Intron-Dependent Accumulation of mRNA in *Coriolus hirsutus* of Lignin Peroxidase Gene the Product of Which Is Involved in Conversion/Degradation of Polychlorinated Aromatic Hydrocarbons

Takashi Yamazaki,1,* Yutaka Okajima,1 Hiroki Kawashima,1 Akira Tsukamoto,2
Jun Sugihara,2 and Kazuo Shishido1,1

1Department of Life Science, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan
2Advanced Technology Research Laboratory, Oji paper Co., Ltd., Shinonome, Koto-ku, Tokyo 135-8558, Japan

Received September 7, 2005; Accepted February 4, 2006; Online Publication, June 23, 2006
[doi:10.1271/bbb.50471]

The homobasidiomycete *Coriolus hirsutus* coding sequences of a lignin peroxidase (LiP) gene (lip, containing six (I–VI) introns), a lip cDNA (lipc), and three lipc derivatives containing one (I), three (I–III), or five (I–V) introns were inserted into chromosome-monomeric strain. The transformant carrying the promoter–lipc–terminator cassette did not contain enough mRNA molecules to be detectable by Northern-blot analysis. On the other hand, all the transformants carrying cassettes of genomic lip and intron(s)-containing lipc sequences contained sufficient amounts of mRNAs to be easily detected by Northern-blot analysis. LiP activities in the culture supernatants of these transformants were found to be about five times as high as those of transformants carrying the lipc cassette (or no cassette). The culture supernatants of the transformants with high LiP activity showed remarkably high conversion activity toward pentachlorophenol (PCP) and degradation activity toward 2,7-dichlorodibenzo-p-dioxin (2,7-DCDD). These results indicate that at least one intron (intron I) is required for accumulation of lip mRNA and its subsequent translational expression in *C. hirsutus*.

Key words: basidiomycete; *Coriolus hirsutus*; intron-dependent mRNA accumulation; lignin peroxidase; polychlorinated aromatic hydrocarbons

Our previous studies showed that cDNA coding sequences of *Pleurotus ostreatus* manganese(II) peroxidase (MnP)11 and *Aspergillus oryzae* endo-(1,4)-β-xylanase XynF12 under the control of the promoter of the *Lentinula edodes* priA gene give efficient expression in the homobasidiomycete *Coprinopsis cinerea* (formerly named *Coprinus cinereus*). The transcripts of these cDNAs were easily detected by Northern-blot analysis. The bacterial endo-xylanase gene was also efficiently expressed in *C. cinerea* under the control of the same promoter.3) Contrary to this, other research groups have reported that in the homobasidiomycete *Schizophyllum commune* and *Phanerochaete chrysosporium*, introns are necessary for efficient expression of at least some homologous and heterologous genes.1,5) The necessity of introns for efficient gene expression has been reported in other eukaryotes such as metazoans and yeast, and splicing has been shown to be required for rapid and efficient nucleocytoplasmic export of mRNA.6–9) In the meanwhile, reports of the two homobasidiomycete fungi were as follows: Lugones et al.4) showed that accumulation of mRNA of the hydrophobin genes *SC3* and *SC6* of *S. commune*, the hydrophobin gene *ABH1* of *Agaricus bisporus*, and the green fluorescent protein (GFP) gene *gfp* of *Aequorea victoria* did not occur in *S. commune* when cDNA coding sequences were introduced. In contrast, mRNAs did accumulate when genomic sequences were used or when an intron was added to cDNA constructs. Ma et al.5) reported that efficient expression in *P. chrysosporium* of the *gfp* gene is observed only when an intron is inserted within it. These observations led us to examine whether the cDNA

References

1. To whom correspondence should be addressed. Tel: +81-45-924-5714; Fax: +81-45-924-5773; E-mail: kshishid@bio.titech.ac.jp

* Present address: Institute of Space and Astronautical Science, Japan Aerospace Exploration Agency, 2-1-1 Sengen, Tsukuba, Ibaraki 305-8505, Japan

** Abbreviations:** aa, amino acid(s); bp, base pair(s); C., *Coriolus* or *Coprinopsis*; cDNA, DNA complementary to RNA; Ch., *Coriolus hirsutus*; DCDD, dichlorodibenzo-p-dioxin; gfp, green fluorescent protein (GFP) gene; gpd, glyceraldehyde-3-phosphate dehydrogenase gene; kb, kilobase(s) or 1,000 bp; Le., *Lentinula edodes*; lip, lignin peroxidase (LiP) gene; lipc, lip cDNA; mnp, manganese(II) peroxidase (MnP) gene; nt, nucleotide(s); P., *Phanerochaete or Pleurotus*; PAGE, polyacrylamide-gel electrophoresis; PCP, pentachlorophenol; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; S., *Schizophyllum*
coding sequence gives efficient expression in homobasidiomycete fungus other than C. cinereae. We chose the white-rot homobasidiomycete Coriolus hirsutus as a recipient and the C. hirsutus lignin peroxidase (LiP) gene (lip) and its cDNA (lipc) as a donor for the following reasons: The transformation system was already established in this fungus, and the Arg auxotrophic monokaryotic strain and the ARG1 gene encoding ornithine carbamoyltransferase (OCTase) were obtained.10 LiP is the key enzyme in the degradation of lignin, the most intractable aromatic polymer on Earth, and is also known to be involved in conversion/degradation of chlorinated aromatic pollutants such as pentachlorophenol (PCP) and mono–tetrachlorodibenzo-p-dioxins (M–TetraCDDs).11–10 LiP converts PCP into tetrachloro-p-benzoquinone, and subsequently into tetrachlorodihydroxybenzene. It opens the dioxin ring and eliminates the chlorine(s) of M–TetraCDDs. Here, we report that the presence of at least one (first) intron in the coding region of lip is required for its efficient expression in C. hirsutus. The level of mRNA accumulation in the case of the lip coding sequence containing only the first intron (designated lipc[ intron I]) was similar to that in the case of the lip gene (containing six [I–VI] introns). Culture supernatants of transformants carrying the lipc[ intron I] or the lip gene showed high LiP activity, and also high conversion activity toward PCP and degradation activity toward 2,7-dichlorodibenzo-p-dioxin (2,7-DCDD).

Materials and Methods

Strains and media. C. hirsutus monokaryotic strain OJ1078 (Arg ‘Leu”) was used as a recipient in transformation experiments.10 MYGC media (1% malt extract, 0.4% yeast extract, 0.4% glucose, 1% casamino acids, pH 5.6) was used for growth of C. hirsutus. The regeneration medium (pH 5.6) used for C. hirsutus protoplasts contained 171.15 g sucrose, 20 g glucose, 0.5 g KH₂PO₄, 1.0 g K₂HPO₄, 1.5 g (NH₄)₂HPO₄, 0.5 g MgSO₄·7H₂O, 6.7 g Yeast Nitrogen Base (Difco; Becton Dickinson, Sparks, MD), 0.5 g L-leucine, 0.12 mg thiamine–HCl (filter sterilized), and 10 g agar per liter. For assessment of LiP activity, PCP conversion, and 2,7-DCDD degradation of C. hirsutus strains, mycelial cells were cultivated at 25 °C in BK medium containing 25 g brewer’s grains and 100 ml Kirk Basal III medium per liter.11 Brewer’s grains were obtained from Kirin Brewery (Tokyo) and Kirk basal III medium was prepared according to the method of Tien and Kirk.12 Construction, propagation, and amplification of recombinant plasmids were carried out in E. coli JM109.13

Plasmid construction. C. hirsutus lipc was isolated from the cDNA library. The lipc-coding region (1,107 bp) from the start codon to the stop codon was amplified by PCR using primer 1, 5’-CCGGATCC(BamHI) ATGGCGTCTAAGGCTTTCTCTC-3’ and primer 2, 5’-CCGGATCC(BamHI) TCAAGAGTGGTGAGG- GG-3’ (see Fig. 1). The amplified DNA fragment was digested with BamHI and inserted into the BamHI site of pUC19, yielding plasmid pUC-lipc. The C. hirsutus genomic lip-coding region (1,452 bp) containing six introns (see Fig. 1) was obtained by PCR using the genomic DNA fragment of C. hirsutus as a template and primers 1 and 2. The amplified DNA fragment was digested with BamHI and inserted into the BamHI site of pUC19, yielding plasmid pUC-lip. The DNA fragment containing the 653-bp promoter region of the C. hirsutus glyceraldehyde-3-phosphate dehydrogenase gene (gpd) was obtained by PCR using the genomic DNA fragment as a template and two primers, 5’-CCGAATTC(EcoRI) AGAGCGGCGGAGGCGCCGTGCGAGC-3’ and 5’- CCGGATCC(BamHI) GATGTGTTGGTGGAGG-GATG-3’. The amplified DNA fragment was digested with BamHI and EcoRI and then inserted between the BamHI and EcoRI sites of pUC19, yielding plasmid pUC-Ch-gpdP. Both termini of the 1.2-kb BamHI–EcoRI fragment containing the Lentinula edodes priA terminator20 were blunted and ligated to HindIII linker [5’-CAAGCTTG-3’]. The resulting DNA fragment was digested with BamHI and HindIII and inserted between the BamHI and HindIII sites of pUC-Ch-gpdP, yielding plasmid pUC-gpdPT. This plasmid was digested with EcoRI to obtain a 1.96-kb EcoRI fragment containing the Ch.gpd promoter and the Le.priA terminator. The 1.96-kb EcoRI fragment was inserted into the EcoRI site of pUCR1, which carries the C. hirsutus ARG1 gene as a selectable marker.10 The resulting recombinant plasmid was named Mlp30 (see Fig. 2A). The BamHI fragments containing the lipc sequence or the lip sequence were inserted between the Ch.gpd promoter and the Le.priA terminator on Mlp30 in the appropriate direction. The resulting plasmids were named Mlp30-lipc and Mlp30-lip respectively (see Fig. 2A, B). lip-coding sequences containing one, three, or five introns at the original positions were constructed by connecting the appropriate parts of lip with the appropriate parts of lipc. The 5’-side sequences of lip containing one (I), three (I–III), or five (I–V) introns were amplified by PCR using primer pairs 1 and 4, 6, or 8, and the 3’-side sequences of lipc were amplified by PCR using primer pairs 2 and 3, 5, or 7 (see Fig. 1 and Fig. 2B). Pairs of appropriate fragments were mixed and a second PCR was carried out to generate three sorts of lip–lipc fused sequences, which were named lipc[ intron I], lipc[ introns I–III], and lipc[ introns I–V] (see Fig. 2B). lipc[ intron I] consisted of exon I, intron I, and the major part of exon II of lip and the appropriate sequence of lipc. lipc[ introns I–III] consisted of the sequences of exon I–5’-part of exon IV of lip and the appropriate sequence of lipc. lipc[ introns I–V] consisted of the sequences of exon I–half part of exon VI of lip and the appropriate sequence of lip. The three fused DNA sequences were inserted between the promoter and the terminator of Mlp30, and the resulting
plasmids were named Mlp30-lip[intron I], Mlp30-lip[introns I–III], and Mlp30-lip[introns I–V] respectively (see Fig. 2A, B). Restriction endonucleases, DNA-modifying enzymes, and the HindIII linker were purchased from Takara Shuzo (Kyoyo) or Nippon Gene (Tokyo), and were used according to the suppliers' instructions.

Transformation of C. hirsutus. Transformation of C. hirsutus was done according to the method of Tsukamoto et al.10

Southern- and Northern-blot analyses. These analyses were done according to the method previously described.21 Total cellular DNA and RNA used for the analyses were prepared from mycelial cells of C. hirsutus strains cultivated in MYGC medium at 25°C for 1 week.

Assay of LiP activity. LiP activity of the culture supernatant of C. hirsutus was assayed according to the method of Tien and Kirk.18 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 of the aforementioned BK medium, and cultured for the indicated times. The reaction mixture (3 ml) contained 100 mM sodium succinate (pH 3.0), 0.4 mM veratroyl alcohol, 0.1% Tween 80, and 100 µl each of the culture supernatants. The reaction was initiated by the addition of H2O2 to give a final concentration of 0.1 mM. LiP activity was determined by measuring the increase in absorbance at 310 nm generated by oxidation of veratroyl alcohol to veratroyl aldehyde.

Assessment of PCP conversion. A reaction mixture (total volume, 1 ml) containing 0.1 mM PCP (Wako, Osaka, Japan), 1 mM MnSO4, 50 mM Na-malonate (pH 4.5), 0.1 mM H2O2, and 100 µl each of the culture supernatants obtained from the cultivation samples, was prepared. One hundred microliters of 3 N HCl was subjected to analysis of the amount of PCP using HPLC. 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.

Assessment of 2,7-DCDD degradation. A reaction
mixture (total volume, 2 ml) containing 0.5 \( \mu \text{M} \) 2,7-DCDD (Cambridge Isotope Lab, Cambridge, MA), 1 mM MnSO\(_4\), 50 mM Na-malonate (pH 4.5), 0.1 mM H\(_2\)O\(_2\), and 1.5 ml each of the culture supernatants obtained from the cultivation samples, was prepared. One hundred microliters of 3 N HCl was added to the reaction mixture, and the acidic mixture was extracted three times with 20 ml of hexane. The collected hexane layers were dried by N\(_2\). The residues were dissolved in acetone and subjected to analysis of the amount of 2,7-DCDD using GC (Shimadzu, Kyoto, Japan) equipped with a DB-5.625 column (J&W Scientific, Folsom, CA).

**Results and Discussion**

**Transformation of C. hirsutus Arg\(^-\)/Leu\(^-\) auxotrophic monokaryotic strain OJ1078 with MIp30-series of plasmids**

MIp30-lipc and MIp30-lip- contain the C. hirsutus lip\(_c\) (intronless) and lip\(_c\) (containing six (I–VI) introns) between the Ch.gpd promoter and the Le.priA terminator respectively (Fig. 2). These recombinant plasmids were introduced into protoplasts of C. hirsutus monokaryotic strain OJ1078 (Arg\(^-\)/Leu\(^-\)), and Arg\(^+\) transformants that showed almost the same growth rates as recipient strain OJ1078 were selected. As a result, the two Arg\(^+\) transformants were obtained for each plasmid. The two Arg\(^+\) transformants derived from the introduction of MIp30-lipc were named TF0-1 and TF0-2, and those from MIp30-lip were named TF6-1 and TF6-2. To ascertain the presence of the introduced expression cassette of lip\(_c\) or lip\(_c\) DNA in the transformants, Southern-blot analysis was done using the \(^{32}\)P-labeled 1.2-kb Le.priA terminator as a probe. In the DNA samples (20 \( \mu \text{g} \)) of the four Arg\(^+\) transformants without restriction enzyme digestion, 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 sample of the control Arg\(^+\) transformant obtained by introduction of MIp30 DNA alone. Hence it was suggested that TF0-1, TF0-2, TF6-1, and TF6-2 carry about five copies of each expression cassette on their chromosomes. Next, all the DNA samples (20 \( \mu \text{g} \)) were digested with EcoRI and subjected to Southern-blot analysis using the 1.2-kb Le.priA terminator probe. As shown in Fig. 3A, TF0-1, TF0-2, TF6-1, and TF6-2 all gave one intense hybridization signal at the position corresponding to the size (3.0 kb or 3.4 kb) of the expression cassette (Ch.gpd promoter–lip\(_c\) [or lip\(_c\)]–Le.priA terminator) (lanes 2–5). No hybridization signal was observed in the case of the control Arg\(^+\) transformant (lane 1). The radioactivities of the 3.0-kb band of TF0-1 (lane 2) and the 3.4-kb bands of TF6-1 and TF6-2 (lanes 4 and 5) were approximately five times higher than the radioactivity of the 3.0-kb band of TF0-2 (lane 3). These data indicate that TF0-1, TF6-1, and TF6-2 carry about five times more copies of the lipc-
C. hirsutus and MIp30-lipc[introns I–V], were introduced into mids MIp30-lipc[introns I], MIp30-lipc[introns I–III], terminator of MIp30. The resulting recombinant plasmid constructs were inserted between the promoter and the positions of lip containing one, three, and five introns at the original lip accumulation, and Methods’ culture conditions used in this study (refer to ‘Materials and Methods’).

To investigate how many introns are necessary for mRNA accumulation, lip [introns I], lip [introns I–III], and lip [introns I–V], which are the lip sequences containing one, three, and five introns at the original positions of lip, were constructed (Fig. 2). These lip constructs were inserted between the promoter and the terminator of Mlp30. The resulting recombinant plasmids Mlp30-lipc [introns I], Mlp30-lipc [introns I–III], and Mlp30-lipc [introns I–V], were introduced into C. hirsutus OJ1078 (Arg−Leu−). As in the cases of Mlp30-lipc and Mlp30-lip, two Arg+ transformants that showed the same growth rates as that of the recipient OJ1078 were obtained for each plasmid. These six transformants obtained by introduction of Mlp30-lipc [introns I], Mlp30-lipc [introns I–III], and Mlp30-lipc [introns I–V] were named TF1-1 and TF1-2, TF3-1 and TF3-2, and TF5-1 and TF5-2 respectively. Southern-blot analysis using the 1.2-kb Le.priA terminator probe indicated that the six transformants carry 2–5 times more copies of the intron(s)-containing the lip expression cassette on their chromosomes than TF0-2 (lanes 2 and 6–11 of Fig. 3A). The results of Northern-blot analysis of the six transformants are shown in lanes 1–11 of Fig. 3B. All of the RNA blots of the six Arg+LiP+ transformants gave one intense signal of the lip transcript, the intensity of which coincided with that of the Southern blot. Hence an efficient mRNA accumulation is considered to occur only in the presence of one intron (intron I) in the coding region of lipc.

**Fig. 3.** Southern-Blot Analysis of EcoRI-Digest of Total Cellular DNA (A) and Northern-Blot Analysis of Total Cellular RNA (B) Prepared from the Ten Arg+LiP+ Transformants and the Control Arg+ Transformant.

A. EcoRI-digested were hybridized with the 32P-labeled probe of the 1.2-kb Le.priA terminator. Lanes: 1, control Arg+ transformant; 2 and 3, TF0-1 and TF0-2; 4 and 5, TF6-1 and TF6-2; 6 and 7, TF1-1 and TF1-2; 8 and 9, TF3-1 and TF3-2; 10 and 11, TF5-1 and TF5-2. B. Total cellular RNA samples were hybridized with the 32P-labeled probe of the 1.1-kb lip sequence. Lanes 1–11 are the same as those in (A). The ethidium bromide-stained ribosomal RNAs (rRNA) bands are shown as an internal control.

**LiP activity in the culture supernatants of the Arg+LiP+ transformants of C. hirsutus**

The Arg+LiP+ transformants TF0-1, TF6-1, TF1-1, TF3-1, TF5-1 and the control Arg+ transformant were cultured in BK medium at 25°C, and their culture supernatants were subjected to analysis of LiP activity. As shown in Fig. 4, the LiP activities of all transformants reached a maximum level at 16d of cultivation, and then they gradually decreased. The LiP activities of the culture supernatants of TF0-1, TF3-1, and TF5-1 were similar to that of TF6-1, about five times higher than those of TF0-1 or the control Arg+ transformant. These data coincided well with those of Northern-blot analysis.

We attempted to analyze LiP protein in the culture supernatants of the Arg+LiP+ transformants by the SDS–PAGE method, but unfortunately we could not
identify it clearly due to the presence on the gel of many protein bands about the size (approximately 40 kD) of the LiP protein.

**Conversion of PCP and degradation of 2,7-DCDD by culture supernatants of the Arg⁺LiP⁺ transformants of**

**C. hirsutus**

The Arg⁺LiP⁺ transformants of TF0-1, TF6-1, TF1-1, TF3-1, TF5-1, and the control Arg⁺ transformant were cultured in the BK medium at 25°C and their culture supernatants were subjected to the analyses. As shown in Fig. 5, the PCP conversion activities of all transformants reached a maximum level at 16 d of cultivation and then gradually decreased, as observed for the LiP activities. The culture supernatants of TF6-1, TF1-1, TF3-1, and TF5-1 showed remarkably high PCP conversion activities: at times when only 19.2% and 21.4% of PCP were transformed by the control Arg⁺ transformant and TF0-1, 80.5%, 68.3%, 70.1%, and 63.3% of PCP were transformed by TF6-1, TF1-1, TF3-1, and TF5-1 respectively.

Similarly, 2,7-DCDD degradation activities were analyzed. As shown in Fig. 6, the culture supernatants of TF6-1, TF1-1, TF3-1, and TF5-1 showed clearly higher 2,7-DCDD degradation activities than that of the control Arg⁺ transformant and TF0-1: at times when only 31.9% and 29.1% of 2,7-DCDD were degraded by the control Arg⁺ transformant and TF0-1, 67.7%, 60.1%, 61.3%, and 63.5% of 2,7-DCDD were degraded by TF6-1, TF1-1, TF3-1, and TF5-1 respectively.

In this paper, we report that the presence of at least one intron (the first intron) is necessary for efficient accumulation of lip mRNA and its subsequent translational expression in *C. hirsutus*. We succeeded in molecular breeding of *C. hirsutus* strains with high LiP activity, which efficiently convert/degrade PCP and 2,7-DCDD. As for the reason a sufficient amount of the transcript of intronless cDNA accumulates, especially in *C. cinerea*, as mentioned in the introduction, one of the possible explanations is as follows: Previous studies with other eukaryotes such as metazoans and yeast[6–9]...
showed that splicing of pre-mRNA and export of mRNA are normally coupled in vivo. Spliced mRNA does form a significantly large mRNA-protein complex as compared with intronless mRNA, and is very rapidly exported to the cytoplasm. That rapid export of mRNA to the cytoplasm by the formation of a large mRNA-protein complex might make possible an escape from digestion by a nuclear RNA-degrading enzyme(s). The total activity of the nuclear RNA-degrading enzyme might be relatively low in total activity of the nuclear RNA-degrading enzyme(s). The protein complex might make possible an escape from export to the cytoplasm. That rapid export of mRNA in vivo are normally coupled.

Acknowledgment

This work was partly supported by research grants from NEDO (the New Energy and Industrial Technology Development Organization) of Japan and the Noda Institute for Scientific Research, and a Grant from the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References


