The Journal of Microbiology, October 2006, p. 515-522
Vol. 44, No. 5

Cloning and Sequence Analysis of a Glyceraldehyde-3-phosphate Dehydrogenase Gene from *Ganoderma lucidum*

Xu Fei, Ming Wen Zhao*, and Yu Xiang Li

College of Life Sciences, Nanjing Agricultural University, Nanjing 210095 and Key Laboratory of Microbiological Engineering of the Agricultural Environment, Ministry of Agriculture, P.R. China

(Received July 19, 2006 / Accepted September 14, 2006)

A cDNA library of *Ganoderma lucidum* has been constructed using a Zap Express cloning vector. A glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) was isolated from this library by hybridization of the recombinant phage clones with a *gpd*-specific gene probe generated by PCR. By comparison of the cDNA and the genomic DNA sequences, it was found that the complete nucleotide sequence encodes a putative polypeptide chain of 338 amino acids interrupted by 6 introns. The predicted amino acid sequence of this gene shows a high degree of sequence similarity to the GPD proteins from yeast and filamentous fungi. The promoter region contains a CT-rich stretch, two CAAT boxes, and a consensus TATA box. The possibility of using the *gpd* promoter in the construction of new transformation vectors is discussed.

Keywords: gyceraldehyde-3-phosphate dehydrogenase gene, *Ganoderma lucidum*, medicinal mushroom, strain improvement, promoter, transformation vector

Ganoderma lucidum, called 'lingzhi' in China and 'reishi' in Japan, is a lamella-less basidiomycete belonging to family Polyporaceae. Medicinal use of this bracket fungus dates back more than 4000 years, and is recorded in the book of traditional Chinese medicine, "benzhao gangmu", written by Li Shizhen (1518-1593) during the Ming dynasty. More recently, *G. lucidum* preparations have been reported to activate the immune system (Wang *et al.*, 1997), inhibit tumor cell growth (Maruyama *et al.*, 1989; Wang *et al.*, 1997), protect against radiation damage (Hsu *et al.*, 1990), and to exhibit anti-HIV-1 (El-Mekkawy *et al.*, 1998), anti-platelet aggregation (Su *et al.*, 1999), hepatoprotective (Kim *et al.*, 1999), anti-microbial (Yoon *et al.*, 1994), anti-inflammatory (Lin *et al.*, 1993), and anti-allergic activities (Tasaka *et al.*, 1988).

Attempts to enhance and extend the medicinal applications of *G. lucidum* through strain improvement have been hampered by the lack of an efficient transformation system (Sun *et al.*, 2002; Kim *et al.*, 2004). In other fungi, one approach to alleviating this shortfall has been the construction of transformation vectors in appropriate hosts using a strong endogenous promoter. However, to our knowledge, no homologous gene promoters have been reported in *G. lucidum*. Previous observations in the yeast species, *Saccharomyces cerevisiae*, and in higher eukaryotes have suggested that the glyceraldehyde-3-phosphate dehydrogenase (GPD, EC 1.2.1.12) gene (*gpd*) is under the control of a highly active promoter. In such cases, the GPD protein is a tetramer composed of identical subunits and comprises up to 5% of the soluble cellular protein (Piechaczyk, 1984). Furthermore, 2-5% of the poly (A)+ RNA in yeast is *gpd* mRNA (Holland and Holland, 1978). The flanking regulatory regions of these *gpd* genes have also been used successfully for the construction of transformation vectors in different fungi (Bitter and Egan, 1984; Balance, 1990; Herzog *et al.*, 1995; Van de Rhee *et al.*, 1996; Waterham *et al.*, 1997; Chen *et al.*, 2000; Hirano *et al.*, 2000).

In order to explore whether the same possibility exists for *G. lucidum*, we have constructed a representative cDNA library and cloned and characterized the fungal *gpd* gene and its adjacent regulatory elements. Gene characteristics, codon usage, and homology with *gpd* genes of other fungi are discussed.

Materials and Methods

Strains, culture conditions, and plasmids

G. lucidum, strain HG, was obtained from the Shanghai Academy of Agricultural Sciences and maintained on

* To whom correspondence should be addressed.
(Tel) 86-25-8439-5602; (Fax) 86-25-8439-5602
(E-mail) mwzhao@njau.edu.cn

potato dextrose agar (PDA). *Escherichia coli*, strain DH5α, was used for transformation and was grown on LB medium at 37°C. PCR products were cloned with the pMD18-T Easy Vector System (Takara, Japan). XL1-Blue MRF' was used for the amplification of the cDNA library and transfection experiments. All molecular cloning techniques were carried out as described by Sambrook *et al.* (1989).

Isolation of genomic DNA

Mycelium from *G. lucidum* cultures grown at 25°C for seven days on potato dextrose broth was harvested, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. Genomic DNA was isolated from this material using the CTAB method (Russo *et al.*, 1992).

Construction of a cDNA library

Total RNA was extracted from powdered mycelium using the Isogen reagent (Promega), and poly (A)+ RNA was purified with the Straight A's mRNA isolation system (Promega). cDNA was synthesized from the poly(A)+ RNA using the Zap Express cDNA Gigapack III Gold Cloning Kit (Stratagene, USA) according to the instructions of the manufacturer, ligated into an *EcoR*I-*Xho*I-predigested dephosphorylated Zap Express cloning vector, and packaged with Gigapack III Gold packaging extracts (Stratagene, USA).

Cloning of the gpd gene

Degenerate oligonucleotide primers were designed on the deduced amino acid sequences of *gpd* genes previously characterized from *Lentinula edodes* (Hirano *et al.*, 1999), *Schizophyllum commune*, *Phanerochaete chrysosporium* (Harmsen *et al.*, 1992), and *Xanthophyllomyces dendrorhous* (Verdoes *et al.*, 1997). The sequences of primers were: primer-F: 5'-GKA TCG GMC GYM YGT CYY CMG HAA TGC-3' (encoding RIGRIVLRNA) and primer-R: 5'-ARG CAR TTG GTH GTG CAH GAA GCR TTY G-3' (encoding SNASCTTNCL). PCR amplification using these primers and *G. lucidum* genomic DNA as the template was carried out using a PTC-200 automated thermal cycler (MJ Research, USA) in 20 μl reaction volumes containing 20 pmol each of primer F and primer R, 1 × Taq DNA polymerase reaction buffer (Stratagene, USA), 200 μM dNTP, 5 U *Taq* DNA polymerase, and 1 μg template. Amplification conditions were: 1 cycle at 95°C for 5 min; 30 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 40 s; then a final extension step at 72°C for 10 min. Amplification products were fractionated by electrophoresis in 1% (w/v) agarose gels, and appropriate bands were excised and purified using a GenElute agarose spin column. The purified DNA was precipitated with ethanol. A 624 bp fragment of the *gpd* gene was amplified by PCR using two degenerate oligonucleotide primers, designed according to deduced *gpd* amino acid sequences reported in other basidiomycetes, and *G. lucidum* genomic DNA as template. The DNA sequence revealed more than 80% identity with other basidiomycete *gpd* genes when with a search was conducted using the NCBI database.

Plaques (6×10^5) from the cDNA library were transferred to Hybond N^+ nylon membranes (Amersham), and an amplified DNA fragment was used as a probe for cloning of the *gpd* gene. Labeling of the DNA probe, hybridization, and signal detection were carried out using the ECL Direct Nucleic Acid Labeling and Detection System according to the instructions of the manufacturer (Amersham).

On the basis of the cDNA sequence of *G. lucidum*, two specific primers were designed as: G1: 5'-CAT CCC CCT CTC AAC ATG-3', which contained the start codon and the 5' untranslated region (UTR) in the cDNA sequence and G2: 5'-TTA GAG AGC GCC GTC CTG-3' (contained the stop codon). PCR amplification using these primers and *G. lucidum* genomic DNA as the template was carried out using a PTC-200 automated thermal cycler (MJ Research, USA) in 20 μl reaction volumes containing 20 pmol each of primers, 1× pfu DNA polymerase reaction buffer (Stratagene, USA), 200 μM dNTP, 5 U *Pfu* DNA polymerase, and 1 μg template. Amplification conditions were: 1 cycle at 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 40 s, 72°C for 2 min 30 s; followed by a final extension step at 72°C for 10 min. Amplification products were fractionated by electrophoresis in 1% (w/v) agarose gels, and appropriate bands were excised and purified using a GenElute agarose spin column. The purified DNA was precipitated with ethanol, and a 1355 bp fragment was obtained.

The 5' flanking region of *G. lucidum* was cloned by means of chromosome walking using the TaKaRa LA PCR™ *in vitro* Cloning Kit according to the instructions of the manufacturer (TaKaRa, Japan). Two gene-specific primers (GSP) were designed from the *G. lucidum gpd* genomic sequence: S-1: 5'-ATG GCA TAG GTA CGA GAA ATG AGT GGC TGC-3' and S-2: 5'-CGG CGG CTC AGA GTC CGA GTC GAG ATT AGA-3', and a 1210 bp fragment was obtained.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using Isogen (Promega, USA). Reverse transcription and subsequent PCR reactions were performed using the TaKaRa One Step RNA PCR Kit (AMV) with primers P1: 5'-CTC CTT CAC GGA GAC ATT-3' and P2: 5'-TAA CAC CGC

AGA CGA ACA-3', which are correspond to amino acids 22-27 and 129-134, respectively. The amplified fragments were cloned in the pMD-18T easy vector (TaKaRa, Japan) prior to sequencing. PCR amplification using these primers and *G. lucidum* RNA as the template was carried out using a PCT-200 automated thermal cycler (MJ Research, USA) in 50 μl reaction volumes containing 20 pmol each of primer F and primer R, 1× one step RNA PCR buffer (TaKaRa), 200 μM dNTP, 40 U RNase inhibitor, 5 U AMV RTase, and 5 U AMV-optimized *Taq* DNA polymerase. Amplification conditions were: 1 cycle of

```
-1209                                                    TCCAAAGCCGCTCTCATGGCATGGCACAGAGGTATATCGTTACAT
-1164  ATATCCGTTTGTGTCAACGTCGAGGGGTTCCCCTCGTTCAAGCCTCTTCAGACATTGGGCCACCCATGCTCAAGATGCCCAAG
-1081  GCCCACGCTGTATATCCATAAGTAAGATATGCGCATCCTCGTTTCTCGCCTTGCAATTGGTGTTCGTTGAACAACTGGTCGCAG
 -997  AGGCAAGAAGTCGAAGTGACTTCTCTTGACTTTCTATGTCCGACCTCAAGGTCGCTCCGGGGCCACAGGCCTAAATACGACC
 -915  ACCATAATCAATGGCCAACAATCACGATGGTCCCGATATATACAATAACCCTTGGTTTTGGCGTAGCAATGCCAGGAAATTGCTA
 -830  GTGTCTCATCTCAATCTGAGCACAGGCATGGAACGTCGGTCTGGAAAGCCGAGGCCGAAGTAGGGTACTTGATGGAAAGCTGA
 -747  ATTCTTGCGAGGCTCAGACACCTGGAGGAGGTTCTTGGCTCGCGCACGAGGGTTCGAACATTATTCAAAGAAGGTGAACAAA
 -665  GGTCGCCCCAAGGCGGTCAACAGGTAATTGGAAGAAGGGAGGTTAACGTTACCCGACCAATTACATAACTAAGAGTGATTCGT
 -582  ATCGATGGATGTCGGTTGGGGTATTGTGTGAGAAAGAGACGAGGTGCAGCTCGACATGGCTGGAGATGGCGGGGAAATGGA
 -500  CGAGTGGGATGGTTGGATTGCAGCAACCAATCCGGGGCCAGCGGATGTGATACCCATCCGCGTCGTCTGACGATGCATATGGCG
 -416  AGACAGGCCGAGTCAAAGATGGCACGTTTGAAGCTTGTGCATGCCGGCAGCTCAGCACAAGTTTCTGTGGTGCTGTTGCAGGC
 -333  ACTGAGGAACGTGAGCAGGCTTTAGAGGGAGCTAGGCTGGGCGACGTTGGTCTGGGTATGCGAGGAAGACATCGGAGAGATG
 -251  GCAAGGGCGAGGGACGGGCCGAGTCTAGGCTGAGGCTGAAGAGTCAGACGCAACTAGCGCTCATTGGCGGGAGACCGCCCCA
 -169  GTCCGGCAAGCGCCGAGTGACGCAGGTGGTGACGCCCGCCAGTCGCCCAGTCCGTAGTAGTGTGCCCGATTGGATCTGCGATA
  -86  ACATCGGTCGAGGCGTATAAATGCTCTCTCTGCCCTCTCTCCTTGTCCAGCCTCATCTTCTTACTCACTCTTCATCCCCCTCTCAAC
    1  ATG CCC gtgagtcctgcatccccatcgtgcaccgtattcacctcatcgtttggccccttctcacag GTC AAG GTC GGA ATC AAC GG gtgagtttcagatctaatctcgactcggactctgagcc
    1   M   P                                                               V   K   V   G   I   N  G
  126  gccgtcctcatctttctc at c cctgtcctttttag T TTC G gtatgtgcatacctctctccatctcatttgtgcagccactcatttctcgtacctatg ccatctag GT CGT ATC GGC CGT ATC GTC CTT CGC
   10                                          F                                                                          G   R   I   G   R   I   V   L   R
  257  AAT GCT CTC CTT CAC GGA GAC ATT GAG GTC GTG GCT GTC AAC GA gtccgtaccttttcctcatgatcgtgtccttcctcaatcgctgaacgttactcacacag T CCT
   20   N   A   L   L   H   G   D   I   E   V   V   A   V   N   D                                                                    P
  364  TTC ATC TCC CTT GAA TAC ATG GTC TAC ATG TTC AAG TAC GAT TCC GTC CAC GGC CGC TTC AAG GGC GCG GTA GAG GCA
   36   F   I   S   L   E   Y   M   V   Y   M   F   K   Y   D   S   V   H   G   R   F   K   G   A   V   E   A
  442  AAG GAC GGC AAG CTC TAT GTT GAG GGG AAG CCC ATC TCC GTC TTC GAG GAG AAG GAC CCT GCC AAC ATT AAC TGG
   62   K   D   G   K   L   Y   V   E   G   K   P   I   S   V   F   E   E   K   D   P   A   N   I   N   W
  517  TCT GGC GTC GGC GCC GAG TAC ATC GTC GAG TCC ACT GGT GTC TTC A CC ACC GTC GAC AA gtgcgcatatatctgc ctttttgtcgcgtacgagcc
   87   S   G   V   G   A   E   Y   I   V   E   S   T   G   V   F   T   T   V   D   K
  613  atctgacgcgttcacgcgctgatag G GCC TCG GCC CAC TTG AAG GGT GGT GCC AAG AAG GTG GTC ATC TCC GCC CCC TCG GCG GAT
  107                              A   S   A   H   L   K   G   G   A   K   K   V   V   I   S   A   P   S   A   D
  699  GCT CCC ATG TTC GTC TGC GGT GTT AAC CTC GAC AAG TAT GAC TCC AAG TAC CAA GTC gtacgtaacagtcacatagccgaagtcaccttttcgtctg
  127   A   P   M   F   V   C   G   V   N   L   D   K   Y   D   S   K   Y   Q   V
  796  acccgccgtatctccaccttcatttag ATA TCC AAC GCT TCC TGC ACC ACC AAC TGT CTG GCT CCT CTC GCA AAG GTC ATC AAC GAC AAC
  146                               I   S   N   A   S   C   T   T   N   C   L   A   P   L   A   K   V   I   N   D   N
  886  TTC GGT ATC GTC GAG GGT CTC ATG ACC ACC GTT CAC TCG ACG ACC GCC ACC CAG CGT ACC GTC GAC GGC CCG TCG
  167   F   G   I   V   E   G   L   M   T   T   V   H   S   T   T   A   T   Q   R   T   V   D   G   P   S
  961  ATG AAG GAC TGG CGT GGT GGC CGT GCC GTT GCC TCC AAC ATC ATC CCC TCG TCG ACC GGT GCC GCC AAG GCC GTC
  192   M   K   D   W   R   G   G   R   A   V   A   S   N   I   I   P   S   S   T   G   A   A   K   A   V
 1036  GGC AAG GTC ATT CCC AGC CTC AAC GGC AAG CTC ACG GGT ATG GCC TTC CGT GTG CCC ACG ATC GAC GTT TCC GTC
  217   G   K   V   I   P   S   L   N   G   K   L   T   G   M   A   F   R   V   P   T   I   D   V   S   V
 1111  GTC GAC CTT GTT GTC CGT CTC GAG AAG GAG GCG ACG TAC GAC GAG ATC AAG GCC GCC GTC AAG TCC GCC GCC ACC
  242   V   D   L   V   V   R   L   E   K   E   A   T   Y   D   E   I   K   A   A   V   K   S   A   A   T
 1186  GGC AAT CTC AAG GGA ATC ATC GAC TAC ACT GAG GAC AAG GTC GTG TCG ACC GAC TTC ACC GGT TCG GAC GCG TCG
  267   G   N   L   K   G   I   I   D   Y   T   E   D   K   V   V   S   T   D   F   T   G   S   D   A   S
 1261  TCG ATC TTC GAC GCC GAG GCC GGC ATC GCG CTT AAC AAG AAC TTC GTC AAG CTC ATC GCA TGG TAC GAC AAC GAG
  292   S   I   F   D   A   E   A   G   I   A   L   N   K   N   F   V   K   L   I   A   W   Y   D   N   E
 1336  TGG GGC TAC TCC CGC CGT GTC TGC GAC CTC CTG GTC TAC GCG GCG AAG CAG GAC GGC GCT CTC TAA
  317   W   G   Y   S   R   R   V   C   D   L   L   V   Y   A   A   K   Q   D   G   A   L   -
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *G. lucidum gpd* gene. The deduced amino acid sequence is indicated below the respective codon. Intron sequences are in lower-case letters; all the other sequences are in upper-case letters. The putative TATA box and CAAT box in the promoter region are single-underlined, and the transcription initiation sites are double-underlined. The genomic DNA sequence data is available in the DDJB/EMBL/GenBank databases under accession number DQ404344, the cDNA sequence data is available in the DDJB/EMBL/GenBank databases under accession number DQ404343, and the promoter region sequence data is available in the DDJB/EMBL/GenBank databases under accession number DQ404345.

50°C for 30 min; 1 cycle of 94°C for 2 min; 30 cycles of 94°C 30 s, 50°C for 30 s, 72°C for 40 s; and a final extension step at 72°C for 10 min.

Sequencing and computer analysis of the gpd gene

gpd sequences were determined in both directions using an Applied Biosystem 3730 DNA sequencer. The BLAST 2 program at the National Center for Biotechnology Information (NCBI) was used for nucleotide sequence analysis, database searches, and amino acid sequence deduction. Protein sequences were aligned using the Clustal W software package (Thompson *et al.*, 1994). *gpd* sequences of other basidiomycetes were obtained from nucleotide sequence libraries (DDBJ/EMBL/GenBank).

Results and Discussion

Cloning and sequence analysis of G. lucidum gpd cDNA and gpd gene

The *gpd* cDNA fragment consisted of a 1198 bp sequence, including 28 bp of the 5' untranslated region, an ATG translation initiation codon, a TAA translation termination codon and a poly (A) tail (data not shown). The *G. lucidum gpd* gene encodes a 337 amino acid protein, and is similar to the previously characterized *gpd* genes of other basidiomycetes. The sequence of the coding region of the genomic DNA showed perfect agreement with that of the cDNA (Fig. 1), suggesting that this *gpd* gene encoded a functional protein. Some fungi such as *Mucor circinelloides* and *Agaricus bisporus*, which have more than one *gpd* gene, contained only one *gpd* mRNA, indicating that there was only one functional *gpd* gene (Harmsen *et al.*, 1992; Wolff and Arnau, 2002). It has been reported that *A. bisporus gpd1, A. bisporus gpd2, Phanerochaete chrysosporium gpd, Schizophyllum commune gpd,* and *Ustilago maydis gpd* contained nine, nine, six, and five introns and one intron, respectively (Smith, 1989; Harmsen *et al.*, 1992). In the *G. lucidum gpd* gene, the presence of six introns was confirmed by comparison of the cDNA and genomic DNA sequences. The length of the intron was found to vary between 59 and 73 nucleotides. All introns had a 5' splice site (GTPu) and 3' splice site (PyAG), which is compatible with the consensus sequences for introns of filamentous fungi. Introns in the *G. lucidum gpd* gene were located after the codons corresponding to the amino acids at the positions 2, 9, 10, 34, 106, and 145. The second intron position was characteristic of the *gpd* genes of most basidiomycetes, but was absent from ascomycetes (Harmsen *et al.*, 1992). Like most of the fungal *gpd* genes, where most of the introns are located near the 5' end of the gene, in the *G. lucidum gpd* gene, they were also positioned at the 5' end of the gene. It is reported that the positions of introns were strongly conserved in the *gpd* genes of the investigated basidiomycetes, although the numbers of introns differed. Likewise, the positions of introns in the *gpd* gene of *G. lucidum* were similar

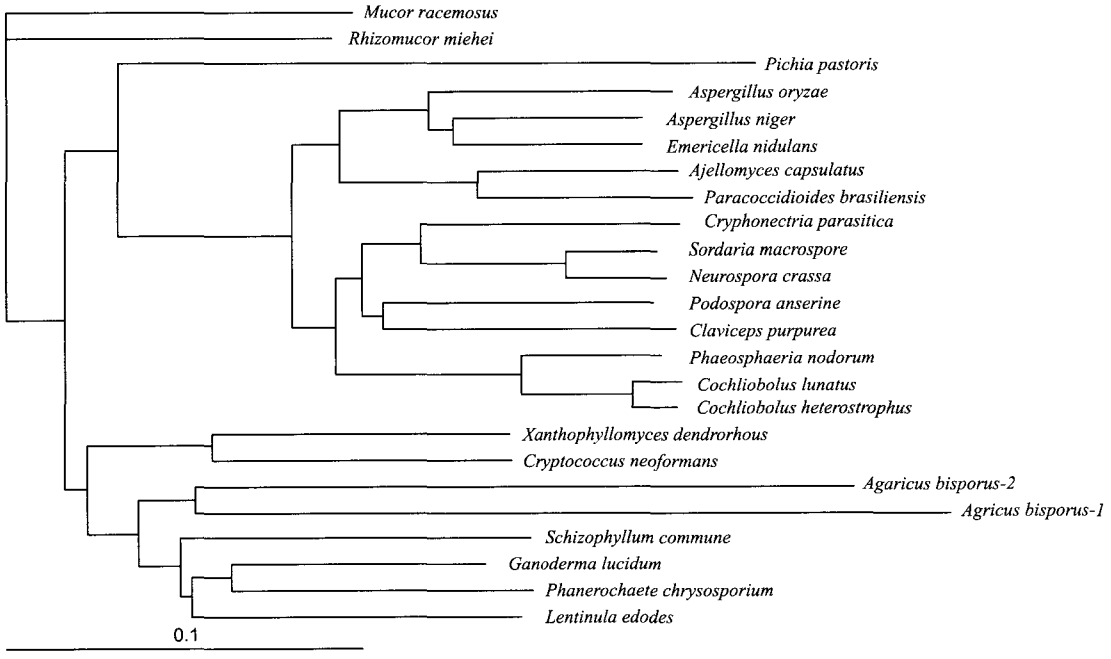

Fig. 2. Phylogenetic analysis of *gpd* sequences using the neighbor-joining method with *M. racemosus* and *R. miehei* as the outgroup. Phylogenetic tree of 24 fungi and yeast derived from the nucleotide sequence of *gpd* gene.

to those in the case of the other basidiomycetes, which may reflect the evolutionary divergence of these mushrooms. The position of only one intron was conserved between basidiomycetes and ascomycetes (Harmsen *et al.*, 1992); these comparisons suggested the existence of a clear boundary between ascomycetes and basidiomycetes with respect to intron positioning with the *gpd* gene.

Comparison of the amino acid sequence of G. lucidum gpd gene with gpd genes of other fungi

The predicted amino acid protein deduced from the cDNA revealed high similarity to the known fungal *gpd* sequences (Harmsen *et al.*, 1992; Hirano *et al.*, 1999; Kuo *et al.*, 2004). Conserved amino acid residues, e.g. Cys -151, which functioned as the binding site of the enzyme in the catalytic region, were also found to be present in this putative protein, like as in the the GPD proteins of *L. edodes*, *A. bisporus*, *Flammulina velutipes*, *P. chrysosporium*, and *S. commune*. The amino acid sequence of the *G. lucidum* GPD gene was aligned with selected sequences from other fungi from basidiomycetes using the Clustal W program. The highest similarity (85.76% identity) of the *G. lucidum* GPD amino acid sequences was found with *P. chrysosporium*. The homology with *L. edodes*, *S. commune*, and *Xanthophyllomyces dendrorhous* was 82.78%, 81.60%, and 77.22%, respectively.

Table 1. Codon usage in *G. lucidum gpd* gene

Codon	Amino acid	GPDa	GPDb	Codon	Amino acid	GPDa	GPDb
TTT	Phe	0.000	0	TAT	Tyr	0.167	2
TTC	Phe	1.000	12	TAC	Tyr	0.833	10
TTA	Leu	0.000	0	TAA	–	1.000	1
TTG	Leu	0.050	1	TAG	–	0.000	0
CTT	Leu	0.250	5	CAT	His	0.000	0
CTC	Leu	0.600	12	CAC	His	1.000	4
CTA	Leu	0.000	0	CAA	Gln	0.333	1
CTG	Leu	0.100	2	CAG	Gln	0.667	2
ATT	Ile	0.136	3	AAT	Asn	0.125	2
ATC	Ile	0.819	18	AAC	Asn	0.875	14
ATA	Ile	0.045	1	AAA	Lys	0.000	0
ATG	Met	1.000	7	AAG	Lys	1.000	26
GTT	Val	0.154	6	GAT	Asp	0.130	3
GTC	Val	0.718	28	GAC	Asp	0.870	20
GTA	Val	0.026	1	GAA	Glu	0.067	1
GTG	Val	0.102	4	GAG	Glu	0.933	14
TCT	Ser	0.038	1	TGT	Cys	0.250	1
TCC	Ser	0.462	12	TGC	Cys	0.750	3
TCA	Ser	0	0	TGA	–	0.000	0
TCG	Ser	0.385	10	TGG	Trp	1.000	4
CCT	Pro	0.273	3	CGT	Arg	0.727	8
CCC	Pro	0.636	7	CGC	Arg	0.273	3
CCA	Pro	0	0	CGA	Arg	0.000	0
CCG	Pro	0.091	1	CGG	Arg	0.000	0
ACT	Thr	0.105	2	AGT	Ser	0.000	0
ACC	Thr	0.684	13	AGC	Ser	0.115	3
ACA	Thr	0	0	AGA	Arg	0.000	0
ACG	Thr	0.211	4	AGG	Arg	0.000	0
GCT	Ala	0.171	6	GGT	Gly	0.400	12
GCC	Ala	0.543	19	GGC	Gly	0.467	14
GCA	Ala	0.086	3	GGA	Gly	0.100	3
GCG	Ala	0.200	7	GGG	Gly	0.033	1

Note : Column GPDa gives the fraction of synonymous codon. Column GPDb presents the total number of specific codons in the GPD gene

Phylogenetic analysis

Previous phylogenetic analyses of yeast and fungal *gpd* sequences (Fig. 2) have shown that clustering of *gpds* from ascomycetous yeast, filamentous ascomycetous, and basidiomyces into distinct groups (Smith, 1989; Harmsen *et al.*, 1992). The phylogenetic tree was constructed using the neighbor-joining method of PAUP 4.0 (Sinauer, UK). With *M. racemosus* and *Rhizomucor miehei* as the outgroup, the *gpd* sequences were divided into four groups: (I) zygomycetes, (II) ascomycetes yeast, (III) filamentous ascomycetes, and (IV) basidiomycetes. Clusters containing species currently classified in the same orders are usually well-supported. The highest similarity of the *G. lucidum gpd* amino acid sequences was found with *P. chrysosporium*.

Codon usage in the G. lucidum gpd gene

Codon usage in the *G. lucidum gpd* gene is show in Table 1. Varying patterns of codon usage have been observed in different organisms, and preference is thought be connected to the abundance of the relevant tRNA molecules. However, no report has yet been published on the codon usage of *G. lucidum*. Highly-expressed genes generally show a more marked codon bias than genes expressed at low levels, and there can therefore be a difference usage bias between genes of the same organism. In the *G. lucidum gpd* gene, 48 out of 61 possible sense codons are used, but only 41 of 61 codons are used more than twice (Table 1). From the data shown in Table 1, it is clear that there is a strong preference for pyrimidine (Py) in the third position, although the choice of purine (Pu) or Py is possible. For two-fold degenerate codons with a Pu or Py in the wobble position, a glycine (G) or cytosine (C) is preferred, respectively. With the exception of the codons for Arg and Gly, a thymine (T) was not frequently used as the third base. The TAA codon is observed the most frequently as a terminator in filamentous fungi, and is also found in the *G. lucidum gpd* gene. The observed codon preference may be useful for the design of *G. lucidum* oligonucleotides for PCR amplification of other interesting *G. lucidum* genes.

Sequence of the 5'-flanking region

There were one typical TATA box (TATAAAA) and two CAAT boxes (CCAAT) located in the 5'- flanking region (Fig. 1). The TATA box was observed 67 bp upstream from the start codon, while the two CAAT boxes were located 470 bp and 605 bp upstream of the initiating ATG codon; these CAAT boxes were located further upstream from the initiating ATG codon than other published basidiomycetous gpd genes (Harmsen *et al.*, 1992; Hirano *et al.*, 1999). A pyrimidine region composed of stretches of thymine nucleotides interrupted by cytosine residues was observed immediately upstream of the transcription initiation site. Using the promoter prediction software (http://www.fruitfly.org/seq_tools/promoter.html), two possible core promoter regions were found at the 5'-flanking region; one was located -33~ -82 bp upstream from the start codon, the other was located -888~ -937 bp further upstream from the start codon. The gpd, pgk, qut, and qa boxes featured in the promoter of the *A. nidulans* and *A. niger* gpd gene (Punt *et al.*, 1990) were not found in the gpd promoter of *A. bisporus* (Harmsen *et al.*, 1992), *L. edodes* (Hirano *et al.*, 1999), or in the present study of *G. lucidum* (Fig. 1).

Expression of the native gpd gene

Expression of the *gpd* gene was detected by RT-PCR (Fig. 3). The specific primer pair, P1-P2, borders a region with a size of 461 bp in the genomic DNA. This fragment contains 2 intron sequences, and the corresponding mRNA fragment is 340 bp long. A fragment with the expected size of the mRNA sequence was amplified from total RNA. After sequencing, it proved to be identical with the cDNA of the *G. lucidum gpd* gene. It also suggested that this *gpd* gene encoded a functional protein and was highly expressed at high levels.

This paper has reported the isolation and characterization of the gene coding for a putative glyceraldehyde-3-phosphate dehydrogenase of *G. lucidum*. The *G. lucidum* GPD protein sequence exhibited the highest homology to the *P. chrysosporium*, as expected

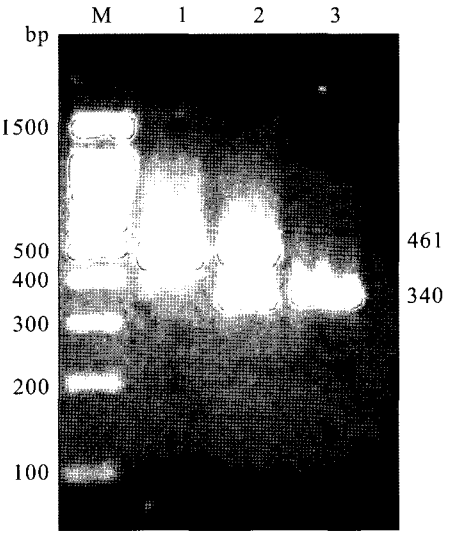

Fig. 3. Detection of the expression of the *G. lucidum gpd* gene with RT-PCR. Lane M: 100 bp+1.5 kb DNA Ladder. Lane 1: PCR amplification from the total genomic DNA. Lane 2: One-step RT-PCR from the total RNA without DNase treatment. Lane 3: One step RT-PCR from the DNase-treated total RNA.

considering the taxonomic relationship between the two species. In expectation of a high constitutive expression, the promoters of *gpd* genes have been used in several examples of the functioning of heterologous regulatory elements, as in most cases, homologous regulatory elements have been found to be more efficient. The data presented here and the availability of the homologous expression signals of the *gpd* gene of *G. lucidum* allow the construction of similar transformation vectors that should extend our understanding of this medically important fungus.

Acknowledgments

We thank Dr. John Buswell of the Chinese University of Hong Kong for his comments and linguistic revision of the manuscript. This work was supported by grants from the National Natural Science Foundation of China (Nos. 30300006 and 30571294) and Innovative Young Scholars of Jiangsu Province in China (BK 2006517).

References

Balance, D.J. 1990. Transformation systems for filamentous fungi and an overview of fungal gene structure. Molecular Industrial Mycology, p. 1-29. *In* S.A. Leong and R.M. Berka (eds.), Dekker, New York, USA

Bitter, G.A. and K.M. Egan. 1984. Expression of heterologous genes in *Saccharomyces cerevisiae* from vectors utilizing the glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Gene* 32, 263-274.

Chen, X., M. Stone, C. Schlagnhaufer, and C.P. Romaine. 2000. A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. *Appl. Environ. Microbiol.* 66, 4510-4513.

El-Mekkawy, S., M.R. Meselhy, N. Nakamura, Y. Tezuka, M. Hattori, N. Kakiuchi, K. Shimotohno, T. Kawahata, and T. Otake. 1998. Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. *Phytochemistry* 49, 1651-1657.

Harmsen, M.C., F.H. Schuren, S.M. Moukha, C.M. van Zuilen, P.J. Punt, and J.G. Wessels. 1992. Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune, Phanerochaete chrysosporium* and *Agaricus bisporus*. *Curr. Genet.* 22, 447-454.

Herzog, R.W., N.K. Singh, C. Schmidt, and P.A. Lemke. 1995. Presence of a P1 bacteriophage sequence in transforming plasmids of *Pleurotus ostreatus*. *Curr. Genet.* 27, 460-465.

Hirano, T., T. Sato, K. Okawa, K, Kanda, K. Yaegashi, and H. Enei. 1999. Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of *Lentinus edodes*. *Biosci. Biotechnol. Biochem.* 63, 1223-1227.

Hirano, T., T. Sato, K. Yaegashi, and H. Enei. 2000. Efficient transformation of the edible basidiomycete *Lentinus edodes* with a vector using a glyceraldehyde-3-phosphate dehydrogenase promoter to hygromycin B resistance. *Mol. Gen. Genet.* 263, 1047-1052.

Holland, M.J. and J.P. Holland. 1978. Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. *Biochemistry* 17, 4900-4907.

Hsu, H.Y., S.L. Lian, and C.C. Lin. 1990. Radioprotective effect of *Ganoderma lucidum* (Leyss. ex. Fr.) Karst after X-ray irradiation in mice. *Am. J. Chin. Med.* 18, 61-69.

Kim, D.H., S.B. Shim, N.J. Kim, and I.S. Jang. 1999. Beta-glucuronidase-inhibitory activity and hepatoprotective effect of *Ganoderma lucidum*. *Biol. Pharm. Bull.* 22, 162-164.

Kim, S., J. Song, and H.T. Choi. 2004. Genetic transformation and mutant isolation in *Ganoderma lucidum* by restriction enzyme-mediated integration. *FEMS Microbiol. Lett.* 233, 201-204.

Kuo, C.Y., S.Y. Chou, and C.T. Huang. 2004. Cloning of glyceraldehyde-3-phosphate dehydrogenase gene and use of the *gpd* promoter for transformation in *Flammulina velutipes*. *Appl. Microbiol. Biotechnol.* 65, 593-599.

Lin, J.M., C.C. Lin, H.F. Chiu, J.J. Yang, and S.G. Lee. 1993. Evaluation of the anti-inflammatory and liver-protective effects of *Anoectochilus formosanus*, *Ganoderma lucidum*, and *Gynostemma pentaphyllum* in rats. *Am. J. Chin. Med.* 21, 59-69.

Maruyama, H., K. Yamazaki, S. Murofushi, C. Konda, and T. Ikekawa. 1989. Antitumor activity of *Sarcodon aspratus* (Berk.) S. Ito and *Ganoderma lucidum* (Fr.) Karst. *J. Pharmacobiodyn* 12, 118-123.

Punt, P.J., M.A. Dingemanse, A. Kuyvenhoven, R.D. Soede, P.H. Pouwels, and C.A. van den Hondel. 1990. Functional elements in the promoter region of the *Aspergillus nidulans* gpdA gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene* 93, 101-109.

Piechaczyk, M., J.M. Blanchard, L. Marty, C. Dani, F. Panabieres, S. El Sabouty, P. Fort, and P. Jeanteur. 1984. Post-transcriptional regulation of glyceraldehyde-3-phosphate-dehydrogenase gene expression in rat tissues. *Nucleic Acids Res.* 12, 6951-6963.

Russo, P., J.T. Juutii, and M. Raudaskoski. 1992. Cloning, sequence and expression of a beta-tubulin-encoding gene in the homobasidiomycete *Schizophyllum commune*. *Gene* 119, 175-182.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, (2nd ed). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA

Smith, T.L. 1989. Disparate evolution of yeasts and filamentous fungi indicated by phylogenetic analysis of glyceraldehyde-3-phosphate dehydrogenase genes. *Proc. Natl. Acad. Sci. USA* 86, 7063-7066.

Su, C.Y., M.S. Shiao, and C.T. Wang. 1999. Differential effects of ganodermic acid S on the thromboxane A2-signaling pathways in human platelets. *Biochem. Pharmacol.* 58, 587-595.

Sun, L., H. Cai, W. Xu, Y. Hu, and Z. Lin. 2002. CaMV 35S promoter directs beta-glucuronidase expression in *Ganoderma lucidum* and *Pleurotus citrinopileatus*. *Mol. Biotechnol.* 20, 239-244.

Tasaka, K., M. Mio, K. Izushi, M. Akagi, and T. Makino. 1998. Anti-allergic constituents in the culture medium of *Ganoderma lucidum*. (II). The inhibitory effect of cyclo-octasulfur on histamine release. *Agents Actions* 23, 157-

160.

Thompson, J.D., D.G. Higgings, and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680.

Van de Rhee, M.D., P.M. Graca, H.J. Huizing, and H. Mooibroek. 1996. Transformation of the cultivated mushroom, *Agaricus bisporus,* to hygromycin B resistance. *Mol. Gen. Genet.* 250, 252-258.

Verdoes, J.C., J. Wery, T. Boekhout, and A.J. Van Ooyen. 1997. Molecular characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of *Phaffia rhodozyma. Yeast* 13, 1231-1242.

Wang, S.Y., M.L. Hsu, H.C. Hsu, C.H. Tzeng, S.S. Lee, M.S. Shiao, and C.K. Ho. 1997. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. *Int. J. Cancer* 70, 699-705.

Waterham, H.R., M.E. Digan, P.J. Koutz, S.V. Lair, and J.M. Cregg. 1997. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* 186, 37-44.

Wolff, A.M. and J. Arnau. 2002. Cloning of glyceraldehyde-3-phosphate dehydrogenase-encoding genes in *Mucor circinelloides (Syn. racemosus)* and use of the GPD1 promoter for recombinant protein production. *Fungal Genet. Biol.* 35, 21-29.

Yoon, S.Y., S.K. Eo, Y.S. Kim, C.K. Lee, and S.S. Han. 1994. Antimicrobial activity of Ganoderma lucidum extract alone and in combination with some antibiotics. *Arch. Pharm. Res.* 17, 438-442.