Isolation and Characterization of the Glyceraldehyde-3-phosphate Dehydrogenase Gene of Lentinus edodes

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The glyceraldehyde-3-phosphate dehydrogenase (GPD) gene of Lentinus edodes was isolated from a genomic DNA library and cDNA corresponding to this gene was isolated from a mycelium cDNA library. The L. edodes GPD gene was found to encode a 337-aa protein. By comparison of the cDNA and genomic DNA sequences, the presence of eight introns in the GPD gene was confirmed. The putative amino acid sequence of the L. edodes GPD gene product showed high similarity to those of other basidiomycetes. The results of Southern blot analyses suggested that only one copy of the GPD gene is present in the genome of L. edodes. The promoter region was found to contain a C-rich stretch, two CAAT boxes and a consensus TATA box. In addition, the transcript of the GPD gene was found to be expressed constitutively and strongly. These results suggest that the promoter of the L. edodes GPD gene may be very useful as a component of transformation vectors.

Key words: Lentinus edodes; glyceraldehyde-3-phosphate dehydrogenase gene; promoter

Lentinus edodes is one of the most important edible basidiomycetes in Japan. An efficient transformation system for expression of heterologous genes in L. edodes has not yet been established. We have investigated methods of gene transfer and have constructed a transformation vector for use in L. edodes. The pLC1 vector, which contains the L. edodes ras gene promoter and priA gene terminator, has been reported to be useful for transformation of Pleurotus ostreatus and Coprinus cinereus. Recently, using the pLC1 vector, we showed that application of the restriction enzyme-mediated integration (REMI) method to achieve gene transfer resulted in efficient transformation of L. edodes. Currently, pLC1 is the only vector available for transformation of L. edodes. Thus, we have sought a constitutive and strong endogenous promoter that can drive the expression of heterologous genes in L. edodes. Glyceraldehyde-3-phosphate dehydrogenase (GPD, EC 1.2.1.12) is a key enzyme in glycolysis and gluconeogenesis. In the former pathway it converts glyceraldehyde-3-phosphate into bisphosphoglycerate, and in the latter pathway it catalyzes the reverse reaction. The GPD protein is a tetramer composed of identical subunits. In Saccharomyces cerevisiae and in higher eukaryotes, the GPD protein comprises up to 5% of the soluble cellular protein. Furthermore, 2-5% of the poly(A) RNA in yeast is GPD mRNA. These observations suggest that the GPD gene is controlled by a highly active promoter.

We have focused on constructing transformation vectors that allow high-level expression of heterologous genes in L. edodes. In this study, we cloned the GPD gene in an effort to isolate a highly active promoter from L. edodes, and analyzed the complete nucleotide sequence of this gene, including the 5' and 3' flanking regions.

Materials and Methods

Materials. Mycelia of strain 57 (a dikaryotic strain) obtained from Hokken Sangyo Co. were used. The strain was grown on MYPG agar medium (0.25% malt extract, 0.1% yeast extract, 0.1% peptone, 0.5% glucose, 1.5% agar) for 2 weeks at 25°C. Then, MYPG liquid medium was inoculated with mycelia, and incubated with gentle agitation for 4 weeks at 25°C.

Construction of a cDNA library. The mycelia were ground in liquid nitrogen with a mortar and pestle. The mycelial powder was placed in a 15-ml tube, and total RNA was extracted using Isogen (Nippon Gene). The poly (A) RNA was purified with the Straight A's mRNA isolation system (Novagen). The RNA concentration in the samples was measured spectrophotometrically. A cDNA library was constructed using the Zap Express cDNA Gigapack III Gold Cloning Kit according to the manufacturer's recommendations (Stratagene). cDNA synthesized from the above-mentioned poly (A) RNA was ligated into an EcoRI-XhoI-predigested dephosphorylated Zap Express cloning vector, and packaged with Gigapack III Gold packaging extracts (Stratagene).

Construction of a genomic DNA library. The phenol/cresol reagent used for extraction of genomic DNA was prepared as follows: 100 g of phenol with 4.7
ml of m-cresol added was dissolved at 50°C, and after addition of 0.05% (w/v) 8-quinolinol, an equal amount of 1 m NaCl was added to the mixture. The mycelia were ground in liquid nitrogen with a mortar and pestle. The mycelial powder was suspended in 20 ml of TESS buffer (0.73 m sucrose, 10 mm Tris-HCl (pH 8.0), 1 mm EDTA, 1% SDS) and incubated for 1 hour at 65°C. After addition of 5 ml of 5 m NaCl, the sample was centrifuged at 8000 × g for 20 min. An equal amount of phenol/cresol reagent was added to the supernatant and the sample was mixed thoroughly. The mixture was centrifuged at 13000 × g for 5 min and the upper aqueous layer was collected. After chloroform extraction and ethanol precipitation, the pellet was dissolved in TE buffer and treated with RNase A, amylase, and protease K. The sample was then extracted with phenol/chloroform (1:1, v/v) and with chloroform, and the DNA was precipitated with 2.5 vol. of ethanol. The DNA was dissolved in TE buffer for use in construction of the genomic DNA library. A genomic DNA library was constructed using the Lambda EMBL3/BamHI Vector Kit (Stratagene) according to the manufacturer’s instructions. Genomic DNA fragments digested with Sau3AI were ligated into the BamHI site of the lambda EMBL3 cloning vector, and packaged with Gigapack III Gold packaging extracts.

Cloning of the GPD gene. Degenerate oligonucleotide primers were designed based on the deduced amino acid sequences of GPD genes previously characterized from other basidiomycetes: primer-U1 (5’-GKAATCGG-MCGMYGTCYCMGHAATGC-3’) anneals to sequences coding for RGRIVLRNA and primer-L1 (5’-ARGCARTTGGHTGTGCA HGAAGCRTTY-3’) anneals to sequences coding for SNASCTTNCL. Using these primers and L. edodes genomic DNA as the template, 30 cycles of PCR amplification were done at 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The amplified DNA fragment was used as a probe for cloning of the GPD gene. For screening of the positive clones, 4 × 10^5 plaques from the cDNA library or 5 × 10^5 plaques from the genomic DNA library were transferred to Hybond N+ nylon membranes (Amersham). Labelling of the DNA probe, hybridization, and signal detection were done by means of the ECL Direct Nucleic Acid Labelling and Detection System according to the manufacturer’s recommendations (Amersham).

Subcloning and sequencing of cDNA and genomic DNA clones. The cDNA clones were subcloned into pBK-CMV phagemid vectors by in vivo excision according to the instruction manual of the Zap Express cDNA Gigapack III Gold Cloning Kit. The phagemids into which the GPD gene had been inserted were purified by the RPM kit (BIO 101). The lambda phage DNAs including the genomic DNA clones were purified by the Wizard Lambda Prep DNA Purification System (Promega). Sequencing of these clones was done using the ABI Prism Dye Terminator cycle sequencing kit (Perkin Elmer). The reaction mixture was analyzed using the ABI Prism 310 Genetic Analyzer (Perkin Elmer).

Location of the transcription start point. The transcription start point of the isolated GPD gene was found using the Smart PCR cDNA Synthesis Kit (Clontech), known as the CapFinder method. Using total RNA from mycelia as a template, reverse transcription was done at 42°C for 1 hour with Superscript II MMLV reverse transcriptase (Gibco BRL) and an oligo d(T)12-18 primer with the SMART II oligonucleotide (Clontech) which has an oligo (G) sequence at its 3’ end. Only full-length GPD cDNA with the SMART II oligonucleotide sequence at its 5’ end was amplified by PCR using the Smart PCR primer (Clontech) together with the GPD gene specific primer (5’-GCTTCTTTGACAGCACGT-3’) and the above-mentioned synthesized cDNA as a template. The amplified DNA fragments were subcloned into the plasmid vector pCR2.1 (Invitrogen) and sequenced.

Southern blot analysis. Genomic DNA was extracted by the method described above in the section on construction of the genomic DNA library. Approximately 5 µg of genomic DNA digested with EcoO109I, StuI or HhaI was size-fractionated by electrophoresis on a 1% agarose gel. After depurination of the digested DNA fragments within the gel in 0.25 M HCl, the DNA fragments were transferred to a Hybond N+ nylon membrane (Amersham) using 20 × SSC. Using a UV cross linker, the DNA fragments were fixed to the membrane. A genomic DNA fragment amplified by PCR using two GPD gene specific primers, 5’-tagatgcagcggagaccgc-3’ and 5’-ggagagcggagcggaga-3’, was used as a probe. Labelling of the DNA probe, hybridization, and signal detection were done by the ECL Direct Nucleic Acid Labelling and Detection System (Amersham).

Northern blot analysis. Total RNA was extracted from mycelia, primordia and stipes, gills, and pilei of mature fruiting bodies by the method described above in the section on construction of the cDNA library. An equal amount of each of the total RNA samples (10 µg) was electrophoresed on 2.2 m formaldehyde/1.5% agarose gels and transferred to Hybond N+ nylon membranes (Amersham) using 20 × SSC. Using a UV cross linker, the RNA was fixed to the membranes. The 800-bp fragment of the GPD cDNA and the 880-bp fragment of coding region of the ras genomic DNA were used as the probes. Labelling of the DNA probes, hybridization, and signal detection were done by the AlkPhos Direct DNA/RNA Labelling and Detection System according to the manufacturer’s instructions (Amersham).

Results and discussion

Isolation of the L. edodes GPD gene

To isolate the L. edodes GPD gene, two degenerate oligonucleotide primers were designed according to the deduced amino acid sequences of previously characterized GPD genes from the other basidiomycetes, Agaricus bisporus, Phanerochaete chrysosporium, and Schizothyrium commune. Using these primers, a fragment approximately 700 bp in size was amplified by
PCR from the \textit{L. edodes} genomic DNA template. The amplified DNA fragment was subcloned and sequenced, and the nucleotide sequence of the fragment showed greater than 60% identity when compared to the GPD genes of the three above-mentioned basidiomycetes. Then, using this amplified DNA fragment as a probe, we isolated the GPD gene of \textit{L. edodes} from the genomic DNA library and a cDNA corresponding to this gene from the mycelium cDNA library. Finally, five cDNA clones and three genomic clones were isolated from the corresponding DNA libraries. The coding regions in these clones were found to be identical.

***Structure of the \textit{L. edodes} GPD gene***

The \textit{L. edodes} GPD gene encodes a 337-aa protein (Fig. 1, 2), and is similar to the previously characterized GPD genes of other basidiomycetes, except for the \textit{A. bisporus} GPD2 gene.\textsuperscript{13,16} The sequence of the coding region of the genomic DNA clone agreed perfectly with that of the cDNA clone. It has been reported that \textit{A. bisporus} GPD1, \textit{A. bisporus} GPD2, \textit{P. chrysosporium} GPD, \textit{S. commune} GPD, and \textit{Ustilago maydis} GPD contain nine, nine, six and five introns and one intron, respectively.\textsuperscript{13,16} In the \textit{L. edodes} GPD gene, the presence of eight introns was confirmed by comparison of the cDNA and genomic DNA sequences. The methionine initiation codon was immediately followed by the first intron. Harmsen et al.\textsuperscript{19} reported that the positions of introns were strongly conserved in the GPD genes of the investigated basidiomycetes, although the number of introns differed. Likewise, the positions of introns in the GPD gene of \textit{L. edodes} are similar to those in the case of the other basidiomycetes.

In \textit{A. bisporus}, the GPD2 gene is strongly expressed in both mycelia and fruiting bodies, but transcripts of GPD1 are not detected in mycelia or fruiting bodies.\textsuperscript{17} The deduced amino acid sequence of the \textit{L. edodes} GPD gene shows higher similarity to \textit{A. bisporus} GPD2 than to GPD1 (Fig. 2). Also, the similarity between the putative amino acid sequence of the \textit{L. edodes} GPD gene and that of \textit{P. chrysosporium} or \textit{S. commune} is 81.3% and 80.7%, respectively. In pig muscle, lobster muscle, and yeast, a main catalytic residue, the site for binding of glyceroldehyde-3-phosphate, is the 149th residue, which is a cysteine residue.\textsuperscript{18} In the GPD genes of \textit{A. bisporus}, \textit{P. chrysosporium}, and \textit{S. commune}, the corresponding site is a cysteine residue in the 151st posi-

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**Fig. 1.** Nucleotide Sequence and Deduced Amino Acid Sequence of the \textit{L. edodes} GPD Gene.

The deduced amino acid sequence is indicated below the respective codons. Exon sequences are capitalized. The major transcription start point and the position of the 3' end of the mRNA are indicated by arrows. Numbers in both margins indicate positions relative to the major transcription start point. Single and double underlines indicate TATA and CAAT boxes, respectively. Single underlines indicate pyrimidine-rich sequences.

**Fig. 2.** Alignment of the Deduced Amino Acid Sequences of the GPD Proteins of \textit{L. edodes}, \textit{A. bisporus}, \textit{P. chrysosporium}, and \textit{S. commune}.

The deduced amino acid sequences of the GPD proteins of \textit{A. bisporus}, \textit{P. chrysosporium}, and \textit{S. commune} are quoted from the report of Harmsen et al.\textsuperscript{19} Identical residues in all five sequences are marked under the aligned sequence. The values in parentheses indicate the extent of similarity to the amino acid sequence of the \textit{L. edodes} GPD protein.
tion. Likewise, in the putative GPD gene product of L. edodes, the 151st aa residue is cysteine (Fig. 2). In addition, the surrounding regions are strongly conserved. These results suggest that this GPD gene encodes a functional protein.

The sequences of the 5’- and 3’-flanking regions

A number of transcription start points of filamentous fungal genes appear in or immediately downstream from a CT-rich stretch. In the case of the L. edodes GPD gene, there is a CT-rich stretch located at 42 nt to 78 nt upstream from the translation start codon (Fig. 1). The transcription start points were found by sequence analysis of genomic GPD clones and by the CapFinder method. The major transcription start point is located 66 bp upstream from the translation start codon, within the above-mentioned CT-rich stretch (Fig. 1). Two CAAT boxes (CCAAT), which are located at −151 nt to −147 nt and −137 nt to −133 nt from the major transcription start point, and a consensus TATA box (TATAAAA), which is located at −31 nt to −25 nt, are each followed by a CT rich stretch (Fig. 1). A gpd box, pgk box, gut box, and qa box conserved in A. nidulans and A. niger GPD promoter regions are not present in the promoter region of the L. edodes GPD gene, or those of other basidiomycetes. The sequence around the translation start codon (ATG) has been reported to be important in determining the efficiency of initiation of translation in eukaryotes. The sequence (AAAATGG) of the L. edodes GPD gene is identical to consensus sequences found in the case of genes encoding glycolytic enzymes, such as the hexokinase, enolase and glyceraldehyde-3-phosphate dehydrogenase genes, of S. cerevisiae. The 3’-end of the GPD mRNA was located by analyzing the sequences of the isolated cDNA clones. In three out of five of the cDNA clones, the poly (A) track started 149 bp downstream from the translation stop codon (TAA), and in the other two clones, it started 185 bp downstream (Fig. 1). However, the consensus sequence serving as a polyadenylation signal (AATAAA) in higher eukaryotes is absent in the case of the GPD gene. It has been reported that this signal sequence is lacking in most yeast and filamentous fungal genes.

Southern and northern blot analyses

The two GPD genes of A. bisporus are arranged tandemly in the genomic DNA, and only a 223-bp region separates the transcription start point of the GPD2 gene from the translation stop codon of the GPD1 gene. In addition, the GPD2 gene is strongly expressed in both mycelia and fruiting bodies of A. bisporus, while transcripts of the GPD1 gene are not detected in mycelia or fruiting bodies. Thus, Southern and northern blot analyses were done to investigate the copy number and the transcription level of the L. edodes GPD gene. The expected signals specific for the GPD gene were detectable in the Southern blot analysis (Fig. 3). This result suggests that only one copy of the GPD gene is present in the genome of L. edodes. In the northern blot analysis, the strong signals were detected in all tissues analyzed, indicating that the GPD gene of L. edodes is expressed constitutively (Fig. 4). The regulatory sequence of L. edodes ras gene is used as the promoter of the pLC1 vector, which has been reported to be the only vector available for transformation of L. edodes. The GPD gene was transcribed more strongly than the ras gene in all tissues. This result suggests that the GPD gene may be regulated by a promoter more active than the ras gene.
Conclusions
We cloned the GPD gene of *L. edodes* to isolate an active promoter for use in construction of transformation vectors for *L. edodes*. The 5'-flanking region of the *L. edodes* GPD gene was found to contain a consensus TATA box and two CAAT boxes. Furthermore, a C-rich stretch is present around the major transcription start point. Northern blot analyses indicated that the GPD gene is transcribed constitutively and strongly in all tissues of *L. edodes*. These results suggest that the promoter of the GPD gene promoter may be very useful as a component of transformation vectors.

References