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SHORT CONTRIBUTION

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

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Abstract Two chromosome-integrating vectors, pLC1 and pLC2, were used. The former is the pUC19-based vector carrying the *Lentinus edodes ras* gene promoter and *priA* gene terminator, and the latter is the pBR322-based vector carrying the promoter and terminator of the *priA* gene. The manganese (II) peroxidase (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 *Coprinus 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 the *mnpc* sequence on their chromosomes. One Trp^+ MnP^+ transformant (named TF2-7), which was derived from the introduction of pLC2-mnp and carried the highest number of 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 TF2-7.

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Introduction

We have succeeded in constructing the chromosome-integrating vector pLC1, which is very useful for the expression of foreign genes in various basidiomycetous fungi (Kajiwara 1993; Yanai et al. 1996). The *ras* gene promoter and *priA* gene terminator, derived from the popular edible basidiomycete *Lentinus edodes*, were used in the construction of pLC1 (see Fig. 1). The *L. edodes ras* gene was transcribed at similarly higher levels during vegetative growth and all stages of fruiting development (Hori et al. 1991; Kajiwara and Shishido 1992). The *L. edodes priA* gene was actively transcribed in earlier developmental stages of fruiting and contained the terminator, consisting of the sequences required for both transcription termination and polyadenylation (Zaret and Sherman 1982), and the site for polyadenylation (Kajiwara et al. 1992; Kajiwara 1993; Yanai et al. 1996). By using pLC1, the *Escherichia coli* β-glucuronidase gene (*GUS*), the aminoglycoside phosphotransferase I gene (*aphI*) derived from the *E. coli* transposon Tn903, and the *Streptomyces hygroscopicus* bialaphos-resistance gene (*bar*) were expressed in the basidiomycetes *Pleurotus ostreatus* and *L. edodes* (Kajiwara 1993; Yanai et al. 1996; our unpublished results). The basal promoter region of the *priA* gene was also found to be useful for an expression of the foreign genes in vegetatively growing mycelia of *P. ostreatus* and *L. edodes* (our unpublished data), constructing the vector pLC2, which contains the basal promoter and terminator of *priA* (see Fig. 1).

We have recently isolated genomic and complementary DNAs coding manganese(II) peroxidase from *P. ostreatus* in which the enzyme is a key peroxidase in the degradation of lignin (Asada et al. 1995). Lignin occurs as one of the major components of woody and

herbaceous plants and is the most abundant renewable aromatic polymer on earth.

There is considerable interest in the application of the biological degradation of lignin to industrial processes such as biomechanical pulping, bleaching of paper, production of feedstock from wood and agricultural wastes and conversion of lignocellulosic materials to fuel and chemicals. This led us to attempt to introduce the *P. ostreatus* Mn(II) peroxidase cDNA (designated *mnpc*) into protoplasts of the rapidly growing *Coprinus cinereus* by using the vectors pLC1 and pLC2, and hence to produce strains with remarkably high lignin-decolorization and -degradation activities. We now report the successful experimental results.

Materials and methods

Strains and media

C. cinereus monokaryotic strain LT2-44 (*trp1-1*, *trp1-6*), kindly provided by Dr. S. Yanagi of NFRI at Tsukuba (originally provided by Dr. L. A. Casselton of University of Oxford), was used as the recipient in transformation experiments. *P. ostreatus* IFO30160, a dikaryotic strain, was from the culture collection of the Institute of Fermentation Osaka. MYG medium (1% malt extract, 0.4% yeast extract, 0.4% glucose, pH 5.6), containing 100 μg tryptophan/ml when required, was used for the growth of *C. cinereus* and *P. ostreatus*. Regeneration medium (pH 6.0), used for *C. cinereus* protoplasts, contained 171 g sucrose, 5 g glucose, 2 g asparagine, 40 μg thiamine, 1 g KH_2PO_4, 2.25 g $Na_2HPO_4 \cdot 12H_2O$, 1.5 g NH_4Cl, 0.29 g Na_2SO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, and 5 g soluble starch l^{-1}. Construction, propagation, and amplification of recombinant plasmids were carried out in *E. coli* JM109 (Yanisch-Perron et al. 1985). *E. coli* was cultured in Luria-Bertani medium.

Plasmid construction

pLC1 (6.4 kb) is the pUC19-based vector carrying the *L. edodes ras* gene promoter (2.5 kb) and the *priA* gene terminator (1.2 kb) and containing the *Bam*HI cloning site between the promoter and terminator (Kajiwara 1993; Yanai et al. 1996) (Fig. 1). pLC2 (5.6 kb) is the pBR322-based vector carrying the *priA* basal promoter (0.37 kb) and its terminator and it has the *Bam*HI cloning site (Fig. 1). Plasmid pMOSBlue-*mnp*, containing the 1274-bp sequence of Mn(II) peroxidase cDNA (*mnpc*), derived from *P. ostreatus* IFO30160 between the *Xba*I and *Bam*HI sites on the pMOSBlue T-vector (Amersham), was digested with *Xba*I and ligated to a *Bam*HI linker [5′-d(CCGGATCCGG)-3′] after the ends had been filled-up. Thus the *P. ostreatus mnpc* was obtained as a 1.3-kb *Bam*HI fragment and inserted into the *Bam*HI site of pLC1 and pLC2, yielding pLC1-mnp and pLC2-mnp (Fig. 1). Plasmid pCc1001, used for co-transformation with pLC1-mnp or pLC2-mnp, is the pUC9-based vector carrying the 6.5-kb *Pst*I genomic fragment containing the *C. cinereus* tryptophan synthetase gene (*TRP1*) (Binninger et al. 1987).

Transformation of *C. cinereus*

Oidia (approx. 10^9 cells) were harvested from a single plate culture of LT2-44 and germinated at 30 °C for 48 h without shaking in 30 ml MYG medium supplemented with tryptophan. The cells were harvested by centrifugation at 750 g for 5 min at room temperature and washed once with 10 ml MM buffer (0.5 M mannitol in 50 mM maleate, pH 5.5). The washed cells were suspended in 1 ml the MM buffer containing 10 mg Novozyme 234 and 1 mg Chitinase-RS (Pias Corp., Osaka, Japan) and incubated at 30 °C for 2 h. The protoplasts were separated from cell debris by filtering through 60-μm-pore nylon mesh, washed once with 10 ml MM buffer and once with 10 ml MMC (MM containing 50 mM $CaCl_2$), and resuspended in 1 ml MMC to give $(1–2) \times 10^8$ cells/ml. Transformations were essentially carried out as described by Binninger et al. (1987). To 200 μl protoplast suspension were added 20 μl plasmid DNA (4 μg) in buffer containing 10 mM TRIS/HC1, 1 mM EDTA, pH 8.0, and 50 μl polyethylene glycol solution (25% PEG4000, 50 mM $CaCl_2$ in 10 mM TRIS/HC1 pH 7.5) in 15-ml polypropylene conical tubes. The tubes were placed in ice for 20 min and then 2 ml PEG was added. The protoplasts were incubated at room temperature for 5 min. To regenerate the protoplasts, 1.0-ml aliquots were mixed with 10 ml regeneration medium containing 0.7% agar (kept molten at 50–55 °C) and poured over a layer of the regeneration medium containing 1.5% agar in 9-cm plastic plates. The plates were inverted and incubated at 30 °C. Colonies were examined after 10–14 days.

DNA isolation and Southern-blot analysis

Genomic DNAs of *C. cinereus* and *P. ostreatus* were isolated as described by Zolan and Pukkila (1986). Plasmid DNA was isolated from *E. coli* by the alkaline lysis method and purified by centrifugation to equilibrium in cesium chloride/ethidium bromide gradients (Sambrook, Fritsch and Maniatis 1989). The undigested and *Bam*HI-digested genomic DNA (20 μg each) were fractionated on a 0.8% agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham) by the method of Southern (1975). DNA probe labelling with ^{32}P, hybridization, and signal detection were done according to the procedures previously published (Ishii et al. 1991).

Assessment of lignin degradation

A 10 ml sample of MYG medium, containing 10 mg lignin (Tokyokasei Kôgyo, Japan) and 2.5 mg $MnCl_2$ in an L-shaped tube, was inoculated with three ($7 \times 7 \times 1$ mm) cubes of the MYG/agar colonized with *C. cinereus* mycelium and cultivated at 30 °C for the indicated periods with shaking. Degradation of lignin was determined by the decrease of the absorbance at 275 nm of the supernatant, which shows the degradation of the aromatic rings. The decrease of the absorbance at 480 nm was measured for decolorization of lignin (brown color). The absorbance of the cultured medium containing 10 mg/10 ml lignin was 27.15 at 275 nm and 2.19 at 480 nm, and these values were taken as 100%.

Results and discussion

Co-transformations of the *C. cinereus* Trp^- auxotrophic strain LT2-44 (trp1-1, 1-6) with pCc1001 and pLC1-mnp or pLC2-mnp

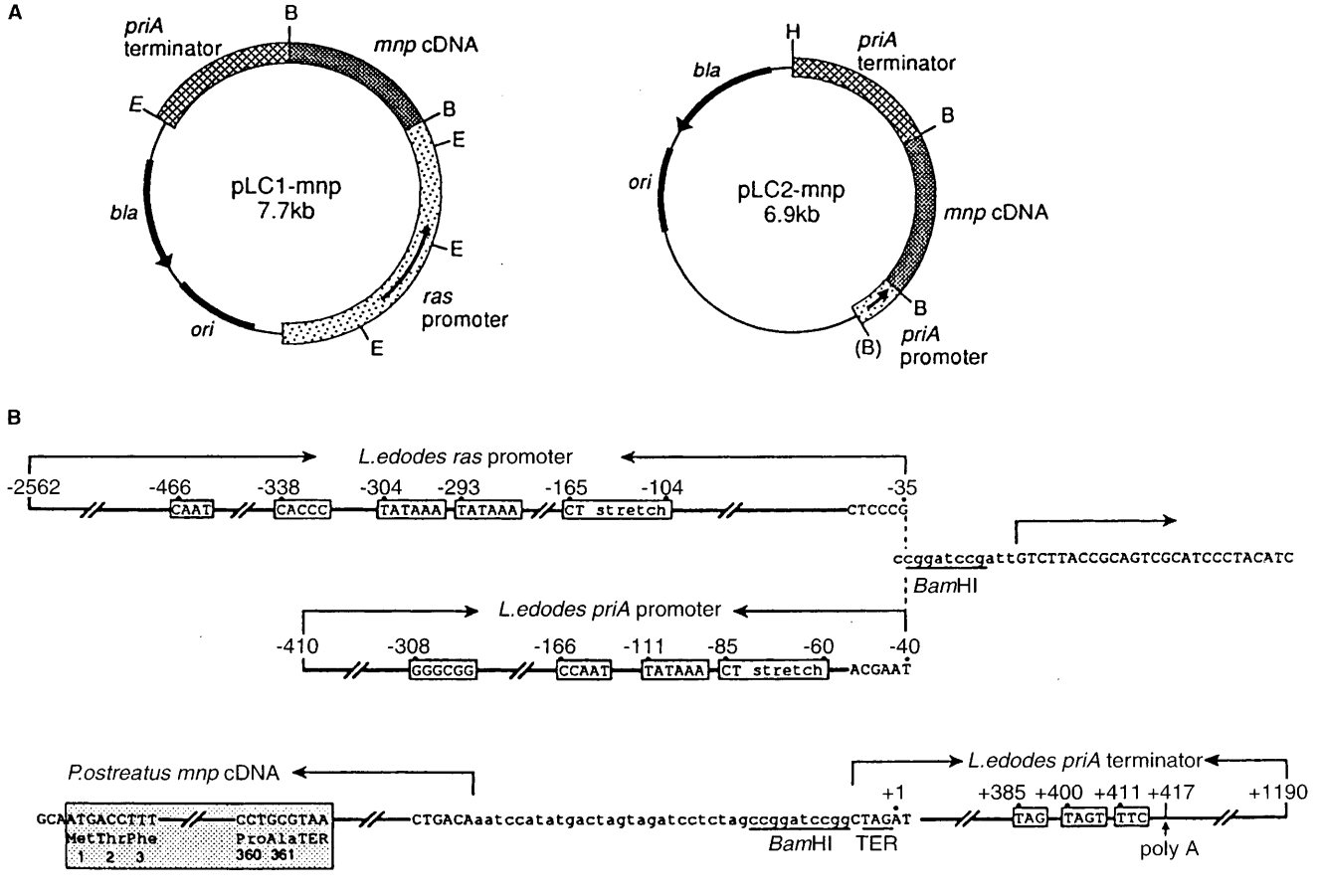
pLC1-mnp and pLC2-mnp contain the manganese(II) peroxidase (MnP) cDNA (*mnpc*) derived from *P. ostreatus between* the *L. edodes ras* gene promoter and the *priA* gene terminator or between the basal promoter and terminator of the *priA* gene respectively (see Fig. 1). These recombinant plasmids were introduced into the *C. cinereus* LT2-44 (*trp1-1*, *1-6*) genome by co-transforming with pCc1001 carrying the *C. cinereus TRP1* gene, and selecting for Trp^+ transformants. For each experiment 4 μg each of pCc1001 and pLC1-mnp or pLC2-mnp was presented to $(2–4) \times 10^7$ total protoplasts. From two independent co-transformations a total

of about 60 Trp^+ colonies were obtained at a frequency 5–10/μg transforming pCc1001 DNA for both pLC1-mnp and pLC2-mnp. For both types of transformant, thirteen Trp^+ colonies were selected at random and cultivated on MYG/agar plates. The mycelial colonies grown on the plates were screened for a lignin-decolorization activity in MYG liquid medium containing 0.1% lignin and 0.025% $MnCl_2$, producing two Trp^+ transformants for each plasmid that showed activities clearly higher than that of the recipient. The two Trp^+ MnP^+ transformants derived from the introduction of pLC1-mnp were named TF1-2 and TF1-4 and those from pLC2-mnp were TF2-1 and TF2-7.

To ascertain the presence of the introduced DNA in the transformants, Southern-blot analysis was done using the ^{32}P-labeled 1.3-kb *Bam*HI fragment containing *P. ostreatus mnpc* as a probe (see Fig. 1). In the DNA samples of the four Trp^+ MnP^+ transformants without restriction enzyme digestion, specific hybridization signals were observed in the high-molecular-mass DNA region corresponding to the chromosomal DNA (data not shown). No specific hybridization signal was found in the DNA sample of the Trp^+ transformant obtained by introduction of pCc1001 DNA alone. Similar results were obtained by using the ^{32}P-labeled probe of pUC19 or pBR322.

Next, all the DNA samples were digested with *Bam*HI and subjected to Southern-blot analysis using the aforementioned ^{32}P-labeled 1.3-kb *mnpc* probe. As shown in Fig. 2, TF2-1, TF2-7, TF1-2, and TF1-4 all gave one intense hybridization signal at the position corresponding to the size (1.3 kb) of the probe and one faint signal at around 2.6 kb (lanes 1–4). No hybridization signal was observed in the case of the Trp^+ transformant (lane 5). The *Bam*HI digest of the DNA of *P. ostreatus* IFO30160, from which the *mnpc* was derived, was also analyzed. It gave a single faint band at the 9-kb position (lane 6). These results suggest that the introduced pLC1-mnp and pLC2-mnp DNA had been integrated into chromosomal DNA of the four Trp^+ MnP^+ transformants. The simplest explanation for the

Fig. 1A,B Structures of the *Pleurotus ostreatus* manganese(II)-peroxidase expressing recombinant plasmids pLC1-mnp and pLC2-mnp (**A**), the structural features of the *ras* and *priA* promoters and *priA* terminator, and the nucleotide sequences at the fusion junction between the promoters, *P. ostreatus mnp* cDNA and the terminator (**B**). **A** *Thin lines* pUC19 (for pLC1-mnp) or pBR322 (for pLC2-mnp). *B BamHI*, *E EcoRI*, *H HindIII*. **B** *Boxes* the conserved sequences of eukaryotic promoter and terminator. The numbers in the promoter regions and terminator region refer to the nucleotide positions relative to the first nucleotide (+1) of the start codon and to the last nucleotide (+1) of the termination codon respectively. The sequence data for *ras* and *priA* will appear in the DDBJ/EMBL/GenBank nt sequence databases under accession numbers D01208 and X60956 respectively



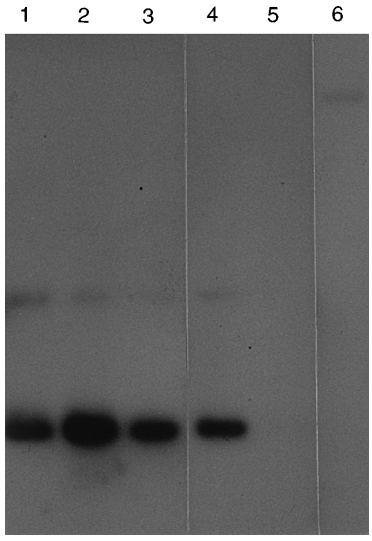


Fig. 2 Southern-blot analysis of the *Bam*HI digests of total DNA prepared from the four Trp^+ MnP^+ transformants of *Coprinus cinereus*. Lanes: *1* TF2-1, *2* TF2-7, *3* TF1-2, *4* TF1-4, *5* DNA from the control Trp^+ transformant of *C. cinereus*, *6* DNA from *P. ostreatus* IFO30160. The 1.3-kb *Bam*HI fragment containing the *P. ostreatus mnpc* was used as a probe

generation of a faint, larger 2.6-kb band in all the *Bam*HI digests of the four Trp^+ MnP^+ transformants is as follows. A small part of the introduced plasmid DNA was cleaved at the site within the *mnpc* sequence, close to the *Bam*HI site at the junction of the *ras* or *priA* promoter and the *mnpc* sequence, and integrated into the defined site on the chromosomes. The nearest *Bam*HI site on the chromosomes is 2.6 kb from the *Bam*HI site at the junction of the *mnpc* sequence and the *priA* terminator.

Comparison of the total radioactivity of the intense (1.3-kb) and faint (2.6-kb) hybridization bands with that of the single band of *P. ostreatus* IFO30160 suggests that TF2-1, TF2-7, TF1-2, and TF1-4 carry 6, 10, 6, and 5 copies of the *mnpc* sequence. The same amount of DNA as used in the above experiments was taken from each of the DNA preparations of the transformants and *P. ostreatus* IFO30160 and all were digested with both *Xho*I and *Sal*I. The digests were subjected to Southern-blot analysis using the ^{32}P-labeled 1.3-kb *Xho*I–*Sal*I genomic fragment containing the *C. cinereus ras* gene (Ishibashi and Shishido, 1993) as the probe. The four Trp^+ MnP^+ transformants and control Trp^+ transformant all gave one faint signal at around 1.3 kb (data not shown). Their intensities were almost the same and similar to the intensity of the 9-kb signal in the *Bam*HI digest of *P. ostreatus* IF030160 (lane 6 of Fig. 2). These results suggest equal loading and transfer of DNA preparations and satisfactory estimates of the copy numbers of the *mnpc* sequence for the four transformants.

The four Trp^+ MnP^+ transformants were cultivated in MYG medium without tryptophan for 3 months, and then total DNA were prepared. Southern-blot analysis of these samples showed the presence of the introduced DNA on chromosomes and the same hybridization patterns as shown in Fig. 2 (data not shown), suggesting that all the transformants were mitotically stable.

The lignin-decolorization and -degradation activities of the *C. cinereus* Trp^+ MnP^+ transformants

TF2-7, containing the highest number of copies of the *mnpc* sequence and the control Trp^+ transformant, were cultured in 10 ml MYG liquid medium containing 0.1% lignin and 0.025% $MnCl_2$ in an L-shaped tube at 30 °C with shaking. Triplicate tubes were prepared and used

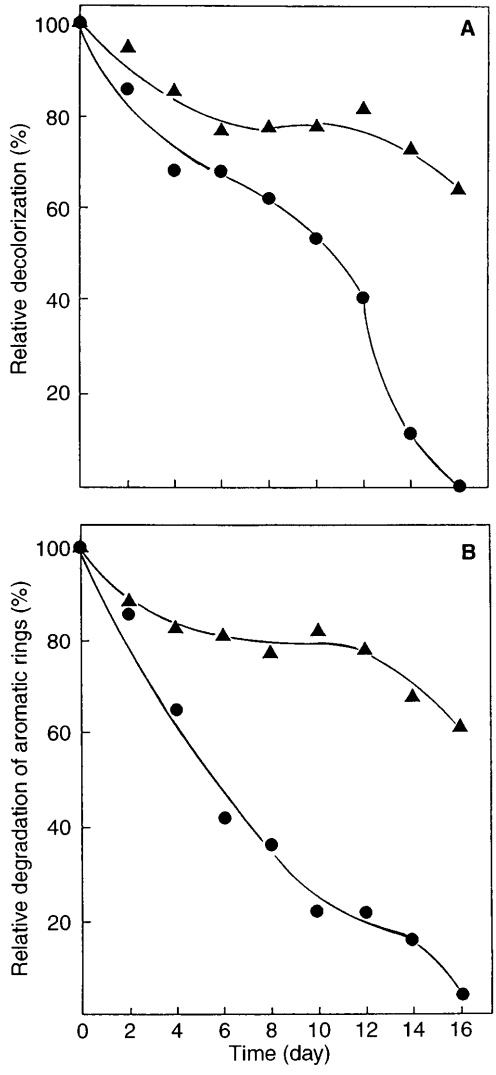


Fig. 3A, B Decolorization (**A**) and degradation (**B**) of lignin during the cultivations of the Trp^+ MnP^+ transformant TF2-7 (●) and the control Trp^+ transformant (▲) in the lignin-containing malt extract/yeast extract/glucose (pH 5.6) medium. The decolorization and degradation of lignin are shown by the decrease of the absorbance at 480 nm and 275 nm respectively, the absorbance at the start time of cultivation being taken as 100%. For more details, refer to text

for cultivation. After the indicated periods, 0.3-ml aliquots were taken out from each of the three cultures, mixed and then centrifuged. The supernatants were investigated for decolorization of the brown lignin by measuring the decrease of the absorbance at 480 nm (Fig. 3A) and for degradation of the aromatic rings of lignin by measuring the decrease of the absorbance at 275 nm (Fig. 3B). Decolorization and degradation of lignin by TF2-7 were completed after 16 days of cultivation, while only 35%–40% was decolored and degraded by the control. Similar results were obtained in both MY medium (without glucose) and protoplast regeneration medium without sucrose (see Materials and methods). The ligninolytic activities in the supernatants of the cultures of TF2-7 and the Trp^+ control were determined by rapid and sensitive assay using Remazol brilliant blue R dye (RBBR, Ulmer et al. 1984), demonstrating that the activity of TF2-7 is about 30 times as high as the control.

Results analogous to those with TF2-7 were obtained in the experiments using the TF2-1, TF1-2, and TF1-4, though their lignin-decolorization and -degradation activities were slightly lower than those of TF2-7 (data not shown). Several other Trp^+ colonies, obtained by the introduction of pCc1001 and the recipient LT2-44, gave the same results as the Trp^+ control used.

In this paper we have described the molecular breeding of the *C. cinereus* strains that carry high copy numbers of the *P. ostreatus* manganese(II) peroxidase cDNA sequence on their chromosomes and show markedly higher lignin-decolorization and -degradation activities, by using novel vectors containing the promoters and terminator of the genes derived from *L. edodes*. This is first report on the efficient expression of a foreign gene in a *C. cinereus* host, as far as we know. The *C. cinereus* strains constructed in this study are considered to be useful for the elimination of lignin from lignocellulosic materials.

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References

Asada Y, Watanabe A, Irie T, Nakayama T, Kuwahara M (1995) Structures of genomic and complementary DNAs coding for *Pleurotus ostreatus* manganese (II) peroxidase. Biochim Biophys Acta 1251: 205–209

Binninger DM, Skrzynia C, Pukkila PJ, Casselton LA (1987) DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J 6: 835–840

Hori K, Kajiwara S, Saito T, Miyazawa H, Katayose Y, Shishido K (1991) Cloning, sequence analysis and transcriptional expression of a *ras* gene of the edible basidiomycete *Lentinus edodes*. Gene 105: 91–96

Ishibashi O, Shishido K (1993) Nucleotide sequence of a ras gene from the basidiomycete *Coprinus cinereus*. Gene 125: 233–234

Ishii S, Murakami T, Shishido K (1991) Gyrase inhibitors increase the content of knotted DNA species of plasmid pBR322 in *Escherichia coli*. J Bacteriol 173: 5551–5553

Kajiwara S (1993) Molecular genetical studies on *ras* gene and fruiting genes of eubasidomycetes. PhD Thesis. Tokyo Institute of Technology

Kajiwara S, Shishido K (1992) Characterization of the promoter region of the basidiomycete *Lentinus edodes Leras* gene. FEMS Microbiol Lett 92: 147–150

Kajiwara S, Yamaoka K, Hori K, Miyazawa H, Saito T, Kanno T, Shishido K (1992) Isolation and sequence of a developmentally regulated putative novel gene, *priA*, from the basidiomycete *Lentinus edodes*. Gene 114: 173–178

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517

Ulmer DC, Leisola MSA, Fiechter A (1984) Possible induction of the ligninolytic system of *Phanerochaete chrysosporium*. J Biotechnol 1: 13–24

Yanai K, Yonekura K, Usami H, Hirayama M, Kajiwara S, Yamazaki T, Shishido K and Adachi T (1996) The integrative transformation of *Pleurotus ostreatus* using bialaphos resistance as a dominant selectable marker. Biosci Biotechnol Biochem 60: 472–475

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene 33: 103–119

Zaret KS, Sherman F (1982) DNA sequence required for efficient transcription termination in yeast. Cell 28: 563–573

Zolan ME, Pukkila PJ (1986) Inheritance of DNA methylation in *Coprinus cinereus*. Mol Cell Biol 6: 195–200