EXPRESSION OF GENES FOR THE GLUCOAMYLASES (GLYCOSIDE HYDROLASE FAMILY 15, GH15) IN EDIBLE MUSHROOMS

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ABSTRACT

The glycoside hydrolase family 15 (glucoamylases) comprises exohydrolases that catalyze the release of β-glucose units from the non-reducing ends of polymersaccharides. A glucoamylase gene (*PnGlu1*) from *Pholiota nameko* was cloned and characterized. The 1743 bp coding region of *PnGlu1* encoded a 581-amino acid polypeptide with a signal peptide comprising 17 amino acids at the N-terminal end. In addition to showing high homology with the glucoamylase from *Laccaria bicolor*, the *PnGlu1* gene encodes a protein with an N-terminal catalytic domain and a C-terminal starch-binding domain. Quantitative reverse transcription-PCR (qRT-PCR) was used to examine the role of the glucoamylase gene in the growth of mycelia when the fungus was cultured in minimal medium containing glucose or soluble starch, as well as the growth of mycelia and tissue development in fruit bodies at different stages. The findings suggested that expression of *PnGlu1* in the dikaryon strain was higher than observed in the monokaryon strain, with *PnGlu1* expression induced by soluble starch. However, during cultivation on sawdust medium, the expression of *PnGlu1* decreased drastically in dikaryotic mycelia after mycelial stimulation (*kinkaki*). Conversely, although *PnGlu1* expression was not observed during fruit body development, the glucoamylase enzyme activity was stable and the glucose content increased dramatically. These results suggested that at least two glucoamylases are produced by *P. nameko*; the one cloned in this study, which only catalyzed the conversion of soluble starch into glucose and which, in turn, increased mycelial growth, and another glucoamylase that may be involved in fruit body formation.

Keywords: Fruit body formation; Glucoamylase; Quantitative reverse transcription-PCR; Southern hybridization

INTRODUCTION

In saprophytic fungi, such as *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo and *Lentinus edodes* (Berk.) Singer, three glycoside hydrolase families have been found related with the degradation of cellulose. The GH6 and GH7 families are specially for degradation of cellulose, and the ability of GH 15 (glucoamylase) to cleave terminal α-1, 4 glucosidic bonds and α-1, 6 branch bonds is important for the complete degradation of starch to glucose, and thus, for

the provision of a simple, soluble carbon source for nutrition [1-3]. However, in mycorrhizal fungi, such as *Laccaria bicolor* (Maire) P.D. Orton and *Tricholoma matsutake* (S. Ito & S. Imai) Singer, only the GH15 family has been identified [4, 5]. The observation that GH15 is common to both saprophytic and mycorrhizal fungi suggests that the glucoamylase gene may play an important role in the morphogenesis of basidiomycetous fungi.

Starch, an abundant glucose polymer in nature, has been shown to strongly induce glucoamylase gene expression during fruit production in basidiomycetous fungi [2, 6]. GH15 catalyzes starch hydrolysis and thus, the release of β-glucose units from the nonreducing ends of starch molecules. *L. edodes*, *Flammulina velutipes* (Curtis) Singer, and *Pholiota nameko* (T. Ito) S. Ito & S. Imai are economically important edible mushrooms in Japan. Thus, in order to study the genetic processes involved in the fruiting of *P. nameko*, we cloned and characterized *PnGlu1* in mono- and dikaryotic *P. nameko* cultured on different starch media using quantitative reverse transcription-PCR.

MATERIALS AND METHODS

Amplification of GH15 genes. Initially, fragments of genomic DNA encoding the putative glucoamylase gene (*PnGlu1*) were amplified by PCR with degenerate oligonucleotide primer pairs F-15-GP2-AF and F-15-GP2-BR (Table 1). The F15-GP2-AF and F15-GP2-BR primers were designed based on the amino acid sequences GLGEPKF and FDLWEEI, respectively, which have been reported to be conserved in the glucoamylase protein of *L. edodes* [3]. PCR reactions were conducted in 50 µl reaction volumes containing 1 x Ex *Taq* buffer (Takara Bio Co., Shiga, Japan), 50 ng of genomic DNA, 50 pmol of each primer, 0.2 mM of each dNTP, and 1.25 U of Ex *Taq* polymerase (Takara Bio Co.). PCR reactions were performed on a Takara PCR Personal Thermal Cycler (Takara Bio Co.) using an initial denaturation of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 2 min at 50 °C, and 30 s at 72 °C. Finally, the temperature of the reaction mixture was maintained at 72 °C for 10 min. The PCR fragments, which were approximately 400 bp in length, were cloned into the pMD20 T-vector (Takara Bio Co.) and sequenced. The partial *PnGlu1* sequences thus obtained were used to identify the *PnGlu1* flanking regions by cassette amplification using several sets of PCR primers. These primer sets were designed based on the partial nucleotide sequences of *PnGlu1* obtained from the initial PCR. All of the fragments were subcloned into pMD20 T-vector (Takara Bio Co.) and directly sequenced to determine the sequence of the flanking region.

The nucleotide sequences of *PnGlu1* from monokaryon NGW19-6 were deposited into the DNA Data Bank of the Japan database under the accession no. AB639794.

Real-time PCR assay. Total RNA from monokaryon NGW19-6 and dikaryon NGW19-6/12-163 was used as template material for all RT-PCR reactions performed in this study. The *actinI* gene was used as the housekeeping gene. A partial *actinI* gene fragment in monokaryon NGW19-6 was cloned using the degenerate PCR primers, UnivActF1 and UnivActR1 [7]. Primers for *PnGlu1* and actin used for the real-time PCR assay were designed according to their cDNA sequences using GENETYX (ver. 10.0.3, Genetyx). Primers were designed based on the principles of primer design, with 3 to 6 bases of the 3' splice site designed to cross the intron in the primer spanning the intron. All primers were tested to ensure that they amplified a single band with no primer-dimers. Plasmid extraction was performed according to a modification of

Birnboim [8] and five plasmid dilutions (10^4 to 10^8) were used to construct standard curves. Real-time PCR was conducted using the one step RNA-direct SYBR green Real-time PCR master mix (Toyobo) and Linegene (BioFlux, Hangzhou, China). Each reaction was performed in duplicate. The PCR cycle conditions consisted of 90 °C for 30 s to activate thermostable DNA polymerase, 61 °C for 20 min for reverse transcription, 95 °C for 30 s for predenaturation, followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 74 °C for 30s. Melting curves were determined according to the manufacturer's instructions. To confirm amplification specificity, the real-time RT-PCR samples were then run on a 1.5% agarose gel and analysis was performed according to manufacturer's instructions. Relative gene expression was expressed as the ratio of the target gene (*PnGlu1*) concentration to the housekeeping gene concentration, with the obtained values representing mean gene expression obtained from at least two separate PCR experiments using the same RNA preparation.

Table 1: Primers used in this study

Primer	Sequence	Remark
F-15-GP2-AF	5'- GGNYTNGGNGARCCNAARTT -3'	Used for initial amplification
F-15-GP2-BR	5'- ATYTCYTCCCANARRTCRAA -3'	
Realtime Gulco F	5'-GCAACAAGTATCTAACCCCAGTG-3'	Used for real-time RT-PCR of *PnGlu1*
Realtime Gulco R	5'-GTAAGGCAGGTCCATCCCGT-3'	
Actin forward	5'-TCGGTCTTGAGGCTGCTGGT-3'	Used for real-time RT-PCR of actin
Actin reverse	5'-AGTCAACTCCTTCTGCATACGGTC-3'	

RESULTS AND DISCUSSION

Characterization of glucoamylase gene from *P. nameko.* In *P. nameko*, the nucleotide sequence from the initial ATG to the stop codon of the coding region consisted of 1743 bp and encoded 581 amino acids. The locations of the exons and introns of the gene were determined from the nucleotide sequences of RT-PCR products. All of the introns started with GT and ended with AG, and the coding region was interrupted by ten introns varying in size from 52 to 66 bp (Fig. 1). The deduced amino acid sequence from *P. nameko* was 32-70% similar to glucoamylase genes from other fungi, with the highest similarity obtained between the sequences from *P. nameko* and *L. bicolor* S238N-H82 [4]. Predictions obtained using the PSORT II program revealed that the sequence coded an extracellular protein of the cell wall, and that the first 17 amino acids coded a putative signal peptide found in the N terminal region of the protein. The SOSUI program predicted that the coded protein was soluble. Comparisons of the amino acid sequence of the putative glucoamylase with other fungal glucoamylases suggested that the structure of the glucoamylase protein from *P. nameko* comprises an N-terminal catalytic domain that was related to enzyme activity and a C-terminal region containing the starch binding domain, linked by a glycosylated hinge region.

Real-time PCR analysis of *PnGlu1* gene expression at different fungal developmental stages. Expression of the *PnGlu1* gene at different developmental stages (vegetative mycelial growth, appearance of primordia and formation of fruiting bodies) was examined when *P. nameko* was cultured on sawdust medium. *PnGlu1* gene expression was highest immediately before mycelial stimulation and decreased gradually thereafter. Thirty days after mycelium

stimulation, *PnGlu1* gene expression in both the mycelia of primordia and in the fruit bodies of different sizes decreased to very low levels.

Figure 1: Nucleotide and amino acid sequences of the *Pn-Glu1* gene from *P. nameko.*
Capital and lower case letters indicate the exons and introns, respectively. The amino acid sequences corresponding to the putative signal peptide and glucoamylase active site are underlined. The nucleotide consensus sequence of yeast MAT a and α homeodomain protein binding site are boxed. Italic case letters indicate the putative poly A(+) signal.

Quantitative reverse transcription-PCR assays of glucoamylase expression in the mono- and dikaryon of *P. nameko* grown on glucose and on soluble starch showed that soluble starch is the optimum carbon source for supporting the growth of *P. nameko*; starch induced glucoamylase expression in both the mono- and dikaryon. In order to study the regulation of the glucoamylase gene, we also cloned the upstream sequence of the open reading frame. In doing so, we found consensus sequences of mating type protein binding sites upstream of the start codon (Fig.1), which are necessary for homeodomain protein formation. Mating type protein binding sites have also been reported in the mycorrhizal fungus *T. matsutake* [5]. The observation that glucoamylase expression levels were higher in the dikaryon and transformant strains compared with the monokaryon strain implies that the glucoamylase gene is regulated by mating-type loci. In this study, we attempted to elucidate the function of glucoamylase in fruit body formation. However, the finding that the putative glucoamylase gene cloned in this study was expressed only during the mycelial growth stage indicated a need for further research aimed at identifying a new glucoamylase gene expressed specifically during fruiting body formation.

CONCLUSIONS

The nucleotide sequence of a *PnGlu1* gene was identified and characterized. The consensus nucleotide sequence of yeast MAT a and α homeodomain protein binding sites were observed in an untranslated region upstream of the ATG codon of *PnGlu*. *PnGlu1* gene expression in the dikaryon and transformants with the *hox* gene was higher than in the monokaryon. *PnGlu1* was expressed in the fungal mycelium but not in the fruiting body.

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