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Isolation and Sequence Analysis of *GpdII* Promoter of the White Button Mushroom (*Agaricus bisporus)* from Strains Holland737 and IM008

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Abstract
Many recent studies have shown that glycosylation patterns of *Agaricus bisporus* are similar to those of mammalians, so that this organism is a good candidate for the expression of glycosylated pharmaceutical protein. To achieve constant interested gene expression in all cells of the organism, proper promoter isolation is necessary. To isolate this promoter, PCR with specific primers was performed on extracted DNA of the white button mushroom strains Holland737 and IM008. The PCR amplified 290 bp fragments of gpdII promoters. IM008 gpdII promoter was used to construct pCAMBIAH8 plasmid. Comparison of isolated promoters among sequence records at NCBI demonstrated high similarity between IM008 gpdII promoter and previously reported gpdII promoter. Sequence analysis of isolated promoters revealed several point mutations on this promoter. TACAAA promoter sequence in −65 site acts as TATA box. Among the three CAAT candidate sequences, one is functional, which is located at position −108. Transformation of the white button mushroom with constructed pCAMBIAH8 plasmid was successfully performed.

Keywords: CAAT box, pharmaceutical protein, TATA box, transgenic mushroom.

Abbreviations: gpdII, glyceraldehyde-3-phosphate dehydrogenase II; **hph,** *hygromycine phospho transferase*; **PCR,** polymerase chain reaction; **CTAB,** cetyltrimethylammonium bromide; **TBP,** TATA-binding protein**; PIC,** preinitiation complex; **IPTG**, isopropyl β-D-1-thiogalactopyranoside.

Introduction

The glyceraldehyde-3-phosphate dehydrogenase (GPD, E.C.1.2.1.12) is one of the key enzymes in the glycolysis pathway. Because of its critical role in every living cell, its expression in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and other eukaryotic organisms is high, representing up to 5% of the soluble cellular proteins (Piechaczyk *et al.,* 1984; Punt *et al.,* 1990; Harmsen *et al.,* 1992). The *gpd* gene is regulated by a constitutive and active promoter, and this was proved by expressing heterologous genes in *S. cerevisiae* (Bitter *et al.,* 1984), *Pichia pastoris* (Döring *et al.,* 1998), *Lentinula edodes* (Hirano *et al.,* 2000), *Mucor circinelloides* (Wolff *et al.,* 2002), *Flammulin avelutipes* (Kuo *et al.,* 2004), and *A. nidulans* (Upshall *et al.*, 1987) by their native *gpd* gene promoters. Two

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studies by Chen and coworkers (2000) and Burns and coworkers (2006) revealed that the best promoter for the transformation of *Agaricus bisporus* is homologous gpdII promoter.

In recent years, researches have shown that some mushrooms have medicinal properties, and it is believed the white button mushroom may produce several compounds with medicinal properties (Burns *et al.,* 2005). There is also a special interest in using *A. bisporus* because its glycosylation is similar to that of mammalian cells as biological factors for producing recombinant proteins (Velcko *et al.,* 2004, Zhang *et al.,* 2004). Although this organism has great importance in biotechnological advances, limited reports about production of recombinant proteins in this organism is available (Sonnenberg *et al.,* 2000). In recent years, construction of synthetic promoter for a higher, better, and stable expression of heterologous genes has been performed. To synthesize a promoter with the abovementioned properties, it is important to investigate transcription binding sites in promoter sequence.

The core promoter is the minimal stretch of contiguous DNA sequence that is sufficient to direct the accurate initiation of transcription by the RNA polymerase II machinery (Butler *et al.,* 2002). It plays a critical role in the regulation of transcription. The best-characterized core promoter element is the TATA box, which is recognized by the TBP subunit of TFIID and nucleates transcription PIC formation. However, many promoters do not contain a TATA box, and thus, alternative mechanisms are used to initiate PIC formation (Deng *et al.,* 2005). The development of transfection and *in vitro* transcription assays made it possible to demonstrate that mutations in the TATA box usually reduce or abolish the activity of cellular and viral promoters. Recent database analyses of *Drosophila* genes revealed that the TATAAA consensus sequence or a sequence with one mismatch from the consensus was present in 43% of 205 core promoters or, in another study, in 33% of 1941 potential promoters (Smale *et al.,* 2003). The CAAT sequence is one of the most common *cis* elements present in the promoter regions of numerous eukaryotic genes. A statistical analysis of over 500 promoters revealed that the CCAAT sequence is an element in approximately 30% of eukaryotic promoters (Kato, 2005).

Materials and Methods

The white button mushroom strains Holland737 and IM008 were obtained from the Department of Industrial Fungi Research Center of Jahad-e-Daneshgahi, Mashhad, and cultured in liquid CYM-CE medium (Molloy, 2004) at 25°C and incubated in dark for 15 days.

Escherichia coli strain DH5α was used as host to keep plasmids containing gpdII promoter and *Agrobacterium tumefaciens* strain LBA4404 was used as gene transformer into *A. bisporus*.

DNA extraction

Genomic DNA of *A. bisporus* was extracted by modified CTAB method (Nazrul *et al.,* 2010). Bacterial plasmid was isolated by miniprep plasmid extraction kit according to the manufacturer's instructions (Fermentas).

Promoter isolation

Based on *A. bisporus* gpdII promoter sequence (NCBI gene id 68160332), PCR technique was used to isolate upstream sequence of ATG in IM008 and Holland737 strains. First primer set, F1: 5′-GAAGAAGAATTCAGAGGTCCGC -3′ and R1: 5′-AGACAAACCATGGCGATAAGC - 3′ was used to amplify promoter region in the *A. bisporus gpdII* gene and then cloned into pTZ57R plasmid. Second primer set, F2: 5′-CTGCCACCATGTTGGTAATTCAGAGGTCCGC -3′ and R2: 5′-ATAGCACCTGCAGCGATAAG -3′ was

used to amplify cloned gpdII promoters. Underlined primer nucleotides represent *BstX*I and *Pst*I restriction enzyme sites. PCR was performed for interested promoter amplification by both primer sets by the following thermal program: a) one cycle for first denaturing at 95°C for 5 min, b) 30 cycles, denaturing at 94°C for 50 sec, annealing at 52°C for 45 sec, and extension at 72°C for 60 sec, and c) final extension step at 72°C for 5 min.

Hph selection marker gene isolation from pCAMBIA1304 plasmid

pCAMBIA1304 selection marker gene, *hph*, was isolated with specific primer set, Fh: 5′-CACATCTCGAGTCGGCATCTA-3′ and Rh: 5′-GCACTGCAGATGAAAAAGCC-3′, containing *Xho*I and *Pst*I restriction sites, which are underlined. This following thermal program was used to amplify *hph* gene: a) one cycle for first denaturing at 95°C for 5 min, b) 30 cycles, denaturing at 94°C for 50 sec and annealing at 53°C for 50 sec and at 72°C for 90 sec, and c) final extension at 72°C for 5 min.

Each reaction (25 µl) consisted of 50 ng of DNA template, 1× incubation buffer, 1 mM $MgCl_2$, 200 µM each dNTP (Fermentas), 4 pmol of each primer (metabion), and 1 unit mixed Taq and pfu DNA polymerase (4:1) (Vivantis technology). PCR products were resolved in 1% (w/v) agarose gel prepared with 1× TAE buffer (Sambrook *et al*., 2001).

Construction of cloning and expression vectors

PCR products of first primer set were cloned into pTZ57R T/A cloning vector according to the manufacturer's instructions (Fermentas). To construct pBlue8 and pBlue7 cloning vectors, pBluescript SK+ containing ampicillin resistance gene as a bacterial selection marker was used. First, PCR products obtained from second primer set and pBluescript SK+ were digested by *Bstx*I and *Pst*I, then desired fragments were isolated on 1% agarose gel, and finally ligation reaction was performed. Construct pBlueH8 was created by insertion of the *hph* gene into the pBlue8. To construct pCAMBIA1304-based expression vector, "pCAMBIAH8," original *hph* gene and its promoter (35S) of pCAMBIA1304 were removed and H8 fragment of pBlueH8 was inserted into pCAMBIA1304 by *BstX*I and *Xho*I. This construct consisted of Kanamycine resistance and *hph* genes as selection markers in *E. coli* and *A. bisporus*, respectively. All ligation reactions were performed with molar ratio of 3:1 according to the manufacturer's instructions (Fermentas).

Bacterial transformation and selection

Heat-shock method was used to transform *E. coli* DH5α and *A. tumefaciens* strain LBA4404 (Sambrook *et al*., 2001). LB agar medium with 100 $mg \cdot L^{-1}$ ampicillin and 50 $mg \cdot L^{-1}$ kanamycin was used as a selection medium to select transformed bacteria with pTZ57R, pBluescript SK+ derivatives, and pCAMBIA1304 derivatives, respectively. This selection medium also contains IPTG, Xgal. Transformed bacteria were incubated on selection medium at 37°C for 12 h.

Some of the white colonies belonging to transformed bacteria with manipulated pTZ57R, pBlue8, and pBlue7 constructs containing gpdII promoter were opted as candidate, and then colony PCRs were performed with first primer set for pTZ57R and second primer set for pBlue8 and pBlue7. Some of white bacterial colonies transformed with pBlueH8 construct were chosen for colony PCR with primers Fh and F2. The bacterial plasmids were extracted with miniprep plasmid extraction kit (Fermentas). Double digest reaction with *Bstx*I and *Xho*I was performed to prove H8 fragment insertion into pCAMBIAH8 construct.

Sequencing and phylogeny analysis

Cloned gpdII promoters into the pTZ57R were sequenced by Bioneer Company (Korea) with M13 (−20) primer. Sequence and phylogeny analyses were performed with Mega 5.05 software. The maximum likelihood statistical method and Tamura-Nei model were used for phylogeny analysis (bootstrap = 1000). Sequences were aligned with ClustalW by Bioedit software. All motifs were obtained from other studies (Harmsen *et al*., 1992; Kilaru *et al*., 2005) and manually searched.

Transformation of A. bisporus strain Holland737 with plasmid pCAMBIAH8

Transformation was performed with pCAMBIAH8 plasmid containing gpdII promoter of *A. bisporus* strain IM008 for expression of the *hph* selection marker gene as indicated by Romaine and Schlagnhaufer protocol (Romaine *et al*., 2007). Obtained colony transformation was proved by PCR with *hph* gene primer set on genomic DNA of colonies as a template.

Results

Promoter isolation

PCR with first primer set on both strain genomic DNA, as expected, amplified 290 bp fragments (Fig. 1A). Also, white bacterial colony PCR product lengths with first primer set were 290 bp fragments demonstrating successful transformation of manipulated pTZ57R with gpdII promoter (Fig. 1B). PCR with second primer set on desired white bacterial colonies, transformed with pBlue8 and pBlue7 as templates, amplified the 290 bp fragments (Fig. 2).

Isolation of hph selection marker gene of plasmid pCAMBIA1304

PCR with specific *hph* gene primer set on pCAMBIA1304 as template amplified 1049 bp fragments as expected (Fig. 2).

Construction of cloning and expression vectors

PCR on candidate white bacterial colonies, harboring the pBlueH8, amplified 1330 bp fragments and confirms bacterial transformation with pBlueH8 (Fig. 3). Double digestion by *Bstx*I and *Xho*I on pCAMBIAH8 released two fragments of size 1325 and 10481 as expected and proved insertion of H8 into pCAMBIA1304 (Fig. 3).

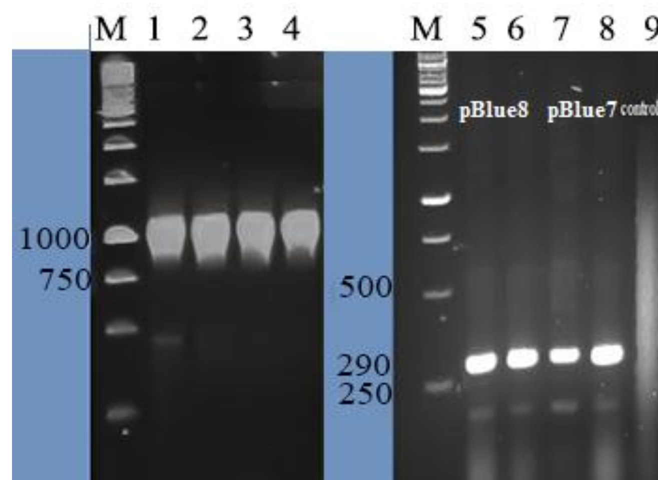

Fig.1. A. PCR product of the white button mushroom extracted genomic DNA. (M) Size marker 100 bp plus (Fermentas) (1, 2) Experimental repeats of PCR product on extracted DNA of IM008. (3, 4) Experimental repeats of PCR product on Holland737 genomic DNA. (5) Negative control (enzyme + enzyme buffers + primers + water). B. Colony PCR on candidate white bacteria harboring manipulated pTZ57R plasmid that contains gpdII promoter. (M) Size marker 1 kb (Fermentas). (1, 2, 3, 4) Experimental repeats. (5) Negative control (enzyme + enzyme buffers + primers + water). (6) Control (enzyme + enzyme buffers + primers + blue colony of bacteria).

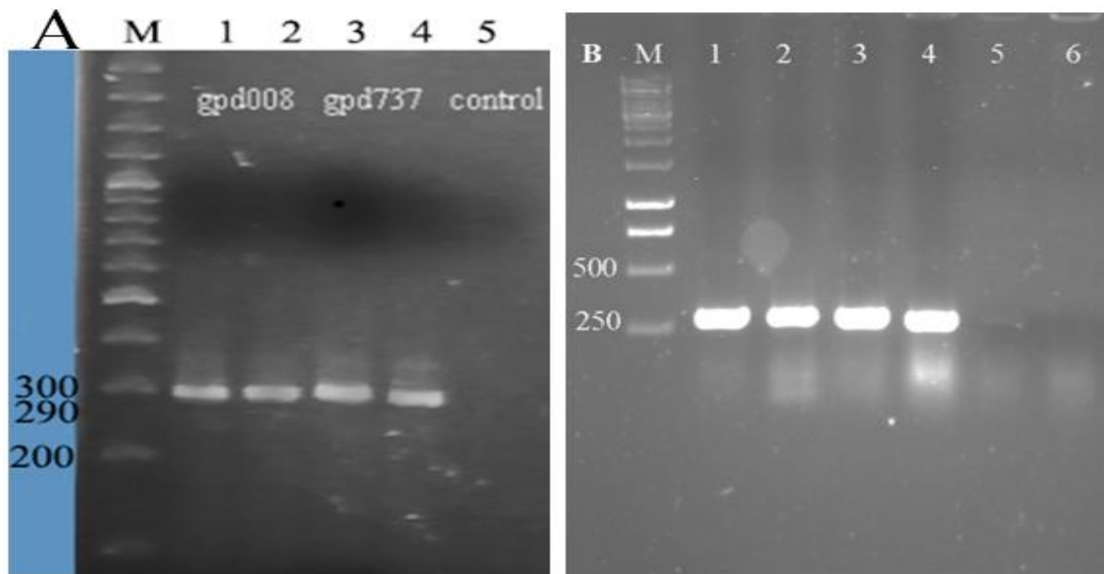

Fig. 2. PCR products of *hph* and gpdII of IM008. M: Size marker 1 kb. (1, 2, 3, 4): Repeats of amplified *hph* gene by specific primer set. (5, 6, 7, 8): Amplification of IM008 gpdII promoter by second primer set on candidate white bacterial colonies as template. (9): Negative control.

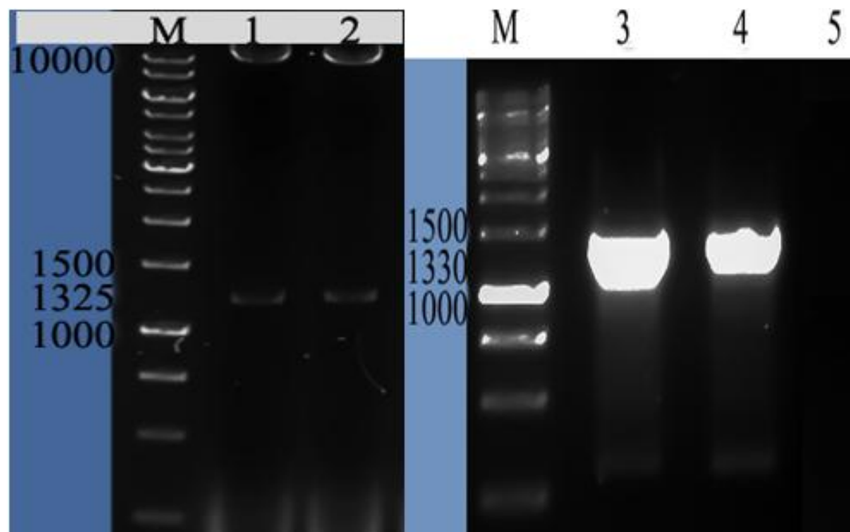

Fig. 3. Double digestion of pCAMBIAH8 and PCR products of pBlueH8. M: Size marker 1 Kb. (1, 2): Digested fragments of pCAMBIAH8 by *Xho*I and *BstX*I. (3, 4): Amplification of H8 by Fh and F2 primers on candidate white bacterial colonies as template. (5): Negative control.

Sequencing and phylogeny analysis

Sequencing results demonstrate that *gpdII* gene promoter region is isolated correctly. Sequence analysis revealed that there is a small difference between housekeeping gpdII promoters isolated from *A. bisporus* strains IM008 and Holland737. Phylogeny analysis revealed that recorded gpdII promoter at NCBI and IM008 has more similarity than recorded gpdII promoter at NCBI and Holland737, and gpdII promoter of *Coprinus cinereus* was used as an out-group (Fig. 4). Alignment of recorded gpdII promoter at NCBI with isolated promoters indicated that Holland737 gpdII promoter has 3 point mutations at

nucleotide −146, −213, and −227 sites and IM008 gpdII promoter has one point mutation at nucleotide −158 site. This alignment also demonstrated variation only at 5′ end of the gpdII promoter and no variation identified on the 3′ end. Restriction site analysis revealed that point mutation at −213 site created recognition site for *Xho*I at Holland737 gpdII promoter (Fig. 5).

According to other studies, we found two sequences as candidate for TATA box at −15 and −65 sites and three sequences as candidate for CAAT box at −58, −108, and −222 sites (Fig. 5).

Transformation of A. bisporus strain Holland737 with pCAMBIAH8 construct

We successfully transformed *A. bisporus* by the use of pCAMBIAH8 construct, which uses gpdII promoter of *A. bisporus* strain IM008 for the expression of *hph* selection marker gene. PCR with specific primers of *hph* gene amplified 1049 bp fragments that confirm the presence of H8 fragment and transgenic mushrooms (Fig. 6).

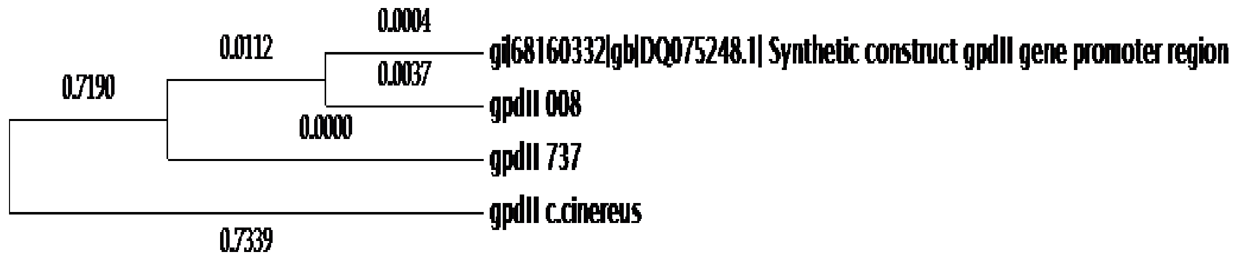

Fig. 4. Phylogeny analysis of the isolated gpdII promoter with sequence recorded at NCBI and gpdII *Coprinus cinereus*

Fig. 5. Alignment of *A. bisporus* strains IM008 and Holland737 gpdII promoter with sequence recorded at NCBI and *C. cinereus* gpdII promoter. Green shadows: candidate TATA box. Purple shadows: candidate CAAT box. Red arrows: point mutations detected in Holland737 gpdII promoter. Blue arrow: point mutation detected in IM008 gpdII promoter.

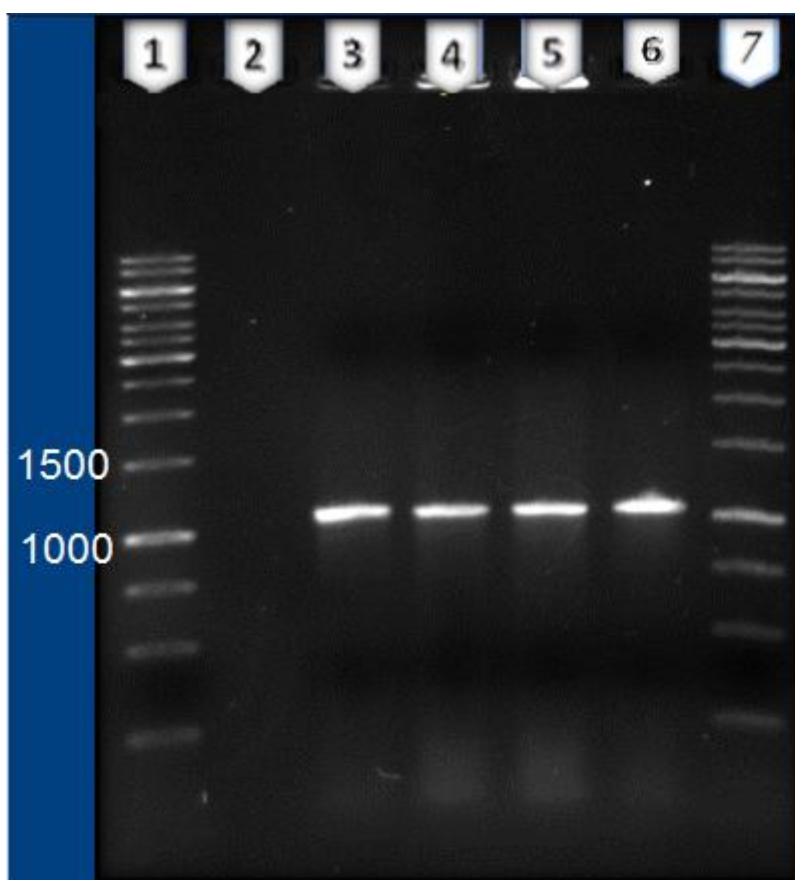

Fig. 6. Verification of the transformed mushrooms by PCR with hph primer set. (1 and 7): Size marker 1 kb. (2): Untransformed mushroom strain Holland737. (3): pCAMBIAH8 construct. (4, 5, 6): Transformed Holland737 clones.

Discussion

gpdII Promoter of both strains was isolated and successfully sequenced, although construction of pBlueH7 and pCAMBIAH7 was not successful because Holland737 gpdII promoter contains a restriction site for *Xho*I, which is the result of point mutation at −213 site. Two possible TATA box sequences are present on gpdII promoter, and according to sequence analysis conducted by Kilaru and coworkers (2005), it was shown that instead of TATA box TATA-like box exists in basidiomycetes' promoter (Kilaru *et al*., 2005). In other eukaryotes, TATA box is located between −25 and −30 sites of transcription initiation site, but in yeast, it is located between −40 and −120 (Esser *et al*., 2004). The TACACACA is located between −15 and −23 sites that match neither with eukaryotic TATA box site nor with yeast TATA box site. The TACAAAAA is located at −65 site of gpdII promoter that matches with both the locations of TATA box and has only one mismatch in comparison with TATA box consensus sequence. As mentioned earlier, we can consider that this sequence acts as a TATA box. This result is similar to the results of Harmsen and coworkers (1992) and Kilaru and coworkers (2005). Our analysis showed that this motif is conserved among gpdII promoters of *A. bisporus* strains.

Three CAAT sequences were detected at positions −58, −108, and −222 nucleotides, but the eukaryotic consensus CAAT box motif, which is CCAAT, will be found only at position −42 of gpdII promoter. In other eukaryotic promoters,

this motif is located at upstream (−80) of the initial transcription site, before the TATA box, whereas in *A. bisporus*, this motif is located after TATA-like box sequence at position −58. As this candidate CAAT box is located after TATA-like box, it cannot be considered as CAAT box. However, Harmsen and coworkers (1992) and Kilaru and coworkers (2005) analyzed −184 upstream of gpdII promoter and showed that CAAT sequence at position −108 is the active CAAT box of gpdII promoter, which like other eukaryotic promoters is located before TATA-like box. Based on these observations, gpdII promoter CAAT box of *A. bisporus*, similar to TATA-like box, does not follow the consensus sequence of CAAT box. Third sequence that was the candidate as CAAT box is located at position −222. As analyzed by Kilaru and coworkers, we found that CAAT box in other basidiomycetes' promoters is located between the positions −93 and −203 in *Phanerochaete chrysosporium* and *L. edodes*, respectively, so that it cannot be considered as CAAT box.

The nucleotides located exactly before ATG have a major effect on binding of transcription factors to promoter (Burns *et al.*, 2005). Sequencing and sequence analysis of isolated promoters showed that differences are on the 5′ end of the isolated promoter and any difference detected on promoter 3′ end. As Burns and coworkers results since TATA-like box located at the 3′ end of the promoter that has major effect on detection of promoter region, binding of transcription factor elements for transcription initiation, the 3′ end region is conserved.

We used 271 bp of *A. bisporus* gpdII promoter fragment for the expression of *hph* gene, and as Kilaru and Kues revealed, only 265 and 277 bp in length of gpdII promoter are sufficient to be functional in *A. bisporus* and *C. cinerea* (Kilaru *et al.*, 2005). After *A. bisporus* transformation with pCAMBIAH8, 3 colonies were achieved (data not shown), which showed that 271 bp fragment of gpdII promoter is sufficient for gene expression in *A. bisporus*, and this observation confirmed that important parts of gpdII promoter are located at the 3′ region. This finding is similar to that observed by Kilaru and Kues (2005).

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References

1. 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 32:263–74.

2. Burns, C., K. E. Gregory, M. Kirby, M.K. Cheung, M. Riquelme, T.J. Elliott, M. P. Challen, A. Bailey, and G.D. Foster. 2005. Efficient GFP Expression in the Mushrooms *Agaricus Bisporus* and *Coprinus Cinereus* Requires Introns. Mol. Biotechnol. 42:191–99.

3. Butler, J.E.F, and J.T. Kadonaga. 2002. The RNA Polymerase II Core Promoter: A Key Component in the Regulation of Gene Expression. Gene. Dev. 16:2583–2592.

4. 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. Microb. 66:4510–13.

5. Deng, W., and S.G.E. Roberts. 2005. A Core Promoter Element Downstream of the TATA Box That Is Recognized by TFIIB. Gene. Dev. 19:2418–2423 .

6. Döring, F., M. Klapper, S. Theis, and H. Daniel. 1998. Use of the Glyceraldehyde-3-Phosphate Dehydrogenase Promoter for Production of Functional Mammalian Membrane Transport Proteins in the Yeast *Pichia Pastoris*. Biochem. Biophys. Res. Commun. 250:531–35.

7. Harmsen, M.C., F.H.J Schuren, S.M. Moukha, C.M. van Zuilen, P.J. Punt, and J.G.H. 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.

8. 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. M. G. G. 263:1047–52.

9. Kato, M. 2005. An Overview of the CCAAT-Box Binding Factor in Filamentous Fungi: Assembly, Nuclear Translocation, and Transcriptional Enhancement. Biosci. Biotechnol. Biochem. 69:663–72.

10. Kilaru, S., and U. Kues. 2005. Comparison of *gpd* Genes and Their Protein Products in Basidiomycetes. Fungal. Genet. Newsl. 52:1-23.

11. 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. Biot. 65:593–599.

12. Molloy, S. 2004. Sugar Transport and Water Relations of *Agaricus bisporus*. PhD Diss. Univ. of Bedford, Cranfield, Institute of Bioscience and Technology.

13. Nazrul, M.I., and B.Y. Bing. 2010. ISSR as New Markers for Identification of Homokaryotic Protoclones of *Agaricus bisporus*. Curr. Microbiol. 60:92–98.

14. Piechaczyk, M., J. M. Blanchard, L. Marty, C. Dani, F. Panabieres, S. E 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.

15. Prize, B. and C. Lang. 2004. Genetics and Biotechnology, p. 130. U. Kück (eds.) Gene Regulation in Yeast. Springer Science & Business Media, Berlin.

16. Punt, P.J., M.A. Dingemanse, A. Kuyvenhoven, R.D.M. Soede, P.H. Pouwels, and C.A.M.J.J. 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.

17. Romaine, C. P., and C. Schlagnhaufer. 2007. Mushroom (*Agaricus bisporus*). Methods. Mol. Biol. In *Agrobacterium* Protocols. 2:453–63 .

18. Sambrook, J., and D.W. Russell. (ed.) 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2344 p.

19. Smale, S.T., and J.T. Kadonaga. 2003. The RNA Polymerase II Core Promoter. Annu. Rev. Biochem. 72:449–79.

20. Sonnenberg, A. S. M., and L.J.L.D. van Griensven. 2000. Genetics and Breeding of *Agaricus bisporus*. In Science and Cultivation of Edible Fungi. Congr. on the Science and Cultivation of Edible Fungi, May15-19, 2000. pp. 25–39 .

21. Upshall, A., A.A. Kumar, M.C. Bailey, M.D. Parker, M.A. Favreau, K.P. Lewison, M.L. Joseph, J.M. Maraganore, and G.L. McKnight. 1987. Secretion of Active Human Tissue Plasminogen Activator from the Filamentous Fungus *Aspergillus nidulans*. Nat. Biotechnol. 5:1301–1304.

22. Velcko, A.J., R.W. Kerrigan, L.A. MacDonald, M.P. Wach, C. Schlagnhaufer, and C.P. Romaine. 2004. Expression of Novel Genes in *Agaricus bisporus* Using an Agrobacterium-Mediated Transformation Technique. I.S.M.S. 16:591–598.

23. 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.

24. Zhang, C., V. Odon, H.K. Kim, M.P. Challen, K.S. Burton, D. Hartley, and T.J. Elliott. 2004. Mushrooms for Molecular Pharming. I.S.M.S. 16:611–618.