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Isolation and Characterization of Glyceraldehyde-3-phosphate Dehydrogenase Gene of *Trichoderma virens* UKM1

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Abstract: In this study, the isolation and characterization of *Trichoderma virens* glyceraldehyde-3-phosphate dehydrogenase gene (GPD1) and its promoter is described. A cDNA clone of a partial GPD1 had been identified from an ongoing work on *T. virens* Expressed Sequence Tag (EST) analysis. This led to the isolation of a 2.9 kb *T. virens* GPD1 that encompasses the 5'-regulatory flanking region (1,364 bp), open reading frame (1,448 bp) and 3'-regulatory flanking region (31 bp) by DNA walking. Based on this sequence, a 1.017 kb cDNA fragment encompassing the Open Reading Frame (ORF) that encodes for GPD1 was subsequently isolated by reverse transcription-polymerase chain reaction. Comparison of the GPD1 and its cDNA sequences demonstrated that the complete gene sequence encodes a polypeptide chain of 338 amino acids interrupted by 2 introns. Sequence comparison analysis of the 5' non-coding region with the 5' flanking sequences of other fungal GPD genes show several regions of similar sequence. The segments from positions -68 bp relative to the start codon is potentially a transcription start site and is mapped within the pyrimidine rich region. The presumptive TATA and CAAT boxes are mapped at -363 to -358 and -109 to -105 from the initiation of translation sites, respectively. The deduced protein product is 71 to 96% identical to glyceraldehyde-3-phosphate dehydrogenases of other filamentous fungi. Phylogenetic analysis based on deduced amino acid sequences shows that GPD1 of *T. virens* forms a cluster with filamentous ascomycetes. The sequence of this gene and its promoter can be used for the development of genetic tools in molecular studies of *T. virens* and in the expression of heterologous genes.

Key words: *Trichoderma virens*, glyceraldehyde-3-phosphate dehydrogenase, promoter

INTRODUCTION

Trichoderma virens is a mycoparasitic filamentous fungus and ubiquitous in the soil. It parasitizes a wide range of phytopathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum* and *Botrytis cinerea* by forming a mycoparasitic coil around the host hyphae and secretes lytic enzymes, for example chitinases (Baek *et al.*, 1999; Chet and Chernin, 2002). The effect of coiling and secretion of cell wall degrading enzymes destroys some of the resting structures of the host and reduce their inoculum potential in soil (Harman *et al.*, 2004). Besides mycoparasitism, *T. virens* was also reported to be active against root-knot nematode (Meyer *et al.*, 2001) and

reduced weed emergence (Hutchinson, 1999). In addition, this fungus could secrete small proteins that can induce systemic resistance of plants towards pathogen through the activation of plant defense system (Djonovic *et al.*, 2006). Due to its ability to compete and destroy other fungal pathogens and subsequently protect plants against these pathogens, *T. virens* has been commercialized as a biocontrol product for plant protection (Fravel, 2005).

Glyceraldehyde-3-phosphate dehydrogenase (GPD, EC 1.2.1.12) is an essential enzyme in glycolysis and gluconeogenesis. In the glycolysis pathway, it converts glyceraldehyde-3-phosphate into bisphosphoglycerate and in the gluconeogenesis pathway, it catalyzes the

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reverse reaction. The GPD mRNA accounts for 2-5% of the poly(A)⁺ RNA present in yeasts (Holland and Holland, 1979). This suggests that the GPD is regulated by a highly active promoter. Vectors carrying the promoter region of the GPD have been reported to be very efficient in directing the expression of heterologous genes. For example, the promoter of this gene in *Pichia pastoris*, the GAP promoter, is used to regulate the expression of heterologous proteins in *P. pastoris* and is available commercially (Vassileva *et al.*, 2001; Menéndez *et al.*, 2004). Homologous and heterologous protein production using the GPD promoter was also reported in other fungal families including the Ascomycetes, *Aspergillus niger* (Halaoui *et al.*, 2006), Zygomycetes, *Mucor circinelloides* (Larsen *et al.*, 2004) and Basidiomycetes, *Lentinus edodes* (Nitta *et al.*, 2004).

The GPD is also considered a multifunctional protein in microorganisms displaying diversity of functions besides enzymatic reactions in glycolysis. For example, it functions as a transferrin-binding protein in *Staphylococcus epidermidis* (Modun and William, 1999), surface antigen in *Schistosoma mansoni* (Goudout-Crozel *et al.*, 2004) and as cell surface protein that assist the adhesion of pathogen to host tissue, as in *Paracoccidioides brasiliensis* (Barbosa *et al.*, 2006). As a housekeeping gene, the amino acid sequence showed conserved structure between various prokaryote and eukaryote organisms, allowing evolution analysis to be carried out (Johannesson and Stenlid, 2003; Douhan and Rizzo, 2005).

This study reports on the isolation and characterization of *T. virens* UKM1 GPD1 which encodes for glyceraldehyde-3-phosphate dehydrogenase and its promoter sequence. *T. virens* UKM1 used in this study was isolated from forest soil and has been investigated for its chitinase production (Abd-Aziz *et al.*, 2008a, b). The information on the GPD1 sequence can be applied towards developing genetic tools for molecular biology studies, it can be utilized as an internal control during gene expression analysis in *T. virens*. In addition, the GPD1 promoter has great potential to be developed as a strong promoter to over-express heterologous genes for functional studies or for the production of recombinant proteins in *T. virens*.

MATERIALS AND METHODS

Fungal strain and cultivation: The *T. virens* UKM1 used in this study was taken from the fungal stock culture collection, School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia. The fungus was maintained on a Potato Dextrose Agar (PDA; Difco,

France) at 30°C and subcultured monthly. Fungal cultivation was carried out by excising approximately 1 cm³ of mycelia on PDA and inoculated onto fresh PDA plate. The cultures were incubated for 6 days at 30°C. For genomic DNA isolation, fungal mycelia were grown in Potato Dextrose Broth (PDB; Difco, France) and incubated with shaking at 180 rpm, 30°C for 4 days.

Genomic DNA isolation: The genomic DNA extraction was carried out using a modified protocol first described by Pich and Schubert (1993). Fungal mycelia (2 g) were frozen in liquid nitrogen, ground with mortar and pestle and transferred into 10 mL extraction buffer (containing 500 mM NaCl, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA and 1% (v/v) freshly prepared β -mercaptoethanol). Ice-cold 20% stock solution of polyvinylpyrrolidone (PVP) was added to a final concentration of 6%. Solid SDS was then added to a final concentration of 2% (w/v). The extract was incubated in a water bath at 65°C for 10 min. Subsequently, 0.1 volume of 5 M potassium acetate was added followed by 30 min incubation on ice and centrifugation at 13,000 rpm (Sigma 3-18K, UK), 4°C for 10 min. The supernatant was transferred to a new tube and 5 μ L of RNase A (10 mg mL⁻¹) was added and incubated at 37°C for 30 min. Standard methods of DNA precipitation using isopropanol and DNA purification were carried out (Sambrook and Russell, 2001). The purified DNA was subsequently stored at -20°C until further usage.

Isolation and cloning of the *T. virens* GPD1 gene: DNA Walking SpeedUp™ Premix Kit (Seegene, Korea) was used to amplify the upstream region of a partial *T. virens* GPD1 cDNA sequence generated from a concurrent *T. virens* expressed sequence tag (EST) analysis. This technique involves the use of the *T. virens* genomic DNA as template. The kit is composed of a PCR (Polymerase Chain Reaction) Master Mix and unique DNA Walking ACP (DW-ACP) primers provided by the manufacturer. The Target-Specific Primers (TSPs) were designed and purchased from 1st BASE (Malaysia). Primers used for DNA walking procedures are shown in Table 1. In DNA Walking ACP-PCR™ Technology, one of the four ACP primers (DW-ACP1-4) and the target-specific primer (TSP) were used to amplify the target region which in this case are the upstream sequences of the partial GPD1 cDNA fragment that encompasses the 5' regulatory region. The TSPs were designed as reverse primers to amplify the 5' upstream sequences of GPD1 based on known sequences whereas DW-ACP primers were used as the corresponding forward primers targeted to anneal to unknown 5' regions of *T. virens* GPD. In each DNA

Table 1: Primers used for PCR amplification

Primer name	Primer sequence (5'→3')	Description
TSP1-A	AAGGACACGGCGGGAGTA	TSP primers for first round amplification of GPD1 5'-upstream region via DNA walking
TSP2-A	GGACCATCGGCAGCCTTCTT	
TSP3-A	CCCTCTTGATGGTCTCGGTGA	
TSP1-B	CGTTCTCGTTGACACCCATC	TSP primers for second round amplification of GPD1 5'-upstream region via DNA walking
TSP2-B	AGTCTCGCTCCACTTGATGTTG	
TSP3-B	GGGTCACGCTCGGTGTAGAAG	
TSP1-C	GCITGTGCCATTGCTGA	TSP primers for third round amplification of GPD1 5'-upstream region via DNA walking
TSP2-C	GGATTGCGTTGGGCTGA	
TSP3-C	GCGGCTCACAGCATAGGT	
TSP1-D	GATGAGTTTGCGTGGAAG	TSP primers for fourth round amplification of GPD1 5'-upstream region via DNA walking
TSP2-D	CGAACAGCAGTCGTAGCGT	
TSP3-D	GGGCTGGAGTTGATACGAG	
Fwd GPD	GGCTTGTTGATGGATTGGC	Primer pair for the amplification of GPD1 open reading frame along with its promoter region
Rev GPD	CAGCTGGGCTCATCTAATCA	

walking experiment, three PCR amplifications were carried out. The first PCR involves the TSP1 primer and one of the four ACP primers. The amplicon from this first PCR was subsequently used as template for the second PCR. The second PCR then uses the DW-ACPN primer and the TSP 2 primer (first nested PCR) to re-amplify the target from the first PCR product. The third PCR (the second nested PCR) uses the Universal and TSP 3 primers and the second PCR product as the template to re-amplify the target region. Amplicons obtained from PCR of this DNA walking strategy were gel purified using MEGAquick-spin™ Agarose Gel DNA Extraction System (Intron Biotechnology, Korea), ligated to pGEM®-T Easy vector (Promega, USA) and transformed into *Escherichia coli* DH5α. Sequencing was carried out to verify the positive transformants carrying the targeted fragments. Ultimately, the final 2.843 kb GPD1 sequence (encompassing the 5' regulatory sequences, the ORF and a partial 3' flanking regulatory region) was amplified using Expand High Fidelity PCR System (Roche, USA) by forward (Fwd GPD) and reverse primers (Rev GPD) (Table 1). The PCR amplicon was ligated into pCR® 2.1-TOPO® vector (Invitrogen, USA), transformed into TOP10 One Shot® Chemically Competent cells and sequenced.

mRNA isolation and cDNA synthesis: Total RNA from mycelium was isolated using TRIzol reagent according to manufacturer's instructions (Invitrogen, USA). cDNA was synthesized from mRNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) and 2 µL of cDNA was used in the PCR reactions. The PCR reactions (20 µL) contained 2 µL cDNA, 10x PCR Buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl), 2.5 mM MgCl₂, 0.5 mM (each) of dNTPs, 1 µM of each forward primer (5' ATGGCTCCCATCAAGGTCGG 3') and reverse primer (5' TTATTTGGAGGCATCGACCTTGG 3') and 2.5U Taq polymerase. The cDNA amplification

conditions consisted of denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 30 sec and 72°C for 1 min, followed by final elongation step at 72°C for 10 min.

DNA sequencing analysis: The plasmid of the positive clone was subjected to restriction enzyme analysis and DNA sequencing using universal M13F, M13R, T7 and SP6 primers. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA).

Bioinformatics analysis of the sequence: The nucleotide sequence obtained was analyzed using the BLAST program from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) to identify and confirm the identity of the amplified fragment with other GPD gene sequences in the GenBank. Sequence analyses were carried out with AUGUSTUS and SoftBerry software. AUGUSTUS is a gene prediction tool for eukaryotes (<http://augustus.gobics.de/>; Stanke *et al.*, 2004). FGENESH is gene structure prediction software from SoftBerry (<http://www.softberry.com/berry.phtml>). The predicted amino acid sequence of the *T. vires* GPD1 was deduced using TRANSLATE (<http://www.expasy.ch/tools/dna.html>) and was compared with GPD amino acids from other fungi using ClustalW (<http://www.ebi.ac.uk/clustalw/>; Larkin *et al.*, 2007) and BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Several software were used to predict the presence of signal peptide such as SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>; Emanuelsson *et al.*, 2007), SIG-Pred (http://www.bioinformatics.leeds.ac.uk/prot_analysis/Sig.html) and SIGFIND (<http://139.91.72.10/sigfind/sigfind.html>). The TFSEARCH program was used for predicting transcription factor binding sites (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and the molecular

weight of the predicted GPD1 was estimated using the ProtParam tool (<http://www.expasy.ch/tools/protparam.html>). A phylogenetic tree was constructed using the neighbor-joining method of PHYLIP package (version 3.67) with Jones-Taylor-Thornton (JTT) model (<http://evolution.genetics.washington.edu/phylip.html>; Felsenstein and Churchill, 1996). The statistical significance of groups in the neighbor-joining tree was assessed by the bootstrap probability with 1000 replications. Molecular Evolutionary Genetics Analysis (MEGA) software (version 3.0) was used for tree editing and display (<http://www.megasoftware.net/>; Kumar *et al.*, 2004).

RESULTS AND DISCUSSION

Gene and cDNA isolation: A partial cDNA fragment which contained polyadenylation tail encoding *T. virens* GPD1 was obtained from concurrent work on *T. virens* EST analysis (GenBank accession No. EE595314). The cDNA sequence revealed more than 80% identity to other Ascomycetes GPD genes when search on GenBank was performed (data not shown). To isolate the full-length gene encompassing its promoter, a DNA walking strategy was carried out. A total of four rounds of DNA walking was performed before the full length gene along with its promoter region was identified. Subsequently, a DNA fragment of approximately 2.9 kb containing the GPD1 open reading frame, its 5'-flanking region and 3'-flanking region was amplified, cloned and sequenced (Fig. 1a). Using this sequence information, the corresponding cDNA of 1,017 bp was synthesized from mRNA isolated from fungal mycelia (Fig. 1b). The cDNA was cloned and sequenced to completion.

Structural gene analysis: Following the alignment of the sequences of the cDNA and the gene, the 2,843 bp DNA fragment obtained represented the GPD1 promoter (1,364 bp), GPD1 open reading frame (1,448 bp) and GPD1 3'-regulatory region (31 bp) (Fig. 2). This sequence was submitted to GenBank with accession number EU573217. In addition, by comparing the cDNA sequence and the sequence of the gene, two introns of 364 bp and 67 bp were identified (Fig. 2). Both introns had the filamentous fungi canonical 5' (GTPu) and 3' (PyAG) splice sites. The cDNA of 1017 bp encodes for 338 amino acids and was comparable to other Ascomycetes such as *Hypocrea koningii* (338 amino acids, GenBank accession No. BAA03391), *Neurospora crassa* (338 amino acids, GenBank accession No. EAA27741), *Aspergillus nidulans* (336 amino acids, GenBank accession No. P20445) and *Magnaporthe grisea* (336 amino acids, GenBank accession No. AAX07728).

Computer assisted analysis of the promoter region revealed a putative CAAT (CCAAT) box at position-09 bp from the start codon (Fig. 2). The position of the CAAT box coincided with the CAAT box predicted for *Gliocladium virens* by Xu *et al.* (1996). A presumptive TATA (TATTAA) element is found at position -363 to -358 from the start codon based on the homologous alignment with other GPDs of *G. cingulata*, *A. nidulans*, *U. maydis*, *S. commune*, *P. crysosporium* and *G. virens*. A CT-rich sequence is found just after the TATA element as well as two other possible CT-rich regions and the transcription start point is deduced at position-68 from the start codon, within the CT-stretches based on this alignment shown in Table 2. In this gene, the sequence around the AUG codon (ACAAUGG) closely resembles the Kozak sequence, which is ACCAUGG in higher

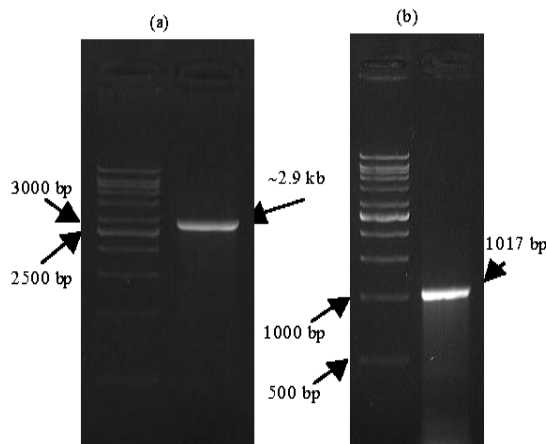


Fig. 1: (a) Amplification of GPD1 open reading frame along with 5' and 3' flanking regions (~2.9 kb) and (b) amplification of GPD1 cDNA (~1 kb). Lanes M: 1 kb DNA ladder

GGCTTGTGATGGATTTGGCCCCCTCTGGCAGTCTAACAGCCAACAAAAAATAATACGGCCGGGCAGCACCGGTGAAAC 83/-1364

GTGGGATGGGCTCAGCTCTGGCCGTGGCCATGAGCATGAGCGGATGGCGGCATCATGTCAGATGGTGTTCCTCGCTCGGG 166/-1281
RORa (-) *GATA/NIT2*

GTTCCAAGAACATCTGCCATGACCTCATATTCCTGATAGACGGCGGATGGCGAGCCCTTGCTTCTGACACGTCCCTTTTTTT 249/-1198

CTTGCCCTTCTTCTTTCCGCTCCCTTTATTGAACTCGGCCGGCGTTGTCCGGTGGAGTCGACAATTGAGGGCCGGGGCGTT 332/-1115

TGTTGGGAGGCGAATTCCTGGAAGCAAAACGCCCGCGGTTCCGTAAAAAACAGCGCAGCGCCTTCGCTAGGAGC 415/-1032

AGCTTGGCTGTGGCCCTAGCCTTACGGCGGGGATTAGACAACGGATGTGGAATCGTTGAGATCGAATGTTTGAATCTTGG 498/-949
GATA/NIT2

GAGCTGTAGTTCGAGGCTGTCCGGTGTGCTGGTTGTCAGGGCGTTGCATGATGGGAAAGGGCATGGACGGGCTGATGATACGG 581/-866
NIT2 *GATA (-)/NIT2*

GTGCCGTGGGCGATATAGGCTATCAATGCTGCGATATCAATGATGACTGCTAACCGGGTGGAAAGCTGCTTGCCTCAGTTGC 664/-783
GATA (-) / C/EBP *CREB (-) RORa* *CREB (-)*

CTTATCAACAAGCACCTCCCGGATTGTACGTCAAGGTCACGTCAAAGCTACCGCGCGTACCTGGTTGTAGCTGCCAGGTA 747/-700
ADRI (-) *ADRI*

GCGCCTCGCCCGCGCGCAAGGGCAGCAACTCGGCGATACTCGCACCCCCCAAGGCGATACCACCGGCAGCTGGAGGGCT 830/-617

ACCACACAGCTACTGCTGAGCTGTTAGTTGCTCGTTTACCTACTATGATTGCAATGGATGCCATGCCATGCCATGCCCTGTAG 913/-534
StuA *HSF*

CAGTAGCGGGGCCAGTGTGTTAGCGCTCCAGCCAGGCATTAATGTCGCTCATCCGATGCCGCATCACCGTTGTGTCAGA 996/-451
HSF (-)

AAACTATTAATCTTTCCACCTCCCTCCTGATCTCTTCCCTTTCTCTCCCTCCATACCAGCCCCAGCAAGCATCGTA 1079/-368

TCTCCCTCGTATCAACTCCAGCCAGGTTAGTATACCATTGATTCGATTGATACCACTACCCTACCCTGCCACTGCCAT 1162/-285
STRE *Sp1 (-)*

GTTGATGTTGGCGCAACGCTACGACTGCTGTTCCGCTCCCTCCCTCCTCCCTGCGCCTTGCCAGTAGCTACCCCGCCAG 1245/-202
API *STRE*

TCCGCGCTCACAATTTTTTTTTCTCCTTACTTATTTACCCCCCTCCCTCCCGCCTGAAGCAGAGACTGAAGCTTACCA 1328/-119

CGAAACTCATCAATCGCAGTCACAAATCAGCTACAATGGCTCCCATCAAGGTGCGCATCAACGGCTTCGGTTCGA 1403/-36
 M A P I K V G I N G F G R

ATTGGACGTATCGTCTTCCGCAACGCTGTTGAGCACCCCGATATCGAGGTCGTGCGCGTCAACGACCCCT 1472
 I G R I V F R N A V E H P D I E V V A V N D P

TTCATCGAGACCACCTATGCTgtgagcgcgctccccctcttttctagaattgcccctcctcagcccaa 1541
 F I E T T Y A

cgcaatccccctcggattctcacccaattgagggtgagaaagagacgataataagagttaaactgcttc 1610

aatgtcgtctttgatgccatgatattggaggttccatccatctattgttatgatggcgagaaatctttt 1679

cagcaatggcacaagctgcgaatggcattgtcgacagatggcgcataatgtctcaaaaaaaaaaaaaa 1748

aaaaaaaaaagccatcaaggtcgcaatgccaagctccctcactctcaccactaccctcacttcatgt 1817

gcagcttcattgttcatccgctaaccggagacatcgctagGCCTACATGCTCAAGTATGATTCTCCAC 1887
 A Y M L K Y D S S H

GGTCTCTTCAAGGGCGATGTTGAGGTGATGGCAAGGACCTCGTTGTGAACGGCAAGAAGGTCCGCTTC 1956
 G L F K G D V E V D G K D L V V N G K K V R F

TACACCGAGCGTGACCCTGCCAACATCAAGTGGAGCGAGACTGGCGCCGACTACATTGTGCGAGTCCACC 2025
 Y T E R D P A N I K W S E T G A D Y I V E S T

GGTGTCTTCAACCACCACCGAGAAGGCCAAGGCTCACTTGGTTGGCGGTGCCAAGAAGGTCATCATCTCT 2094
 G V F T T T E K A K A H L V G G A K K V I I S

GCCCCCTCTGCCGATGCCCCATGTACGTGATGGGTGTCAACGAGAACGACTACAACGGCTCTGCCGAT 2163
 A P S A D A P M Y V M G V N E N D Y N G S A D

GTCATCTCCAACGCTTCTTGCACCACCAACTGCCTGGCTCCCTCGCCAAGGTCATCAACGACAACCTAC 2232
 V I S N A S C T T N C L A P L A K V I N D N Y

Fig. 2: Continued

eukaryotes and AAAAUGG in glycolytic genes of *S. cerevisiae* (Kozak, 1989). The Kozak sequence plays a major role for efficient translation initiation in eukaryotes. Analysis of the partial GPD1 cDNA and terminator, polyadenylation signal sequence (AATAAA) was predicted 67 bp downstream from the stop codon followed by a 20 nucleotide poly-A tract 93 bp further downstream (not shown). The presence of the consensus AATAAA sequence and the downstream sequences are important for efficient and correct polyadenylation of the gene (Wahle and Rueggeger, 1999).

Promoter analysis: From the TFSEARCH analysis, several DNA elements in the *T. virens* GPD1 promoter sequence were identified as potential binding sites that could be relevant for the regulation of GPD1. There were multiple potential binding sites for Sp1 (Stimulating Protein 1), GATA (GATA binding protein), CREB (cAMP-response-element-binding protein), StuA transcription factor, NIT2 (nitrogen regulatory protein), ADR1 transcription factor, RORa (Retinoid related Orphan Receptor alpha), AP1 (Activator Protein 1), STRE (Stress

Response Element), HSF (Heat Shock Factor) and C/EBP (CCAAT/enhancer binding protein) (Fig. 2). This suggests that some of these DNA elements may serve as the binding site for relevant transcription factor which in turn may regulate the expression of *T. virens* GPD1. Potential binding sites for StuA transcription factor, NIT2, ADR1, heat shock elements and GATA-factor binding sites were also found within the GPD promoter sequence reported in other basidiomycetes (Kilaru and Kües, 2005). In addition, elements such as Sp1, HSF, STRE and RORa were reported to play an important role in the lipogenesis regulation of ovine GPD (Lalotitis *et al.*, 2007). Comparison in the first ~950 bp of *T. virens* GPD1 promoter to widely used GPD promoter (for gene over expression) of *A. nidulans* (M33539) and *A. niger* (X99652) showed the presence of Sp1, HSF, CREB, STRE, StuA, ADR1 and NIT2. Based on this analysis, it is possible that these motifs may be important to regulate the expression of GPD in these filamentous fungi.

Amino acid analysis: Sequence analysis using BLAST programme confirmed the identity of this gene. The

<i>T. virens</i>	1	M A P I K V G I N G F G R I G R I V F R N A V E H P D I E V V A V N D P F I E T T Y A A Y M L K Y D S S H G L F K G D V
<i>H. koningii</i>	1	M A P I K V G I N G F G R I G R I V F R N A V E H P D I E V V A V N D P F I E T T Y A A Y M L K Y D S S H G L F K G E V
<i>H. jecorina</i>	1	M A P I K V G I N G F G R I G R I V F R N A V E H P E I E V V A V N D P F I E T S Y A A Y M L K Y D S S H G L F K G D V
<i>G. zeae</i>	1	M A P I K V G I N G F G R I G R I V F R N A V E H P D I E V V A V N D P F I E P H Y A V Y M L K Y D S S H G T F K G E V
<i>H. lixii</i>	1	- M S I K V G I N G F G R I G R I L L S N A L E K P E T S V V A V N D P F I E P T Y A A Y M L K Y D S S H G L F K G D I
<i>T. virens</i>	61	E V D G K D L V V N G K K V R F Y T E R D P A N I K W S E T G A D Y I V E S T G V F T T T E K A K A H L V G G A K K V I
<i>H. koningii</i>	61	E V D G K D L V V N G K K V R F Y T E R P A D I K W S E T G A E Y I V E S T G V F T T T E K A K A H L V G G A K K V I
<i>H. jecorina</i>	61	T V D G K D L V V N G K K V R F Y T E R D P A N I K W S E T G A E Y I V E S T G V F T T T E K A K A H L G G A K K V I
<i>G. zeae</i>	61	G H Q G N D L V V N G K T I R F Y S E R D P A N I K W S E T G A D Y I V E S T G V F T T T D K A K A H L G G A K K V I
<i>H. lixii</i>	60	E V D G Q N L V V N G K P I R F Y S E R D P A N I K W S E T G A E Y I V E S T G V F T T T D K A K A H L G G A K K V I
<i>T. virens</i>	121	I S A P S A D A P M Y V M G V N E N D Y N G S A D V I S N A S C T T N C L A P L A K V I N D N Y G I V E G L M T T V H S
<i>H. koningii</i>	121	I S A P S A D A P M Y V M G V N E S D Y D G S A D V I S N A S C T T N C L A P L A K V I N D N Y G I V E G L M T T V H S
<i>H. jecorina</i>	121	I S A P S A D A P M Y V M G V N E K D Y D G S A D V I S N A S C T T N C L A P L A K V I E D N Y G I V E G L M T T V H S
<i>G. zeae</i>	121	I S A P S A D A P M Y V V G V N E N K Y D G S A D I S N A S C T T N C L A P L A K V I N D K R G I V E G L M T T V H S
<i>H. lixii</i>	120	I S A P S A D A P M V M G V N H K D Y D G P T V I S N A S C T T N G L A P L V K I V N D N E G I V E G L M T T V H S
<i>T. virens</i>	181	Y T A T Q K T V D G P S A K D W R G G R G A A Q N I I P S S T G A A K A V G K V I P E L N G K L T G M S I R V P T A N V
<i>H. koningii</i>	181	Y T A T Q K T V D G P S A K D W R G G R G A A Q N I I P S S T G A A K A V G K V I P E L N G K L T G M S I R V P T A N V
<i>H. jecorina</i>	181	Y T A T Q K T V D G P S A K D W R G G R G A A Q N I I P S S T G A A K A V G K V I P E L N G K L T G M S I R V P T A N V
<i>G. zeae</i>	181	Y T A T Q K T V D G P S S K D W R G G R G A A Q N I I P S S T G A A K A V G K V I P E L N G K L T G M S R V P T A N V
<i>H. lixii</i>	180	Y T A T Q K T V D G P S S K D W R G G R G A A Q N I I P S S T G A A K A V G K V I P A M N G K T G M S R V P S A N V
<i>T. virens</i>	241	S V V D L T V R L E K G A Y A E I T E T I K K A A D G P L K G V L A Y T D D V V S S D M L G N T N S S I F D I K A G
<i>H. koningii</i>	241	S V V D L T V R L E K G A S Y E E I T E T I K K A A D G P L K G V L A Y T G D D V V S S D M L G N T N S S I F D I K A G
<i>H. jecorina</i>	241	S V V D L T V R L E K G A S Y D E I T E T I K K A A D G P L K G I L A Y T D D V V S S D M L G N T N S S I F D I E A G
<i>G. zeae</i>	241	S V V D L T V R L E K G A S Y D Q H K Q V I K E A S E G D L K G V L A Y T E D D V V S S D L N G N T N S S I F D I K A G
<i>H. lixii</i>	240	S V I D L T V R L E K A A S Y E E I T S A I K K A A D G E L K G I H A Y T S D E V V S S D M L G N M N S S I F D I K A G
<i>T. virens</i>	301	I S L N E N F V K L V S W Y D N E W G Y S R R V L D L L A H V A K V D A S K
<i>H. koningii</i>	301	I S L N E N F V K L V S W Y D N E W G Y S R R V L D L L A H V A K V D A S K
<i>H. jecorina</i>	301	I S L N E N F V K L S W Y D N E W G Y S R R V L D L L A H V A K V D A S K
<i>G. zeae</i>	301	I S L N E N F V K L V S W Y D N E W G Y S R R V L D L L A H V A K V D A S K
<i>H. lixii</i>	300	I S L N E N F V K L V S W Y D N E W G Y S R R V L D L L E H V A K V D A S K

Fig. 3: Multiple alignment of GPD amino acid sequence among *T. virens*, *H. koningii* (BAA03391), *H. jecorina* (ABK33667), *G. zeae* (XP_386433) and *H. lixii* (CAA73141). The alignment was constructed using the ClustalW (<http://www.ebi.ac.uk/clustalw/>) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) software. The active site cysteine residue is shown in asterisk

GPD sequences (100%). Filamentous ascomycetes and basidiomycetes GPDs form monophyletic cluster with the bootstrap value of 99 and 94% simultaneously. The GPD1 of *T. virens* clusters with other filamentous ascomycetes GPD sequences such as *H. koningii*, *H. jecorina* and *H. livii*. The close relationship between filamentous ascomycetes and basidiomycetes GPDs 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). Studies by Harmsen *et al.* (1992) also suggested that the relationship between GPDs of filamentous ascomycetes and basidiomycetes is higher and quite distinct from ascomycetes yeasts.

CONCLUSION

The *T. virens* UKM1 glyceraldehyde-3-phosphate dehydrogenase gene (GPD1) of 2.9 kb was amplified and cloned using DNA walking technique. This GPD1 was deduced to contain an ORF of 1,448 bp, interrupted by two introns and encodes for 338 amino acids. The phylogenetic tree analysis showed relatively close relationship between *T. virens* GPD1 with GPD of other *Trichoderma* species. A total of 1,364 bp of GPD1 promoter sequence was also cloned and analyzed. The promoter has potential for gene overexpression in *T. virens* and will be used in the construction of expression vectors for the homologous and heterologous protein expression in this fungus.

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