Full Length Research Paper

Cloning and molecular characterization of glyceraldehyde-3-phosphate dehydrogenase gene from thermotolerant mushroom, *Lentinus polychrous*

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This study describes the cloning and expression analysis of the heat shock protein gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in thermotolerant mushroom *Lentinus polychrous*, one of the most widely cultivated commercial edible mushroom in Thailand. The complete GAPDH coding sequence contained 1,011 bp, encoding for a polypeptide of 337 amino acid residues with the calculated molecular mass of 36.2 kDa and pI of 5.64. Southern blot analysis revealed the presence of a single copy of the *GAPDH* gene in the *L. polychrous* genome. The putative amino acid sequence of the *L. polychrous* GAPDH-encoded protein shared significant sequence identity with other GAPDH proteins from basidiomycetes. Phylogenetic analysis clustered the *L. polychrous* GAPDH protein with other homobasidiomycetes. Expression analysis of the *GAPDH* gene by RT-PCR showed that this gene was highly induced not only by heat, but also by cold, ethanol and salt stresses. The *GAPDH* gene was expressed in both mycelia and fruiting bodies, suggesting that the *GAPDH* gene product is a heat shock protein which might be involved in the developmental phase of the *L. polychrous*.

Key words: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), *Lentinus polychrous*, heat shock protein gene, basidiomycetes, gene expression.

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is one of the key enzymes in glycolysis and gluconeogenesis. In the former pathway, it catalyses the oxidative phosphorylation of glyceraldehydes-3-phosphate into 1,3-biphosphoglycerate in the presence of nicotinamide adenine dinucleotide and inorganic phosphate, and in the latter pathway, it catalyses the reverse reaction (Niu et al., 1994; Hirano et al., 1999). In addition to these long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes such as surface antigen (Goudout-Crozel et al., 1989), membrane transport and fusion, nuclear RNA transport (McDonald and Moss, 1993; Singh and Green, 1993; Sirover, 1999), transferrin-binding protein (Modun and Williams, 1999), transcription activation, initiation of apoptosis (Tarze et al., 2007) and cell surface protein to assist the adhesion of pathogen on host tissue (Barbosa et al., 2006; Egea et al., 2007). The GAPDH is a tetramer composed of identical subunits.

In yeast, 2-5% of the poly (A)$^+$ RNA is GAPDH mRNA (Holland and Holland, 1978), while in *Aspergillus nidulans*, the GAPDH protein comprises up to 5% of the soluble

cellular proteins (Redkar et al., 1998; Hirano et al., 1999). These observations suggest that the *GAPDH* gene is controlled by a highly active promoter. The promoter sequences of native GAPDH-encoding genes have proven useful for efficient expression of heterologous genes in several yeasts and fungi such as *Pichia pastoris* (Vassileva et al., 2001; Menendez et al., 2004), *Mucor circinelloides* (Wolff and Arnau, 2002), *Lentinus edodes* (Nitta et al., 2004) and *Aspergillus niger* (Halaouli et al., 2006).

Lentinus polychrous, commonly known as hed kradang, is one of the most widely commercial cultivated edible mushrooms of the Northeast and Northern part of Thailand. This basidiomycetous mushroom is classified in the order Poreales, family Lintinaceae (Ruksawong and Flegel, 2001). In addition to high concentration of protein, carbohydrate, calcium and phosphorus, this mushroom also contains high quantities of special substances, referred to as nutriceuticals, such as eritadenine, germanium and ergosterol. These nutriceutical compounds can modulate the immune system, lower blood pressure, reduce cholesterol and act as antithrombotic (Pegler, 1983; Waser and Weis, 1997).

Unlike *Lentinula edodes* (formally known as *L. edodes*) or shiitake mushroom in which fruiting body is typically induced by cold shock at 4°C, the formation of fruiting body in *L. polychrous* is activated by heat shock at 40-45°C (Nariso, 2004). It is well known in a wide variety of organisms that this circumstance favors the synthesis of a specific set of proteins, known as heat shock proteins (HSPs) (Lindquist and Craig, 1988). In a previous study, we found that heat shock conditions induce the synthesis of several heat shock proteins in this mushroom (unpublished results). Among them, a protein with the apparent molecular mass of 36 kDa was chosen and analyzed. N-terminal amino acid sequences analysis revealed that this protein is GAPDH which has been previously reported as a heat shock protein in *Saccharomyces cerevisiae* (Thanonkeo et al., 2003). Although gene encoding this enzyme has been cloned and characterized in several filamentous fungi such as *Cryphonectria parasitica* (Choi and Nuss, 1990), *L. edodes* (Hirano et al., 1999), *M. circinelloides* (Wolff and Arnau, 2002), *Phaeosphaeria nodorum* (Ueng et al., 2003), *Pleurotus sajorcaju* (Jeong et al., 2000), *Rhizomucor miehei* (Vastag et al., 2004), *Ganoderma lucidum* (Fei et al., 2006), *Penicillium marneffei* (Thirach et al., 2008), *Moniliophthora perniciosa* (Lima et al., 2009) and *Trichoderma virens* (Oh et al., 2009), to our knowledge this is the first study to isolate and characterize the *GAPDH* gene in *L. polychrous*. We report here that GAPDH is a heat shock protein gene that might be involved in the developmental phase of this mushroom.

MATERIALS AND METHODS

Strain, culture conditions and plasmid

L. polychrous KPM3 isolated from national park at Puvieng, Khon Kaen (Nariso, 2004) was used in this study. The culture was maintained on potato dextrose agar (PDA) at 4°C and subculturing was performed every 4 weeks. *Escherichia coli* DH5α was used for gene transformation. It was grown in LB medium at 37°C. Vector used for gene transformation was pGEM T-easy vector (Promega, Madison, WI, USA). All molecular cloning techniques were carried out as described by Sambrook and Russell (2001).

Genomic DNA and total RNA isolation

Mycelium from *L. polychrous* cultures grown in potato dextrose broth at 30°C for 7 days was harvested by filtration through four layers of cheesecloth, immediately frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Genomic DNA was isolated from the fungal mycelia using the CTAB method (Russo et al., 1992), whereas total RNA was extracted from this fungal mycelia using RNeasy Plant Mini Kit (QIAGEN, Germany). The extraction of total RNA was carried out essentially as recommended by the manufacturer's instruction except that RNase-free DNase I was added to a final concentration of 0.1 mg/ml to remove any contamination of genomic DNA.

Cloning and sequencing of *GAPDH* gene

An internal fragment encoding the amino acid sequence of the *GAPDH* gene product was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA isolated from *L. polychrous* as a template and degenerated oligonucleotide primers synthesized based on the *GAPDH* gene from other basidiomycetous fungi in the GenBank database. The sequences of the two degenerated oligonucleotide primers were forward GAPDH-Flen (5'-TACATGGTYTACATGTTCAA-3') and reverse GAPDH-Rlen (5'-TCRTTGTCGTACCADGMVA-3'). The RT-PCR reaction was carried out using OneStep RT-PCR Kit (QIAGEN, Germany).

The reaction mixture (50 μl) consisted of 10 μl of 5xQIAGEN OneStep RT-PCR buffer, 400 μM dNTP, 0.6 μM of each forward and reverse primer, 2 μl of QIAGEN OneStep RT-PCR enzyme mix and 1 μg of RNA template. The RT reaction was carried out at 45°C for 45 min. Following an initial denaturation of the template cDNA at 95°C for 15 min, 35 cycles of the following temperatures were used: denaturation at 94°C for 1 min, annealing at 48°C for 2 min and extension at 72°C for 5 min. The amplified product was separated on 0.7% agarose gel and target DNA was excised from the gel and purified using the NucleoSpin Extract II Kit (Macherey-Nagel, Germany).

The purified PCR product was cloned into pGEM T-easy vector (Promega, Madison, WI, USA) and the resulting plasmid was transformed into the competent *E. coli* JM109 using the Rapid DNA Ligation and Transformation Kit (Fermentas, USA). The plasmid DNA was isolated from a positive clone and target DNA was sequenced by the dideoxy chain termination methods using the MegaBACE 1000 automated DNA sequencer (Pharmacia Biotech, Sweden). The sequences of the N-terminus and the C-terminus of *GAPDH* gene was amplified using the SMART™ RACE cDNA Amplification Kit (ClonTech, USA). The reaction was carried out essentially as recommended by the manufacturer's instruction.

The complete full length open reading frame (ORF) of the *GAPDH* gene was confirmed by RT-PCR using the forward and reverse primers synthesized based on the known sequences of the 5'- and 3'-end of the *GAPDH* gene. These two primers were forward GAPDH-F1len (5'-ATGGCCGTCAAAGTCGGTATC-3') and reverse GAPDH-R1len (5'-TTTGTCCTACCCTTGGAGATT-3'). After RT-PCR reaction, the amplified product was separated on 0.7% agarose gel, purified, cloned and sequenced as previously described. The sequence of the *GAPDH* gene and deduced amino acid sequence was analyzed using GENETYX (Software Development,

Tokyo, Japan), while homology searching was performed using FASTA and BLAST program in the GenBank and DDBJ databases. The nucleotide sequence reported in this study was deposited in GenBank under the accession number GU356537.

Southern hybridization analysis

The *GAPDH* gene in the *L. polychrous* genome was investigated by Southern blotting. Genomic DNA (20 μg) was digested with *Sal*I, *Pst*I, *Hin*dIII, *Bam*HI and *Eco*RI, electrophoresed on 0.7% agarose gel and transferred to Hybond N⁺ positively charged nylon membrane (Schleicher and Schuell) by overnight capillary transfer. The DNA was cross-linked to the membrane by UV irradiation and baked at 80°C for 2 h. All procedures were performed according to the standard methods as described by Sambrook and Russell (2001).

To prepare a probe for Southern hybridization analysis, an internal fragment encoding the amino acid sequence of the *GAPDH* gene product was amplified by RT-PCR using total RNA isolated from *L. polychrous* as a template and forward GAPDH-Flen and reverse GAPDH-Rlen primers. The GAPDH PCR fragment was alkaline phosphatase labeled using Gene Images AlkPhos Direct Labeling and Detection System (AlkPhos DirectTM, Amersham). All procedures for DNA labeling were carried out as recommended by the manufacturer.

The Hybond N⁺ positively charged nylon membrane with cross-linked DNA was prehybridized at 50°C for 2 h in hybridization oven (Hybrid limited equipment class I) with gentle rotation. After incubation, the labeled-DNA probe was added and subsequently incubated at 55°C an overnight. The membrane was washed according to the standard procedure for Southern blot analysis. After washing, the CDP-*Star* detection reagent (AlkPhos Direct™, Amersham) was added onto the membrane and left at room temperature for 5 min. The membrane was then exposed to X-ray film (Hyper film, Amersham) for 1 h, thereafter it was developed and fixed with developer and fixer solution (Kodak), respectively.

RT-PCR analysis

The expression level of the *L. polychrous GAPDH* gene was determined by RT-PCR. Total RNA was isolated from fungal mycelia grown under various stress conditions including heat shock (45°C), cold shock (10°C), ethanol shock (6% v/v) and salt stress (1 M NaCl) for 6 h using RNeasy Plant Mini Kit (QIAGEN, Germany). In order to determined the possible physiological role of *GAPDH* gene in the formation of fruiting bodies of *L. polychrous*, total RNA was extracted from fungal fruiting bodies and used as template in RT-PCR analysis. RT-PCR was carried out using OneStep RT-PCR Kit (QIAGEN, Germany) with the forward GAPDH-F1len and reverse GAPDH-R1len primers synthesized based on the 5'- and 3'-region of the *L. polychrous GAPDH* gene. The reaction mixture (50 μl) consisted of 10 μl of 5xQIAGEN OneStep RT-PCR buffer, 400 μM dNTP, 0.6 μM of each forward and reverse primer, 2 μl of QIAGEN OneStep RT-PCR enzyme mix and 1 μg of RNA template. As a control, 10 μg samples of total RNA were subjected to agarose gel electrophoresis (0.9% agarose) and stained with ethidium bromide. Actin gene was used as an internal control. A thermocycler was used to perform 1 cycle of 45 min at 50°C for reverse transcription followed by 40 cycles of 1 min at 94°C, 2 min at 50°C and 3 min at 72°C. The amplified products at the 25th, 28th, 31st, 34th, 37th and 40th cycle were electrophoresed on a 0.9% agarose gel. After staining with ethidium bromide, the relative amounts of the products were compared using the Gel Image Master (Pharmacia Biotech). The experiment was repeated at least twice. Under these conditions, the OneStep RT-PCR was able to specifically detect

mRNA because no band was observed when reverse transcriptase was omitted.

RESULTS AND DISCUSSION

Cloning of the *L. polychrous GAPDH* gene

Using degenerated oligonucleotide primer GAPDH-Flen (coding for YMVYMFK) and GAPDH-Rlen (coding for IAWYDNE), a 0.8-kb internal fragment of the *GAPDH* gene was obtained from *L. polychrous*. This fragment was purified and ligated into pGEM T-easy vector for nucleotide sequencing analysis. DNA sequence of this fragment showed high homology to other *GAPDH* genes of basidiomycetous fungi (data not shown). To isolate full-length GAPDH cDNA, RT-PCR amplification using the SMART™ RACE cDNA Amplification Kit (ClonTech, USA) was performed using total RNA isolated from mycelia of *L. polychrous* as a template. Nucleotide sequencing analysis revealed that the resulting 1.0-kb PCR product was full-length GAPDH cDNA of *L. polychrous*.

Sequence analysis of the *L. polychrous GAPDH* gene

The *L. polychrous* GAPDH cDNA contained 1,011 bp of the coding sequence, encoded a protein of 337 amino acid residues (Figure 1). A typical translation initiation codon (ATG) and translation termination codon (TAA), the most frequently found codon in filamentous fungi, were identified in the GAPDH cDNA, indicating a full-length coding sequence of the gene. The calculated molecular mass of the deduced polypeptide is 36.2 kDa and the predicted isoelectric point is 5.64. Sequence analysis revealed the presence of a potential site for substrate binding (ASCTTNCF), as described for GAPDH sequence (Goudot-Crozel et al., 1989), at position 149-156. Amino acids potentially associated with catalysis were found at amino acid positions 151 (C) and 178 (H). Potential phosphorylation sites were located at positions 102-106, 183-185, 191-194, 240-242, 245-250, 292-295, 311-314 and 321-329. The amino acid residues at positions 34 (D) and 315 (N) corresponded to the putative nicotinamide adenine dinucleotide binding sites. Amino acids at positions 150 (S), 152 (T), 196 (R), 210 (T) and 225 (G) were found to be probable sites for inorganic phosphate binding. Positions 181, 233 and 247 were found to be amino acid residues that putatively related to the binding of the phosphate from the substrate (T, R and R) (Thirach et al., 2008).

The fungal *GAPDH* genes contained diverse number of introns and the average number of introns in *GAPDH* genes of Basidiomycetes is higher than that of Asco-mycetes (Harmsen et al., 1992). It has been reported that *Agaricus bisporus GPD1*, *A. bisporus GPD2*, *L. edodes GPD*, *M. perniciosa* GAPDH, *Phanerochaete chryso-sporium GPD*, *G. lucidum GPD* and *Schizophyllum*

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ATGGCCGTCAAAGTCGGTATCAACGGATTCGGTCGTATCGGACGTACCGTCCTCAGGAAT      60
 M   A   V   K   V   G   I   N   G   F   G   R   I   G   R   T   V   L   R   N
GCTCTCCTCCACGGAGACATCAACGTCGTCGCCGTGTACGACCCCTTCATCGACCTTGAG     120
 A   L   L   H   G   D   I   N   V   V   A   V   Y   ***D***  P   F   I   D   L   E
TACATGGTTTACATGTTCAAGTACGACTCCGTCCACGGTCGCTTCAAGGGCACTGTCGAG     180
 Y   M   V   Y   M   F   K   Y   D   S   V   H   G   R   F   K   G   T   V   E
GCTAAGGACGGCAAGCTCTTGGTCGAGGGAAAGCCCATTTCCGTCTTCGAGGAGAAGGAT     240
 A   K   D   G   K   L   L   V   E   G   K   P   I   S   V   F   E   E   K   D
CCCGCCAACATCAACTGGGCCTCTGTCGGCGCTGAGTACATCGTCGAGTCCACGGGTGTC     300
 P   A   N   I   N   W   A   S   V   G   A   E   Y   I   V   E   S   T   G   V
TTCACCACCACCGAAAAGGCCTCTGCTCATTTGAAGGGCGGTGCCAAGAAGGTCATCATC     360
 F  T   T   T   E   K  A   S   A   H   L   K   G   G   A   K   K   V   I   I
TCTGCGCCCTCCGCTGATGCGCCAATGTTCGTCTGCGGTGTCAACTTGGAAGCTTACGAC     420
 S   A   P   S   A   D   A   P   M   F   V   C   G   V   N   L   E   A   Y   D
CCGAAGTACGACGTCATCTCCAACGCTTCCTGCACCACCAACTGCTTCGCGCCCCTCGCA     480
 P   K   Y   D   V   I   S   N  (A  S   C   T   T   N   C   F)  A   P   L   A
AAGGTCATCCACGACAACTTCGGCATTGTCGAGGGCCTCATGACCACCGTCCATGCCACC     540
 K   V   I   H   D   N   F   G   I   V   E   G   L   M   T   T   V   H   A   T
ACCGCCACCCAGCGGACCGTCGACGGTCCGTCGCACAAGGACTGGCGTGGGAGGCCGTGCC     600
 ***T***  A   T  Q   R   T   V   D   G   P  S   H   K   D   W   R   G   G   R   A
GTGGGCAACAACATCATCCCCTCGTCGACTGGTGCCGCCAAGGCCGTGGGCAAGGTCATC     660
 V   G   N   N   I   I   P   S   S   T   G   A   A   K   A   V   G   K   V   I
CCCAGCCTGAACGGCAAGCTCACGGGCATGTCCTTCCGTGTCCCCACGATCGACGTTTCC     720
 P   S   L   N   G   K   L   T   G   M   S   F   ***R***  V   P   T   I   D   V   S
GTCGTCGACCTCGTTGTCCGCCTCGAGAAGCCTGCGACATACGACGAGATCAAGGACGCC     780
 V   V   D   L   V   V   ***R***  L   E   K  P   A   T   Y   D   E   I   K   D   A
ATCAAGGCCGCCGCCGCGGGCCCTTTCAAGGGCATCCTCGACTACACCGAGGAGAAGGTC     840
 I   K   A   A   A   A   G   P   F   K   G   I   L   D   Y   T   E   E   K   V
GTGTCGACCGACTTCACCGGCAACGACGCATCCTCGATCTTCGATGCTGAGGCCGGAATC     900
 V   S   T   D   F   T   G   N   D   A   S  S   I   F   D  A   E   A   G   I
GCGCTCAACAACAACTTTGTCAAGCTGATTGCATGGTACGACAATGAATGGGGATACTCC     960
 A   L   N   N   N   F   V   K   L   I  A   W   Y   D  ***N***  E   W   G   Y   S
CACCGTGTCTGCGACTTGGTGGACTACGCTGCGAAACAGGATGGGAACCTCTAA          1020
 H   R   V   C   D   L   V   D   Y  A   A   K   Q   D   G   N   L   *
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Figure 1. Nucleotide and deduced amino acid sequences of the *L. polychrous GAPDH* gene. Nucleotides are numbered from the first nucleotide from 5' end of the sequence. Amino acids are indicated below the nucleotide sequence in single-letter codes. Translation stop codon is indicated by an asterisk. The sites putatively related to inorganic phosphate binding are indicated by boxes around the amino acids. Amino acids potentially associated with catalysis are in shaded boxes. Amino acid residues related to the binding of phosphate from substrate are in bold. Amino acid putatively related to the NAD⁺ binding is in bold italic. The substrate-binding site is marked with a bracket. The putative phosphorylation sites are indicated by underlines.

commune GPD contained nine, nine, eight, eight, six, six and five introns, respectively (Smith and Leong, 1990; Harmsen et al., 1992; Fei et al., 2006; Thirach et al., 2008; Lima et al., 2009). However, a rare intron organization has also been reported for the *GAPDH* genes of *A. nidulans*, *Claviceps purpurea*, *Blumeria graminis* f. sp. *hordei* and *Pseudozyma flocculosa*, with one intron positioned outside the structural region (Punt et al., 1988; Christiansen et al., 1997; Neveu et al., 2007). Although the number of introns may vary in different *GAPDH* genes, some of the intron positions seem to be strongly conserved between the Basidiomycetes and Ascomycetes *GAPDH* genes (Harmsen et al., 1992).

Apart from their evolutionary significance, introns from *GAPDH* genes may have specific functions, e.g., involve-

ment in the protein expression (Kuo and Huang, 2008) or important for identification of some ectomycorrhizal Basidiomycetes (Kreuzinger et al., 1996). With respect to the number and possible physiological roles of intron in the *L. polychrous GAPDH* genes, further studies such as cloning and sequencing of the genomic DNA and target disruption of the *GAPDH* gene have to be carried out in order to clarify these information and these are now under our investigation.

Southern blot analysis

Southern hybridization of digested *L. polychrous* genomic DNA with the PCR fragment of the *GAPDH* gene revealed
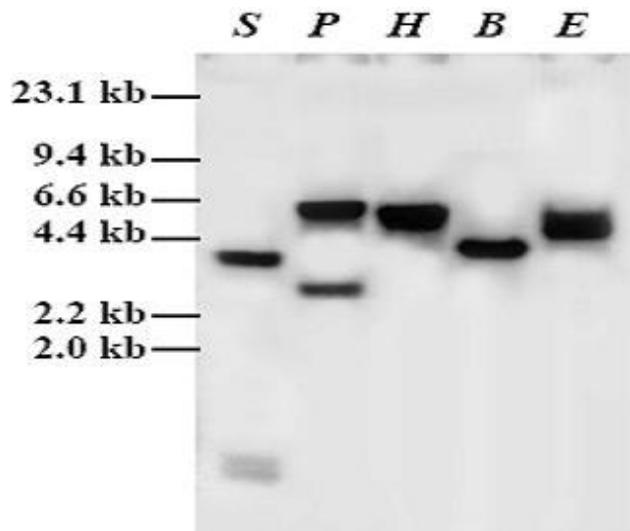
Figure 2. Southern blot analysis of the genomic DNA of *L. polychrous*. Genomic DNA isolated from fungal mycelia was digested with *Sal*I (S), *Pst*I (P), *Hin*dIII (H), *Bam*HI (B) and *Eco*RI (E), electrophoresed on 0.7% agarose gel and transferred to nylon membrane. The membrane was hybridized with an internal fragment of the *GAPDH* gene amplified with the primer GAPDH-Flen and GAPDH-Rlen.

a single band in each digestion (*Sal*I, *Hin*dIII, *Bam*HI and *Eco*RI), except that for *Pst*I digestion in which two sites of restriction enzyme might be present, suggesting that the *GAPDH* gene is present as a single copy in the genome of *L. polychrous* (Figure 2). This result agrees with that of *A. nidulans* (Punt et al., 1988), *S. commune*, *P. chrysosporium*, *A. bisporus* (Harmsen et al., 1992), *L. edodes* (Hirano et al., 1999), *Flammulina velutipes* (Kuo et al., 2004), *P. flocculosa* (Neveu et al., 2007), *Beauveria bassiana* (Liao et al., 2008), *M. perniciosa* (Lima et al., 2009) and *T. virens* (Oh et al., 2009) in which a single copy of the *GAPDH* gene has been reported.

Comparison of the amino acid sequence of the *L. polychrous GAPDH* gene with *GAPDH* genes of other fungi

The *L. polychrous* GAPDH protein showed high sequence identity to the known fungal GAPDH sequences from Basidiomycetes: 88% identity with the GAPDH of *P. chrysosporium* and *G. lucidum*, 82% with *Volvariella volvacea*, 81% with *L. edodes*, 78% with *S. commune* and 77% with *P. sajor-caju*. The *L. polychrous* GAPDH protein was also highly similar to the GAPDH protein of some plant and animals (data not shown). The main conserved amino acid residues, that is, Cys-151, which functions as the binding site of the enzyme in the catalytic region was presented in this putative protein (Figure 3), like that found in the GAPDH proteins of *P. chrysosporium*, *G. lucidum*, *S. commune*, *L. edodes* and *V.*

volvacea. However, this catalytic amino acid residue was changed in *P. sajor-caju*, Ser instead of Cys. This difference might be due to strain variation. The residues surrounding the Cys-151 active site, Ala-Ser-Cys^{151}-Thr-Thr-Asn-Cys, also matched the consensus sequence that is conserved in prokaryotic and eukaryotic GAPDH proteins (Olsen et al., 1975).

Phylogenetic analysis

An evolutionary tree based on the GAPDH amino acid sequence of 45 species representative of Ascomycetes yeasts, filamentous Ascomycetes, Zygomycetes and Basidiomycetes was performed using the neighbor-joining method (Saitou and Nei, 1987). The results revealed a separation of the Basidiomycetes, Zygomycetes and Ascomycetes, and confirmed that the *L. polychrous* GAPDH clustered with other homobasidiomycetes (Figure 4). The close relationship between filamentous Ascomycetes and Basidiomycetes GAPDH proteins in comparison to those of Ascomycetes yeasts was in agreement with earlier studies and may be due to the fact that both filamentous Ascomycetes and Basidiomycetes share a relatively recent common ancestor (Smith, 1989; Lima et al., 2009; Oh et al., 2009). Studies by Harmsen et al. (1992) also suggesting that the relationship between GAPDH of filamentous Ascomycetes and Basidiomycetes is higher and quite distinct from Ascomycetes yeasts.

Differential expression of the *L.* polychrous *GAPDH* gene

To investigate whether or not the *GAPDH* gene becomes activated by environmental stresses such as heat, cold, ethanol and salt stresses, *L. polychrous* mycelia that had been cultured in PD broth for 7 days at 30°C were transferred to high temperature (45°C), low temperature (10°C), ethanol (6% v/v) and NaCl solution (1 M) for 6 h. The change of GAPDH expression was detected by RT-PCR using RNA isolated from various stress-treated *L. polychrous* mycelia. All four stress conditions increased the expression level of *GAPDH* gene (Figure 5a). The heat and cold treatments increased the expression level of *GAPDH* gene approximately six-fold, whereas ethanol and salt treatments increased the expression of the gene about three-fold, as compared to the control condition. This result agrees with that of *P. sajor-caju* in which the expression of *GAPDH* gene is highly induced not only by heat but also by salt, dehydration and cold stresses (Jeong et al., 2000), suggested that the GAPDH is stress-responsive gene. Heat shock is known to alter expression of glycolytic genes, suggesting that primary carbon metabolism is one of the pathways that respond to changing environmental condition (Yang et al., 1993; Laxalt et al., 1996). These findings suggested that the
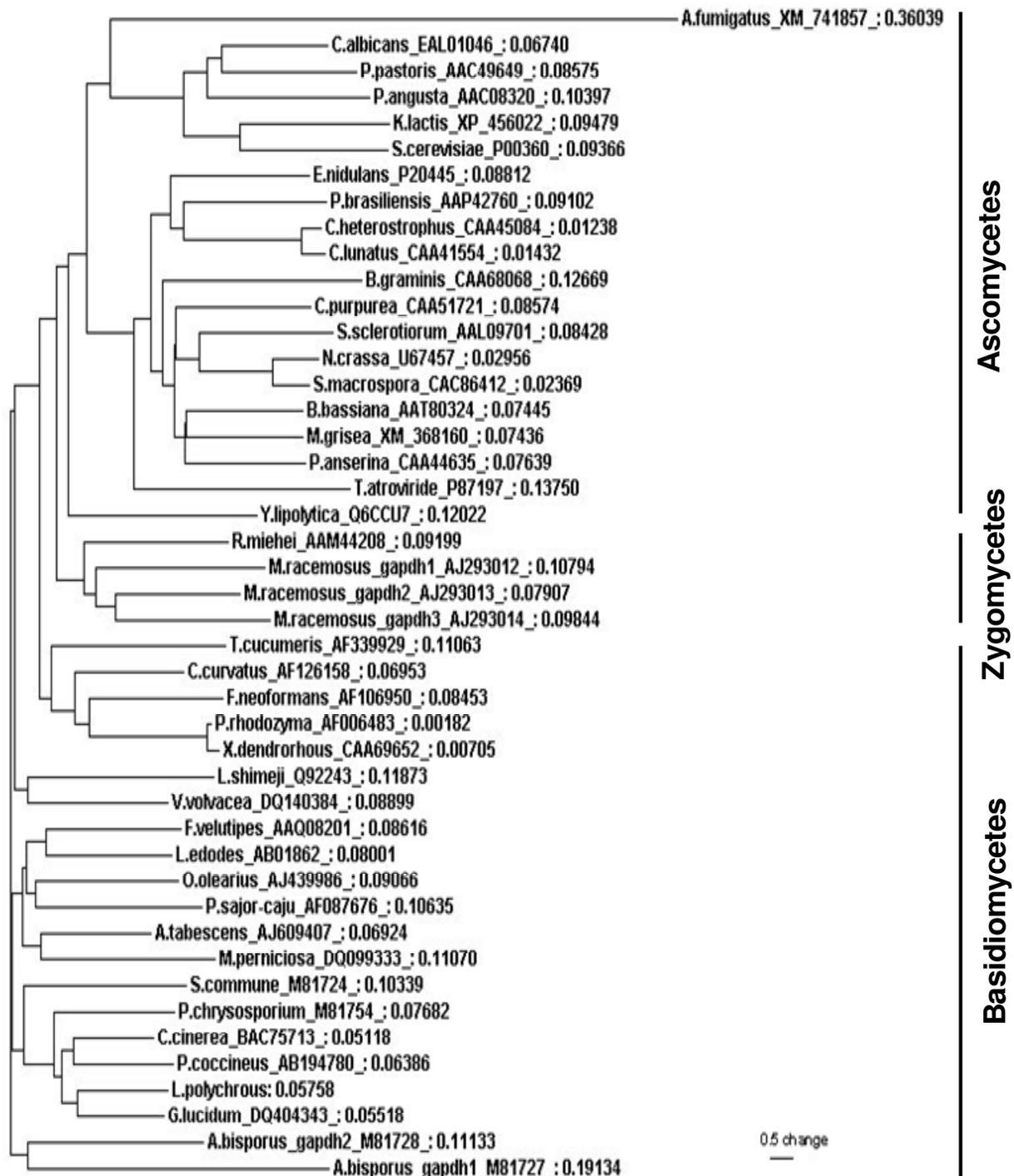
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L.polychrous      MAVKVGINGFGRIGRTVLRNALLHGDINVVAVYDPFIDLEYMVYMFKYDSVHGRFKGTVE 60
G.lucidum         MPVKVGINGFGRIGRIVLRNALLHGDIEVVAVNDPFISLEYMVYMFKYDSVHGRFKGAVE 60
P.chrysosporium   MPVKAGINGFGRIGRIVLRNALLHGDIDVVAVNDPFIDLEYMVYMFKYDSVHGRFKGSVE 60
S.commune         MAVKVGINGFGRIGRIVLRNALQLGNIEVVAINDPFIALDYMVYMFKYDTVHGRYKGTVE 60
L.edodes          MAVKVGINGFGRIGRIVLRNALLNPEVNVVAVNDPFIALEYMVYMFKYDSVHGRFKGTVE 60
V.volvacea        MVVNVGINGFGRIGRIVYRNAVENDQINVVAINDPFIDLAYMVYMFKYDSVHGRFKGTVE 60
P.sajor-caju      -MVNVGINGFGRIGRIVFRNALEVGGVDVVAINDPFIDLDYMVYMFKYDSVHGRFKGSIE 59
                   *:.********** * ***:      ::***: **** * *********:****:**::*


L.polychrous      AKDGKLLVEGKPISVFEEKDPANINWASVGAEYIVESTGVFTTTEKASAHLKGGAKKVII 120
G.lucidum         AKDGKLYVEGKPISVFEEKDPANINWSGVGAEYIVESTGVFTTVDKASAHLKGGAKKVVI 120
P.chrysosporium   AKDGKLYVEGKPIHVFAEKDPANIPWGSVGAEYIVESTGVFTTTEKASAHLKGGAKKVII 120
S.commune         VKDGKLVVDGHAITVFAEKNPADIKWGSAGADYIVESTGVFTTVEKASLHLQGGAKKVVI 120
L.edodes          TKGGKLIVDGKEISVFGEKDAGAIPWSSVGAEYIVESTGVFTTIEKASAHLKGGAKKVII 120
V.volvacea        SKDGKLVINGKSITVFSEKDPAAIPWGTAGADYIVESTGVFTTTEKASAHLKGGAKKVII 120
P.sajor-caju      AKDGKLIVEGKPIHIFAEKDPANIPWGSVGADYVVESTGVFTTIDKASAHLKGGEK-VVI 118
                   *.*** ::*: * :* **:.. * *. .**:*:********* :*** **:** * *:*


L.polychrous      SAPSADAPMFVCGVNLEAYDPKYDVISNAS**C**TTNCFAPLAKVIHDNFGIVEGLMTTVHAT 180
G.lucidum         SAPSADAPMFVCGVNLDKYDSKYQVISNAS**C**TTNCLAPLAKVINDNFGIVEGLMTTVHST 180
P.chrysosporium   SAPSADAPMFVVGVNLDAYDSKYKVISNAS**C**TTNCLAPLAKVIHDKFGIVEGLMTTVHAT 180
S.commune         SAPSADAPMFVVGVNLDKYDSKYQVISNAS**C**TTNCLAPLAKVIHDKYGIAEGLMTTVHAT 180
L.edodes          SAPSADAPMYVCGVNLDSYDSQHAVISNAS**C**TTNCLAPLAKVIHDKFGIVEALMTTVHAT 180
V.volvacea        SAPSADAPMFVVGVNLDAYDPKYTVISNAS**C**TTNCLAPLAKVIHDEFGIVEGLMTTVHAT 180
P.sajor-caju      SAPSADAPMFVCGVNLETYDPKYKVISNAS**S**TTNCLTPLAKVIHDKFGIVEGLISTIHAT 178
                   *********:* ****: **.:: ******.****::******:*::**.*.*::*:*:*


L.polychrous      TATQRTVDGPSHKDWRGGRAVGNNIIPSSTGAAKAVGKVIPSLNGKLTGMSFRVPTIDVS 240
G.lucidum         TATQRTVDGPSMKDWRGGRAVASNIIPSSTGAAKAVGKVIPSLNGKLTGMAFRVPTIDVS 240
P.chrysosporium   TATQKTVDGPSNKDWRGGRSVGNNIIPSSTGAAKAVGKVIPSLNGKLTGLAFRVPTVDVS 240
S.commune         TATQKTVDGPSHKDWRGGRSVNNNIIPSSTGAAKAVGKVIPSLNGRLTGLAFRVPTLDVS 240
L.edodes          TATQKTVDGPSNKDWRGGRSVNGNIIPSSTGAAKAVGKVIPSLNGKLTGLAFRVPTLDVS 240
V.volvacea        TATQKTVDGPSHKDWRGGRSVANNIIPSSTGAAKAVGKVIPSLNGKLTGMSFRVPTLDVS 240
P.sajor-caju      TATQKTVDGPSHKDWRGGRSVNNNIIPSSTGAANWVGKVIPSLNGKLTGLSFRVPTLDVS 238
                   ****:****** *******:* .**********: **********:***::*****:***


L.polychrous      VVDLVVRLEKPATYDEIKDAIKAAAAGPFKGILDYTEEKVVSTDFTGNDASSIFDAEAGI 300
G.lucidum         VVDLVVRLEKEATYDEIKAAVKSAATGNLKGIIDYTEDKVVSTDFTGSDASSIFDAEAGI 300
P.chrysosporium   VVDLVVRLEKPASYDEIKQAIKEASETTHKGILGYTEEKVVSTDFTGNDNSSIFDADAGI 300
S.commune         VVDLVVRLEKEASYDEIVATVKEASEGPLKGILGFTDESVVSTDFTGANESSIFDSKAGI 300
L.edodes          VVDLVCRTEKSATYDEIKAAVKEASKGPLKGILGYTEDHVVSTDFIGDNHSSIFDATAGI 300
V.volvacea        VVDLVVRLEKPTEYSAIKAALKKAAEGEYKGIIDYTEDPVVSTDFIGNPASSIFDASAGI 300
P.sajor-caju      VVDLVVRLEKPADYKEIVAAIKEASETTHKGILGFTSDSVVSTDFVGSTDSSIFDSTAGI 298
                   ***** * ** : *. * ::* *:    ***:.:*.: ****** *   *****: ***


L.polychrous      ALNNNFVKLIAWYDNEWGYSHRVCDLVDYAAKQDGNL 337
G.lucidum         ALNKNFVKLIAWYDNEWGYSRRVCDLLVYAAKQDGAL 337
P.chrysosporium   ALNKNFVKLISWYDNEWGYSRRVCDLLAYAAKVDGAL 337
S.commune         AISKSFVKLIAWYDNEWGYSRRVCDLLVYAAKQDGAL 337
L.edodes          QLNKNFVKLIAWYDNEWGYSGRVVDLLVFAAKKDGAL 337
V.volvacea        QLNPNFVKLISWYDNEWGYSKRVCDLVVYVAGKDGNL 337
P.sajor-caju      QLNPNFVKLIAWYDNEYGYSRRVVDLLKYVAEKDGAQ 335
                   :. .*****:*****:*** ** **: :.*   **
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Figure 3. Multiple alignment of the deduced amino acid sequence of the *L. polychrous* GAPDH and GAPDH protein from *G. lucidum* (DQ404343), *P. chrysosporium* (AB272086), *S. commune* (M81724), *L. edodes* (AB012862), *V. volvacea* (DQ140384) and *P. sajor-caju* (AF087676). Amino acid residues identical and similar between each other are shown by asterisks and dots, respectively. Gaps introduced to maximize the alignment are indicated by a horizontal dash. The conserved active site cysteine residue that is important for the binding of glyceraldehydes-3-phosphate dehydrogenase in the catalytic site is shown by bold letter.

GAPDH enzyme may function as a mediator of the stress-induced metabolic responses and other integrated metabolic changes during abiotic stress. It is well established that under stress conditions, cellular adjustment requires additional energy for growth and expression is

known of several genes involved in mitochondrial ATP formation. Thus, the increased GAPDH activity mobilizes carbon into the pathway leading to glycolysis and ATP formation (Jeong et al., 2000).

It has been demonstrated in *A. bisporus* that the *GPD2*

Figure 4. An unrooted phylogenetic tree showing the relationship between GAPDH protein from *L. polychrous* and other fungi. GenBank accession numbers are indicated behind the organism name. The tree was built using the neighbor-joining method.

gene is strongly expressed in both mycelia and fruiting bodies (Hirano et al., 1999). Similar experiment was performed in this study using RNA isolated from mycelia and fruiting bodies of *L. polychrous* cultured in PD broth as described by Nariso (2004), as template for RT-PCR analysis. As shown in Figure 5b, the *GAPDH* gene was expressed in both mycelia and fruiting bodies of *L. polychrous*, suggesting that the *GAPDH* gene product is a heat shock protein involved in the developmental phase of the fungus.

A

B
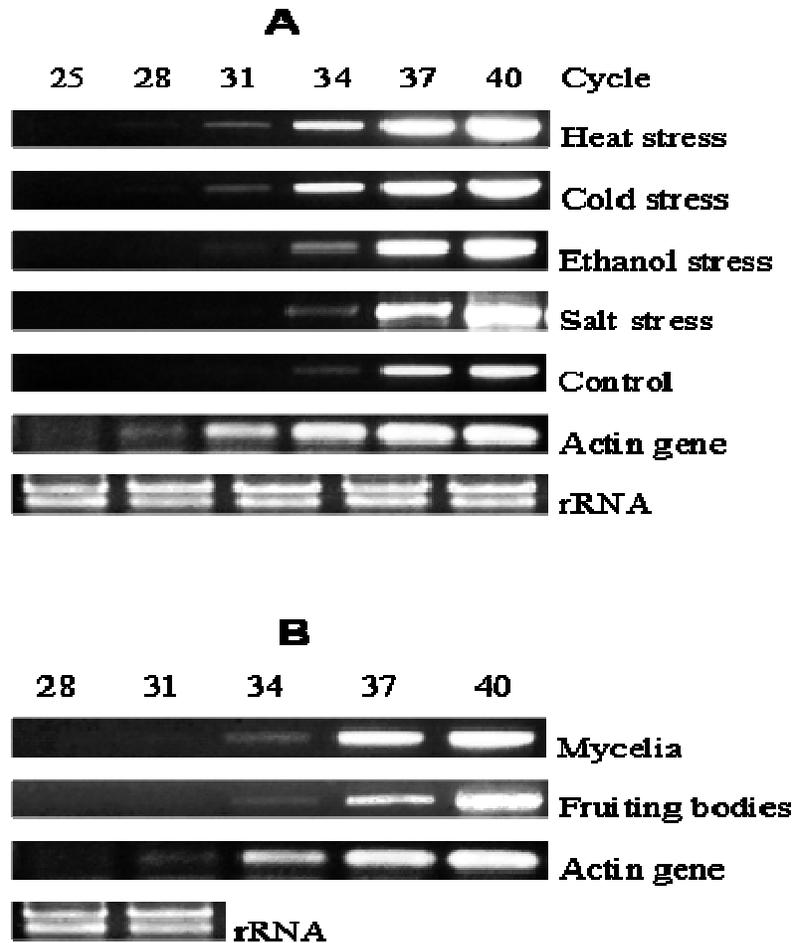
Figure 5. RT-PCR analysis of the *L. polychrous GAPDH* gene expression upon various abiotic stresses (A) and in different growth stages of the fungus (B). Total RNAs were isolated from fungal mycelia exposed to heat (45°C), cold (10°C), ethanol (6%), salt stress (1M NaCl) for 6 h and from mycelia and fruiting bodies of the fungus cultured in PD broth, and then subjected to RT-PCR analysis with primers specific for the *GAPDH* as described in materials and methods. Total RNAs isolated from fungal mycelia cultured in PD broth at 30°C was used as a control condition. Actin gene was used as an internal control for RT-PCR system. The numbers above the lanes represent the number of PCR cycles. Ethidium bromide-stained rRNA was used as a control to normalize the amount of total RNAs (10 µg).

The formation of fungal fruiting bodies is complex, and several candidate genes such as alcohol dehydrogenase, pyruvate decarboxylase, glucose-6-phosphate isomerase, citrate synthase, serine/threonine protein kinase, riboflavin aldehyde-forming enzyme gene and phosphatidylserine decarboxylase (PSD) genes have been proposed to be involved in this process, since their expressions are specifically expressed in primodia or fruiting bodies of the fungi (Hirano et al., 2004; Otto et al., 2004; Miyazaki et al., 2005). Some of them have been reported as the heat shock protein genes such as alcohol dehydrogenase and pyruvate decarboxylase (Miyazaki et al., 2005). Since the *GAPDH* gene is expressed in fruiting bodies of the *L. polychrous*, it might play specific roles in the course of

fruiting bodies development. We can not determine the biological role of this gene at the present, but the direct involvement of the *GAPDH* gene in fruiting bodies formation could be elucidated by gene disruption experiments or the introduction of a promoter which enables the over-expression of the *GAPDH* gene in this mushroom.

ACKNOWLEDGEMENTS

University) under the Project for Graduate Study and Research in Science and Technology.

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