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SHORT COMMUNICATION

Molecular cloning and sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase gene from the violet root rot fungus, *Helicobasidium mompa*

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Abstract The glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene was cloned from the violet root rot fungus, *Helicobasidium mompa*, and characterized. Only one copy of the gene was present in the genome of *H. mompa*. The *H. mompa gpd* gene was found to encode a protein of 335 amino acids and its putative protein showed high homology with GPD protein from other heterobasidiomycetes. Although the promoter region contains a TATA box and several common motifs, these sequence positions were very different from that of other basidiomycetes. Moreover, mRNA expression showed that the *gpd* gene was transcribed constitutively in *H. mompa*.

Keywords Heterobasidiomycete · Inverse PCR · Promoter · RT-PCR

The violet root rot fungus *Helicobasidium mompa* Tanaka is a commercially important soil-borne root pathogen affecting a wide range of plant species. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grapevines, apple and pear trees, and other crops. Valuable knowledge about the molecular basis of the pathogenicity of *H. mompa* could be gained from genetic studies such as gene insertional mutagenesis. However, understanding the genetic basis of its pathogenicity has been limited by the lack of a suitable transformation system.

Glyceraldehyde-3-phosphate dehydrogenase (GPD, E.C.1.2.1.12) plays an important role in both glycolysis and gluconeogenesis. It catalyzes the nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent reversible oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. Because GPD is essential for all living organisms, its amino acid sequence is highly conserved and it should be highly and constitutively expressed (Holland and Holland 1979; Piechaczyk et al. 1984). The promoter sequence of the *gpd* gene has been used to express a wide variety of heterologous genes in fungi (Chen et al. 2000; Kuo et al. 2004; Vastag et al. 2004; Van Bogaert et al. 2008). In order to obtain a promoter with a high expression level, the *H. mompa gpd* gene has been cloned and its 5′-upstream promoter sequence has been characterized.

H. mompa strain V18 obtained from Dr. Hitoshi Nakamura (National Institute of Agro-Environmental Sciences, Japan) was used in the present study. Mycelia for DNA and RNA extraction were grown in 1/5 oatmeal broth (12 g/L oatmeal) at 25°C for 1 week. DNA was extracted from the *H. mompa* strain V18 using the hexadecyltrimethylammonium bromide (CTAB) procedure as described previously (Kano et al. 2011). The degenerate primers GAPDHF (5′-GCNGTNAAYGAYCCNTTYAT-3′) and GAPDHR (5′-TANCCCCAYTCRTTRTCRTA-3′) were designed, based on the amino acid sequences AVNDPFI and YDNEWG, respectively, which are conserved regions of *gpd* genes from *Schizophyllum commune, Phanerochaete chrysosporium, Agaricus bisporus, Lentinus edodes, Ganoderma lucidum*, and *Pseudozyma flocculosa* (Harmsen et al. 1992; Hirano et al. 1999; Fei et al. 2006; Neveu et al. 2007).

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Using these degenerate primers and *H. mompa* genomic DNA as the template, the *gpd* gene was partially cloned by polymerase chain reaction (PCR) amplification. To obtain the complete sequence of the *gpd* gene, inverse PCR was carried out using gene-specific primers which were designed from the partial *H. mompa gpd* gene sequence. Template DNA was prepared using a TaKaRa LA PCR in vitro Cloning Kit according to the instructions of the manufacturer (TaKaRa Bio, Shiga, Japan). All amplified DNA fragments were subcloned into a pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Procedures were carried out according to the manufacturer's recommendations. Reverse transcription and subsequent PCR (RT-PCR) reactions were performed using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa Bio) with primers HgpdRTF1 (5′-GACCTCG CGTACATGGTATACATG-3′) and HgpdRTR1 (5′-TTGGAGCTCGTAGGAACGCGG AA-3′). First-strand cDNA was synthesized by AMV Reverse Transcriptase XL with an Oligo dT-Adaptor Primer; 3′- and 5′-rapid amplification of cDNA ends (RACE) were performed using an RNA PCR kit (AMV) Ver. 3.0 and a TaKaRa 5′-Full RACE Core Set (TaKaRa Bio), respectively, according to the manufacturers' recommendations. Specific primers for 3′ and 5′-RACE were designed on the junctions between exons and introns in order to avoid amplification of genomic DNAs.

DNA sequencing was carried out in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA, USA) using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The nucleotide sequence has been deposited at the DNA Data Bank of Japan (DDBJ) under the accession number AB548815.

To determine the copy number, genomic DNA from *H. mompa* V18 was digested with the appropriate restriction endonucleases (*Pst*I, *Sac*I, and *Hae*III). Procedures for probe labeling, hybridization, and detection were carried out using the Gene Image random primed nucleic acid labeling and detection system (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's recommendations. To amplify the DNA of *gpd* as the hybridization probe, the primers GAPDHUp (5′-TACAGGGCTTGACCTTTG AGCCA AC-3′) and GAPDHDn (5′-CGCTTCTTGCACC ACTAATGTCAGAGCGT-3′) were designed based on the nucleotide sequence of the *gpd* gene from *H. mompa*.

An approximately 1,200 bp DNA fragment was obtained using the degenerate primers. The regions adjacent to the fragment obtained were cloned using an inverse PCR method and 3′- and 5′-RACE. As a result, we obtained 2,904 bp of the *gpd* gene of *H. mompa* (Fig. 1). The transcription start site and the position of the 3′-end of the

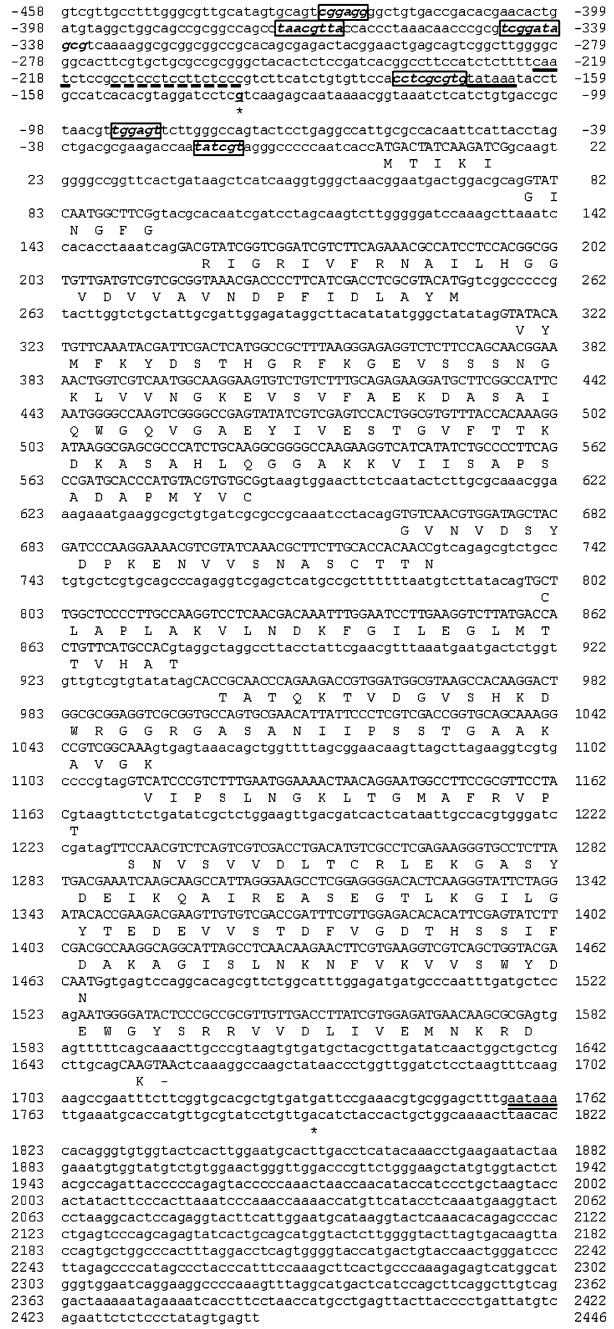

Fig. 1 Nucleotide sequence and deduced amino acid sequence of GPD protein from *Helicobasidium mompa*. Intron sequences and non-protein-coding region are shown in *lowercase letters*. The deduced amino acid sequence of GPD protein is shown *below the nucleotide sequence*. The major transcription start point and the position of the 3′-end of the mRNA are each marked with an *asterisk*. The putative TATA box and putative CAAT box in the promoter region are *underlined*, and a *dashed underline* indicates the pyrimidine-rich sequence. A *double underline* indicates the putative poly(A) signal. *Squares* indicate several conserved motifs including two ADR1 motifs (NGGRGK), a CRE motif (TGACGTYA), a GATA motif (NNNGATRNNN), a potential NIT2 site (TATCTM) and a StuA-like motif (NWWCGCGWN). The DNA Data Bank of Japan (DDBJ) accession number of the sequence is AB548815

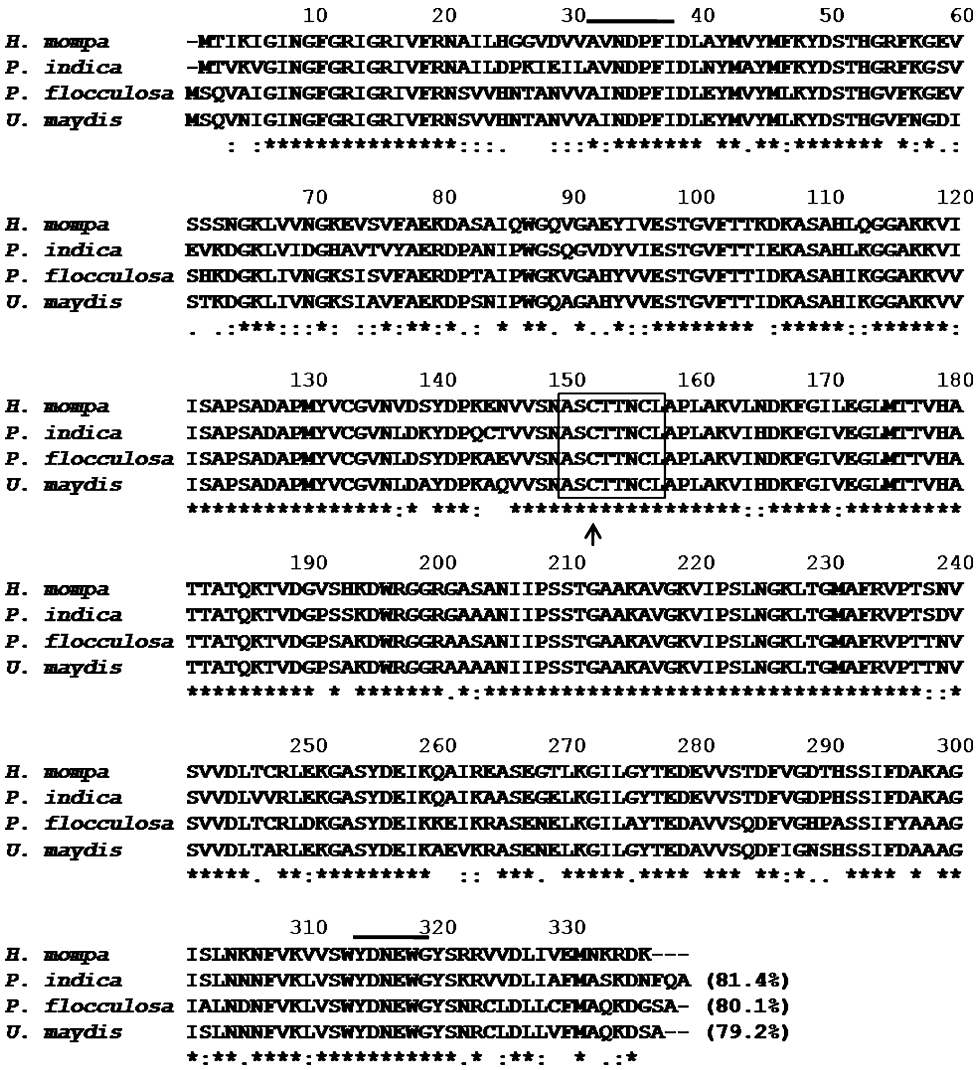

Fig. 2 Multiple alignment of the deduced amino acid sequences of the GPD proteins of *H. mompa*, *Piriformospora indica*, *P. flocculosa*, and *Ustilago maydis*. Identical residues in all four sequences are marked by *asterisks* under the aligned sequence. The *values* in *parentheses* indicate the extent of similarity to the amino acid sequence of the *H. mompa* GPD protein. Two conserved regions for the degenerate oligonucleotide primer design are indicated with *lines* above them. The substrate binding region is marked by a *square*. The main catalytic amino acid residue cysteine at position 151 is indicated by an *arrow*

mRNA were presumed by 3′- and 5′-RACE. A single initiation site located 137 bp upstream of the start codon was found. The *gpd* gene contains ten introns in the coding region and is shown schematically in Fig. 3a. All introns started with the nucleotide pair GT and ended with AG, except for the first intron, which started with the nucleotide pair GC and ended with AG. The 3′ region of the *H. mompa gpd* gene contained one possible sequence: AATAAA 101 bp downstream of the TAA stop codon.

A typical CAAT box and a putative TATA box were found 221 and 169 bp upstream of the ATG start codon, respectively (Fig. 1). Furthermore, one CT-rich region was observed 212 bp upstream of the ATG start codon. A search of the TRANSFAC database with the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) found several conserved motifs (Fig. 1). These motifs were also observed in *Tremella fuciformis* (Sun et al. 2009). Various basidiomycete promoter regions of *gpd* genes were compared by Kilaru and Kües (2005). In various GPD promoter sequences currently available from basidiomycetes, such as *A. bisporus, P. chrysosporium, S. commune*, and *Coprinopsis cinerea*, a TATA box is consistently located between −63 and −97 bp from the start ATG. However, in *H. mompa*, the TATA box is further from the ATG than in various GPD sequence from those basidiomycetes (Fig. 1). While several common motifs from those of basidiomycetes are located less than −300 bp from the ATG, in *H. mompa*, these are located more than −300 bp away. From these observations, it appears that the promoter region of *H. mompa* is quite different from those of various basidiomycetes.

The *gpd* gene reading frame is translated into a protein of 335 amino acids with a calculated size of 35.9 kDa and an estimated *pI* value of 8.2. The deduced amino acid sequence of the GPD protein in *H. mompa* showed high sequence similarity to that of heterobasidiomycetes. The highest similarity of the *H. mompa* GPD amino acid sequence was found with *Piriformospora indica*, which is a

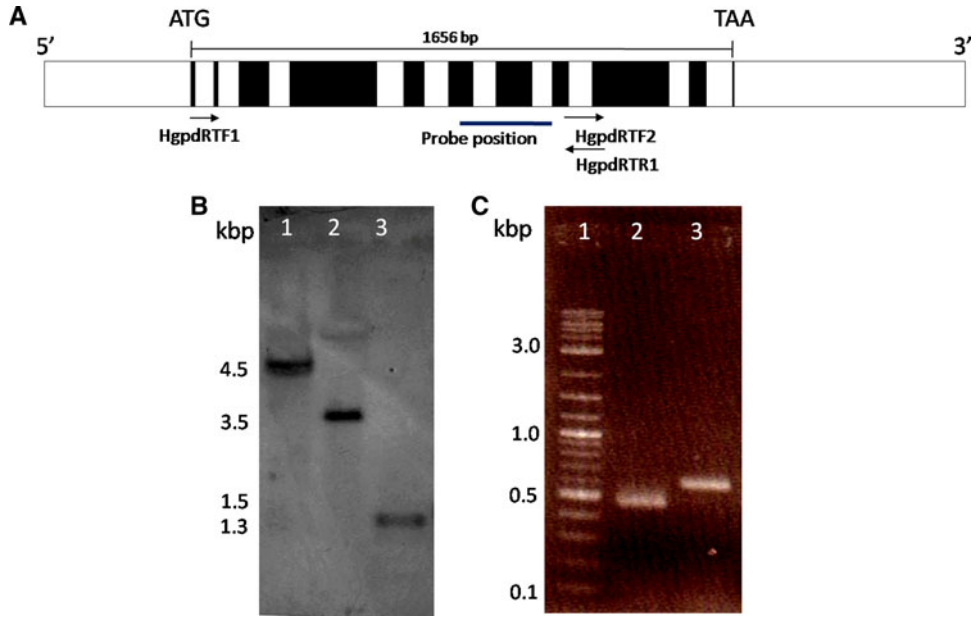

Fig. 3 Schematic diagram, Southern hybridization and transcription of the GPD gene in *H. mompa*. **a** Schematic diagram of the exon and intron positions in the gene encoding GPD from *H. mompa*. The *black boxes* represent the exons and the *open boxes* represent the introns. The position indicated by the *bar* shows the region used as a probe for Southern hybridization. *Arrows* indicate the positions of the primers used for reverse transcription-polymerase chain reaction (RT-PCR) analysis. **b** Genomic DNA (1 μg) from *H. mompa* was digested with restriction enzymes: *Pst* I (*lane 1*); *Sac*I (*lane 2*); *Hae*III (*lane 3*). The positions of molecular DNA size markers (in kilobases) are shown on the *left*. **c** Expression of gene encoding GPD from *H. mompa* analyzed by RT-PCR. *Lane 1* DNA molecular size markers (in kilobases) are indicated on the *left*; *lane 2* 0.45-kbp fragment of the GPD cDNA using primers HgpdRTF2 and oligo(dT) primer; *lane 3* 0.6-kbp fragment of the GPD cDNA using primers HgpdRTF1 and HgpdRTR1

root colonizing fungus (81.4% identity). Homology with the phytopathogenic fungi *P. flocculosa* and *Ustilago maydis* was 80.1 and 79.2%, respectively (Fig. 2). The GPD consensus substrate binding region is found in all GPD enzymes (PROSITE; http://au.expasy.org/prosite/): [ASV]-S-C-[NT]-T-x(2)- [LIM]. This pattern is present in the *H. mompa* protein as ASCTTNCL. The cysteine, which is found at the 151st residue, is essential for the enzymatic activity since it functions as the binding site in the catalytic region (Hirano et al. 1999).

Southern hybridization of digested *H. mompa* genomic DNA with the fragment of the *H. mompa gpd* gene revealed a single band in each digestion, indicating the presence of only one copy for this gene (Fig. 3b). A similar result was also found in *P. flocculosa*, *Flammulina velutipes*, *L. edodes*, and *S. commune* (Harmsen et al. 1992; Hirano et al. 1999; Kuo et al. 2004; Neveu et al. 2007). On the other hand, in some other organisms such as *Saccharomyces cerevisiae*, *A. bisporus*, and *Pilobolus crystallinus*, two or three slightly different *gpd* genes are differentially expressed, suggesting a distinct cellular role (McAlister and Holland 1985; Harmsen et al. 1992; Kubo 2010).

For the RT-PCR analysis, PCR product signals were detected using two primer sets. The expected sizes of the cDNA fragments were observed, indicating that the *gpd* gene is expressed constitutively in *H. mompa* (Fig. 3c).

In *H. mompa*, the Pgpd promoter from the *A. bisporus gpd* gene was employed to direct expression of a hygromycin B resistance gene in an *Agrobacterium tumefaciens* transformation system; however, we obtained low transformation efficiency (Kano et al. 2011). Though there are several examples of the functioning of heterologous regulatory elements, in most cases, such as in *A. bisporus*, *Rhizomucor miehei*, and *F. velutipes*, homologous regulatory elements have been found to be more efficient (Chen et al. 2000; Vastag et al. 2004; Kuo et al. 2004). Based on these results, we suggest that the transformation efficiency of *H. mompa* could similarly be enhanced when using homologous regulatory elements.

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