was decolored and degraded by the control Trp^+ transformant obtained by the introduction of pCc1001 alone, almost all of the lignin was decolored and degraded by CcTF2-7(Po.MnP) [14].

ii) Molecular breeding of the *C. cinereus* strains with high xylan-degradation activities using vectors pLC1 and pLC2

The *Bacillus subtilis* endo-(1,4)-β–xylanase structural gene (*xyn*) was trimmed away from its signal

sequence and then fused after the signal sequence of the basidiomycete *P. ostreatus* MnP cDNA. The resulting modified gene (xyn') was inserted between the promoter and terminator of pLC1 and pLC2. These recombinant plasmids pLC1-xyn' and pLC2-xyn' were introduced into protoplasts of the monokaryotic *C. cinereus trp1* strain with pCc1001. For each plasmid, one Trp^+ Xyn^+ transformant, showing a significantly high xylan-degrading activity was obtained and it was named CcTF1-16(Bs.Xyn) (derived from pLC1-xyn') or CcTF2-11(Bs.Xyn) (derived from pLC2-xyn'). CcTF1-16(Bs.Xyn) and CcTF2-11(Bs.Xyn) were estimated to carry about 6 and 10 copies of the xyn' sequence, respectively, on their chromosomes. The supernatants of CcTF1-16(Bs.Xyn) and CcTF2-11(Bs.Xyn) obtained from their 18-day cultures contain a xylanase activity seven or nine times as high as that of the control Trp^+ transformant [15].

iii) Efficient isolation of cellulose from rice straw by using CcTF2-7(Po.MnP) and CcTF2-11(Bs.Xyn)

To examine the usefulness of the molecular-bred *C. cineres* strains to isolate cellulose from waste plant biomass, we first tested the growth of CcTF2-7(Po.MnP) and CcTF2-11(Bs.Xyn) on solid medium containing rice straw, sawdust of beech wood, or fallen leaves as a waste plant biomass, revealing that rice straw is the most suitable source for culture medium. The two *C. cineres* strains were mixed-cultured at 27° C in the liquid-medium containing 0.5% (w/v) cut rice straw and 0.025% $MnCl_2$. After 3 weeks, the culture supernatant was extensively treated with crude cellulase, showing the presence in it of 9.3% of the total cellulose of rice straw [16]. When rice straw treated with 0.1 N NaOH or cultured with *Elfvingia applanata* were used, the recoveries of the cellulose increased up to 29% [16]. The same experiments were done by using a non-bred control strain, showing the recoveries of the cellulose from the treated or cultured rice straw to be 8%. These results indicate the success in the conversion of approx. 12% of total weight of rice straw into no-precipitable forms of cellulose. This is meaningful and interesting from point of view of obtaining a source of energy from waste material and recycling of natural resources.

Ⅱ-2. Efficient degradation of polychlorinated aromatic hydrocarbons by using molecular-genetically bred *Coriolus hirsutus* strains with high lignin peroxidase activity

Polychlorinated aromatic pollutants have been one of the most important public concerns. Lignin-degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) produced in white-rot basidiomycetous fungi are known to be involved in the degradation of polychlorinated aromatic hydrocarbons (PCAHs). So we attempted to produce *Coriolus hirsutus* strains with high LiP activity and examine the degradation of the PCAHs by them. The chromosome-integrating vector MIp30 carrying the *C. hirsutus gpd* gene promoter—*L. edodes priA* gene terminator and the selectable marker of *C. hirsutus ARG1* gene was constructed. The *C. hirsutus* LiP gene (*lip*) was fused between the promoter and terminator of MIp30 and the resulting recombinant plasmid MIp30-lip was introduced into protoplasts of monokaryotic *C. hirsutus arg1* strain, followed by selection of Arg^+ Lip^+ colonies. Southern-blot analysis revealed that two of the Arg^+ Lip^+ transformants, named ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), possess the plural number of copies (applox. 5) of the promoter—*lip*—terminator cassette on their chromosomes [17,18]. Northern-blot analysis showed that both Arg^+ Lip^+ transformants contained large amounts of *lip*

transcripts. The mycelial cells of the transformants were cultivated in BK medium containing 25 g brewer's grains and 100 ml Kirk Basal III medium per liter and grown at 25°C. The LiP activities of the ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) in their culture supernatants were found to be about five times as high as that of the Arg^+ control [17,18].

The degrading activities of PCAHs, i.e., pentachlorophenol (PCP) and 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) were analyzed as follows. The reaction mixtures containing PCP or 2,7-DCDD and the culture supernatants were incubated for 20 hr at 25°C and the remaining PCP and 2,7-DCDD were extracted by hexane, followed by gas chromatographic analysis. The supernatants of ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) showed remarkably high PCP degradation activities: at the time when only 22.0 % of PCP was degraded by the control Arg^+ transformant, 80.5 % and 78.2 % of PCP were degraded by ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), respectively [17,18]. The 2,7-DCDD degrading activities of the supernatants of ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP) were also higher than that of the control Arg^+ transformant: at the time when only 33.5 % of 2,7-DCDD was degraded by the control Arg^+ transformant, 73.7 % and 63.5 % of 2,7-DCDD were degraded by ChTF3-1(Ch.LiP) and ChTF3-2(Ch.LiP), respectively [17,18].

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