

# Development of Transformation and Recombinant Gene Expression Systems in *Pleurotus ostreatus*\*<sup>1</sup>

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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 (MnPs). Their features have been extensively investigated and many genetic and enzymatic data are available<sup>1,2</sup>.

*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<sup>3-5</sup>. 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<sup>6-13</sup>. 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<sup>14</sup>. Property of MnPs produced by genus *Pleurotus* is difficult to that of *P. chrysosporium* MnPs<sup>15-17</sup>. 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 an 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*<sup>18-22</sup>. 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<sup>18,19,21,22</sup>. Whereas, homologous expression system was developed for *P. chrysosporium*<sup>23,24</sup>. The system provides us active and native enzyme of interest. Property of the enzymes were analyzed using these expression systems<sup>25-30</sup>. In *Pleurotus*, Ruiz-Duenas *et al.* have reported expression of *P. eryngii* MnP in *A. nidulance*. But the productivity is lower level than that in *P. eryngii*<sup>31</sup>. 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<sup>32</sup>. 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<sup>33,34</sup>. 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 carboxin resistance can occur in *P. ostreatus*<sup>35</sup>. 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 confers carboxin resistance in this species.

Carboxinilides are potent inhibitors of the mitochondrial complex II from fungi, bacteria, and animal tissues<sup>36-39</sup>. 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.*<sup>40</sup>). 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<sup>36</sup>. 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<sup>33,34</sup>.

#### I-1.2 Materials and methods

##### *Strains, media and plasmid*

Through this study, *Pleurotus ostreatus* #261 (ATCC66376) 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<sup>41</sup> 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<sup>42</sup>. Poly A tailed RNA was prepared from mycelium 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 -43 and the other at -53 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 basidiomycetous 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

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-58 :   #           #
      atatttctctcttccaccatcGTCCAGCAGCTCCCGGGAACACACAATCATTGAACC
                               SalI

   1 : ATGCAGGGCTCACCTCCAGGTCGTTGGCTCGCTCATCTCGCTCGATTCTGCTTTCTCC
      M Q A L T S R S L A R S S R S I R A F S
   61 : ACCTCGCCTGGAAGATGGCAGGCTGAGCCCTCCAGAAGCCCTCTCCAGAAAGATTC
      T S P G R W Q A E P L Q K P V L Q K E F
  121 : AAGATCTACCGTGGGTGAGCTGAGACCCCTTGGATATCCAGACGTGTGCTCACTCGCGG
      K I Y R W
      C                               T
  181 : TACAGAACCCAGATGAGCCCGCAAGAAGCCTCATCTCCAATCGTACACCATTTGACTTGA
      N P D E P A K K P H L Q S Y T I D L
      G
  241 : ACCAGACAGGCCCATGGTACGTACAATTCAAAGGCGATTGTCTCCATGCTCAGGGGCTC
      N Q T G P M
      C
  301 : GTAGATTCTGGATGCTCTTATCAAGATCAAGAACGAAATTTGATCTACGCTCACATTCGG
      I L D A L I K I K N E I D P T L T F R
  361 : TCGTTTCGTGCAGAGAGGGGATCTCGCGCTCGTGTCCGATGAACATTGACGGACAGAACAC
      R S C R E G I C G S C A M N I D G Q N T
  421 : GTGGCTTGCCTGTGCCGAATTGACCGCAACGCCAGGACAGCAAGATCTACCCCTTT
      L A C L C R I D R N A S K D S K I Y P L
  481 : GCCGCACAGTATGACATGTCTCCAGCTCCTAATTCATCGCTGACAGTCGGCAACAGT
      P H M
  541 : GTACATCGTGAAGACCTCGTACCCGATCTCACCCCTGTTCTACAAGCAGTACAAGTCCAT
      Y I V K D L V P D L T L F Y K Q Y K S I
  601 : CAAGCCTTACCTGCAGAACGACAATGTTCCCGAGAGGGAGCACCTCCAGTCCGCCAGAGGA
      K P Y L Q N D N V P E R E H L Q S P E D
  661 : CCGCAAGAAGCTGGATGGGATGTATGAATGCATCTGTGCCCGCTGCTGCAGCACGTCGTG
      R K K L D G M Y E C I L C A C C S T S C
      T
  721 : CCCCAGTTATTTGGTGAACCAAGACGAGTATCTGGGGCCGGCTGCATTTGATGGCTGCATA
      P S Y W W N Q D E Y L G P A A L M A A Y
  781 : CAGGTGGATTGCCGACTCCGCGAGTGCCTTGTGATGTCTGACGCGCATACTCCCATAGACT
      R W I A D S R
  841 : AACATCTCGTAGGATACGTACGCCCGCAACCGAAGAACACTTCCAGAACGAGCTGAGT
      D T Y G A Q R K E H F Q N E L S
      T
  901 : CTGTTCGGCTGCCACACAATCTTCAATTGTAAGTGGCTTTTCGCCCTTGTATTACCGAGC
      L F R C H T I F N
  961 : CTAATCATATCCGTTATCAAGGCTCTTCGCACTTGTCCAAAGGGCCCTCAACCTGCCAAAG
      C S R T C P K G L N P A K
      G
  1021 : CCATTGCCGAAATCAAGCTCGCGCTTGCCACGGAGTAAACACAAGTTAACAGCCACGGAT
      A I A E I K L A L A T E *
      T
  1081 : TAAAAGCACCGAGTCAGAGGCAGATTTCTTTCTGTAGCAGTTGACAGTTCTTTCCACTT
  1141 : CATCATACAGTGTCCATCACGACATCAAATCATATTCAATCTATAACatccacttggtc
  1201 : ttgagcctgtctgaggtaggtataccctcggtttcacttggatgctgtacgaaacaaga
  1261 : acggatataattctatgggacatctaacggctcaggcaattcatagtctactggtatcaac
  1321 : ggcgggagcgaagaaggggtacgacgtatagccctcggggtccgtgctgccagggtctata
  1381 : atacttctctatcgctttctccaccggccaaccattcttcagtgcgacggcgaattc
  1441 : gggctatagtcggcactcgatc
                               EcoRI

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Fig. 1-1. Nucleotide and deduced amino acid sequence of the *P. ostreatus* sdil gene. The nucleotide sequences marked by underline are exons and the corresponding amino acid sequences are also indicated below. Capital letters in nucleotide sequence indicate the region of which both of the genomic and cDNA clones were obtained. Sharps and an asterisk indicate transcriptional start points and termination codon, respectively. The putative polyadenylation signal is italicized. Restriction enzyme recognition sites used for cloning of genomic clone by the cassette-primer PCR, *SalI* and *EcoRI* sites, are also indicated. The letters above the nucleotide sequence are eight nt substitutions in the second type cDNA sequence. The binding positions of primers, M1 and M2 are marked by (—>) and (<—), respectively.

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>R</sup>*, was isolated from a drug resistant mutant and used to develop plasmids for transformation<sup>43)</sup>. The *Cbx<sup>R</sup>* mutation is in the gene encoding the Ip subunit and causes a single amino acid substitution (His257→Leu) in the third cysteine-rich cluster<sup>34)</sup>. The corresponding histidine residue is conserved in the *P. ostreatus* polypeptide (His239) 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-

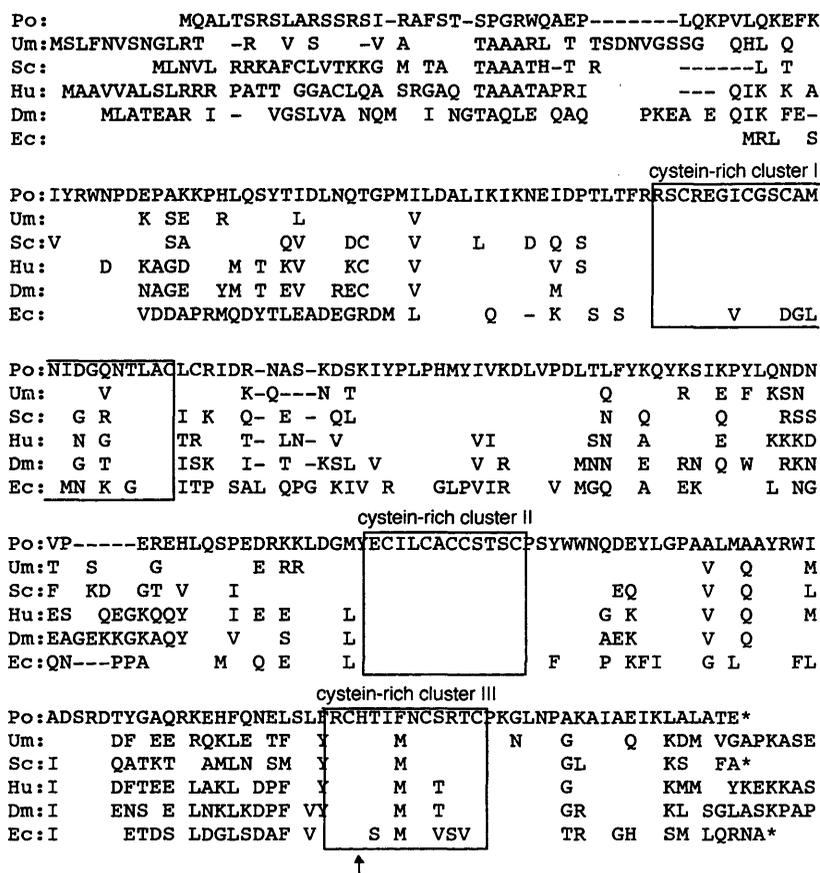


Fig. 1-2. Comparison of amino acid sequence of Ip subunits. Only the positions where the amino acid sequence differs from that of the *P. ostreatus* Ip subunit are listed for the other species. Hyphens and asterisks indicate gaps and C-terminal end, respectively. Three cysteine-rich clusters required 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*. Po, *P. ostreatus*; Um, *U. maydis*, Sc, *S. cerevisiae*, Hu, human, Dm, *D. melanogaster*, Ec, *E. coli*.

nilides. Cloning and sequence determination of the *sdil* gene from the flutolanil-resistant mutants is in progress.

## I-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*

### I-2.1 Introduction

In generally, promoters are reported to be less compatible in the basidiomycetes<sup>44)</sup>. 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 *sdil* promoter region and the genomic clone of the allelic sequence.

### I-2.2 Materials and methods

#### Monokaryotic strains

Several monokaryotic progenies which were isolated from basidiospores from *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 primers, R1 (5'-CAC ACA AAT CAT TGA ACC ATG C-3') and R3 (5'-AGC ATC GCA AGT GAA ACC GA-3') (Fig. 1-5). The amplified PCR products were cloned and sequenced. Determined nucleotide sequences appear in DDBJ, EMBL and GenBank data bases with accession number AB009845 and AB009846.

#### Southern blot analysis

Southern blot analysis of the *P. ostreatus* haploid genome DNA digested with restriction enzymes *EcoR* I, *Sac* I, *Kpn* I, *Apa* I, *Sal* I or *Sph* I, was performed using the PCR amplified partial sequence of *sdil* as a probe (Fig. 1-4). The labeling, hybridization and signal detection were done

-1300: TCCGATGACACTGCCAACGACTACGCTTATGCGTATTGGCGGGACAAAGTCCGGCAGCGG  
 -1240: ATTCACAAGGTCGAGCTCGTAGAGAACTTGCCCTACCATCAAGATGCATCCCTTTGGG  
 -1180: ACGAAGCGTCCAGCTTTGGAGCAGGTGAGTGGCCGAATGGCTTCTATCTCTTTGCAAT  
 -1120: CTGACACCAAGCGCCGTTAGCACCTTTACGAAGCCTTTAACCAAGATAATGTCACCTTGG  
 -1060: TTGACCTCAACGAAAGCCCATTTGATGAGATTACATCTACTGGGGTTTCCGTACCAAGGA  
 -1000: TGGGACAGAATATGCTCTCGACCTGTTGGTCATGGCCACGGGGTTCGATATGGGAACATGG  
 - 940: TGGCTATAGGACATCGAAATCGTAGGGACAAACGGGGCCGCTTTCGCGATAAATGGGC  
 - 880: CAATGGGGTAAAGTCATATCTGGGCATGTTGGCGTCTGGGTTTCCAAACATGTTTATGGG  
 - 820: TATACGGCCCTCATGCACCCAGCGGCTTTACCAATGCACCTACATGTGCTGTGAGTAGCT  
 - 760: CTTTCATTGACTTTAAGGTGGGCATCGGTAATTCGACGAAACGGTCAGGAATGCAAGTT  
 - 700: GATTGGATAACCAATTCGATTGAAATATGATGAAGAATTTCGCTCGCTTCGATCGAAGCA  
 - 640: AGTAAAAAGCGGAACATGGATTGGACTCAACGAATAGATGAAATCGGTGCCAGGGGGCTT  
 - 580: TCGAATCGGGCAAACCTCGTGGTACAGAGGTGCGAACGTCACGCAAGGTTATGGAGCAT  
 - 520: ATGTTTTGGGCTGGAGGATGTCCTGCTATCAAAAGATTTGCGAAGAAGTCGTCGAAAGT  
 - 460: GGATACGATGGAATCATGTTTCAATAAGACGCCCTGACTACCCTCTCAAGATAACAACAAT  
 - 400: ATGTATCGTCTGCTCACGTACCTTGAGACTTCCTAGGTGCTCCTACATGTACGCCCTTACT  
 - 340: ATTGAAGGGAGTACAATTCGACGCAAAGATTATCGATTGCTCGTATCGTTGCTCTTCTGT  
 - 280: CTTACTTTATTGTCATCAAAGTAACGTGGATGTGTATCATCTACTCAATACCCCA  
 - 220: ATTCTACATGTACTTCTCTGGCCTGGAACCAAATACGAAGCCTATGAACTTCCCTTAAG  
 - 160: TCCACCGGCATTTTCCGAAGAGTAACACACCCCTTCTGATTGGTCCGGGCCACGAGCCTT  
 - 100: CCGCTCCGTTCCCGAATTCACGCGATCCCGCCGAGAAATGATATTTCTCTCTTACC  
 - 40: ATCGTCGACGACGTCCTCCGGGAACACACAATCATTGAACatgcaggcgctcacctccag

Fig. 1-3. Nucleotide sequence of the promoter region of *P. ostreatus* *sdil*. 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.

using DIG DNA Labeling and Detection Kit, BOEHRINGER MANNHEIM, according to the reference<sup>45</sup>.

**I-2.3 Results**

*Cloning and sequence analysis of the sdil promoter region*

The sequence of the promoter region of *sdil* was determined as described in Fig. 1-3. The promoter region contained putative CAAT and TATA boxes, and a binding site motif for transcription factor Adr1p which is required for maximal expression of *ADR2* encoding an isozyme of alcohol dehydrogenase, ADHII, in yeast, *Saccharomyces cerevisiae*<sup>46,47</sup>. Although the function of these putative expression signals are to be confirmed by further experiments, it is plausible that they should participate in the regulation of *sdil* gene expression. The isolated

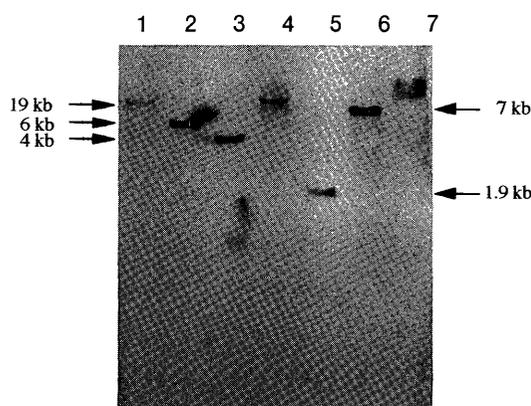


Fig. 1-4. Southern blot analysis of genomic DNA extracted from a monokaryotic progeny of strain #261. Five mg of DNA samples applied in each lane were probed with PCR product containing *sdil*. The DNA samples were digested with; lane 1, *EcoRI*; lane 2, *SacI*; lane 3, *KpnI*; lane 4, *ApaI*; lane 5,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

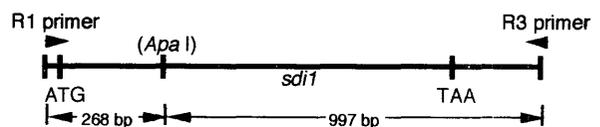
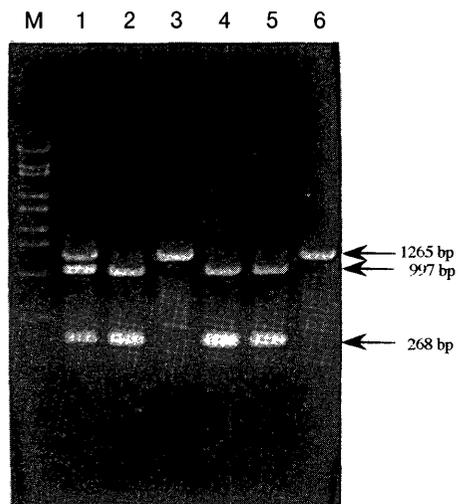


Fig. 1-5. *Apa I* assay and confirmation of allelic sequence in *P. ostreatus* strains. PCR fragments containing *sdil* sequence from each strain amplified with primers, R1 and R3 (see Materials and methods) were digested with *Apa I* and electrophoresed on a 0.7% agarose gel. Lane 1, parental dikaryon,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

promoter region of *sdil* provides us a useful component to develop a expression system for recombinant DNAs in *P. ostreatus*.

*Characterization of the second-type Ip subunit gene*

The PCR amplification of *P. ostreatus* #261 genomic DNA

using specific primers resulted in the isolation of a novel sequence which was very similar to *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 *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 *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.

#### *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 progenies derived from basidiospores of #261. When genomic DNA fragments digested with various restriction enzymes were probed with *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 *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 *sdil* and the second-type sequence, further experiment was performed as described below.

The second-type gene contained an *Apa I* site which did not exist in *sdil*. So *Apa I*-digests of the PCR-amplified fragment of *sdil* could be used to distinguish which type(s) of genes was/were contained in a certain *P. ostreatus* strain. This restriction fragment length polymorphism assay was designated as *Apa I* assay (Fig. 1-5). Using progenies derived from basidiospores of #261, a series of *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 *Apa I* assay consisted with the grouping of the monokaryons by the Southern blot analysis. This confirmed that *sdil* and the second-type gene were allelic and not the distinct members of a gene family.

Using an *Apa I*-assay, a linkage between the *sdil* genotypes and other characteristics can be analyzed easily. Especially, analysis for a linkage between the *sdil* genotypes and the flutolanil-resistance phenotype is useful to investigate whether the drug-resistance links to *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 *sdil* genotypes was observed among the progenies of this mutant strain (data not shown).

#### **I-2.4 Discussion**

This is the first report on cloning of a promoter sequence of the *P. ostreatus* genes which are expressed in the primary metabolic phase. It was shown that *sdil* has no homologous genes in the haploid genome of *P. ostreatus*. From this result, it is unlikely that *P. ostreatus* contains

more than one isoforms of Ip subunit protein for complex II, which is the case in some other organisms such as *Haemonchus contortus*<sup>48)</sup>. In this context, it is conceivable that *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 *P. ostreatus*. According to this idea, we have constructed selectable markers consisting of the *sdil* promoter and terminator and heterologous drug-resistance genes. Examination of *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 *sdil* genotypes classified by *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-subunit proteins. In fact, existence of the another nuclear locus, *oxr-2*, conferring carboxin resistance has been reported in *U. maydis*<sup>33)</sup>. Anyway, the *Apa I*-assay provides a useful method to screen a *P. ostreatus* mutant strain with the flutolanil-resistant phenotype linked to an *sdil* genotype. Furthermore, an attempt to transform *P. ostreatus* with a modified *sdil* gene containing the same amino-acid substitution as in *U. maydis Cbx<sup>R</sup>* is also being undertaken.

### **I-3 Stable transformation of *P. ostreatus* to hygromycin B resistance and effect of restriction enzyme-mediated integration**

#### **I-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. Thereby 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 *P. ostreatus* to hygromycin B resistance using plasmids pPO1 and pPO2 containing an insert of bacteriophage origin and *E. coli* hygromycin B phosphotransferase gene (*hph*) fused by *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<sup>49)</sup>. 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 *P. ostreatus* to stable hygromycin B resistance using pLG-*hph* containing *hph* fused signals of *Lentinula edodes* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) as a marker. It was previously reported that pLG-*hph* was utilized for *L. edodes* transformation successfully<sup>50)</sup>.

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

#### **I-3.2 Materials and methods**

##### *Transformation of 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 (CALBIOCHEM), 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 CaCl<sub>2</sub>, 50 mM maleate buffer (pH 5.5)), followed by resuspension in MMC buffer to give a final concentration of 1×10<sup>9</sup> protoplasts/ml. 50 μl of protoplast solution (1×10<sup>8</sup> protoplasts) aliquot was mixed with 12.5 μl of PEG buffer (25% PEG 4000, 10 mM Tris (pH 7.5), 25 mM CaCl<sub>2</sub>) 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 CaCl<sub>2</sub>). 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 *Bgl*II-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<sup>45</sup>.

*Measurement of growth rate and stability of hydromycin 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\* until the hypha 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.

**I-3.3 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 state. 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

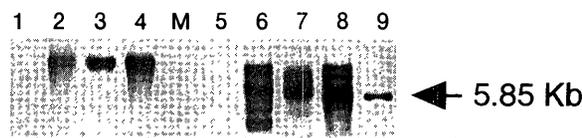


Fig. 1-6. Southern blot analysis of pLG-hph transformants (TL1, 2, 11). Lane 1-4 represents undigested DNA and Lane 5-9 represents *Bgl* II digested DNA: wild-type DNA (lane 1, 5), TL1(lane 2, 6), TL2 (lane 3, 7), TL11 (lane 4, 8), pLG-hph (lane 9). The size of pLG-hph is 5.85 kb.

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 chromosome 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<sup>50</sup>. The effect of *Bgl* II amounts on transformation efficiency were measured. The most optimal condition is 20 unit of *Bgl* II in which transformation efficiency is as 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).

**I-3.4 Discussion**

We constructed stable and reappearant 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

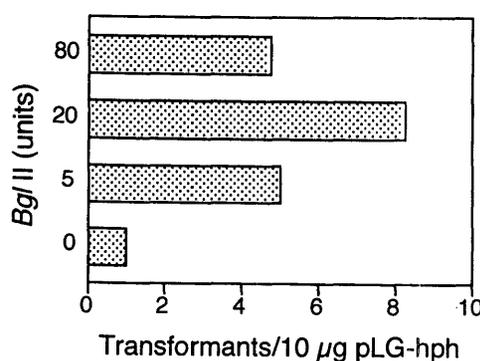


Fig. 1-7. Effects of the restriction enzyme on transformation efficiency. The indicated efficiencies were average of 4 experiments.

without REMI (Fig. 1-7).

A number of germinating protoplast 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 germinant 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 *Cbx<sup>R51</sup>*, but there is sufficient significance in the alternative method.

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

##### I-4.1 Introduction

In I-1 and I-2, cloning and characterized of *sdil* were reported. Subsequently, the single amino acid substitution (His257→Leu) in Center 3 of *sdil* was introduced to construct carboxin resistant marker, *Cbx<sup>R</sup>*. A homologous transformation system for *P. ostreatus* using *Cbx<sup>R</sup>* reported in reference (Honda, 2000 #18). However the system is stable and useful, the transformation efficiency was not so high, about 5 transformants/ $\mu$ g 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.

##### I-4.2 Materials and methods

###### Preparation of carrier DNA

$\lambda$  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  $\mu$ l of *P. ostreatus* protoplast solution ( $1 \times 10^8$  protoplasts) was mixed with 12.5  $\mu$ l of PEG buffer (25% PEG 4000, 10 mM Tris (pH 7.5), 25 mM CaCl<sub>2</sub>) and 1  $\mu$ g of pTM1. Then, the 50  $\mu$ g of ds or ss  $\lambda$  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 CaCl<sub>2</sub>). The mixture was spreaded on SMYM (SMY containing 0.6 M mannitol as an osmotic stabilizer) plate containing 1.5% agar and 2  $\mu$ g/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 *Hind*III-digested DNA extracted from the transformants was performed using  $\lambda$  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<sup>45)</sup>.

##### I-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  $\mu$ g of pTM1, 1–5 transformants/ $\mu$ g DNA were obtained in these condition. The number of the transformants increased as the amount of pTM1 increased to 5  $\mu$ g. While ds  $\lambda$  DNA was added to the transforming mixture, 13–41 transformants were obtained per 1  $\mu$ g of pTM1. Moreover, ss  $\lambda$  DNA exhibited further increases in the efficiency, up to 190 transformants/ $\mu$ g 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  $\lambda$  DNA as a probe. For all the five transformants (T1–T5) obtained from the experiments using ss  $\lambda$  DNA as a carrier, significant signals were

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

	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>P. ostreatus</i> MnP MnP3	1	64.8	67.4	45.6	54.3	54.5	54.4	59.3	59.5	59.9	55.2	58.4	56.5
<i>P. ostreatus</i> MnP Mnp	2		55.4	44.6	49.0	48.7	48.4	51.6	55.6	52.7	50.7	52.2	53.5
<i>T. versicolor</i> MnP MPG1	3			42.1	48.0	49.6	49.5	57.8	61.7	59.5	58.0	69.0	57.6
<i>C. subvermispora</i> MnP Cs-MnP	4				69.2	69.2	71.3	40.6	39.4	42.4	40.7	40.4	38.4
<i>P. chrysosporium</i> MnP MnP1	5					83.2	81.0	47.2	45.5	48.5	46.4	45.9	41.9
<i>P. chrysosporium</i> MnP MnP2	6						81.5	46.2	46.9	47.6	44.7	47.2	42.3
<i>P. chrysosporium</i> MnP MnP3	7							46.4	46.8	46.2	46.0	45.2	42.3
<i>P. chrysosporium</i> LiP LPOA	8								72.1	87.9	61.9	63.2	61.1
<i>P. chrysosporium</i> LiP GLG4	9									72.4	58.7	65.1	61.4
<i>P. chrysosporium</i> LiP GLG6	10										61.5	61.6	60.0
<i>P. radiata</i> LiP	11											62.0	59.6
<i>T. versicolor</i> LiP LPG I	12												61.4
<i>B. adusta</i> LiP	13												

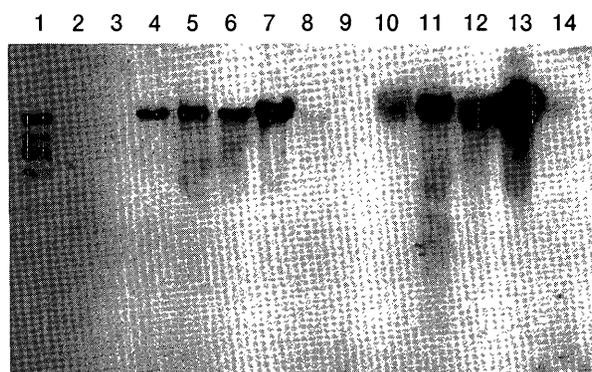


Fig. 1-8. Southern blot analysis of *P. ostreatus* transformants using ss carrier DNA, T1-T5. 1-DNA sequence was used as a probe. Lane 1 represents *Hind*III digested 1-DNA. Lane 2 represents *Apa*I digested pTMI. Lane 3 to 8 represent *Hind*III digested genomic DNA: wild type (lane 3), T1-T5 (lane 4-8). Lane 9-14 represent undigested genomic DNA: wild type (lane 9), T1-T5 (lane 10-14).

detected comigrated with chromosome DNA (Fig 1-8). It was demonstrated that the ss carrier DNA was integrated in the host chromosome.

#### I-4.4 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 increase transformation efficiencies. The ds carrier DNA effect was perhaps due to protection against nuclease because it occurred only when transforming DNA was very small<sup>52,53</sup>. 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<sup>54</sup>. 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 or transforming DNA and is not a good substrate for uptake<sup>55</sup>. 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/ $\mu$ g vector plasmid<sup>6-9,11-13</sup>. We constructed the most high efficient transformation system for *P. ostreatus* using ss carrier DNA.

This method is useful for genetical experiments which require high transformation efficiency. We are studying whether ss carrier DNA enhances the efficiencies in protoplast transformations of other mushrooms.

## Chaptes II Cloning and homologous expression of *P. ostreatus* MnP3

### 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

MnPs are H<sub>2</sub>O<sub>2</sub>-requiring heme glycoprotein enzymes<sup>56,57</sup> and oxidize Mn<sup>II</sup> to Mn<sup>III</sup>. Using transient-state kinetics, it was suggested that the actual substrate of MnP is Mn<sup>II</sup> forming a complex with an organic dicarboxylic acid such as oxalate, rather than MnII itself<sup>57,58</sup>. The substrate oxidized by MnP then oxidizes phenolic substituents of lignin<sup>57,58</sup> and possibly nonphenolic substituents via specific mediator molecules such as a peroxidized lipid<sup>59</sup>. Generally, MnPs occur as a series of isozymes encoded by a family of genes<sup>2</sup>. Many MnP isozymes have been purified and thier genes were cloned from various white-rot basidiomycetes including *Trametes versicolor*<sup>60</sup>, *Ceriporiopsis subvermispora*<sup>61</sup> and *P. chrysosporium*<sup>62</sup> X-ray crystallographic and site-directed mutagenesis studies have defined the Mn binding site in the *P. chrysosporium* MnP1 (H3) isozyme<sup>26,28,63</sup>.

*P. ostreatus* has been reported to secrete a series of MnP isozymes 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<sup>14</sup>. 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 *mnp* genes from *Pleurotus ostreatus* strain IFO 36160 has been isolated and sequenced<sup>64</sup>. In our recent experiments, another strain, IS1, has been shown to secrete at least three isoforms 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-peptone medium (glucose 2%, polypeptone 0.5%, yeast extract 0.2%, KH<sub>2</sub>PO<sub>4</sub> 0.1%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%) supplemented with 0.5 mM MnSO<sub>4</sub> 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

RNA was prepared from the mycelia using the RNA Isolation Kit (Stratagene), and poly (A)<sup>+</sup> RNA was purified with Oligotex<sup>TM</sup>-dT30 (Daiichi Pure Chemicals).

*Cloning of the cDNA and the genomic fragments*

For cloning *mnp3* cDNA, 3'-RACE and 5'-RACE were

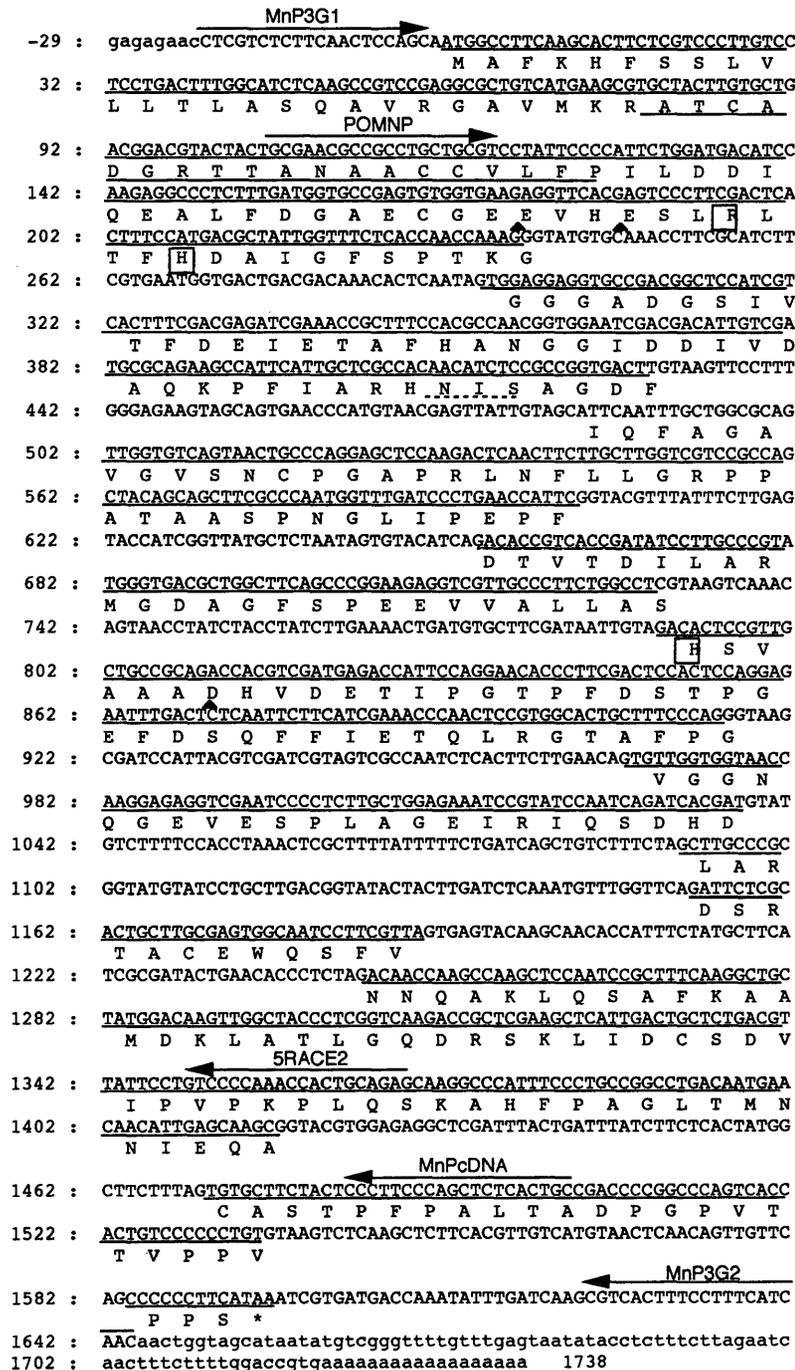


Fig. 2-1. Nucleotide sequence of the cDNA and structural gene encoding *P. ostreatus* MnP3. The deduced amino acid sequences are shown below the corresponding nucleotide sequence. Arrows indicate the positions and directions of the oligonucleotide primers used for PCR. The underlined amino acid sequence 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.

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

Experiment	pTM1 (1 $\mu$ g)	pTM (5 $\mu$ g)	pTM1 (1 $\mu$ g)+ds IDNA (50 $\mu$ g)	pTM1 (1 $\mu$ g)+ss IDNA (50 $\mu$ g)
1	1	—	13 (13)	198 (198)
2	5	26	41 (8)	194 (39)
3	5	25	38 (8)	178 (36)
Average	3.7	25.5	30.7 (8)	190 (51)

The parenthesized numbers indicate what times for that without carrier.

performed<sup>42)</sup> using primers, MnPcDNA (5'-GCA GTG AGA GCT GGG AAG GG-3'), 5RACE2 (5'-CTC TGC AGT GGA TTG GGG AC-3'), dT17adapter (5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT-3') and POMNP (5'-GCN AAY GCN GCN TGY TGY GT-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.

### II-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 mnp*<sup>64)</sup>. 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 endpeptidase. 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 *P. chrysosporium* MnP isozyme<sup>26,28)</sup> were also conserved (Fig. 2-1). The calculated molecular weight of the unprocessed and processed protein were 37400 and 34600, respectively. The estimated Mr of the purified MnP3 isozyme was 42 kDa (unpublished data), and this difference was most likely due to glycosylation of the protein.

### II-1.4. Discussion

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<sup>15,16,65)</sup>. MnP3 has component amino acids of a access channel of heme cavity (Val-177, Gln-215, Phe-142, His-76, Glu-140)<sup>66)</sup>, proposed lignin-binding site (His-232) and the possible electron transfer pathway (Asp-231) shown in LiP<sup>67)</sup>.

Overall sequence homology analysis (Table 2-1) revealed that MnP3 had a higher degree of homology to *T. versicolor* MP2 and the previously cloned *P. ostreatus* MnP (67.4% and 64.6%), medium homology to LiPs from various species (59.3–59.9%)<sup>60,68–72)</sup>, and a low level of homology to MnPs from *P. chrysosporium*<sup>62,73,74)</sup> and *C. subvermispora* (45.6–54.5%)<sup>61)</sup>. 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 phylogenetic tree. It has been reported that the positions of ten cysteine residues involved

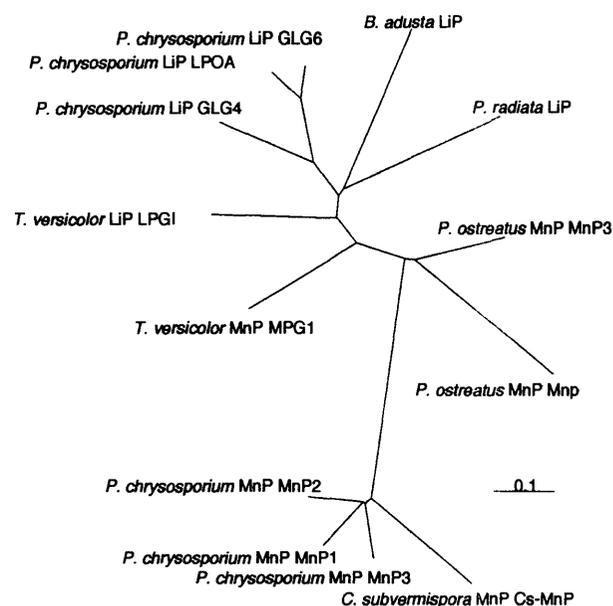


Fig. 2-2. Unrooted phylogenetic tree based on the distances among primary peptide sequences of LiPs and MnPs from various white rot basidiomycetes. Reference numbers are bracketed. The tree was generated by the neighbor-joining method, using CLUSTAL W.

in disulfide bond formation to maintain the tertiary structure of the enzyme are conserved in the MnPs from *P. chrysosporium* and *C. subvermispora*<sup>61,75</sup>. The last two of these cysteine 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<sup>60</sup>. MnPs from *P. chrysosporium* and *C. subvermispora* have a 7–10 amino acid residues insertion around residue number 248 (*P. chrysosporium* MnP3 numbers)<sup>61,62,73,74</sup>, which is not present in the amino acid sequences of the LiPs or *T. versicolor* MP2<sup>60</sup>. Neither of the *P. ostreatus* MnPs contain this insertion sequence (Fig. 2-1)<sup>64</sup>. It has been demonstrated that *P. chrysosporium* MnP1 has an *N*-glycosylation site at Asn131<sup>75</sup>, whereas *T. versicolor* MP2 and LPG1 have such sites at a different position, Asn103 (numbered according to MP2)<sup>76</sup>. 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)<sup>64</sup>.

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 Mn<sup>II</sup>-dependent way, many MnP enzymes from the genus *Pleurotus* react directly with several aromatic compounds in the absence of Mn<sup>II</sup><sup>16,17,77,78</sup>. The versatile functions of the *Pleurotus* MnPs may reflect the strong degradative activity for lignin and a variety of organic pollutants of these fungi<sup>3–5</sup>. Through sequence analysis of the gene<sup>79</sup>, it was demonstrated that MnP3 has component amino acids of the access channel of the heme cavity (Val-177, Gln-215, Phe-142, His-76, Glu-140)<sup>66</sup>, the proposed lignin-binding site (His-232) and the possible electron transfer pathway (Asp-231) shown in *P. chrysosporium* LiP<sup>67</sup>, in addition to the Mn<sup>II</sup> binding site (Glu-37, Glu-40, Asp-178) shown in *P. chrysosporium* MnP<sup>26,28</sup>. It is of interest to elucidate whether these amino acid residues are important to each of the functions of the enzyme.

A recombinant gene expression system is very effective for overproducing and/or characterizing the gene product. It also permits one to analyze the contribution of each amino acid residue to the activity of the product using site-directed mutagenesis techniques. *P. chrysosporium* MnPs and LiPs have been tried to express in heterologous expression systems including *E. coli*, yeast and *Aspergillus*

*oryzae*<sup>22,26,28</sup> as well as homologous systems<sup>23,24</sup>. 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<sup>32</sup>. 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*<sup>80</sup>. On the other hand, an isozyme of *P. eryngii* MnPs was reported to be expressed in *Aspergillus nidulans*<sup>31</sup>. 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 *Cbx<sup>R</sup>* system are reported.

### II-2.2 Materials and methods

#### Construction of *pIpMg* and *pIpMg*

The fragment of *sdil* terminator was amplified with SDH-pro-*NcoI* (5'-GCG CCT CCA TGG TTC AAT GAT TTG TGT GTT CC-3') which adapts a *NcoI* site to the *sdil* promoter and SDH-ter-*KpnI* (5'-GGG GTA CCA CAC AAG TTA ACA GCC ACG G-3') which adapts a *KpnI* site to *sdil* terminator, and digested with *KpnI* and *NcoI*. The coding sequences of *mnp3* genomic DNA and cDNA were amplified by PCR using the primers MnP3-*NcoI* (5'-CTTCAACTCCAGCCATGGCC-3') which adapts a *NcoI* site at the start codon and MnP3-*KpnI* (5'-GGG GTA CCG TCA TCA CGA TTT ATG AAG G-3') which adapts a *KpnI* 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 *NcoI* and *KpnI* site to produce pGEM-T+*mnp3*+*sdil* terminator. The fragment of *sdil* promoter was amplified with SDH-pro-*NcoI* and SDH-pro *SphI* (5'-aca tgc atg etc cga tga cac tgc caa cga c-3'). The amplified fragment was ligated with the pGEM-T+*mnp3*+*sdil* terminator fragment using the introduced *NcoI* and *SphI* site to produce the plasmids, *pIpMg* which contains genomic *mnp3* and *pIpMc* 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 5.5)) containing 0.2% Novozyme<sup>TM</sup> 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

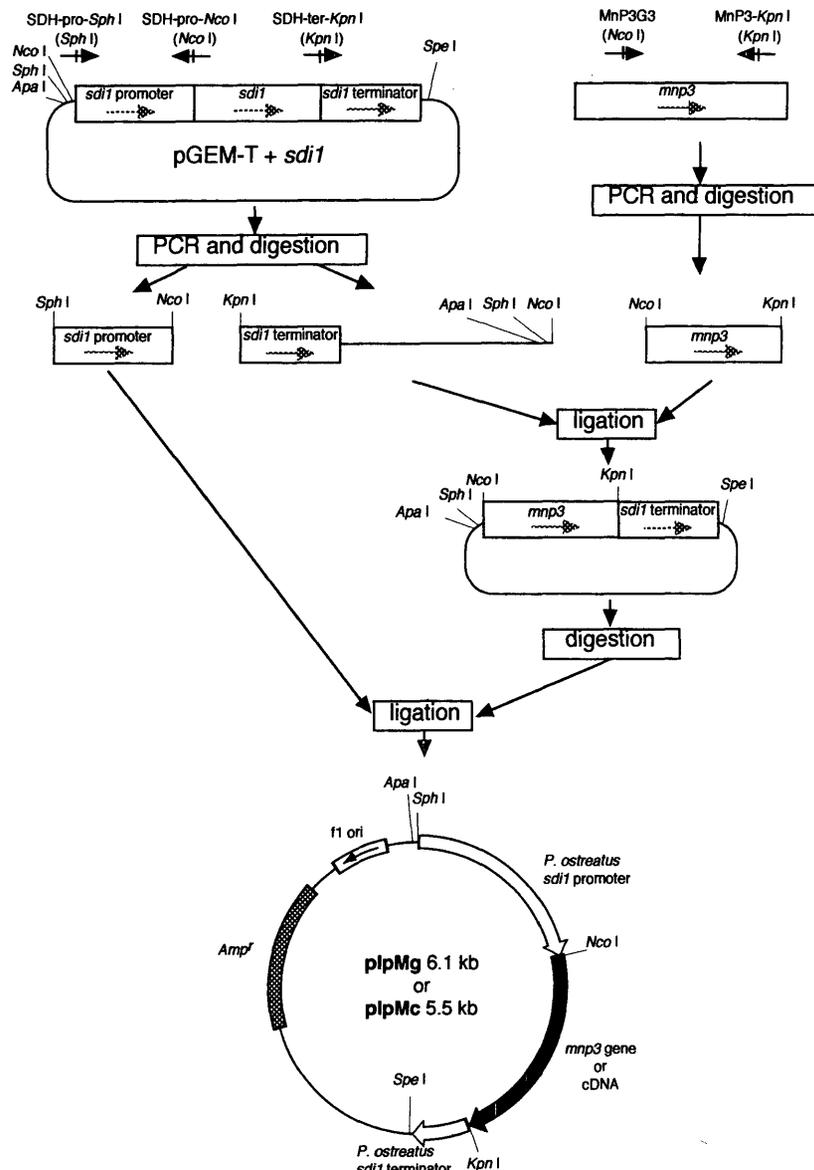


Fig. 2-3. Physical map of the MnP3 expression plasmids, pIpMg and pIpMc. Closed arrow represents location and transcriptional direction of the genomic or cDNA sequence encoding MnP3. Open arrows indicate expression signals from *sdi1*. Thin line and misted arrow indicate pGEM-T vector and *Amp<sup>r</sup>* maker sequence.

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 CaCl<sub>2</sub>, 50 mM maleate buffer (pH 5.5)), and resuspended in MMC buffer to a final concentration of 1×10<sup>9</sup> 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 CaCl<sub>2</sub>), 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 CaCl<sub>2</sub>). 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<sup>45</sup>. Primers used were MnP3G1 and MnP3G2<sup>79</sup>. Intact and *Apa*I-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 MnSO<sub>4</sub>, and 0.1 mM H<sub>2</sub>O<sub>2</sub>. MnP

activity was calculated by subtracting the activity in the absence of Mn(II) from that in the presence of 0.2 mM MnSO<sub>4</sub>. 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)<sup>12-18</sup> was used as a primer for the RT reaction. 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 TG-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 0.8% 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 pIpMg and pIpMc were introduced into *P. ostreatus* #261 strain by co-transformation with pTM1 containing *Cbx<sup>R</sup>* 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 pIpMg and pIpMc sequence, respectively and collected for further analysis. Southern hybridization of the co-transformants of pIpMg 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 *mnp* 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 2-2. MnP activity (on 4th day) and karyotype of the transformants.

Strain	MnP (U/flask)	Karyotype
TMG1	0.195	di
TMG5	0.975	(2n)
TMG7	nd	di
TMG8	nd	di
TMG9	1.560	mono
TMC1	0.195	mono
TMC3	0.390	mono
TMC5	nd	di
wt	nd	di

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

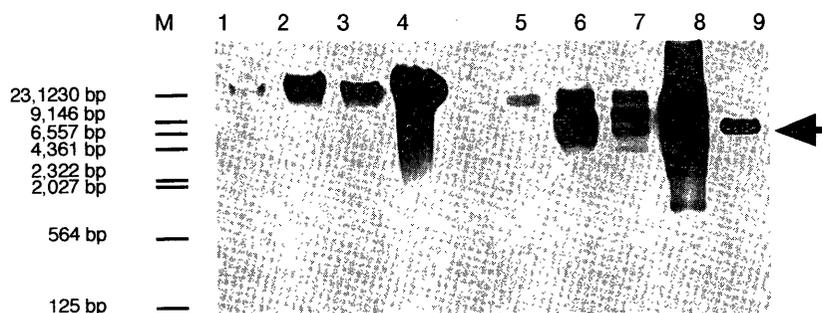


Fig. 2-4. Southern blot analysis of the co-transformant DNA probed with *mnp3* sequence. Lanes 1 to 4 represent undigested DNA and lanes 5 to 9 represent *ApaI*-digested DNA. Samples analysed are: wild type (lane 1 and 5), TMG5 (lane 2 and 6), TMG8 (lane 3 and 7), TMG9 (lane 4 and 8) and pIpMg as a control (lane 9). The positions of the *Hind* III-digested DNA were indicated as a size marker.

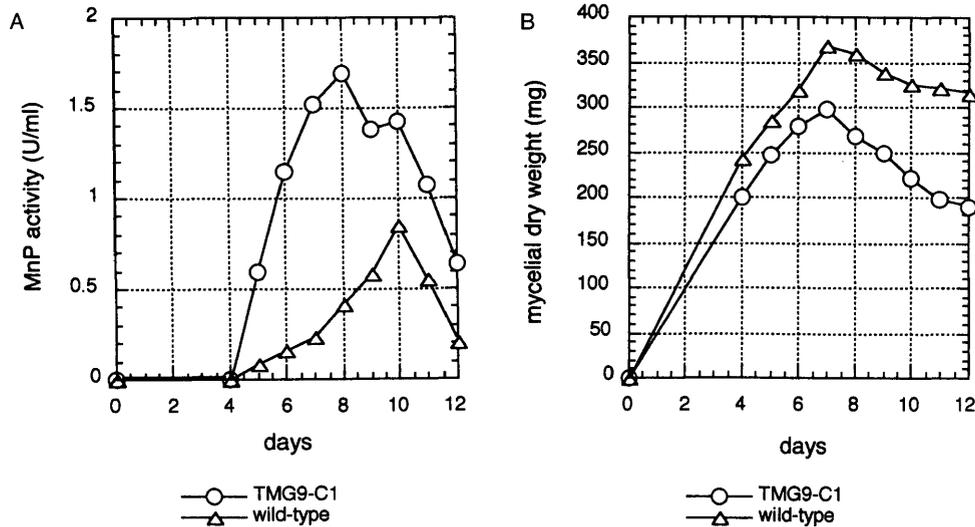


Fig. 2-5. A time course assay of extracellular MnP activity (A) and mycelial dry weight (B) of TMG9-C1 and wild-type #261 strains. Symbols used are: square, TMG9-C1 ; triangle, #261.

transformants 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 TMG5 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.*<sup>81)</sup>.

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

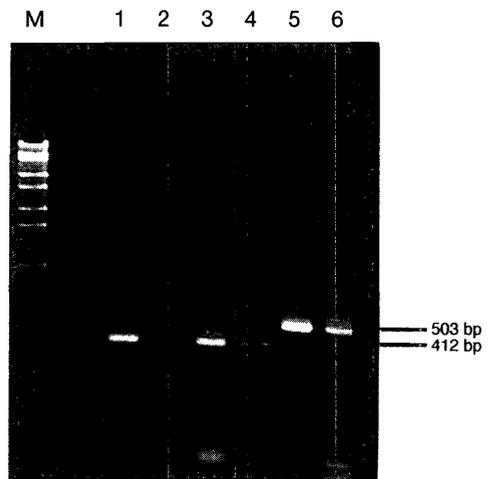


Fig. 2-6. Detection of the *mnp3* transcripts in TMG9-C1 (lane 4-6) and wild-type control #261 (lane 1-3). RT-PCR amplifications of 412-bp endogenous *mnp3* (lane 1 and 4), 503-bp recombinant *mnp3* (lane 2 and 5) cDNA fragments were carried out using total RNA extracted from 6 days old mycelium as a 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 sdi1* 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 *sdi1* 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*<sup>82,83</sup>.

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, *sdi1* 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/ $\mu$ g vector plasmid, single-strand carrier DNA gave a about 40 fold increase (about 200 transformants/ $\mu$ g 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/ $\mu$ g 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 sdi1* 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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