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Kyoto University
Development of Transformation and Recombinant Gene Expression Systems in *Pleurotus ostreatus*\(^1\)

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**Keywords**: basidiomycete, fungus, mushroom, *Lentinula edodes*, lignin, Ligninolytic enzymes, manganese peroxidase, *mnp3*, carboxin, hygromycin B, restriction enzyme mediated transformation (REMI), protoplast

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**Preface**

White-rot basidiomycete degrade lignin more extensively and rapidly than any other known group of organism. In contrast to other fungi and bacteria, white-rot fungi are capable of completely degrading lignin to carbon dioxide and water. The species are widely distributed, occurring in tropical and temperate environments. White-rot fungi are also well adapted for utilizing other plant components and species vary substantially with regard to their relative cellulolytic versus ligninolytic efficiency. Recently, their ability to degrade aromatic pollutant is also attended in bioremediation. The most studied ligninolytic white-rot basidiomycete *Phanerochaete chrysosporium* secretes a large number of ligninolytic heme glycoproteins, lignin peroxidases (LiPs) and manganese peroxidases (MnP). Their features have been extensively investigated and many genetic and enzymatic data are available\(^1,2\).

*Pleurotus ostreatus* is one of the most major edible mushroom eaten widely in the world and one of the white-rot basidiomycete which is attended in utilization for biobleaching, bioremediation and catalysis of difficult chemical transformation\(^3-5\). So use of *P. chrysosporium* in field is difficult due to plant prevention in Japan, our laboratory have studied property of *P. ostreatus*. Construction of DNA mediated transformation system for basidiomycete is very important to investigate its traits. Some transformation systems have been developed. But their efficiency is low in comparison with that in ascomycetous fungi\(^6-13\). Many genetical manipulation are not available due to the low transformation efficiency. In chapter I, development of high efficient transformation system for *P. ostreatus* is reported.

Typical LiP activity is not detected in *P. ostreatus* and MnP is considered as the key enzyme of ligninolytic system in this fungus\(^14\). Property of MnPs produced by genus Pleurotus is difficult to that of *P. chrysosporium* MnPs\(^15-17\). Chapter II-1 reports that it is also suggested by amino-acid sequence predicted by the gene. Enzymatic analysis of *P. ostreatus* MnP is prevented by low productivity. A recombinant gene expression system is very effective to overproduce a enzyme encoding by the gene. It also permits us to analyze contribution of each amino acid residue for the enzyme activity using the site-directed mutagenesis technique. To date MnPs and LiPs from *P. chrysosporium* have been tried to express in heterologous expression systems including *E. coli*, yeast and *Aspergillus oryzae*\(^18-22\). Some of them gave trace activity and it was reported refolding *E. coli* proteins were effective to recover the enzyme activity. However the rate of recovery was about 10% of the total enzyme purified\(^18,19,21,22\). Whereas, homologous expression system was developed for *P. chrysosporium*\(^23,28\). The system provides us active and native enzyme of interest. Property of the enzymes were analyzed using these expression systems\(^25-30\). In *Pleurotus*, Ruiz-Duenas *et al.* have reported expression of *P. eryngii* MnP in *A. nidulans*. But the productivity is lower level than that in *P. eryngii*\(^31\). Ogawa *et al.* have reported

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that cDNA of *P. ostreatus* MnP isozyme, MnP fused with *Lentinula edodes* expression signals were introduced to *Coprinus cinereus*. Then, high lignin-decolorization and -degradation active recombinants were obtained. In our laboratory, expression of MnP3 in *C. cinereus* was tried using recombinant mnp3 under control of *C. cinereus* tubulin expression signals, however no MnP activity was observed in extracellular and intracellular preparation of the recombinants (unpublished data). In chapter II-2, construction of recombinant genes which constitute mnp3 gene or cDNA fused with *P. ostreatus* housekeeping-gene promoter and terminator, cotransformations of *P. ostreatus* using the constructed transformation system reported in chapter I and characterization of the recombinant strains overexpressing MnP are reported.

Chapter I Development of high efficient transformation system for *P. ostreatus*

I-1 Cloning and characterization of the gene encoding iron-sulfur protein of succinate dehydrogenase from *P. ostreatus*

I-1.1 Introduction

We are interested in developing techniques for gene cloning and transformation in *P. ostreatus* that can be used for strain improvement and permit us to combine molecular and biochemical analysis of the enzymes involved in lignin degradation. To this end, we have set out to isolate a gene that may be used to develop a transformation system. In the plant pathogenic species *Ustilago maydis*, mutation in the gene that encodes the iron-sulfur protein (Ip) subunit of succinate dehydrogenase has been shown to confer a dominant resistance to the systemic fungicide carboxin. A similarly modified gene from *P. ostreatus* would provide a potentially valuable selective marker for the development of transformation vectors. We have shown that dominant mutations to carboxanilide resistance can occur in *P. ostreatus*. In this section, we describe the isolation and characterization of the gene encoding the *P. ostreatus* Ip subunit as a first step towards determining whether mutations in this gene also conferers carboxin resistance in this species.

Carboxanilides are potent inhibitors of the mitochondrial complex II from fungi, bacteria, and animal tissues. Complex II generally contains four polypeptide subunits encoded by nuclear genes. Two large subunits constitute the succinate dehydrogenase (SDH) part of the complex II and these are flavoprotein (Fp) and iron-sulfur protein (Ip). Cytochrome *b*, which is an integral membrane protein, represents the two small subunits and is required for the reduction of ubiquinone. The Ip subunit contains three iron-sulfur centers known as centers 1, 2 and 3, each made up of clusters of cysteine residues within the peptide, non-heme iron and labile sulfides (reviewed by B.A.C. Ackrell et al.). Although the precise mechanism of carboxin inhibition is still unclear, these inhibitors have been assumed to act on the ubiquinone reduction by interrupting electron transfer between Center 3 and ubiquinone. The mutation in *U. maydis*, that confers resistance to carboxin is a single amino acid substitution (His257→Leu) in Center 3 of the Ip subunit.

I-1.2 Materials and methods

**Strains, media and plasmid**

Through this study, *Pleurotus ostreatus* #261 (ATCC66576) was used. This strain was grown in Potato Dextrose Agar (Difco) for maintenance. *Escherichia coli* JM109 was used for routine recombinant DNA experiments that required a bacterial host and grown in Luria-Bertani medium. The pGEM-T vector (Promega) was used for cloning of PCR products.

**Cloning the genomic fragments**

*P. ostreatus* Ip subunit gene, *sdil* was cloned using normal PCR reaction and the cassette-primer PCR technique. TaKaRa Ex Taq DNA polymerase, Takara Biochemicals, which ensures high sequence fidelity during the polymerase reaction was used for the normal PCR reaction. The TaKaRa LA PCR in vitro cloning kit, Takara Biochemicals was used for the cassette-primer PCR technique. The sequence appears in DDBJ, EMBL and GenBank nucleotide sequence database with the accession number AB007361.

**Cloning the cDNA fragments**

The actual coding sequence of *sdil* was confirmed by cDNA isolation using the rapid amplification of cDNA ends (RACE) technique. Poly A tailed RNA was prepared from mycerium cultured in liquid medium and served as template in RT-PCR using appropriate primers.

**I-1.3 Results and discussion**

Comparison of genomic and cDNA sequences identified an open reading frame encoding a polypeptide of 268 amino acids which is interrupted by five introns ranging in size from 47-58 nt. An AATAAA sequence is present 20 nt downstream of the stop codon and represents a putative polyadenylation site. Two classes of 5’RACE products were consistently identified, one which initiated at -45 and the other at -55 nucleotides upstream of the first ATG codon. This suggests that *sdil* may have two transcriptional start sites. The sequences appear in DDBJ, EMBL and GenBank nucleotide sequence database with the accession number AB007362 and AB007363.

Two distinct cDNA species were identified in the RACE products that differed in 8 nucleotide positions (indicated in Fig. 1-1) indicating that strain #261 of *P. ostreatus* contains two distinctive *sdil* genes. Both cDNAs encoded exactly the same protein sequence so these were unlikely to be the products of two genes encoding different isoforms. Strain #261 is a dikaryon and, therefore, contains two genetically different nuclei. It remains to be determined whether there is more than one gene, or the cDNAs represent the products of two different alleles of *sdil*.

When the *P. ostreatus* Ip subunit amino acid sequence is compared to that of the corresponding protein from *U. maydis*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* and human there is seen to be 66, 66, 58% and 61% identity, respectively. This high conservation extends to the prokaryotic Ip subunit from *E. coli* in which there is 58% identity. In particular, the highest correspondence, 89-96%, is in the regions containing the cysteine-rich clusters. From these data we conclude that the basi-diomycetous fungi utilize the same succinate : ubiquinone oxidoreduction system as other eukaryotic species.

Dominant drug resistance markers have proved to be very useful for developing transformation systems in fungi.
and avoid the need to isolate auxotrophic strains and clone the appropriate metabolic genes that will complement their defects. In *U. maydis*, a mutant gene conferring resistance to carboxin, *Cbx*<sup>5</sup>, was isolated from a drug resistant mutant and used to develop plasmids for transformation<sup>43</sup>. The *Cbx*<sup>5</sup> mutation is in the gene encoding the Ip subunit and causes a single amino acid substitution (His<sub>239</sub> → Leu) in the third cysteine-rich cluster<sup>34</sup>. The corresponding histidine residue is conserved in the *P. ostreatus* polypeptide (His<sub>239</sub>) and is marked by an arrow in Fig. 1-2. It will be interesting to determine whether or not an equivalent substitution in the *P. ostreatus* polypeptide confers resistance to carboxin. We have successfully isolated several *P. ostreatus* strains resistant to the carboxanilide fungicide flutolanil<sup>35</sup>. In most of these mutants the resistance is dominant and maintained stably during mitotic and meiotic cell divisions. Analysis of the succinate: ubiquinone oxidoreduction system in these mutants promises to provide a better understanding of the mechanism of inhibition and binding sites of carboxa-
Fig. 1-2. Comparison of amino acid sequence of Ip subunits. Only the positions where the amino acid sequence differs from that of the *P. ostreatus* Ip subunit are listed for the other species. Hyphens and asterisks indicate gaps and C-terminal end, respectively. Three cysteine-rich clusters required for the formation of iron-sulfur centers are indicated by boxes. Arrow indicates the conserved histidine residue responsible for the determination of carboxin sensitivity in *Ustilago maydis.*

nP. ostreatus; Um, *U. maydis,* Sc, *S. cerevisiae,* Hu, human, Dm, *D. melanogaster,* Ec, *E. coli.*

1-2 Isolation and sequence analysis of the promoter and an allelic sequence of iron-sulfur protein subunit gene from a white-rot fungus, *Pleurotus ostreatus*

1-2.1 Introduction

In generally, promoters are reported to be less compatible in the basidiomycetes. In this context, isolation of a promoter sequence from *P. ostreatus* has been desired because it will provide us effective expressions of various genes, including selectable markers and genes encoding industrially useful enzymes in this species. We have cloned the structural gene of *sdil* and its transcript from a wild-type *P. ostreatus* strain, shown in section I. In the course of this experiment, the second-type cDNA sequence was also isolated. The second-type cDNA was different in eight nucleotide positions from *sdil* cDNA but the deduced amino-acid sequence was identical to that of *sdil.* We report here the isolation and sequence analyses of the *sdi I* promoter region and the genomic clone of the allelic sequence.

1-2.2 Materials and methods

**Monokaryotic strains**

Several monokaryotic progeny which were isolated from basidiocarps of *P. ostreatus* #261 were used in Southern blot and the genetic analysis of *sdil.*

Cloning of *sdil* promoter and another Ip subunit gene

We attempted to clone the promoter region of *sdil* using the normal PCR and the cassette-primer PCR. The 5'-flanking sequences of *sdil* and the second-type sequence were cloned. The structural gene for the second-type cDNA sequence was amplified from the genomic DNA using the normal PCR and the cassette-primer PCR. The 5'-flanking sequences of *sdil* and the second-type sequence were cloned. The structural gene for the second-type cDNA sequence was amplified from the genomic DNA using the normal PCR and the cassette-primer PCR. The 5'-flanking sequences of *sdil* and the second-type sequence were cloned. The structural gene for the second-type cDNA sequence was amplified from the genomic DNA using the normal PCR and the cassette-primer PCR. The 5'-flanking sequences of *sdil* and the second-type sequence were cloned. The structural gene for the second-type cDNA sequence was amplified from the genomic DNA using the normal PCR and the cassette-primer PCR.
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**Fig. 1-3.** Nucleotide sequence of the promoter region of *P. ostreatus* sdi1. Small letters indicate the coding sequence and the first ATG is bolded. Putative CAAT and TATA boxes are indicated by boxes. Motifs of the binding site for *S. cerevisiae* Adr1p are underlined.

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**Fig. 1-4.** Southern blot analysis of genomic DNA extracted from a monokaryotic progeny of strain #261. Five mg of DNA samples applied in each lane were probed with PCR product containing sdi1. The DNA samples were digested with: lane 1, *EcoR*I; lane 2, *SacI*; lane 3, *KpnI*; lane 4, *ApaI*; lane 5, *SalI*; lane 6, *SphI*. Undigested DNA was applied in lane 7.

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**Fig. 1-5.** *Apa I* assay and confirmation of allelic sequence in *P. ostreatus* strains. PCR fragments containing sdi1 sequence from each strain amplified with primers, R1 and R3 (see Materials and methods) were digested with *Apa I* and electrophoresed on a 0.7% agarose gel. Lane 1, parental dikaryon, #261; lane 2–6, monokaryotic progenies from strain #261. Lane M; 1/4*EcoT14* as a marker DNA.

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**Fig. 1-6.** Southern blot analysis of genomic DNA extracted from a monokaryotic progeny of strain #261. Five mg of DNA samples applied in each lane were probed with PCR product containing sdi1. The DNA samples were digested with: lane 1, *EcoR*I; lane 2, *SacI*; lane 3, *KpnI*; lane 4, *ApaI*; lane 5, *SalI*; lane 6, *SphI*. Undigested DNA was applied in lane 7.

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**Fig. 1-7.** *Apa I* assay and confirmation of allelic sequence in *P. ostreatus* strains. PCR fragments containing sdi1 sequence from each strain amplified with primers, R1 and R3 (see Materials and methods) were digested with *Apa I* and electrophoresed on a 0.7% agarose gel. Lane 1, parental dikaryon, #261; lane 2–6, monokaryotic progenies from strain #261. Lane M; 1/4*EcoT14* as a marker DNA.
using specific primers resulted in the isolation of a novel sequence which was very similar to \textit{sdil}. A comparison between the nucleotide sequence of this PCR fragment and the previously cloned second-type CDNA revealed that there was no difference in the coding sequences and each of this gene and \textit{sdil} was interrupted by five introns at the same positions.

All of the introns found in this gene abode by GT-AG splicing rule and nucleotide sequences of the introns differed at 11 positions from \textit{sdil} intron sequences. A stretch of ATTAAA which was similar to the consensus sequence of the polyadenylation signal, AATAAA, is also present at the 20 nucleotides downstream of the stop codon.

\textit{Southern blot analysis and Apa I assay}

In order to determine the copy number of Ip subunit genes, Southern blot analysis was performed using a monokaryotic progeny derived from basidiospores of \#261. When genomic DNA fragments digested with various restriction enzymes were probed with \textit{sdil} sequence, a single hybridization signal was detected in every digest of every monokaryon’s DNA. The representative result for a monokaryotic progeny of \#261 is shown in Fig. 1-4. The monokaryons were divided into two groups according to the hybridization patterns and the hybridization patterns of parental strain, \#261 contained the patterns of both groups (data not shown). This suggested that \textit{sdil} and the second-type gene were allelic in \#261 and no similar sequences other than them exist. To confirm that these hybridized bands were \textit{sdil} and the second-type sequence, further experiment was performed as described below.

The second-type gene contained an \textit{Apa} I site which did not exist in \textit{sdil}. So \textit{Apa} I-digests of the PCR-amplified fragment of \textit{sdil} could be used to distinguish which type(s) of genes was/were contained in a certain \textit{P. ostreatus} strain. This restriction fragment length polymorphism assay was designated as \textit{Apa} I assay (Fig. 1-5). Using progenies derived from basidiospores of \#261, a series of \textit{Apa} I assay demonstrated that the monokaryotic strains contained only one of the two genes, while the parental dikaryon contained both of them. The classification of monokaryons by the \textit{Southern blot analysis}. This confirmed that \textit{sdil} and the second-type gene were allelic and not the distinct members of a gene family.

Using an \textit{Apa} I-assay, a linkage between the \textit{sdil} genotypes and other characteristics can be analyzed easily. Especially, analysis for a linkage between the \textit{sdil} genotypes and the flutolanil-resistance phenotype is useful to investigate whether the drug-resistance links to \textit{sdil} or not in a particular mutant strain. Using monokaryotic progenies of a flutolanil-resistant mutant MA206, such an assay was performed. However, no linkage between the drug resistance and the \textit{sdil} genotypes was observed among the progenies of this mutant strain (data not shown).

1-2. Discussion

This is the first report on cloning of a promoter sequence of the \textit{P. ostreatus} genes which are expressed in the primary metabolic phase. It was shown that \textit{sdil} has no homologous genes in the haploid genome of \textit{P. ostreatus}. From this result, it is unlikely that \textit{P. ostreatus} contains more than one isoforms of Ip subunit protein for complex II, which is the case in some other organisms such as \textit{Haemonchus contortus}\textsuperscript{40}. In this context, it is conceivable that \textit{sdil} should be expressed constitutively through the aerobic growth of this organism. The promoter region should be useful to construct selectable marker genes for transformation and to overexpress various enzymes in the primary metabolic phase in \textit{P. ostreatus}. According to this idea, we have constructed selectable markers consisting of the \textit{sdil} promoter and terminator and heterologous drug-resistance genes. Examination of \textit{P. ostreatus} transformation with these constructs are in progress.

In a flutolanil-resistant mutant strain MA206, it was shown that the drug resistance was not linked to the \textit{sdil} genotypes classified by \textit{Apa} I digestion. It is conceivable that, in this strain, the mutation leading to the drug resistance occurred in some other genes, such as structural genes for remaining complex II-subunits. In fact, existence of the another nuclear locus, \textit{oxr-2}, conferring carboxin resistance has been reported in \textit{U. maydis}\textsuperscript{35}.

Anyway, the \textit{Apa} I-assy provides a useful method to screen a \textit{P. ostreatus} mutant strain with the flutolanil-resistant phenotype linked to an \textit{sdil} genotype. Furthermore, an attempt to transform \textit{P. ostreatus} with a modified \textit{sdil} gene containing the same amino-acid substitution as in \textit{U. maydis} \textit{Cbx}\textsuperscript{3} is also being undertaken.

1-3 Stable transformation of \textit{P. ostreatus} to hygromycin B resistance and effect of restriction enzyme-mediated integration

1-3.1 Introduction

Most transformation system for basidiomycete are based on nutritional complementation of auxotrophic mutations with equivalent wild-type homologous or heterologous genes. However, isolation and characterization of such mutants is time consuming. Therefore the extension of this strategy to a wider range of species is limited. An alternative selection strategy is the use of drug resistance genes as selective markers. Peng et al. transformed \textit{P. ostreatus} to hygromycin B resistance using plasmids pPO1 and pPO2 containing an insert of bacteriophage origin and \textit{E. coli} hygromycin B phosphotransferase gene (\textit{hph}) fused by \textit{Aspergillus} signals as a marker used. The plasmids was extrachromosomally replicated and unstable. However integrative type transformation events were rarely found, those transformants were also unstable and lost the hygromycin B-resistant phenotype\textsuperscript{40}. Because this transformation system has not been reappeared in other group containing our laboratory and can not be use for strain improvement due to instability, we try to transform \textit{P. ostreatus} to stable hygromycin B resistance using pLG-hph containing \textit{hph} fused signals of \textit{Lentinula edodes} glyceraldehyde-3-phosphate dehydrogenase gene (\textit{gpd}) as a marker. It was previously reported that pLG-hph was utilized for \textit{L. edodes} transformation successfully\textsuperscript{50}.

Here, the successful transformation of \textit{P. ostreatus} with pLG-hph is reported. Moreover, restriction enzyme-mediated integration (REMI) was tested to increase transformation efficiency.

1-3.2 Materials and methods

Transformation of \textit{P. ostreatus}

Strain \#261 was selected as the host strain. The fungus
was stationary cultivated in SMY liquid medium for three days at 28°C. The mycelia was collected by gauze, washed by water and transferred to filter paper to remove extra moisture. The mycelia was suspend in MM buffer (0.5 M mannitol, 50 mM maleate buffer (pH 5.5)) containing 0.2% Novozyme™ 234 Cell Wall Lysing Enzyme (CALIBIOCHEM), 0.05% Zymolyase (SEIKAGAKU CORPORATION), 0.01% Chitinase (SIGMA). Incubation was done for 1 hour at 28°C with constant agitation at 75 r.p.m. Protoplasts were separated from hyphal debris by filtration through a G-2 glass filter, collected by centrifugation at 500 G for 10 min at 4°C, washed using MM buffer and rewashed using MMC buffer (0.5 M mannitol, 25 mM CaCl₂, 50 mM maleate buffer (pH 5.5)), followed by resuspension in MMC buffer to give a final concentration of $1 \times 10^8$ protoplasts/ml. 30 µl of protoplast solution ($1 \times 10^8$ protoplasts/ml) aliquot was mixed with $12.5 \mu l$ of PEG buffer (25% PEG 4000, 10 mM Tris (pH 7.5), 25 mM CaCl₂) and 5-10 µg of pLG-hph. When REMI, various amount of Bgl II was added to the mixture. The mixture was placed on ice for 15 minutes, mixed with 1 ml of PEG buffer and incubated for 5 minutes at room temperature and mixed with 1 ml of STC buffer (1.2 M sorbitol, 10 mM Tris (pH 7.5), 10 mM CaCl₂). Then the mixture was spreaded on SMYM (SMY containing 0.6 M mannitol as an osmotic stabilizer) plate containing 1.5% agar and 100 µg/ml hygromycin B. The plates were incubated for 1–2 weeks at 28°C.

**Southern blot analysis**

Southern blot analyses of intact and BglII-digested DNA extracted from the transformants TL1, 2, 11 were performed using the PCR amplified partial sequence of hph as a probe. The labeling, hybridization and signal detection were done using DIG DNA Labeling and Detection Kit, BOEHRINGER MANNHEIM, according to the reference[4](#). Measurement of growth rate and stability of hygromycin B resistance of the transformants

pLG-hph transformant was subcultivated. It was inoculated to edge of potato dextrose agar (PDA) plate supplemented no hygromycin B and incubated at 28°C until the mycelia reaches to opposite edge of the plate. Then mycelium of the opposite edge was pick up and inoculated to new PDA plate for subcultivation and to PDA plate containing 100 µg/ml hygromycin B to assay stability of hygromycin B resistance. These operations were repeated for 3 months.

**1-3. Results**

**Transformation of P. ostreatus**

Transformation mixture was poured on selective plate and incubated for about 5–7 days, then a number of germinating protoplast were microscopically observed (data not shown). But, the most of them did not grow more than the stage. Successively growing clones were defined as transformants. The transformation efficiency was about 1 transformant/10 µg pLG-hph. Growth rate of the transformants was generally low in comparison with that of wild-type strain (data not shown).

**PCR and Southern blot analysis of pLG-hph transformants**

To investigate the fate of transforming DNA, PCR which amplified hph fragment using transformant DNA as a template was performed. In the result, the fragments of purpose size were amplified in all transformants (data not shown). Moreover, southern blot analysis of the Bgl II digested and undigested genomic DNA using hph sequence as a probe was performed. Without digestion, specific signals were detected and comigrated with chromosomes DNA. With digestion, many hybridization bands were found in addition to the band at 5.85 kb which was size of pLG-hph. These indicate that many copies of the pLG-hph were integrated in the host chromosome DNA (Fig. 1-6).

**Stability of transforming DNA and its phenotype**

All tested transformants were stable in hygromycin B resistance during mitotic cell division. On the other hand, the strains derived from basidiospores of pLG-hph transformants were hygromycin B resistant (data not shown). It indicates that the phenotype was also stable during meiotic cell division.

**REMI**

To increase transformation efficiency, we tested REMI method. pLG-hph contain unique restriction site for Bgl II represents undigested DNA and Lane 5–9 represents Bgl II digested DNA: wild-type DNA (lane 1, 5), TL1 (lane 2, 6), TL2 (lane 3, 7), TL1 (lane 4, 8), pLG-hph (lane 9). The size of pLG-hph is 5.85 kb.

To increase transformation efficiency, we tested REMI method. pLG-hph contain unique restriction site for Bgl II[30]. The effect of Bgl II activity on transformation efficiency was measured. The most optimal condition is 20 unit of Bgl II in which transformation efficiency was is 8 times as that without REMI. Decrease in the number of transformants were found in the presence of the higher amount of restriction enzymes (Fig. 1-7).

**1-3.4 Discussion**

We constructed stable and reapprarent transformation system for P. ostreatus using hygromycin B-resistant marker. However the transformation efficiency is low (about 1 transformant/10 µg plG-hph), REMI method gave a 8 fold increase over transformation efficiency
without REMI (Fig. 1-7).

A number of germinating protoplasts were microscopically observed on early days of cultivation but the most of them did not grow more than the state. No germinating protoplast was observed in protoplasts transformation-treated without pLG-hph (data not shown). These results suggest that introduced hph was transiently expressed and not fixed in the most of germline clones. On the other hand, the drug resistance of the clones defined the transformant was stable in mitotic and meiotic cell division.

This novel transformation system is suitable for improvement of P. ostreatus strain. We already constructed stably transformation system for P. ostreatus using CbxR51, but there is sufficient significance in the alternative method.

1-4 Enhancement of transformation efficiency in basidiomycete P. ostreatus by single-strand carrier DNA

1-4.1 Introduction

In 1-1 and 1-2, cloning and characterized of sdiI were reported. Subsequently, the single amino acid substitution (His257→Leu) in Center 3 of sdiI was introduced to construct carboxin resistant marker, CbxR.

A homologous transformation system for P. ostreatus using CbxR reported in reference (Honda, 2000 #18). However the system is stable and useful, the transformation efficiency was not so high, about 5 transformants/μg vector plasmid.

Here we report single-strand (ss) carrier DNA gave about a 51-fold increase over transformation efficiency without carrier in the system. This is the first report that ss DNA has an effect on increase of transformation efficiency using fungal protoplasts.

1-4.2 Materials and methods

Preparation of carrier DNA

λ phage DNA (TAKARA BIOMEDICALS) was used as a carrier DNA either in double-strand (ds) and ss form. The DNA was dissolved in TE buffer at a concentration of 10 mg/ml. Heat denaturation was carried out for ss DNA preparation.

Transformation of P. ostreatus

P. ostreatus #261 was selected as the host strain. Protoplast formation and transformation of P. ostreatus was carried out according to the method as described by section III. 50 μl of P. ostreatus protoplast solution (1×10⁷ protoplasts) was mixed with 12.5 μl of PEG buffer (25% PEG 4000, 10 mM Tris (pH 7.5), 25 mM CaCl₂) and 1 μg of pTM1. Then, the 50 μg of ds or ss λ DNA was added to the transformation mixture as a carrier DNA. The mixture was placed on ice for 15 minutes, mixed with 1 ml of PEG buffer and incubated for 5 minutes at room temperature and mixed with 1 ml of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM CaCl₂). The mixture was spreaded on SMYM (SMY containing 0.6 M mannitol as an osmotic stabilizer) plate containing 1.5% agar and 2 μg/ml carboxin (Accu Standard Inc.). After the incubation at 28°C for 10 days, the number of colonies was counted.

Southern blot analysis

Southern blot analysis of intact and HindIII-digested DNA extracted from the transformants was performed using λ DNA sequence as a probe. The labeling, hybridization and signal detection were done using DIG DNA Labeling and Detection Kit, BOEHRINGER MANNHEIM, according to the reference#35).

1-4.3 Results

The number of the carboxin-resistant transformants in the presence or absence of the carrier DNA were summarized in a Table 1-1. When P. ostreatus was transformed with 1 μg of pTM1, 1–5 transformants/μg DNA were obtained in these condition. The number of the transformants increased as the amount of pTM1 increased to 5 μg. While ds λ DNA was added to the transforming mixture, 13–41 transformants were obtained per 1 μg of pTM1. Moreover, ss λ DNA exhibited further increases in the efficiency, up to 190 transformants/μg pTM1 DNA in average. The effect of the ss carrier DNA was 51-fold increase over transformation levels using pTM1 alone. The drug resistance phenotype of the transformants which were obtained with the carrier DNA was stably maintained over several subculture on non selective media (data not shown).

To check if the carrier DNA was also incorporated into the host cell, we carried out Southern blot analysis using DIG-labeled λ DNA as a probe. For all the five transformants (T1–T5) obtained from the experiments using ss λ DNA as a carrier, significant signals were shown.)

Table 2-1. The percent identity of amino-acid sequences among known LiPs and MnPs.

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<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>10</th>
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<td>P. ostreatus MnP MnF</td>
<td>64.8</td>
<td>67.4</td>
<td>45.6</td>
<td>54.3</td>
<td>54.5</td>
<td>54.4</td>
<td>59.3</td>
<td>59.5</td>
<td>59.9</td>
<td>55.2</td>
<td>58.4</td>
<td>56.5</td>
<td></td>
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<tr>
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<td>44.6</td>
<td>49.0</td>
<td>48.7</td>
<td>48.4</td>
<td>51.6</td>
<td>55.6</td>
<td>52.7</td>
<td>50.7</td>
<td>52.2</td>
<td>53.2</td>
<td>53.5</td>
<td></td>
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<td>C. subermispora MnP Cs-MnP</td>
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<td>69.2</td>
<td>93.1</td>
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<tr>
<td>P. chrysosporium MnP MnF</td>
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<td>46.9</td>
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<tr>
<td>P. chrysosporium MnP MnF</td>
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<td>61.1</td>
<td>61.0</td>
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<tr>
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<td>91.3</td>
<td>91.4</td>
<td>91.5</td>
<td>91.6</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>T. versicolor LiP LPG1</td>
<td>61.4</td>
<td>61.3</td>
<td>61.2</td>
<td>61.1</td>
<td>61.0</td>
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<td>60.6</td>
<td>60.5</td>
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<td>B. adusta LiP</td>
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<td>60.6</td>
<td>60.5</td>
<td>60.4</td>
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</table>
detected co-migrated with chromosomal DNA (Fig 1-8). It was demonstrated that the ss carrier DNA was integrated in the host chromosome.

Discussion

Carrier DNA was utilized in transformations for other fungi. Orr-Weaver et al. and Austin et al., used ds carrier DNA in the transformations of yeast protoplast and Neurospora crassa protoplast, respectively. These resulted in increased transformation efficiencies. The ds carrier DNA effect was perhaps due to protection against nuclease because it occurred only when transforming DNA was very small. Schiestl et al. reported that ds carrier DNA also had effect in intact cell transformation of yeast. Moreover, they found that ss carrier DNA was more effective than ds carrier DNA in the method. But, ss carrier DNA did not yield any increased transformation efficiency to induce DNA uptake in the transformation of yeast protoplast, indicating a difference in the mechanism of transformation with the two methods. Gietz et al. proposed that carrier DNA competes with transforming DNA for binding sites on the cell wall, resulting in more transforming DNA being available for uptake and higher transformation efficiency in intact cell transformation of yeast. Moreover, the increase of transformation efficiency using ss carrier DNA was explained by that ss carrier DNA binds more effectively to the cell wall than ds carrier DNA and is not a good substrate for uptake. In our experiment, ds carrier DNA also increased the efficiency of the transformation for P. ostreatus protoplast. Furthermore, surprisingly, ss carrier DNA gave a 6-fold increase over transformation levels with ds carrier DNA in this method. The fact that cell wall was removed in this experiment indicates the mechanism different to that proposed Gietz et al.

Several transformation systems for edible mushrooms containing P. ostreatus have been reported to date. But their efficiencies are low, 1–5 transformants/µg vector plasmid (1–15). We constructed the most high efficient transformation system for P. ostreatus using ss carrier DNA. This method is useful for genetic experiments which require high transformation efficiency. We are studying whether ss carrier DNA enhances the efficiencies in protoplast transformations of other mushrooms.

II-1 Isolation of cDNA and genomic fragments encoding the major manganese peroxidase isozyme from the white-rot basidiomycete Pleurotus ostreatus

II-1.1 Introduction

MnP is H2O2-requiring heme glycoprotein enzymes and oxidize MnIII to MnII. Using transient-state kinetics, it was suggested that the actual substrate of MnP is MnII forming a complex with an organic dicarboxylic acid such as oxalate, rather than MnII itself. The substrate oxidized by MnP then oxidizes phenolic substituents of lignin and possibly nonphenolic substituents via specific mediator molecules such as a peroxidized lipid. Generally, MnPs occur as a series of isozymes encoded by a family of genes. Many MnP isozymes have been purified and their genes were cloned from various white-rot basidiomycetes including Trametes versicolor, Ceriporiopsis subvermispora and P. chrysosporium. X-ray crystallographic and site-directed mutagenesis studies have defined the Mn binding site in the P. chrysosporium MnP1 (H3) isozyme.

P. ostreatus has been reported to secrete a series of MnPs into the culture medium, while no lip activity was observed under various culture conditions. In this organism, MnPs are considered to be the key enzymes in the lignin degradation system. Cloning of genes encoding the MnP isozymes will permit us to determine their primary structures and provide clues into their evolutionary relationships and individual contributions to lignin biodegradation. One of the genes from P. ostreatus strain IFO 36160 has been isolated and sequenced.

In our recent experiments, another strain, IS1, has been shown to secrete at least three isozymes of MnP at high levels into the liquid culture medium. The major isoenzyme, MnP3, was purified and its N-terminal amino acid sequence was determined (unpublished data). Comparison of the sequence with that of the previously cloned MnP from the IFO 30160 strain suggested that these two MnPs are not allelic forms and are encoded by distinct genes. We report here cloning of the cDNA and the structural gene fragments of P. ostreatus MnP3, with the aim of understanding its structure, function and evolutionary relationship with other MnPs or LiPs secreted by various white-rot basidiomycetes.

II-1.2 Materials and methods

Strains and media

P. ostreatus IS1 is a dikaryotic strain which was selected as a good producer of MnP isozymes. To prepare mRNA for RT-PCR, this strain was grown in glucose-pepetone medium (glucose 2%, polypeptone 0.5%, yeast extract 0.2%, K2HPO4 0.1%, MgSO4·7H2O 0.05%) supplemented with 0.5 mM MnSO4 and hot water-extracted wheat bran extract (175 g of wheat bran was autoclaved with 500 ml of water for 20 minutes at 120°C and...
centrifuged at 10,000 r.p.m. for 10 minutes. The recovered supernatant was used as the supplement for 1 liter of medium.

mRNA extraction

Strain IS1 was cultured for 11 days at 28°C. Total mRNA was prepared from the mycelia using the RNA Isolation Kit (Stratagene), and poly (A)+ RNA was purified with Oligotex (Daiichi Pure Chemicals).

Cloning of the cDNA and the genomic fragments

For cloning mp3 cDNA, 5'-RACE and 3'-RACE were performed. The nucleotide sequences of the cDNA and structural gene encoding P. ostreatus MnP3 are shown in Fig. 2-1. The deduced amino acid sequences are shown below the corresponding nucleotide sequence. Arrows indicate the positions and directions of the oligonucleotide primers used for PCR. The underlined amino acid sequence was confirmed by amino acid sequencing of the N-terminal domain of the purified enzyme. The amino acids indicated by arrowheads are putative residues constituting the Mn binding site. The conserved amino acids responsible for the peroxidase function are indicated by boxes. The amino acid sequence marked by a dashed underline is the putative N-glycosylation site.
performed\(^4\) using primers, MnPcDNA (5'-GCA GTG AGA GCT GGG AAG GG-3'), 5RACE (5'-CTC TGC AGT GGA TTT GGG AC-3'), dT17adapter (5'-GAC AGA GCT GGG AAG GG-3'), 5RACE2 (5'-CTC TGC AGT CGA TTA TTA TTA TTA TT-3') and POMNP (5'-GCN AAY GCN GCN TGY TGY GTG-3') (Fig. 2-1). POMNP has a sequence corresponding to the determined N-terminal end of the purified MnP3. The structural gene was amplified using the primers MnP3G1 and MnP3G2 (Fig. 2-1), with genomic DNA extracted from *P. ostreatus* IS1 as the template.

**Nucleotide sequence analysis**

The nucleotide sequences of the cloned fragments were analyzed using an ABI 377 DNA sequencer by the dye-terminator method. The determined cDNA and structural gene sequences appear in DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers AB011546 and AB016519, respectively.

### 11-1.3 Results

**Structure of mnp3 coding sequence**

Sequence analysis indicated that the isolated structural gene, mnp3, contains a coding sequence of 1074 nucleotides which is interrupted by ten introns and encodes a protein of 358 amino acid residues (Fig. 2-1). The number of introns and some of their positions in mnp3 are different from those of the previously cloned *P. ostreatus* mnp5\(^6\). All the 5' RACE products isolated from independent experiments initiated-29 nucleotides upstream of the first ATG codon, suggesting that this was the start site for mnp3 transcription. The open reading frame ended with a TAA terminator codon, followed by 121 nucleotides in the 3' untranslated region of the cDNA. Typical polyadenylation signals were not present, as is sometimes the case with basidiomyceteous genes.

**Deduced amino acid sequence of MnP3**

The predicted amino acid sequence (Fig. 2-1) contains a 26-amino acid signal peptide sequence followed by a sequence identical to the determined N-terminal sequence of the purified MnP3 isozyme (unpublished data). The signal peptide sequence contained specific motifs for removal by a signal peptidase and Kex2-related endopeptidase. The amino acid residues known to be involved in peroxidase functions, i.e. the distal His and Arg, and the proximal His, were conserved in MnP3. The residues which constitute the Mn binding site of *MnP* isozyme were also conserved (Fig. 2-1). The putative MnP3 amino acids sequence revealed that it is new enzyme which have both features of *P. chrysosporium* MnP and LiP, as other *Pleurotus* MnPs\(^5^-^6\). MnP3 has component amino acids of a access channel of heme cavity (Val-177, Gln-215, Phe-76, Glu-140)\(^6\), proposed lignin-binding site (His-232) and the possible electron transfer pathway (Asp-231) shown in LiP\(^2\)

Overall sequence homology analysis (Table 2-1) revealed that MnP3 has a higher degree of homology to *T. versicolor* MP2 and the previously cloned *P. ostreatus* MnP1 (67.4% and 64.6%), medium homology to LiPs from various species (59.3-59.9%)\(^6\), and a low level of homology to MnPs from *P. chrysosporium*\(^6\), \(^7\), \(^7\), \(^43\) and *C. subvermispora* (45.6-54.5%)\(^6\). The unrooted phylogenetic tree based on the distances among amino acid sequences of LiPs and MnPs (Fig. 2-2) indicated that MnP3 has diverged far from MnPs of *P. chrysosporium* and *C. subvermispora*, and is rather close to a group characterized by LiPs and *T. versicolor* MnP.

Three other structural characteristics were found to be consistent with the phylogenetetic tree. It has been reported that the positions of ten cysteine residues involved

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**Table 2-1. Number of the carboxin-resistance transformants in *P. ostreatus* transformation.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pTM1 (1 µg)</th>
<th>pTM (5 µg)</th>
<th>pTM1 (1 µg)+ds IDNA (50 µg)</th>
<th>pTM1 (1 µg)+ss IDNA (50 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>-</td>
<td>13 (13)</td>
<td>198 (198)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>26</td>
<td>41 ( 8)</td>
<td>194 ( 39)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>25</td>
<td>38 ( 8)</td>
<td>178 ( 36)</td>
</tr>
<tr>
<td>Average</td>
<td>3.7</td>
<td>25.5</td>
<td>30.7 ( 8)</td>
<td>190 ( 51)</td>
</tr>
</tbody>
</table>

The parenthesized numbers indicate what times for that without carrier.
in disulfide bond formation to maintain the tertiary structure of the enzyme are conserved in the MnPs from *P. chrysosporium* and *C. subvermispora* 61,75). The last two of these cystein residues are present in the extended C-terminal tail. *P. ostreatus* MnPs lacks the extended C-terminal tail, and hence also the last two cysteine residues as well as the LiPs and *T. versicolor* MP2 69. MnPs from *P. chrysosporium* and *C. subvermispora* have a 7–10 amino acid residues insertion around residue number 240 (*P. chrysosporium* MnP3 numbers) 61,62,73,74), which is not present in the amino acid sequences of the LiPs or *T. versicolor* MP2 69). Neither of the *P. ostreatus* MnPs contain this insertion sequence (Fig. 2-1) 64). It has been demonstrated that *P. chrysosporium* MnP1 has an N-glycosylation site at Asn131 75), whereas *T. versicolor* MP2 and LPG1 have such sites at a different position, Asn103 (numbered according to MP2) 76). The deduced amino acid sequences of both *P. ostreatus* MnPs contained one putative N-glycosylation site at positions corresponding to those of the *T. versicolor* peroxidases (Fig. 2-1) 69).

These results suggested that *P. ostreatus* MnPs and *T. versicolor* MP2 possess characteristics intermediate to those of LiPs and MnPs from *P. chrysosporium*. Since some but not all of the introns share their positions in these genes, it is possible that these fungal peroxidase genes have developed from a common ancestor gene. It seems that in *P. chrysosporium*, peroxidases have gained more specialized properties during evolution than those in *T. versicolor* or *P. ostreatus*. In this context, it is of interest to determine how each enzyme has obtained, or even lost, its specificity for various substrates. Further analysis of the fungal peroxidases, including determination of three-dimensional structure or site-directed mutagenesis of the substrate binding sites, will provide some insight into the evolution of these molecules.

**II-2 Homologous expression of recombinant manganese peroxidase genes in *Pleurotus ostreatus***

**II-2.1 Introduction**

While *P. chrysosporium* MnPs act only in a MnII-dependent way, many MnP enzymes from the genus *Pleurotus* react directly with several aromatic compounds in a Mn-dependent way, many MnP enzymes from the genus *Pleurotus* have been reported to date. *Coprinus cinereus* transformants harboring a recombinant *P. ostreatus* mnp fused to *Lentinus edodes* ras expression signals were isolated and characterized 31). It was reported that decolorization of the soluble lignin by the transformants was observed. However, no data for the expression of the introduced mnp is presented in the literature. In our laboratory, similar trials to express a recombinant *P. ostreatus* mnp3 under the control of *C. cinereus* tub expression signals gave no MnP activities either in the extracellular or intracellular preparation of the *C. cinereus* recombinants 80). On the other hand, an isozyme of *P. eryngii* MnPs was reported to be expressed in *Aspergillus nidulans* 15). Although the purified enzyme showed similar properties to the native enzyme, the yield of the enzyme was not so high with the system.

Here, homologous gene expression system in *P. ostreatus* that uses expression signals of *sdil* and a successful overexpression of MnP3 using the CbxR system is reported.

**II-2.2 Materials and methods**

**Construction of plpMg and plpMg**

The fragment of *sdil* terminator was amplified with SDH-pro-No1 (5'-GGG CCT CCA TGG TTC AAT GAT TGT TGT GTT CC-3') which adapts a No1 site to the *sdil* promoter and SDH-ter-Kpnl (5'-GGG GTA CAC AAG TTA ACA GGC ACG G-3') which adapts a Kpnl site to *sdil* terminator, and digested with Kpnl and No1. The coding sequences of mnp3 genomic DNA and cDNA were amplified by PCR using the primers MnP3-No1 (5'-CTTCAACTCCAGGCTAGGCC-3') which adapts a No1 site at the start codon and MnP3-Kpnl (5'-GGG GTA CCG TCA TCA CGA TTT ATG AAG G-3') which adapts a Kpnl site after the termination codon of the MnP3 coding sequence. The amplified genomic or cDNA sequence were ligated with the pGEM-T+ *sdil* terminator fragment using the introduced No1 and Kpnl site to produce pGEM-T+ mnp3+ *sdil* terminator. The fragment of *sdil* promoter was amplified with SDH-pro-No1 and SDH-pro-SpHl (5'-taca tgc atg ctc tga tca tgc cta cac c-3'). The amplified fragment was ligated with the pGEM-T+ mnp3+ *sdil* terminator fragment using the introduced No1 and SpHl site to produce the plasmids, plpMg which contains genomic mnp3 and plpMc which contains mnp3 cDNA (Fig. 2-3).

**Co-transformation of P. ostreatus**

*P. ostreatus* dikaryotic strain #261 (ATCC 66376) was selected as a host strain. *P. ostreatus* was stationary cultivated in SMY liquid medium for three days at 28°C. The mycelia was collected with gauze, washed with water and transferred to filter paper to remove all moisture. It was suspended in MM buffer (0.5 M mannitol, 50 mM maleate buffer (pH 5.3)) containing 0.2% Novozyme™ 234 Cell Wall Lysing Enzyme (CALBIOCHEM); 0.05% Zymolyase (SEIKAGAKU CORPORATION), and 0.01% Chitinase (SIGMA). Incubation was done for 1 hour at 28°C with constant agitation at 75 r.p.m. Protoplasts were separated from hyphal debris by filtration.

oryzae 22,26,28) as well as homologous systems 23,24). In terms of providing active and native enzymes in abundance, homologous gene expression systems are superior to heterologous ones.

For *Pleurotus* MnPs, two heterologous expression systems have been reported to date. *Coprinus cinereus* transformants harboring a recombinant *P. ostreatus* mnp fused to *Lentinus edodes* ras expression signals were isolated and characterized 31). It was reported that decolorization of the soluble lignin by the transformants was observed. However, no data for the expression of the introduced mnp is presented in the literature. In our laboratory, similar trials to express a recombinant *P. ostreatus* mnp3 under the control of *C. cinereus* tub expression signals gave no MnP activities either in the extracellular or intracellular preparation of the *C. cinereus* recombinants 80). On the other hand, an isozyme of *P. eryngii* MnPs was reported to be expressed in *Aspergillus nidulans* 15). Although the purified enzyme showed similar properties to the native enzyme, the yield of the enzyme was not so high with the system.

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through a G-2 glass filter, collected by centrifugation at 500 G for 10 min at 4°C, washed using MM buffer and rewarshed using MMC buffer (0.5 M mannitol, 25 mM CaCl₂, 50 mM maleate buffer (pH 5.5)), and resuspended in MMC buffer to a final concentration of 1 × 10⁹ protoplasts/ml. A 50 µl aliquot of the protoplasts was mixed with 12.5 µl of PEG buffer (25% PEG #4000, 10 mM Tris·HCl (pH 7.5), 25 mM CaCl₂), 1 µg of pTM1 and 10 µg of pIpMg or pIpMc. The mixture was placed on ice for 15 minutes, mixed with 1 ml of PEG buffer and left for 5 minutes at room temperature, and mixed with 1 ml of STC buffer (1.2 M sorbitol, 10 mM Tris (pH 7.5), 10 mM CaCl₂). The mixture was spread on SMYM (SMY containing 0.6 M mannitol as an osmotic stabilizer) agar plates containing 2 µg/ml carboxin. After incubation at 28°C for 4–8 days, colonies were isolated and transferred onto fresh PDA plates containing the same concentration of carboxin.

**Southern blot analysis**

Southern blot analysis of the transformants was performed using the mnp3 coding sequence as a probe. The coding sequence of mnp3 was amplified and labeled with a DIG DNA Labeling and Detection Kit, BOEHRINGER MANNHEIM, according to the reference 45). Primers used were MnP3G1 and MnP3G2 79). Intact and ApaI-digested genomic DNA prepared from the transformants were hybridized.

**Assay of MnP activity**

MnP activity in the culture filtrates of the transformants was measured spectrophotometrically using a reaction mixture containing 0.4 mM guaiacol, 50 mM lactate buffer (pH 4.5), 0.2 mM MnSO₄, and 0.1 mM H₂O₂. MnP activity was assayed by monitoring the absorbance of the reaction mixture at 470 nm. The absorbance reading was compared to an MnP standard curve to determine the MnP activity in the culture filtrates.
activity was calculated by subtracting the activity in the absence of Mn(II) from that in the presence of 0.2 mM MnSO4. One unit of MnP activity was defined as the amount of enzyme which increased by one absorbance per minute at 465 nm.

**Competitive RT-PCR**

Total RNA was extracted from the mycelium cultivated in GP medium for 6 days, using the RNeasy Mini Kit (Qiagen). The RNA (2 μg) was used for reverse transcription (RT), followed by PCR with Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech). poly d(T)12-18 was used as a primer for the RT. Primers used for the competitive PCR were: CM1 (5′-CGT GGC ACT GCT TTC CCA GG-3′) which has the coding sequence of mnp3, CM2 (5′-TGA TCA AAT ATT TGG TCA TCA CG-3′) which has a sequence corresponding to the 3′ untranslated region of mnp3 mRNA, and sdi-primer-11 (5′-GAT GCT CGT GAT GGA CAC TGG TCA TCA CG-3′) which has a sequence corresponding to the 3′ untranslated region of P. ostreatus sdi1 mRNA. A 412-bp fragment will be amplified from the endogenous mnp3 transcripts using the primers, CM1 and CM2. While a 503-bp fragment will be amplified from the recombinant mnp3 transcripts using the primers, CM1 and sdi-primer-11. Each cycle of PCR consisted of template denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute, and DNA extension at 72°C for 1 minute. After 35 cycles, an additional extension step of 15 minutes at 72°C was included. All the PCR-amplified fragments were analyzed on 1% agarose gels. To check for contamination of genomic DNA, a PCR without the reverse transcription was performed as a negative control for all RT-PCRs according to the manufacturer’s instructions. No amplified fragments were observed in these control experiments (data not shown).

**Determination of karyotype**

The karyotype of the transformants was determined by observing clamp connections under a microscope. Some transformants were further analyzed with DAPI staining and mating tests using tester monokaryons, P. ostreatus #261-20, -21, -22 and -25. These tester strains were derived from basidiospores of P. ostreatus #261 and have different mating types.

**II-2.3 Results**

**Co-transformation of Pleurotus ostreatus**

The recombinant plasmids plpMg and plpMc were introduced into P. ostreatus #261 strain by co-transformation with pTM1 containing CbsR marker gene. Carboxin resistant transformants were observed after incubation at 28°C and confirmed to have drug resistance on a secondary screening plate containing carboxin. To check whether the recombinant mnp3 exists in the transformants, PCR amplification of the recombinant mnp3 gene was performed using specific primers (data not shown). Four and three transformants were demonstrated to be co-transformants of the plpMg and plpMc sequence, respectively and collected for further analysis. Southern hybridization of the co-transformants of plpMg was performed to analyze the mode of the integration (Fig. 2-4). When intact DNA of the co-transformants was probed with the mnp3 coding sequence, signals co-migrated with high molecular-weight DNA, suggesting that the introduced recombinant mnp3 sequence was integrated in the chromosomal DNA of the transformants. With ApaI-digested DNA probed with the same sequence, two bands representing each allelic sequence of the endogenous mnp3 were detected for the dikaryotic wild-type strain. On the other hand, at least four additional bands were observed for the co-transformants. These results suggested that the recombinant mnp3 gene was introduced ectopically with multiple copy numbers and that no gene replacements occurred in these transformants.

**Characterization of the co-transformants**

Microscopic observation revealed that some of the

<table>
<thead>
<tr>
<th>Strain</th>
<th>MnP (U/flask)</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMG1</td>
<td>0.195</td>
<td>di</td>
</tr>
<tr>
<td>TMG5</td>
<td>0.975</td>
<td>(2n)</td>
</tr>
<tr>
<td>TMG7</td>
<td>nd</td>
<td>di</td>
</tr>
<tr>
<td>TMG8</td>
<td>nd</td>
<td>di</td>
</tr>
<tr>
<td>TMG9</td>
<td>1.560</td>
<td>mono</td>
</tr>
<tr>
<td>TMG1</td>
<td>0.195</td>
<td>mono</td>
</tr>
<tr>
<td>TMG5</td>
<td>0.390</td>
<td>mono</td>
</tr>
<tr>
<td>TMG8</td>
<td>nd</td>
<td>di</td>
</tr>
<tr>
<td>wt</td>
<td>nd</td>
<td>di</td>
</tr>
</tbody>
</table>

wt: wild type, nd: not detected, di: dikaryon, mono: monokaryon, (2n): putative diploid (see text).

![Fig. 2-4](image-url) Southern blot analysis of the co-transformant DNA probed with mnp3 sequence. Lanes 1 to 4 represent undigested DNA and lanes 5 to 9 represent ApaI-digested DNA. Samples analysed are: wild type (lane 1 and 5), TMG5 (lane 2 and 6), TMG8 (lane 3 and 7), TMG9 (lane 4 and 8) and plpMg as a control (lane 9). The positions of the Hind III-digested DNA were indicated as a size marker.
transfectants had a thin mycelium and no clamp connections, indicating that they were monokaryons regenerated from the protoplasts during the transformation process (Table 2-2). One of the transformants, TMG5, had a thick dikaryon-like mycelium with no clamp connections, suggesting that the strain was a diploid. This was confirmed by mating tests in which TMG9 mated with all four tester monokaryons with different mating types (data not shown). Regeneration of diploid strains from *P. ostreatus* protoplasts was also reported by Takehara et al. (19).

The productivity of the transformants was measured using culture filtrates on the fourth day of the liquid culture (Table 2-2). No MnP activity was observed for the wild-type control at this point. Significant MnP activities were detected for some of the transformants, suggesting the expression of the introduced recombinant constructs in these strains.

In order to obtain a more productive MnP-producing strain, mating crosses were tried between the monokaryotic transformants. One of the resulting dikaryons, TMG9-C1, which was generated by a cross between TMG9 and TMC1, was chosen for further characterization.

**Overproduction of MnP by TMG9-C1**

The time course of extracellular MnP activity was followed together with mycelial dry weight in the liquid culture of TMG9-C1 and wild-type control #261 (Fig. 2-5). In #261, extracellular MnP activity emerged on the 5th day and reached a maximum on the 10th day. While in TMG9-C1, strong MnP activity was observed on the 5th day with the highest peak on the 8th day. The MnP activity was four times as high as that of the wild-type control at this point. After the 8th day, the activity gradually decreased with a shoulder at the 10th day when the activity of the wild-type control reached maximum. On the other hand, mycelial dry weights of these strains showed similar profiles, especially in the early stage of the culture. It was demonstrated that the difference in MnP activities was caused by the difference in enzyme productivity and not biomass of these strains. These results suggested that the recombinant *mnp3* genes were highly expressed in the early stage of the liquid culture of TMG9-C1 in addition to the endogenous MnP3.

**Detection of the recombinant mnp3 transcripts**

To demonstrate the expression of the recombinant *mnp3* in TMG9-C1, detection of the mRNA was carried out, using RT-PCR techniques (Fig. 2-6). Using the primers specific for the endogenous *mnp3*, trace amounts of the transcripts were detected in TMG9-C1 (lane 4-6) and wild-type control #261 (lane 1-3). RT-PCR amplifications of 412-bp endogenous *mnp3* (lane 1 and 4), 503-bp recombinant *mnp3* (lane 2 and 5) cDNA fragments were carried out using total RNA extracted from 6 days old mycelium as a template. Competitive RT-PCRs for the endogenous and recombinant fragments were also performed (Lane 3 and 6) to compare their expression levels. Lane M contains *Hind* III-digested DNA as a size marker.
cDNA fragments were amplified from both of the RNA samples prepared from TMG9-C1 and the wild-type control, on the 6th day of the liquid culture. With the primers specific for the recombinant construct, a strong signal was detected only in TMG9-C1 using the same RNA preparations. Furthermore, competitive RT-PCR experiments demonstrated that the recombinant mnp3 was transcribed predominantly in TMG9-C1 at this point. These results confirmed that the introduced recombinant mnp3 was expressed remarkably in the early stage of culture of the transformant.

II-2.4 Discussion

With the recombinant constructs under the control of P. ostreatus sd1 expression signals, MnP3 was overexpressed in the transformants. The predominant transcription of the recombinant sequence reflected the higher extracellular enzyme activity in the early stage of culture of the transformant TMG9-C1. Since sd1 encodes a component of the mitochondrial respiratory chain, it is expected to be transcribed in the primary metabolic phase. However, the extracellular MnP activity of TMG9-C1 did not correlate with the initial mycelium growth (Fig. 2-5), suggesting that some post transcriptional factor(s) might be rate limiting for the MnP3 production in the initial stage. A post-transcriptional regulation of MnP production was also proposed in P. chrysosporium.

The homologous expression system developed in this study will be effective for expressing other P. ostreatus proteins in an active and native form. It also permits mutational analysis of the proteins, using in vitro site-directed mutagenesis techniques. Moreover, utilization of recombinant strains overproducing specific enzymes would have a significant impact on the industrial application of the fungus. In this context, it is of interest to assay the ability to degrade lignin and organic pollutants of the recombinant TMG9-C1. Finally, the gene expression system can be also used to express heterologous gene products in P. ostreatus.

Conclusion

P. ostreatus succinate dehydrogenase iron-sulfur subunit gene, sd1 was cloned and characterized. Using the gene as a base, carboxin resistant marker for P. ostreatus transformation was developed. However the efficiency of the transformation system using the plasmid pTM1 containing the marker is low, 1-5 transformants/μg vector plasmid, single-strand carrier DNA gave a about 40 fold increase (about 200 transformants/μg vector plasmid) over it.

P. ostreatus transformation system using integrative type vector plasmid pLG-hph which contains hph fused signals of Lentinula edodes glyceraldehyde-3-phosphate dehydrogenase gene, gpd as a marker was constructed. The efficiency of the transformation system using the marker is 0.1 transformants/μg vector plasmid. The transformants were integrative type and stable. Restriction enzyme-mediated integration 8 fold increased the transformation efficiency.

A major manganese peroxidase (MnP) isozyme gene, mnp3 was cloned and characterized. From the sequence analysis, mnp3, it was demonstrated that MnP3 has a property specific for both of manganese peroxidase and lignin peroxidase from Phanerochaete chrysosporium. For further characterization of the enzyme, expression cassettes were constructed using promoter and terminator sequence of P. ostreatus sd1 gene, followed by insertion of the coding sequence of mnp3. Upon introduction of the constructs by cotransformation with pTM1, strains with higher MnP activity were isolated. One of the recombinants obtained by a mating between two monokaryotic transformants, TMG9-C1 has shown four times higher MnP activity than the wild-type control in the early stage of liquid culture of the mycelium. Predominant transcription of the recombinant mnp3 at the 6th day was demonstrated by a competitive RT-PCR experiment.

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