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FULL PAPER

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# Isolation of a *ras* gene from the basidiomycete *Coriolus hirsutus* and use of its promoter for the expression of *Pleurotus ostreatus* manganese(II) peroxidase cDNA in *C. hirsutus*

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**Abstract** A *ras* gene homologue (named *Ch.ras*) was cloned from the basidiomycete *Coriolus hirsutus*. *Ch.ras* has a coding capacity of 215 amino acids (aa) interrupted by six small introns. The deduced Ch.Ras protein exhibited significant homology (86.5% or 86.0% identical) to the basidiomycete *Coprinus cinereus* Ras (215 aa) and *Lentinula edodes* Ras (217 aa) proteins. The 5′-upstream region of *Ch.ras* contains two GC boxlike sequences, one TATA boxlike sequence, one CCAAT box, and three CT-rich sequences. Primer extension analysis showed the presence of three transcriptional initiation sites: one is located in the most upstream CT-sequence and the other two just after it. By using the 1.4-kb fragment containing the promoter elements and transcriptional initiation sites, we have constructed the chromosome-integrating vector pHRP, which is useful for the expression of foreign genes in *C. hirsutus*. The *Pleurotus ostreatus* manganese(II) peroxidase (MnP) cDNA (designated *mnp*c) was inserted into the downstream of the *Ch.ras* promoter elements of pHRP, yielding pHRP-mnp. We obtained, with pHRP-mnp, *C. hirsutus* strains that show high levels of enzymatic activity of MnP and efficiently degrade pentachlorophenol (PCP), a chlorinated aromatic toxic compound.

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## Introduction

Plant biomass i.e., lignocellulose, is the most abundant renewable organic resource on earth. If we can develop biological methods for isolation of cellulose, which is convertible into ethanol by fermentation, from waste plant biomass, it is meaningful and remarkable from the point of view of obtaining a source of energy. To isolate cellulose from plant biomass, it is required to eliminate lignin, the most intractable aromatic polymer on earth.

White-rot basidiomycete fungi are known to be the only organisms that can degrade lignin. Manganese(II) peroxidase (MnP) and lignin peroxidase (LiP) are key peroxidases in the degradation of lignin (Kirk and Farrell 1987). MnP and LiP are also known to be involved in degradation of chlorinated aromatic toxic compounds such as pentachlorophenol (PCP) and dioxins (Pointing 2001). The knowledge just mentioned led us to attempt the molecular breeding of fungal strains producing a large amount of MnP or LiP. Production in the fungi of MnP and LiP is seen during secondary metabolism and is completely suppressed under conditions of excess nitrogen and carbon. Thus, we constructed the chromosome-integrating vector pLC1 in which *Lentinula edodes ras* gene promoter and *priA* terminator were used for the constitutive expression of foreign genes in basidiomycetes (Kajiwara and Shishido 1992; Kajiwara 1993). The *L. edodes ras* gene was transcribed similarly at high levels during vegetative growth and all stages of fruiting development (Hori et al. 1991). The terminator of *L. edodes priA* gene consisted of the sequences required for both transcription termination and polyadenylation and the site for adenylation (Kajiwara 1993). By using pLC1, we have introduced the *Pleurotus ostreatus* MnP cDNA (designated *mnp*c) into protoplasts of rapidly growing *Coprinus cinereus*, producing strains with remarkably high lignin-decolorization and -degradation activities

(Ogawa et al. 1998). These produced *C. cinereus* strains grew well on solid media containing rice straw and fallen leaves, but grew only slightly on those containing sawdust of beech wood (Kikuchi et al. 2002). *Coriolus hirsutus*, on the other hand, grow well on the sawdust-based media. We attempted to produce *C. hirsutus* strains with a high MnP productivity by using pLC1, but no *C. hirsutus* strains showing constitutively high levels of MnP activity were obtained. Therefore, the *ras* gene was isolated from *C. hirsutus* and its promoter was used for an expression of *P. ostreatus mnp*c in *C. hirsutus*. The successful experimental results are described next.

## Materials and methods

### Strains and media

*Coriolus hirsutus* monokaryotic strain OJ1078 (*arg*$^-$ *leu*$^-$) was used as the recipient in transformation experiments (Tsukamoto et al. 2003). MYGC media (1% malt extract, 0.4% yeast extract, 0.4% glucose, 1% casamino acid, pH 5.6) was used for the growth of *C. hirsutus.* Regeneration medium (pH 5.6) used for *C. hirsutus* protoplasts contained 171.15 g sucrose, 20 g glucose, 0.5 g $KH_2PO_4$, 1.0 g $K_2HPO_4$, 1.5 g $(NH_4)_2HPO_4$, 0.5 g $MgSO_4{\cdot}7H_2O$, 6.7 g Yeast Nitrogen Base (Difco), 0.5 g L-leucine, 0.12 mg thiamine-HCl (filter sterilized) and 10 g agar per liter. For assessments of MnP activity and pentachlorophenol (PCP) degradation of *C. hirsutus* strains, the mycelial cells were cultivated in BK medium containing 25 g brewer's grains and 100 ml Kirk Basal III medium per liter. Brewer's grains were obtained from Kirin Brewery (Tokyo, Japan) and Kirk basal III medium was prepared according to the method of Tien and Kirk (1988). *C. hirsutus* cells were grown at 25°C. Construction, propagation, and amplification of recombinant plasmids were carried out in *Escherichia coli* JM109 (Yanisch-Perron et al. 1985).

### Cloning of genomic and complementary DNA fragments encoding the *C. hirsutus ras* gene

The *C. hirsutus* genomic library was constructed by ligating a size-fractionated *Bam*HI-digest of genomic DNA into pUC19, followed by transformation of *E. coli* JM109. The genomic DNA was isolated as described by Zolan and Pukkila (1986). This library was screened by using a $^{32}$P-labeled probe (Feinberg and Vogelstein 1983) of the 0.3-kb DNA fragment encoding the highly conserved region of eukaryotic *ras* genes amplified with polymerase chain reaction (PCR) using *C. hirsutus* genomic DNA as a template and the following two degenerate primers: primer 1 (5′-GAYGARTATGAYCCKACTATCGA-3′) and primer 2 (5′-ATAYTCTTCCTGNCCGGCRGTATC-3′) (refer to Fig. 1). Previously published conditions were used for the hybridization (Hori et al. 1991). The genomic DNA clones were restriction mapped, and the restriction fragments were analyzed by nucleotide (nt) sequencing after subcloning into pUC19. To isolate the DNA fragment containing the full-length *Ch.ras* cDNA, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using poly(A)$^+$ RNA (1 μg) and the following two oligonucleotide primers: primer 3 (5′-CCTCCGCCATACAGCCATGTCCAG-3′) was identical to the 5′-flanking sequence containing the start codon of *Ch.ras* and primer 4 (5′-TTACGCGACA ACGCAGCCACAGC-3′) was complementary to the sequence encoding the 3′-end of *Ch.ras* (refer to Fig. 1). RT-PCR was carried out using a ReverTra Dash RT-PCR kit (Toyobo, Osaka, Japan) according to the supplier's instruction. The PCR-amplified 0.65-kb DNA fragment was directly inserted into *Hin*cII-digested pUC19. Sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977).

### Primer extension analysis

Total cellular RNA of *C. hirsutus* was isolated as described previously (Yamazaki et al. 2000). Poly(A)$^+$ RNA was prepared from the total cellular RNA using Oligotex-dT30<Super> (Takara Shuzo, Kyoto, Japan) according to the supplier's instruction. Mapping of transcriptional initiation sites by primer extension analysis was done as described previously (Yamazaki et al. 2000) using the following primer: primer 7 (5′-ACGCACCCTGGACATG GCTGTATGG-3′) (refer to Fig. 1). The sizes of the primer extension products were determined from the sequencing ladders (ACGT) by the dideoxy chain termination method of Sanger et al. (1977).

### Plasmid construction

The 1274-bp fragment of manganese(II) peroxidase (MnP) cDNA (*mnp*c) of *P. ostreatus* IFO30160 was isolated by digestion of plasmid pMOSBlue-mnp (Ogawa et al. 1998) with *Bam*HI and *Xba*I, and it was then inserted between the *Bam*HI and *Xba*I sites of pUC19, yielding plasmid pUC-mnp. The 1.4-kb DNA fragment containing the promoter region of the *Ch.ras* gene was obtained by the PCR method using the cloned 3.5-kb genomic DNA fragment as a template and two oligonucleotide primers: primer 5 (5′-GGGGATCCGCTATACCGAAAGGC-3′) and primer 6 (5′-GAGGATCCCGCTGTATGGCGGAGGAGG-3′) (refer to Fig. 1). The amplified DNA fragment was digested with *Bam*HI, of which sites are in two primers. The digest was inserted into the *Bam*HI site of pUC-mnp. The plasmid that the *Ch.ras* promoter sequence was inserted into appropriate direction to *P. ostreatus mnp*c was selected and named pRP-mnp (refer to Fig. 3). The 1.2-kb *Xba*I-*Eco*RI fragment containing the *L. edodes priA* gene terminator sequence (Kajiwara 1993) was inserted between *Xba*I and *Eco*RI sites of pUC19 and the recombinant plasmid was digested with *Eco*RI. The *Eco*RI-linearized DNA was treated with the Klenow fragment, ligated with the *Pst*I linker (5′-GCTGCAGC-3′), and recircularized with T4 DNA ligase. The circularized DNA was digested with *Xba*I and *Pst*I and the 1.2-kb *Xba*I-*Pst*I fragment was inserted

between *Xba*I and *Pst*I sites of pRP-mnp, yielding pHRP-mnp (refer to Fig. 3). Restriction endonucleases, DNA-modifying enzymes, and the *Pst*I linker were purchased from Takara Shuzo or Nippon Gene (Toyama, Japan) and used according to the suppliers' instructions.

### Transformation of *C. hirsutus*

Oidia (≈2 × $10^8$) were harvested from culture plates of OJ1078 (*arg*$^-$ *leu*$^-$) (Tsukamoto et al. 2003). The cells were harvested by centrifugation at 1500*g* for 10 min at room temperature and washed twice with MM buffer (0.5 M $MgSO_4$ in 50 mM maleic acid, pH 5.6). The washed cells were suspended in 2 ml MM buffer containing 1% Yatalase (Takara Shuzo), 0.5% cellulase Onozuka R-10 (Yakult Pharmaceutical, Tokyo, Japan), and incubated at 37°C at 2 h. The protoplasts were harvested by centrifugation at 1000*g* for 10 min at room temperature. They were washed with 1 M sorbitol in 20 mM 2-morpholinoethanesulfonic acid (MES, pH 6.3) and resuspended in this buffer at a concentration of 0.5–1 × $10^7$ cells/100 µl. To 100 µl protoplast suspension, 20 µl plasmid DNA solution (containing 5 µg each of pHRP-mnp and pUCR1 carrying the *C. hirsutus ARG1* gene) (Tsukamoto et al. 2003) was added and placed on ice for 30 min. Then, 120 µl of 50% PEG3350 in 20 mM MES (pH 6.3) was added and placed on ice for 30 min. To regenerate the protoplasts, 10 ml regeneration medium (kept melted, 50°–55°C) used for selecting *C. hirsutus* transformants showing an $Arg^+$ prototrophy were added and mixed. The mixture was poured into 9-cm plastic plates and incubated at 30°C. Colonies were examined after 4–5 days. *C. hirsutus* transformants showing an $Arg^+$ prototrophy were subjected to the following experiments.

### Southern blot analysis

The analysis was done according to the method previously described (Yamazaki et al. 2000).

### Quantitative RT-PCR of the *mnp*c transcripts

Quantitative RT-PCR was done according to the method of Akiyama et al. (2002) with a few appropriate modifications. Total cellular RNA isolated from *C. hirsutus* transformants was used for first-strand cDNA synthesis. Reverse transcription reaction was performed with 1 µg total cellular RNA and 10 pmol oligo $(dT)_{20}$ primer using SuperScript II RNase $H^-$ reverse transcriptase (Invitrogen, Carlsbad, CA, USA). After reverse transcription, synthesized cDNA was used as a template in the PCR with two oligonucleotide primers: the sense primer was identical to the 5′-end of *mnp*c (5′ATGACCTTTGCTTCGCTTTCTGCGCTCGTC) and antisense primer was complementary to the 3′-end of *mnp*c (5′TTACGCAGGTGGGACACGAGGAACAGAGGTGACTGG). PCR was performed with rTaq DNA Polymerase (Toyobo), and the reaction parameters were 30 s at 95°C, 60 s at 55°C, and 120 s at 72°C for 25, 30, 35, and 40 cycles. The amplified DNA products were electrophoresed on 1.0% agarose gels and stained with ethidium bromide. The densitometric measurement of the intensities of electrophoretic patterns was analyzed by NIH (National Institute of Health) image software.

### MnP assay

*C. hirsutus* strains were inoculated with one agar disc (6 mm in diameter and 1 mm thick) to 100-ml Erlenmeyer flasks containing 10 ml BK medium. MnP activity was measured by the formation of $Mn^{III}$-malonate complex according to the method of Wariishi et al. (1992). The samples cultured for the indicated times were centrifuged, and 100 µl culture supernatants were subjected to measuring MnP activities. One unit was defined as the activity of formation of 1 µmol $Mn^{III}$-malonate complex per minute.

### Analysis of PCP degradation

The reaction mixture (total volume, 1 ml) containing 0.1 mM PCP (Wako, Osaka, Japan), 1 mM $MnSO_4$, 50 mM Na malonate (pH 4.5), 0.1 mM $H_2O_2$, and 100 µl culture supernatants obtained from the cultivation sample was prepared. The culture supernatant, boiled for 5 min, was used as a control. Next, 100 µl 3N HCl was added to the reaction mixture and the acidic mixture was extracted three times with an equal volume of chloroform. The collected chloroform layers were dried by $N_2$. The residues were dissolved in methanol and subjected to analysis of the amount of PCP using HPLC (Jasco, Tokyo, Japan) equipped with an ODS column (YMC, Kyoto, Japan). $H_2O$:methanol (2:8) in 0.05% phosphoric acid was used as a solvent at a flow rate of 1.0 ml/min. Chromatograms were spectroscopically recorded at 254 nm, and PCP concentration was determined using a standard curve.

## Results and discussion

### Cloning and sequence analysis of the *Ch.ras* gene

Part of the *Ch.ras* gene was amplified with PCR using *C. hirsutus* genomic DNA as a template and the two degenerate primers (primer 1 and 2 of Fig. 1) designed from the two highly conserved amino acid (aa) sequences among eukaryotic *ras* genes, i.e., DEYDPTIE and DTAGQEEY, respectively. The amplified 0.3-kb PCR product was cloned and sequenced to confirm that it is the *ras* sequence. Next, *C. hirsutus* DNA was digested with *Bam*HI and subjected to Southern blot analysis using $^{32}$P-labeled 0.3-kb PCR product. One hybridization signal was detected at the size corresponding to 3.5 kb. So, we cloned the 3.5-kb *Bam*HI fragment and sequenced. Homology search of the deduced aa sequence of the 3.5-kb *Bam*HI fragment suggested that it contains a whole sequence of *ras* gene. We attempted to isolate the full-length cDNA of *Ch.ras* by RT-PCR method

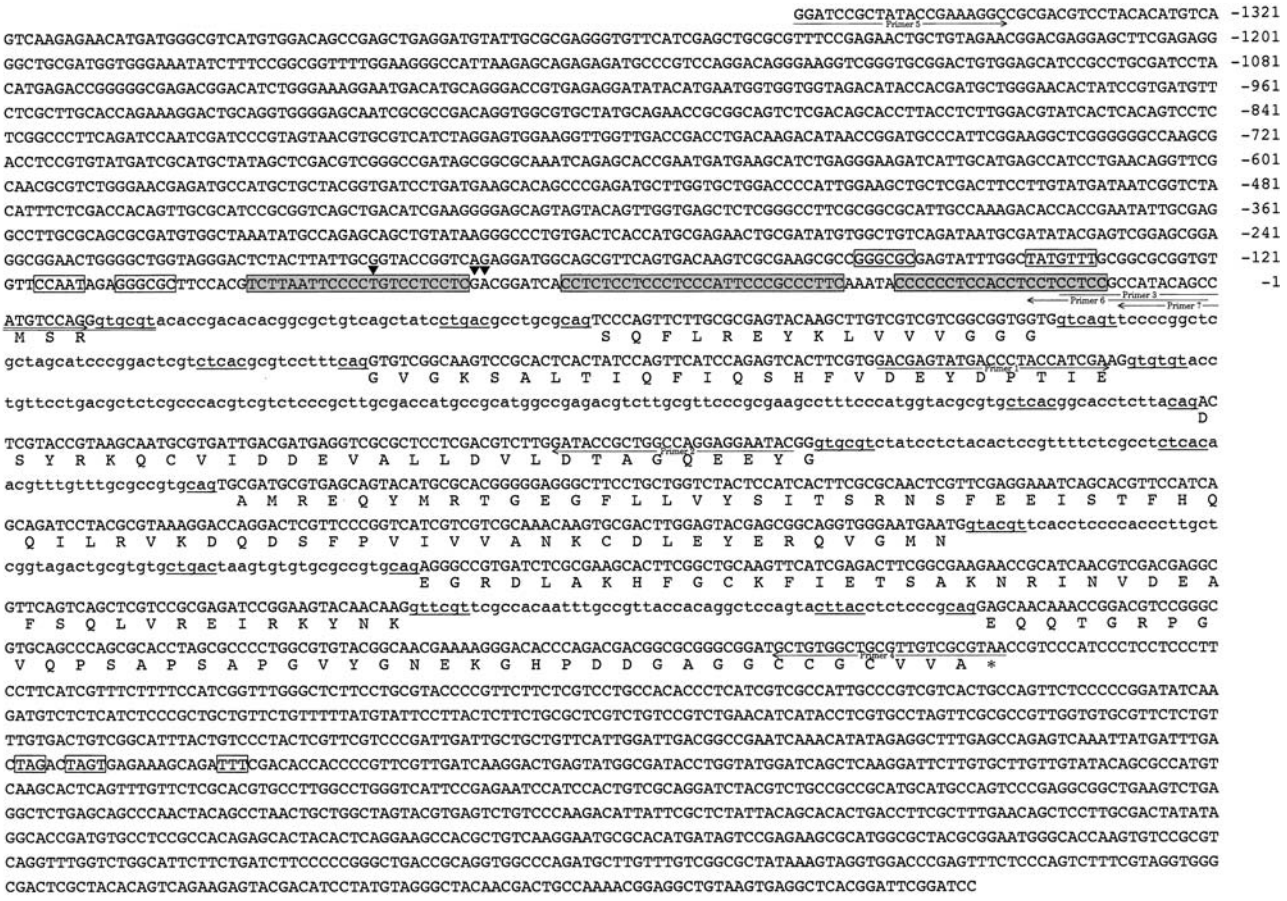

**Fig. 1.** The nucleotide (nt) and deduced amino acid (aa) sequences of the *Coriolus hirsutus ras* gene. The presence of introns in *lowercase letters* was deduced from comparison of the 0.65-kb cDNA sequence, and consensus sequences for splicing are *underlined*. The nt sequence coordinates of the coding region of the *Ch.ras* gene and the 3′-noncoding region are shown on the *left-hand side* and those of the 5′-noncoding region on the *right-hand side*. The aa sequence coordinates are also present on the *left-hand side*, and the start codon is assigned the +1 coordinate. Two GC-boxlike sequences, a TATA boxlike sequence, a CCAAT box, and putative signals for transcriptional termination and poly(A) addition are *boxed*. Three CT-rich sequences (CT motif) are *shaded*. Seven primers (primer 1–7) used for PCR or primer extension analysis are indicated by *horizontal long arrows*. *Closed arrowheads* are transcriptional initiation sites of the *Ch.ras* gene determined in Fig. 2. The nt sequence data of *Ch.ras* will appear in the DDBJ/EMBL/GenBank nt sequence databases under accession no. AB175073

using poly $(A)^+$ RNA of *C. hirsutus* and primer 3 and 4 designed from the putative 5′- and 3′-ends of the *Ch.ras* gene, respectively (see Fig. 1). Cloning and sequencing of the PCR-amplified 0.65-kb DNA fragment revealed that this fragment contains a 648-bp open reading frame with start and stop codons encoding a 215-aa sequence with extensive similarities to other eukaryotic Ras proteins. The sequence of the genomic clone and that of the cDNA clone agreed perfectly with each other, and comparison between these sequences showed that the coding sequence is interrupted by six introns and that the positions of the introns are the same for these for other basidiomycetous *ras* genes. The molecular weight of the predicted polypeptide of the *Ch.ras* gene product was calculated to be 23963. Among various Ras proteins, Ch.Ras was highly homologous to the basidiomycetous Ras proteins *Coprinus cinereus* Ras (Cc.Ras, 215 aa) and *Lentinula edodes* Ras (Le.Ras, 217 aa) in size and aa sequence, and the deduced aa sequence of Ch.Ras protein showed an overall identity of 86.5% and 86.0% to those of Cc.Ras and Le.Ras, respectively. As Ras proteins of other basidiomycetes, the Ch.Ras protein has the conserved C-terminal sequence CysAAX (where A is any aliphatic aa and X is any aa) and two consecutive Cys residues at the positions 2 and 3 aa upstream from the CysAAX box (see Fig. 1).

## 5′- and 3′-flanking nt sequences and transcriptional initiation sites of the *Ch.ras* gene

Sequencing the 3.5-kb genomic clone revealed that the 5′-noncoding region contains two GC boxlike sequences (−156 to −151 and −109 to −104), one TATA boxlike sequence (−139 to −133), one CCAAT box (−117 to −113), and CT motifs extended over 22 (−96 to −75), 28 (−65 to −38), and 21 (−32 to −12) nt (19, 26, and 20 are pyrimidines in these, respectively). Transcriptional initiation sites of the *Ch.ras* gene were analyzed by the primer

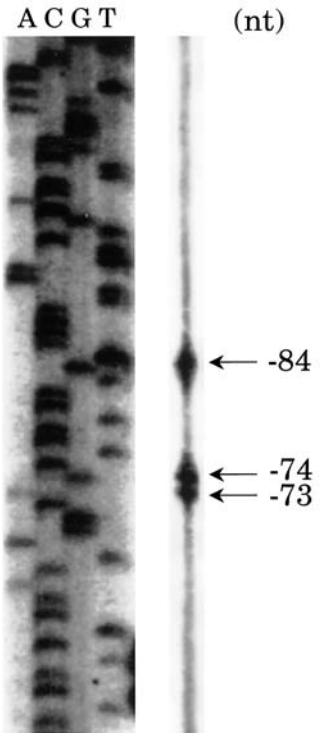


**Fig. 2.** Determination of the transcriptional initiation sites of the *Ch.ras* gene by primer extension analysis. The nt corresponding to the ends of extension products (marked by *arrows*) were determined from the sequencing ladders (*ACGT*), which were derived from the 5′-flanking region of *Ch.ras.* The nt numbers shown in the figure correspond to the nt indicated by the *closed arrowheads* in Fig. 1

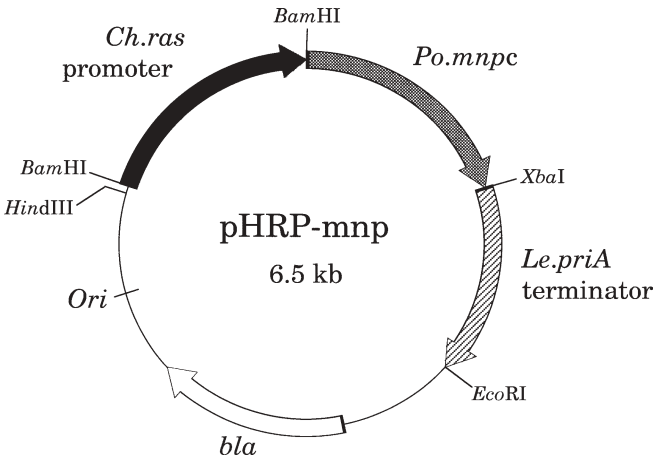


**Fig. 3.** Structure of the *Pleurotus ostreatus* manganese(II) peroxidase (MnP)-expressing recombinant plasmid pHRP-mnp. *Thin line* indicates pUC19, and the *bla* gene and the origin of replication (*ori*) are also indicated. Restriction sites of *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I are indicated

extension method. As shown in Fig. 2, analysis of the *Ch.ras* transcripts gave three bands at the positions corresponding to initiation sites at nt 73, 74, and 84 upstream from the *Ch.ras* start codon. These transcriptional initiation sites fall within the most upstream CT motif and the region just downstream of it. The transcriptional initiation sites of various fungal genes, especially basidiomycetous fungal genes, are often found in or near the CT motifs (Kajiwara 1993). Thus, the CT motifs of the 5′-noncoding region of *Ch.ras* are considered to play an important role for expression of the *Ch.ras* promoter activity. On the other hand, the 3′-flanking region was found to contain sequences TAG···TAGT···TTT required for both transcriptional termination and polyadenylation (Zaret and Sherman 1982) in the region ranging from nt 1442 to nt 1464 (nt 383 to 405 downstream from the termination codon of *Ch.ras*).

## Cotransformation of the *C. hirsutus* Arg$^-$ strain OJ1078 with pUCR1 and pHRP-mnp

The recombinant plasmid pHRP-mnp (Fig. 3), which contains the manganese(II) peroxidase (MnP) cDNA (*mnp*c) derived from *P. ostreatus* between the *Ch.ras* promoter and the *L. edodes priA* terminator, was used for the experiments. Because the poly(A) addition site of the *Ch.ras* gene was not determined, the *L. edodes priA* terminator was used, which contains the sequences required for both transcriptional termination and the polyadenylation (Zaret and Sherman 1982) and the poly(A) addition site. pHRP-mnp was introduced into the *C. hirsutus* OJ1078 genome by cotransforming with pUCR1 carrying the *C. hirsutus ARG1* gene and selecting for Arg$^+$ transformants. Five micrograms each of pHRP-mnp and pUCR1 was presented to 0.5–1 $\times$ $10^7$ protoplasts. Seven Arg$^+$ colonies were obtained. To ascertain the presence of the introduced *mnp*c DNA in the transformants, Southern blot analysis was done using the $^{32}$P-labeled 1.3-kb *mnp*c as a probe. In DNA samples from the Arg$^+$ transformants that had not been subjected to digestion with restriction enzymes, specific hybridization signals were observed in the high molecular mass region corresponding to the chromosomal DNA (data not shown). No specific hybridization signal was found in the DNA of the Arg$^+$ transformant obtained by the introduction of pUCR1 alone. Next, all the DNA samples were digested with both *Bam*HI and *Xba*I (see Figs. 3, 4A) and subjected to Southern blot analysis using the aforementioned probe. Two of seven Arg$^+$ transformants gave the signal for the presence of an entire *mnp*c sequence (data not shown). To estimate the copy number of the expression cassette (promoter-*mnp*c-terminator) on the chromosomes of the two Arg$^+$*mnp*c$^+$ transformants, DNA samples (20 μg each) were digested with both *Eco*RI and *Hin*dIII (see Figs. 3, 4A) and subjected to Southern blot analysis using the $^{32}$P-labeled 1.4-kb genomic DNA fragment containing the 5′-flanking promoter region (nt −1362 to −2) of *Ch.ras* as a probe. As shown in Fig. 4B, both Arg$^+$*mnp*c$^+$ transformants gave two signals at the positions of 4.0 kb and 3.8 kb. The Arg$^+$ transformant obtained by the introduction of pUCR1 alone gave just one signal at the position of 4.0 kb (lane 1, Fig. 4B). The 4.0-kb signals, which have almost the same intensities, are considered to be derived from the intrinsic *Ch.ras* gene, whereas the 3.8-kb signals, which have different intensities, are derived from the introduced *mnp*c expression cassette

consisting of the *Ch.ras* promoter, the *mnp* cDNA, and the *Le.priA* terminator. Comparison of the radioactivity of 4.0-kb and 3.8-kb hybridization signals suggested that the two Arg$^+$*mnp*c$^+$ transformants carry one and three copies of the expression cassette on their chromosomes, and these two transformants were named ChTF-1(Po.MnP) (lane 2) and ChTF-3(Po.MnP) (lane 3) , respectively.

## Quantitative RT-PCR analysis of transcripts of *P. ostreatus mnp*c gene in ChTF-1(Po.MnP) and ChTF-3(Po.MnP)

To analyze transcriptional expression of the *P. ostreatus mnp*c gene in ChTF-1(Po.MnP) and ChTF-3(Po.MnP), Northern blot analysis was done. Total cellular RNAs isolated from vegetatively growing mycelia of the transformants were analyzed using the $^{32}$P-labeled 1.3-kb fragment containing the *mnp*c gene as a probe. Very faint hybridization signals of *mnp*c were detected at around the 1.5-kb position. Because it was difficult to discuss the *mnp*c transcript levels, a quantitative RT-PCR was done using two oligonucleotide primers designed for amplification of the 1.3-kb *mnp*c sequence (Fig. 5). The 1.3-kb PCR product was detected at 35 cycles for ChTF-1(Po.MnP) and 30 cycles for ChTF-3(Po.MnP). At 40 cycles of the reaction, the intensity of the PCR product of ChTF-3(Po.MnP) was clearly higher than that of ChTF-1(Po.MnP). These results coincided with the result of the copy number analysis (see Fig. 4). We tried to detect MnP proteins derived from the *P. ostreatus mnp*c gene in the culture supernatant of ChTF-1(Po.MnP) and ChTF-3(Po.MnP) by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. However, unfortunately we could not identify them clearly because many protein bands appeared at around the size of the *P. ostreatus* MnP protein (about 40 kDa) on the gel (data not shown).

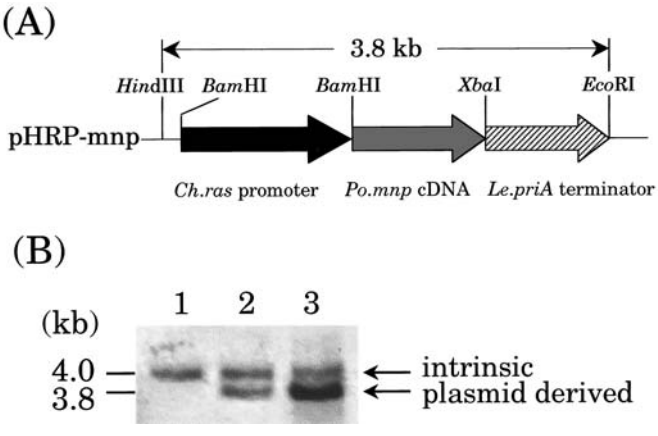


**Fig. 4.** Analysis of total cellular DNA of the *C. hirsutus* transformants. **A** Restriction map of pHRP-mnp. The 3.8-kb *Eco*RI-*Hin*dIII fragment is shown. **B** Southern blot analysis of *Eco*RI + *Hin*dIII digests of total cellular DNA prepared from the control Arg$^+$ transformant (*lane 1*), ChTF-1(Po.MnP) (*lane 2*), and ChTF-3(Po.MnP) (*lane 3*). The $^{32}$P-labeled 1.4-kb genomic DNA fragment containing the 5′-flanking promoter region of *Ch.ras* was used as a probe

## MnP activity and pentachlorophenol (PCP)-degradation activity of ChTF-1(Po.MnP) and ChTF-3(Po.MnP)

ChTF-1(Po.MnP), ChTF-3(Po.MnP), and the Arg$^+$ transformant were cultured in BK medium. The growth rates of these transformants were almost the same. The culture supernatants were examined for the MnP activity by measuring the increase of the absorbance at 270 nm based on the formation of Mn$^{III}$-malonate complex. As shown in Fig. 6, the MnP activities of all transformants reached maximum level at 11 days cultivation and then gradually decreased. The activities at 11 days cultivation of ChTF-3(Po.MnP) and ChTF-1(Po.MnP) were about three and two times higher than that of the control Arg$^+$ transformant.

Next, PCP-degrading activity was analyzed for the supernatants of 11 days cultivation at 25°C. Reaction mixtures containing 0.1 mM PCP and the culture supernatants were incubated for 20 h and the remaining PCP was extracted by chloroform, followed by HPLC analysis. As shown in Fig. 7, 82% and 68% of initial input PCP was degraded by ChTF-3(Po.MnP) and ChTF-1(Po.MnP), respectively, whereas only 30% was degraded by the control Arg$^+$ transformant. The PCP-degrading activities of ChTF-3(Po.MnP) and ChTF-1(Po.MnP) were 2.7 or 2.3 times higher than that of the control Arg$^+$ transformant. These values coincide well with the MnP activities shown in Fig. 6.

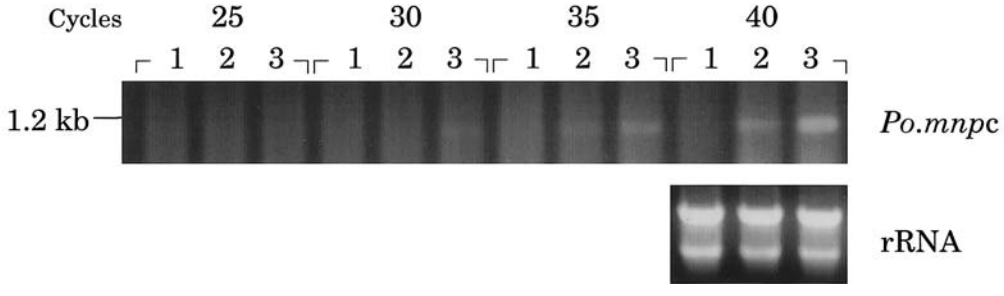


**Fig. 5.** Quantitative RT-PCR of the transcripts from the *P. ostreatus mnp*c (*Po.mnp*c) genes in *C. hirsutus* transformants. PCR cycles are indicated *above* the lanes. Total cellular RNAs (1 μg each) isolated from the three transformants used in Fig. 4B were subjected to the analysis, while these were run on an agarose gel and stained. The bands of ethidium bromide-stained ribosomal RNAs (rRNA) are shown as an internal control

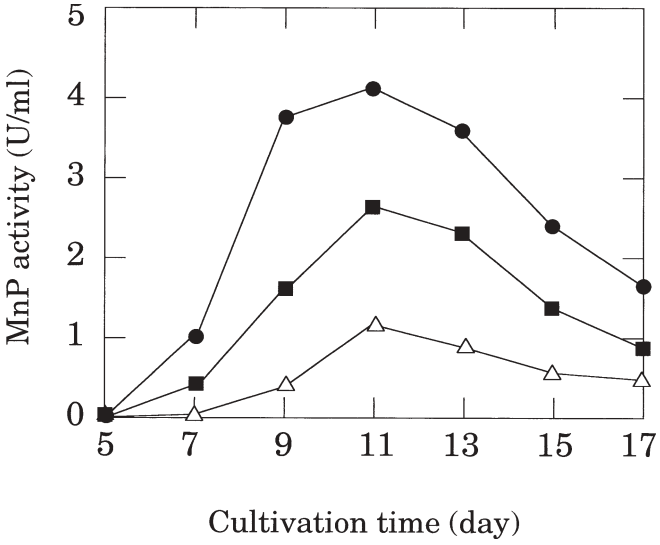


**Fig. 6.** MnP activity in the culture supernatants of the control $Arg^+$ transformant (△), ChTF-1(Po.MnP) (■), and ChTF-3(Po.MnP) (●). One unit was defined as the activity of formation of 1 μmol $Mn^{III}$-malonate complex per minute. MnP activities (U/ml) shown are the average of three independent experiments

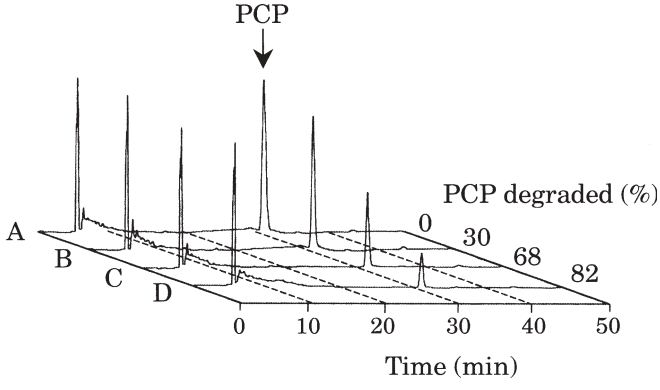


**Fig. 7.** Degradation of pentachlorophenol (PCP) by the culture supernatants of the $Arg^+$ transformant (*B*), ChTF-1(Po.MnP) (*C*), and ChTF-3(Po.MnP) (*D*). As a control (0% degradation), the boiled culture supernatant of the $Arg^+$ transformant (*A*) was used in the experiment. Values of PCP degraded (%) are the average of three independent experiments

In this report, we succeeded in molecular breeding of *C. hirsutus* strains showing high levels of MnP activities using the promoter of *C. hirsutus ras* gene. These strains are considered to be useful for degradation from the point of view of plant biomass reuse and environmental preservation.

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## References

Akiyama R, Sato Y, Kajiwara S, Shishido K (2002) Cloning and expression of cytochrome P450 genes, belonging to a new P450 family, of the basidiomycete *Lentinula edodes.* Biosci Biotechnol Biochem 66:2183–2188

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

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

Kajiwara S (1993) Molecular genetical studies on *ras* gene and fruiting genes of eubasidiomycetes. Ph.D. thesis, Tokyo Institute of Technology, Tokyo

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

Kikuchi M, Ogawa K, Yamazaki T, Kajiwara S, Shishido K (2002) Use of molecular-genetically bred *Coprinus cinereus* strains for an efficient isolation of cellulose from rice straw. Biosci Biotechnol Biochem 66:199–201

Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41:465–505

Ogawa K, Yamazaki T, Hasebe T, Kajiwara S, Watanabe A, Asada Y, Shishido K (1998) Molecular breeding of the basidiomycete *Coprinus cinereus* strains with high lignin-decolorization and -degradation activities using novel heterologous protein expression vectors. Appl Microbiol Biotechnol 49:185–189

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57:20–33

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol 161:238–249

Tsukamoto A, Kojima Y, Kita Y, Sugiura J (2003) Transformation of the white-rot basidiomycete *Coriolus hirsutus* using the ornithine carbamoyltransferase gene. Biosci Biotechnol Biochem 67:2075–2082

Wariishi H, Valli K, Gold MH (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J Biol Chem 267:23688–23695

Yamazaki T, Hasebe T, Kajiwara S, Shishido K (2000) Structure and function of a pyrimidine/purine-biased sequence from the 5′-flanking region of the basidiomycete *Lentinus edodes* gene *priA*. Mol Gen Genet 263:262–270

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

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

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