# Regulation of the starch degradation enzymes during fruiting in edible mushroom *Pholiota microspora*

# 食用きのこナメコにおける子実体形成過程におけるデンプン分解酵素の発現制御に関する研究

By

Gang ZHU

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Supervisor:

Prof. Tadanori AIMI

The United Graduate School of Agricultural Science

Tottori University

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# Chapter1

# General introduction

The edible fungus *Pholiota microspora* (T. Ito) S. Ito & Imai is one of the commercially important cultivated mushrooms in Japan, similar with *Lentinus edodes*, *Flammulina velutipes*, and *Pleurotus ostreatus* (Chang and Hayes 1978). Due to recent advances in bottle cultivation technology in Japan, this mushroom is now available in domestic markets. The artificial substrate for *P. microspora* cultivation is generally used sawdust medium. The addition of a suitable amount of rice bran to sawdust medium increases the yield of the fruit bodies (Chang and Hayes 1978). Rice bran, a byproduct after polished rice processing, is traditionally added to sawdust as a nutrient for production of mushroom (Rossi et al. 2003). In other artificial cultivation of mushrooms, such as *L. edodes*, addition of any amount of rice bran to substrate showed an improvement in mushroom quality and production (Rossi et al. 2003).

In the genus Agaricaceae, such as *Coprinus cinereus* (Ji and Moore 1993), *F. velutipes* (Kitamoto and Gruen 1976; Gruen and Wong 1982) and *C. congregatus* (Robert 1977), fruiting body formation has been shown to be associated with breakdown of polysaccharides in the medium. When a mushroom fungus forms fruiting bodies, large amount of mycelia may be needed either to store the nutrients or to transport the nutrients to the fruit bodies (Ohta 1997). The major carbohydrate polymers present in commercial rice bran are cellulose, hemicellulose, and starch. Starch is not botanically present in the outer pericarp layers, but because of endosperm breakage during milling, it appears in the bran. The quantity varies according to the amount of breakage and degree of milling, but values of 10-20% (*w*/*w*) could be expected. Hemicelluloses have been reported to comprise 8.7-11.4% of the bran. Cellulose in rice bran is reported to range from 9.6 to 12.8% (Marshall and Wadsworth 1993).

## 1-1 Starch degradation enzymes

Starch, which is a polymer of glucose units linked by glycosidic bonds, is the second most abundant carbohydrate on the earth (Buléon et al. 1998). Starch consists of two distinct types of polysaccharide, amylose and amylopectin (Bertoldo and Antranikian 2001). Amylose is a linear polymer consisting of α-1, 4-linked glucopyranose residues, and amylopectine is a branched polysaccharide composed of hundreds of short α-1, 4 -glucan chains, which are interlinked by α-1, 6-linkages. Because of the complex structure of

starch, combinations of enzymes are required to degrade starch. These enzymes can be commonly divided into two groups, endoamylases and exoamylases (Chen et al. 2012).

Endoamylases such as α-amylases (EC 3.2.1.1) belong to the glycoside hydrolase (GH) 13 family, which randomly acts on α-1, 4-glycosidic linkages in starch and related polysaccharides to produce shorter maltooligosaccharides and α-limit dextrins (Pandey et al. 2000). GH13 is the largest GH family and groups together a number of enzymes with different activities and substrate specificities that act on α-glycosidic bonds. It has been divided into 40 official subfamilies by the CAZy (Carbohydrate-Active enZYmes) database curators. Within the GH13 family, α-amylase specificity is present in several subfamilies, such as GH13_1, 5, 6, 7, 15, 19, 24, 27, 28, 32, 36, and possibly a few more not yet defined (Stam et al. 2006).

Exoamylases include glucoamylase (EC 3.2.1.3), α-glucosidase (EC 3.2.1.20) and β-amylase (EC 3.2.1.2). β-Amylases are typically of plant origin; fungi generally produce two types of exoamylase, glucoamylase and α-glucosidase (Bertoldo and Antranikian 2002).

Glucoamylase (EC 3.2.1.3), which belongs to the glycoside hydrolase 15 family, catalyzes hydrolysis of α-1, 4 and α-1, 6-glucosidic linkages to release β-D-glucose units from the non-reducing ends of starch and related polysaccharides. In addition to acting on α-1, 4 linkages, and the enzyme slowly hydrolyzes α-1, 6-glycosidic linkages of starch (Norouzian et al. 2006). Glucoamylases have been identified in most fungi, which implies that glucoamylases are essential enzymes, probably because they are conducive for fungi to obtain glucose by hydrolyzing starch (Norouzian et al. 2006). In the saprophytic fungus *L. edodes*, the expression of the glucoamylase gene (*gla1*) increases during fruiting body formation, which indicates that glucoamylase may play an important role in the morphogenesis of this basidiomycete (Zhao et al. 2000). In the brown-rot basidiomycete *Fomitopsis palustris*, glucoamylase activity is suddenly enhanced when the fruiting body formation initiated in liquid static culture, which suggests that the function of *F. palustris* glucoamylase is related to morphogenesis (Yoon et al. 2006). Thus, glucoamylases are believed to be important in fruiting body morphogenesis of fungi.

α-Glucosidases (EC 3.2.1.20; α-D-glucoside glucohydrolase) are a group of exo-acting enzymes that catalyze the liberation of α-D-glucose from the non-reducing terminus of substrates (Chiba 1988). Various types of α-glucosidases from mammals, insects, plants, fungi and bacteria have been purified and the nucleotide sequences of their genes have been identified (Yamamoto et al. 2004). They were suggested to

be grouped into two major families based on their primary structure, glycoside hydrolase families 13 and 31. Most α-glucosidases from bacteria and insects belong to GH13 and those from plants, animals, and fungi belong to GH31. According to their substrate specificity, these enzymes are conventionally classified into three types. Type I hydrolyze heterogeneous substrates, more efficiently those with sucrose and *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) than homogeneous substrates such as maltose. Type II enzymes are more specific for maltose and have low activity on heterogeneous substrates. Type III enzymes resemble type II, but hydrolyze oligosaccharides and starch at similar rates. Family 13 includes enzymes designated as type I. The α-glucosidase types II and III are classified as family 31 (Henrissat 1991; Henrissat and Bairoch 1993; Chiba 1997).

## 1-2 Fruiting body formation in basidiomycetes

The fruiting body formation is a complex process, which requires the coordination between genetic, environmental, and physiological factors (Wösten and Wessels 2005). An asexual or sexual spore of basidiomycetes generated hyphae grow at their tips, while branching subapically. The anastomosis of hyphae formed mycelium which is a network of hyphae. The vegetative mycelium colonizes and assimilates the substrate, using secretes extracellular enzymes from tips of growing hyphae to degrade polymeric components. After a maximum of submerged mycelium has been formed, hyphae escape the substrate to grow into the air. These hyphae form aggregates, which are called hyphal knots or nodules. Within the knots hyphae aggregate, differentiation of cells occurs, the structures are termed primordia. The lower part will develop into the stipe, while the cap will be formed from the upper part. The stipe elongates and the cap expands, giving rise to a fully developed fruiting body (Wösten and Wessels 2005; Liu et al. 2006).

The fruiting body of mushroom contains relatively large amounts of carbohydrate and fibre, ranging from 51% to 88% and from 4% to 20% (dry weight basis) (Mattila et al. 2000). The mushroom fungal cell walls contain chitin, other hemicellulose and hetero-glucans with β-(1, 3), β-(1, 4), and β-(1, 6) glucosidic linkages (Manzi and Pizzoferrato 2000). Hetero-glucan is the major structural polysaccharide of the fungal cell wall, constituting approximately 50–60% of the wall by dry weight. In general, between 65% and 90% of the cell wall glucan is found to be β-1, 3-glucan. The synthesis of β-1, 3-glucan is required for proper cell wall formation and the normal development of fungi (Zhu et al. 2015). The glucan was synthesized by

glucan synthase, a trans-membrane protein, who accept its substrate UDP-glucose at the cytoplasmic site and extruding its product outside the plasma membrane. By incubating isolated membrane preparations with UDP- glucose in the presence of amylase, a glucan with exclusively β-1, 3-linkages was obtained, i.e. no β-1, 6-linkages could be found. With enzyme preparations contaminated with wall material, a low amount of (1, 6) linkages could be detected (Balint et al. 1976; Lopez-Romero and Ruiz-Herrera 1977).

Fungal cell wall is a highly dynamic structure subject to constant change, for example, during cell expansion and division, and during spore germination, hyphal branching and septum formation in filamentous fungi. Cell wall polymer branching and cross-linking, and the maintenance of wall plasticity during morphogenesis, may depend upon the activities of a range of hydrolytic enzymes found intimately associated with the fungal cell wall. Changes in cell wall metabolism during fruit body morphogenesis have been observed for many fungal species (Stringer and Timberlake 1995; Wessels 1994). In *Agaricus brasiliensis*, yields of β-glucans increased from 42 mg/g [β-glucan/fruiting bodies (dry wt)] in immature stage to 43 mg/g in mature stage with immature spores, and decreased to 40 mg/g in mature stage with spore maturation (Camelini et al. 2005).

## 1-3 Starch utilization in fungi

The fungi are heterotrophs, divided into three groups based on their nutritive adaptations - saprophytic, parasitic and symbiotic fungi. The fungi play an essential role in nutrient cycling, especially as saprotrophs and symbionts. The mycorrhiza is consistent with symbiotic association between fungus and roots, classified into two types: endomycorrhizae and ectomycorrhizae. The plant in mycorrhiza provides carbohydrates, such as glucose and sucrose, to fungi. In return, the plant gains water and mineral by higher absorptive capacity of fungal mycelium.

The saprophytic fungi can decompose polysaccharides such as cellulose and hemicellulose and utilize them as a carbon source, but the ectomycorrhizal fungi have low ability to synthesize the corresponding enzymes (Ohta 1997). Conversely, both ectomycorrhizal fungi and saprophytic fungi have the ability to secrete amylolytic enzymes to decompose starch. The ectomycorrhizal fungus *Tricholoma matsutake* has three types of amylolytic enzymes: α-amylase, glucoamylase, and α-glucosidase (Kusuda et al. 2008), while the ectomycorrhizal fungus *Lyophyllum shimeji* has two types: glucoamylase and α-amylase (Arai et al. 2003). This kind of mushroom has been cultivated in an artificial medium containing barley grain

without a host plant, which revealed that a sufficient quantity of starch used as a carbon source was able to supply a factor inducing fruiting body formation (Ohta 1994). These observations suggest that amylolytic enzymes and starch-hydrolyzing ability are very important for fruiting body formation of ectomycorrhizal mushrooms. In saprophytic mushrooms such as *Coprinopsis cinerea* (Stajich et al. 2010), *F. palustris* (Yoon et al. 2006) and *L. edodes* (Zhao et al. 2000), only the gene encoding glucoamylase has been identified and characterized. Little research has been conducted on the α-amylase and α-glucosidase genes of saprophytic mushrooms.

## 1-4 Purpose of this study

Starch has been shown to strongly induce glucoamylase gene expression during fruit production in basidiomycetous fungi. GH15 catalyzes starch hydrolysis to release of β-glucose units from the non-reducing ends of starch molecules. In order to study the genetic processes involved in the fruiting of *P. microspora,* the glucoamylase gene *PnGlu1* was cloned and characterized in a previous study (Li et al. 2012). Following the completion of sequencing of the genome of *P. microspora*, we identified a different glucoamylase gene in the haploid genome, named *PnGlu2*. We also cloned three α-amylase genes (*PnAmy1, PnAmy2, PnAmy3*) and a α-glucosidase gene (*PnGcs*) from *P. microspora* genome.

In this study, we examined their expression in minimal media supplemented with different carbon sources and in sawdust medium during the development cycle using quantitative reverse transcription polymerase chain reaction (qRT-PCR). We also measured α-glucosidase activity during the developmental cycle of *P. microspora* in sawdust media. Our findings should clarify what role these genes play in *P. microspora*.

# Chapter 2

# Identification, characterization and expression of the second glucoamylase gene from *Pholiota microspora*

## 2-1 Abstract

The glucoamylase 1 gene (*PnGlu1*) from the saprophytic fungus *Pholiota microspora* has been previously cloned and characterized. In this study, a second glucoamylase gene (*PnGlu2*), 1719 bp long and encoding 573 amino acids, was identified in the *P. microspora* genome. PnGlu1 and PnGlu2 show 70% amino acid identity. Transcriptional analyses showed that *PnGlu1* and *PnGlu2* expression in minimal media containing different carbon sources are far lower than that in sawdust medium. In sawdust medium, transcription level of *PnGlu1* is high in vegetative dikaryotic mycelia and that of *PnGlu2* is high in primordia and at the fruiting body stage. These data suggested that the two glucoamylase gene *PnGlu1* and *PnGlu2* may differentially regulate in each developmental stages such as mycelial growth and fruiting body formation, respectively.

## 2-2 Introduction

Starch, which is a polymer of glucose units linked by glycosidic bonds, is the second most abundant carbohydrate in nature (Buléon et al. 1998). Glucoamylase (EC 3.2.1.3), which belongs to the glycoside hydrolase 15 family, catalyzes hydrolysis of α-1, 4 and α-1, 6-glucosidic linkages to release β-D-glucose units from the non-reducing ends of starch and related polysaccharides. In addition to acting on α-1, 4 linkages, the enzyme slowly hydrolyzes α-1, 6-glycosidic linkages of starch (Norouzian et al, 2006). Glucoamylases have been identified in most fungi, which implies that glucoamylases are essential enzymes, probably because they are conducive for fungi to obtain glucose by hydrolyzing starch (Norouzian et al. 2006). In the saprophytic fungus *Lentinula edodes*, the expression of the glucoamylase gene (*gla1*) increases during fruiting body formation, which indicates that glucoamylase may play an important role in the morphogenesis of this basidiomycete (Zhao et al. 2000). In the brown-rot basidiomycete *Fomitopsis palustris*, glucoamylase activity is suddenly enhanced when the fruiting body formation initiated in liquid static culture, which suggests that the function of *F. palustris* glucoamylase is related to morphogenesis

(Yoon et al. 2006). The ectomycorrhizal fungus *Lyophyllum shimeji* has been cultivated in an artificial medium containing barley grain without a host plant, which revealed that a sufficient quantity of starch used as a carbon source was able to supply a factor inducing fruiting body formation (Ohta, 1994). Thus, glucoamylases are believed to be important in fruiting body morphogenesis of fungi.

The basidiomycetous fungus *P. microspora* is an economically important edible mushroom in Japan. In order to study the genetic processes involved in the fruiting of *P. microspora,* the glucoamylase gene *PnGlu1* was cloned and characterized in a previous study (Li et al. 2012). Following the completion of sequencing of the genome of *P. microspora*, we identified a different glucoamylase gene in the haploid genome, named *PnGlu2*. Because previous randomly designed quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers for *PnGlu1* were unfortunately located at a consensus sequence for *PnGlu1* and *PnGlu2*, we designed primers specific for *PnGlu1* for the present study, in which we describe characterization of the genomic DNA and cDNA sequences of the *PnGlu2* gene from *P. microspora.* We also analyzed expression of the *PnGlu1* and *PnGlu2* genes in a minimal medium containing various carbon sources, and in sawdust medium.

## 2-3 Materials and methods

### 2-3-1 Fungal strains and culture condition

Monokaryotic strains of *P. microspora*, NGW19-6 (*A4*, *pdx1*), an auxotrophic mutant for pyridoxine, and NGW12-163 (*A3*, *arg4*), an auxotrophic mutant for arginine, were used in this study (Masuda et al. 1995; Yi et al. 2009). A dikaryotic mycelia obtained by crossing NGW19-6 and NGW12-163 was referred to as NGW19-6/12-163.

To analyze the effect of carbon source on gene expression, strain NGW19-6/12-163 was grown on potato dextrose agar at 25 °C for 1 wk, and then 5 circular agar blocks (5 mm diameter) of mycelia were transferred into 10 mL minimal medium (1.5 g/L $(NH_4)_2HPO_4$, 1 g/L $KH_2PO_4$, 20 g/L glucose, thiamine hydrochloride 25 mg/mL, pH 5.5) in an Erlenmeyer flask and grown at 25 °C for 5 d (days). The mycelia were collected by filtration and washed three times with minimal medium without carbon source. The washed mycelia were suspended in 10 mL minimal medium containing 20 g/L glucose, 20 g/L cellobiose, 20 g/L sucrose, 20 g/L maltose, 20 g/L maltotriose, 20 g/L soluble starch, 20 g/L corn starch, 20 g/L potato

starch, 20 g/L wheat starch, 20 g/L amylose, 20 g/L amylopectin, or no carbon source. After 24 h of incubation, mycelia were harvested by filtration for RNA extraction.

Fruiting bodies of *P. microspora* were cultivated on a sawdust substrate. The sawdust substrate was prepared as follows: beech sawdust was mixed with rice bran at a gravimetric ratio of 5: 1 and adjusted to 65% moisture using tap water, and the medium was put into a 100 mL Erlenmeyer flask and autoclaved at 121 °C for 60 min. After cooling the medium, it was inoculated with circular agar blocks of NGW19-6/12-163 mycelia (5 mm diameter) and incubated at 25 °C. When the mycelia had colonized the substrate (about 40 days after inoculation), the surface layer of the mycelia was scratched by a spatula and then 50 mL sterilized distilled water was poured into the flask. The water was removed after the flasks had been incubated at 15 °C overnight, and then cultivation was continued at 15 °C until the fruiting body developed. We defined the day after removing the water as day 0. Samples for RNA extraction were taken from mycelia in sawdust medium at 0 d, 10 d, 20 d, 30 d, 40 d, 60 d, 80 d, and 100 d, and also from the tissues in primordia (about 120 d) and three different stages of fruiting body (total height of fruiting body: 1 cm, 2 cm and 3 cm) .

### 2-3-2 Genome sequencing and annotation

The complete nucleotide sequence of the genomic DNA of monokaryotic NGW19-6 was determined using Illumina HiSeq 2000 paired-end technology provided by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). This sequencing run yielded 30,935,254 high-quality filtered reads with 101 bp paired-end sequencing. The genomic sequence was assembled using Velvet assembler version 1.1.02 (hash length 85 bp) (Zerbino and Birney, 2008). The final assembly contained 4,770 contigs of total length 33,400,256 bp, with an N50 length of 72,431 bp. The prediction of protein-coding sequences and annotation were performed by the Microbial Genome Annotation Pipeline (http://www.migap.org/), which utilizes the MetaGeneAnnotator (Noguchi et al. 2008), RNAmmer (Lagesen et al. 2007), tRNAScan-SE (Lowe and Eddy 1997), and BLAST algorithms (Altschul et al. 1990).

### 2-3-3 DNA and RNA preparation and cDNA synthesis

Genomic DNA extraction was followed the method of Dellaporta（Dellaporta et al. 1983）. The RNA was extracted using a MagExtractor Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

The cDNA was synthesized using total RNA as template by ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo).

Amplification of cDNA fragments and 3′-rapid amplification of cDNA ends (RACE) were performed with a Takara RNA PCR (AMV) version 3.0 kit (Takara Bio, Shiga, Japan) and 5′-RACE with a 5′-Full RACE Core Set (Takara Bio). PCR was carried out according to the manufacturer's instructions using the oligonucleotide primers listed in Table 2-1. The amplified fragments were subcloned into pMD20 T-vector (Takara Bio) and sequenced.

### 2-3-4 Analysis of nucleotide and protein sequences

Nucleotide and protein sequence data were analyzed using GENETYX ver. 10.0.3 software (GENETYX, Tokyo, Japan). Protein motifs in the glucoamylase amino acid sequence were identified using the web-based MOTIF search program (http://motif.genome.jp/). Subcellular localization of the enzyme was predicted by the PSORT II program (http://psort.ims.u-tokyo.ac.jp /form2.html). A phylogenetic tree was constructed by MEGA 5.05 software using the neighbor-joining method with a bootstrap value of 1,000 replicates. DNA and deduced amino acid sequences were aligned with ClustalX2 software (http://www.clustal.org/clustal2/).

### 2-3-5 qRT-PCR assays

The actin gene (*act2* LC121583) was used as a reference gene. The primer pairs for amplification of cDNA of *PnGlu1, PnGlu2* and *act2* were designed based on their cDNA sequences using GENETYX software. Amplification of genomic DNA was prevented by designing primers for exon-exon junctions. All primers were tested to ensure that they amplified a single band with no primer-dimers, as shown in Table 2-1. Plasmids with the inserted target gene (*PnGlu1* or *PnGlu2*) or the housekeeping gene (*act2*) were extracted according to the method described by Birnboim (Birnboim 1983). Standard curves were constructed using five ten-fold dilutions of plasmid. Real-time PCR was performed using the KOD SYBR qPCR Mix kit (Toyobo). Thermocycling was carried out using a LineGene Real-Time Thermal Cycler (BioFlux, Tokyo, Japan), with an initial incubation for 2 min at 98 °C, followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s and 68 °C for 1 min. Each run was completed with a melting curve analysis to confirm the specificity of amplification and absence of primer-dimers. Data analysis was performed according to the manufacturer's instructions.

## 2-4 Results

### 2-4-1 Nucleotide sequence of *PnGlu1* and *PnGlu2* from *P. microspora*

The genomic DNA sequence of *PnGlu2* was identified using a tblastn search against the draft genome database of *P. microspora* with the glucoamylase protein sequences of *L. edodes* (AAF75523.1) and *Trametes versicolor* (XP_008032332.1).

The nucleotide sequence of *PnGlu2*, which has been deposited in the DDBJ/EMBL/GenBank database under accession number LC066672, is shown in Fig. 2-1. The open reading frame of *PnGlu2*, from ATG to the stop codon, is 1719 bp, which is shorter by 24 bp than that of *PnGlu1*. Comparison of the nucleotide sequence of the complete cDNA of *PnGlu1* and *PnGlu2* shows 65% identity. The locations of intron-exon junctions were determined from the nucleotide sequences of the cDNA amplified by 3′-and 5′-RACE PCR and RT-PCR. All of the introns started with GT and ended with AG. The 9 introns of *PnGlu2* interrupt the coding sequence, which is comprised of 10 exons (Fig. 2-1).

The upstream region (-1 to -2060) of *PnGlu2* (Fig. 2-1) were analyzed and compared to that region in *PnGlu1*. There found a putative TATA box at position -83, two putative CAAT boxes at positions -309 and -158 in the promoter region of *PnGlu2*. The stress responsive element (STRE: 5′-AGGGG-3′), which has been suggested to function in osmotic stress induction (Moskvina et al. 1998), was found in the *PnGlu2* promoter region at position -840, while *PnGlu1* possessed two STRE elements at positions -1943 and -1917. The six carbon catabolite repressor (CREA) binding sites (5′-SYGGRG-3′), identified as a repressor involved in glucose repression (Dowzer and Kelly, 1991), were identified in *PnGlu2* at positions -1149, -882, -855, -731, -713 and -324. Only two CREA binding sites appeared in the *PnGlu1* promoter region at positions -1659 and -834, suggesting that transcription of glucoamylase genes may be repressed by glucose, and that *PnGlu2* expression is repressed more strongly by glucose than *PnGlu1*. XYR1 (xylanase regulator 1), a zinc binuclear cluster protein that binds the core sequence (5′-GGCTAA-3′), is both the major cellulase activator and indispensable for cellulase expression (Mach and Zeilinger, 2003). This consensus sequence was located in the *PnGlu1* promoter region at position -1915, but in *PnGlu2*, the site is lacking. We predict that *PnGlu1* is induced in the presence of cellulose. AmyR is a transcriptional activator with a $Cys_6$ zinc cluster. The AmyR binding site (5′-CGG-$N_8$-CGG-3′) has been found in a

number of promoters from *Aspergillus* genes coding for starch-degrading enzymes (Petersen et al. 1999). This consensus binding sequence was only identified in the promoter region of *PnGlu2* at position -1043. It was inferred that *PnGlu2* is a starch-induced gene. We predict that *PnGlu2* may be more easily induced by starch than *PnGlu1*.

### 2-4-2 Characterization of protein sequence of *PnGlu1* and *PnGlu2* of *P. microspora*

PnGlu1, the first 17 amino acids of which act as a signal peptide, may be an extracellular glucoamylase that belongs to glycoside hydrolase family 15 (Li et al. 2012). The amino acid sequence of PnGlu2 implies the presence of a signal peptide (Fig. 2-1 the first 17 amino acids) based on the PSORT II program, inferring that PnGlu2 may be an extracellular enzyme. The *PnGlu2* gene encodes a protein of 573 amino acid residues with a predicted molecular mass of 60.96 kDa after removal of the signal peptide, while the 1743 bp *PnGlu1* cDNA encodes a 581 amino acid protein with a predicted molecular weight of 61.40 kDa after removal of the signal peptide. PnGlu2 had the highest homology (75% identity) to the glycoside hydrolase family 15 protein of *Laccaria bicolor* (EDR13753.1), and had 70% identity with PnGlu1. The MOTIF program predicted that PnGlu2 also belongs to glycoside hydrolase family 15. Base on the amino acid sequence alignment with other known species as well as PnGlu1 (Zhao et al. 2000), PnGlu2 possesses a catalytic domain at the N-terminal region and a starch-binding domain (SBD) at the C-terminal region, separated by a highly O-glycosylated linker region. Five highly conserved segments (Fig. 2-1 amino acid residues Sl: 53-78, S2: 122-153, S3: 195-216, S4: 321-353, S5: 402-448), which constitute the catalytic domain, were observed at the N-terminal region (Zhao et al. 2000). The SBD of PnGlu2 (Fig. 2-1), which is predicted by the MOTIF program to be categorized in the carbohydrate binding module (CBM) 20 family (Henrissat and Bairoch 1993), appeared at the C-terminal region. PnGlu1 also possessed an SBD belonging to CBM 20 family, indicated that PnGlu1 and PnGlu2 have the ability to attach and degrade raw starch (Machovič and Janeček, 2006). The linker region in PnGlu2 is estimated to comprise amino acid residues 449 to 479, which is 6 amino acid residues shorter than that of PnGlu1 (amino acid residues 450 to 486). The linker regions of glucoamylase enzymes are related to starch degradation, thermostability, and peptide backbone extension (Zhao et al. 2000), but the main function of this region in PnGlu1 and PnGlu2 is not yet known.

### 2-4-3 Phylogenetic relationship between *PnGlu1* and *PnGlu2* of *P. microspora*

The length of the introns of *PnGlu1* ranged between 52 bp and 66 bp (Li et al. 2012), and that of *PnGlu2* ranged from 54 bp to 64 bp. When comparing *PnGlu1* and *PnGlu2* gene structure, all 9 introns are at the same positions (Fig. 2-2). Nucleotide sequence identity between each intron from intron 1 to intron 9 was low (24%–43%). Thus, the positions of intron insertion were evolutionarily conserved, but their nucleotide sequences were variable.

In the other species from the Agaricomycotina, the number and positions of introns among glucoamylase genes in *P. microspora* and *L. bicolor* were identical (Fig. 2-2). Intron I in *AbGlu*, intron IV in *CcGlu1*, introns IV and V in *CcGlu2*, introns I and VII in *PoGlu1*, and intron VII in *PoGlu2* were absent, but introns VIII and IX appeared in all genes (Fig. 2-2) and may be the most ancient introns, which also suggests that these introns existed before speciation. Although various introns are absent in various species, the positions of existing introns are conserved.

The phylogenetic tree of glucoamylases, constructed using 20 glucoamylase sequences is shown in Fig. 2-3. The glucoamylases from the Basidiomycotina and from the Ascomycotina formed two main clades. PnGlu1 and PnGlu2 of the saprophytic basidiomycete *P. microspora* clustered together with the ectomycorrhizal basidiomycete *L. bicolor*, and formed a single clade. Saprophytic basidiomycetes *L. edodes* and *P. ostreatus* clustered with the ectomycorrhizal basidiomycete *T. matsutake* in the same clade. We inferred that glucoamylases from these two types of fungi may share some common functions, such as mycelial growth and fruiting body formation. However, these details are not yet clear.

## 2-4-4 Transcription of *PnGlu1* and *PnGlu2* of *P. microspora*

To investigate the effect of carbon sources on glucoamylase gene expression, *P. microspora* was cultured in a minimal medium containing various carbon sources (see Materials and methods). To investigate glucoamylase gene expression on tissue growing in sawdust medium, we measured it during vegetative mycelial growth stage, at appearance of primordia and during fruiting body formation stage.

In minimal media, the highest level of *PnGlu1* expression was observed when maltose was used as the sole carbon source after incubation for 24 h (Fig. 2-4), while the transcript level of *PnGlu2* was much higher in the amylose culture than in other culture media (Fig. 2-5).

In sawdust medium, transcription of *PnGlu1* then increased rapidly for the first 10 days and reached a peak at 30 days in the mycelial growth stage. Gene expression levels of *PnGlu1* decreased gradually to

very low levels in the remaining mycelial growth and fruiting body development stages (Fig. 2-6). The expression level of *PnGlu2* was much higher at 10 days, and then declined at 20 days in the mycelial growth stage. At the fruiting body formation stage, the expression of *PnGlu2* dramatically increased over the mycelia growth stage. The highest *PnGlu2* gene expression level throughout the entire *P. microspora* developmental cycle was in 1 cm fruiting bodies (Fig. 2-7).

*PnGlu1* and *PnGlu2* gene expression in sawdust medium largely exceeded than that in minimal media containing different carbon sources. The highest *PnGlu1* expression in sawdust medium was 20-fold higher than in minimal medium containing maltose, while the highest *PnGlu2* expression in sawdust media was 10-fold higher than in minimal medium supplemented with amylose. We inferred that pure starch cannot dramatically induce *PnGlu1* and *PnGlu2* gene expression in minimal media. Other inducible factors in sawdust media are required for the strikingly high glucoamylase gene expression in *P. microspora*.

## 2-5 Discussion

Glucoamylases are present in many fungi and show a conservative pattern in evolution. This conserved evolution in glucoamylases reflects their important roles in fungi, and suggests that they may be essential (Chen et al. 2012). In the present study, a new glucoamylase gene, *PnGlu2*, was identified and characterized from *P. microspora*. This is the first report of two distinct glucoamylase genes located in the *P. microspora* genome. Based on intron position and amino acid sequence identity, *PnGlu1* and *PnGlu2* are likely duplicated genes that originated from a common ancestor. The clustering of *PnGlu1* and *PnGlu2* on the genome sequence, separated by less than 2 kb (data not shown), strongly supports such an event. PnGlu1 and PnGlu2 possess glycoside hydrolase family 15 conserved regions, and the major difference in their amino acid sequences lies in the 6 amino acids absent in the PnGlu2 linker region compared to PnGlu1. However, it is currently unclear if this absence leads to different functions of the two enzymes.

Expression of *PnGlu1* and *PnGlu2* is distinguished by the type of culture used. The two genes are markedly expressed in sawdust medium but little expressed in minimal media containing different carbon sources. Growth of *P. microspora* in sawdust medium is closer to natural conditions than that in a minimal medium, so gene expression presumably more closely resembles that under natural conditions. Recent findings showed that expression of the *A. oryzae* glucoamylase gene *glaB* is greatly enhanced in solid-state culture but repressed in submerged culture. Starch is not the only inducer of *glaB* expression; other

inducing factors, such as low water activity, elevated temperature and physical barriers to hyphal extension, are essential for maximal expression of *glaB* in solid-state culture (Ishida et al. 1999). The STRE was found in the promoter region of *PnGlu1* and *PnGlu2*; thus we supposed that these two genes maybe are dramatically induced by physical factors like water activity and physical barriers to hyphal extension in sawdust medium.

Although *PnGlu1* and *PnGlu2* are clustered and display a high degree of homology, they present a very dissimilar expression pattern, especially in sawdust medium. The differential expression of these two glucoamylase genes during the development of fruiting bodies in sawdust medium suggest that the roles played by *PnGlu1* and *PnGlu2* in the mushroom developmental cycle are different. The highest expression of *PnGlu1* was in the mycelial growth stage (Fig. 2-6) and that of *PnGlu2* was in the fruiting body development stage (Fig. 2-7). The molecular mechanism behind the regulation of gene expression might be very complicated in sawdust medium. Binding sites for the transcriptional factor XYR1, which is involved in cellulase regulation, were identified in the promoter region of *PnGlu1* but not *PnGlu2*. We supposed that cellulose may induce *PnGlu1* expression (Yoon et al. 2006). AmyR is a transcriptional activator, which induces starch degradation genes expression (Petersen et al. 1999). The binding sequence of AmyR was only identified in *PnGlu2* promoter region, which was inferred that *PnGlu2* is a starch-induced gene. Our hypothesis is as follows: *P. microspora* produces cellulases that degrade the cellulose of sawdust medium at the mycelial stage, after which reaction products such as cellooligosaccharides maybe induce transcription of *PnGlu1* at the mycelial stage. At the primordia and fruiting body formation stages, all glucose and other nutrients may have been used for β-glucan synthesis (Li et al. 2012). When the amount of glucose decreases, the starch maybe is hydrolyzed to glucose by PnGlu2, which is one reason why *PnGlu2* gene expression increased at that stage.

We show here strong expression of glucoamylase gene *PnGlu2* in the fruiting stage of *P. microspora*, sugessting that PnGlu2 releases glucose from starch to provide nutrients for fruiting and therefore plays an important role in the morphogenesis of this basidiomycetous fungus.

Table 2-1. PCR primers used in this study

| Primer | Sequence | Use |
|---|---|---|
| 3RGlu2 | 5'-GTCTCTAGATAATGCTCTCGCTTTG-3' | 3'-Race |
| 5RGlu2P | 5'-pGCAGTTTGTGCACTG-3' | |
| 5RA1Glu2 | 5'-TGTGAATAGATAATTAGGATTCGTG-3' | |
| 5RA2Glu2 | 5'-CCTTGGCGACATACAAATCG-3' | 5'-Race |
| 5RS1Glu2 | 5'-GACTATCATCGATCAATTCACAAC-3' | |
| 5RS2Glu2 | 5'-ACACAACCTTGCGACCATTG-3' | |
| qGlu1F | 5'-ACTACTGCCTCTCGCAATAGTATTG-3' | Real-time PCR for amplification of *PnGlu1* |
| qGlu1R | 5'-ATTGCTCGATGACAGCAATAAAG-3' | |
| qGlu2F | 5'-ACGACATTCGTGACCAACT-3' | Real-time PCR for amplification of *PnGlu2* |
| qGlu2R | 5'-CCCATAGATCGAAAGTAGACTG-3' | |
| qActinF | 5'-CTTCACCACCACCGCCGA-3' | Real-time PCR for amplification of *Actin* |
| qActinR | 5'-CTTCAGGAGCACGGAATCGC-3' | |

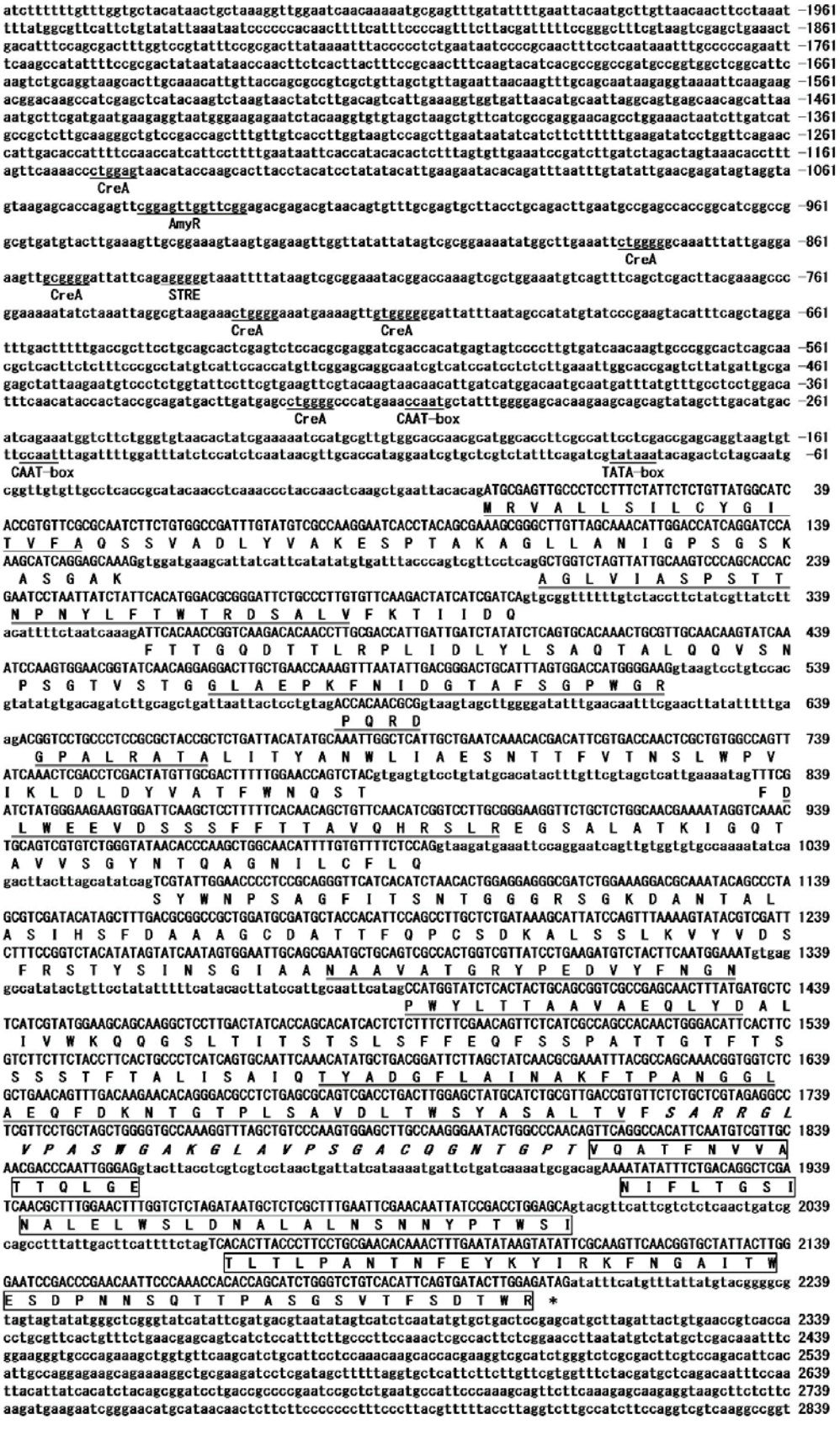

Fig. 2-1 Nucleotide and amino acid sequences of *PnGlu2* from *P. microspora*. *Capital letters* indicate exons and amino acid. Introns, 5'-upstream, and 3'-downstream are shown in *lowercase letters*. The predicted stop codon is indicated by an *asterisk*. The putative signal sequence is underlined. Five conserved sequences in catalytic domains are *double underlined*. The linker region is shown as *bold italics*. The consensus amino acids of the starch binding domain (SBD) are boxed. The putative responsive elements in the promoter region are also underlined with the abbreviations. STRE: stress responsive element; CreA: carbon catabolite repressor binding sites; AmyR: transcriptional activator of starch induced genes binding sites.

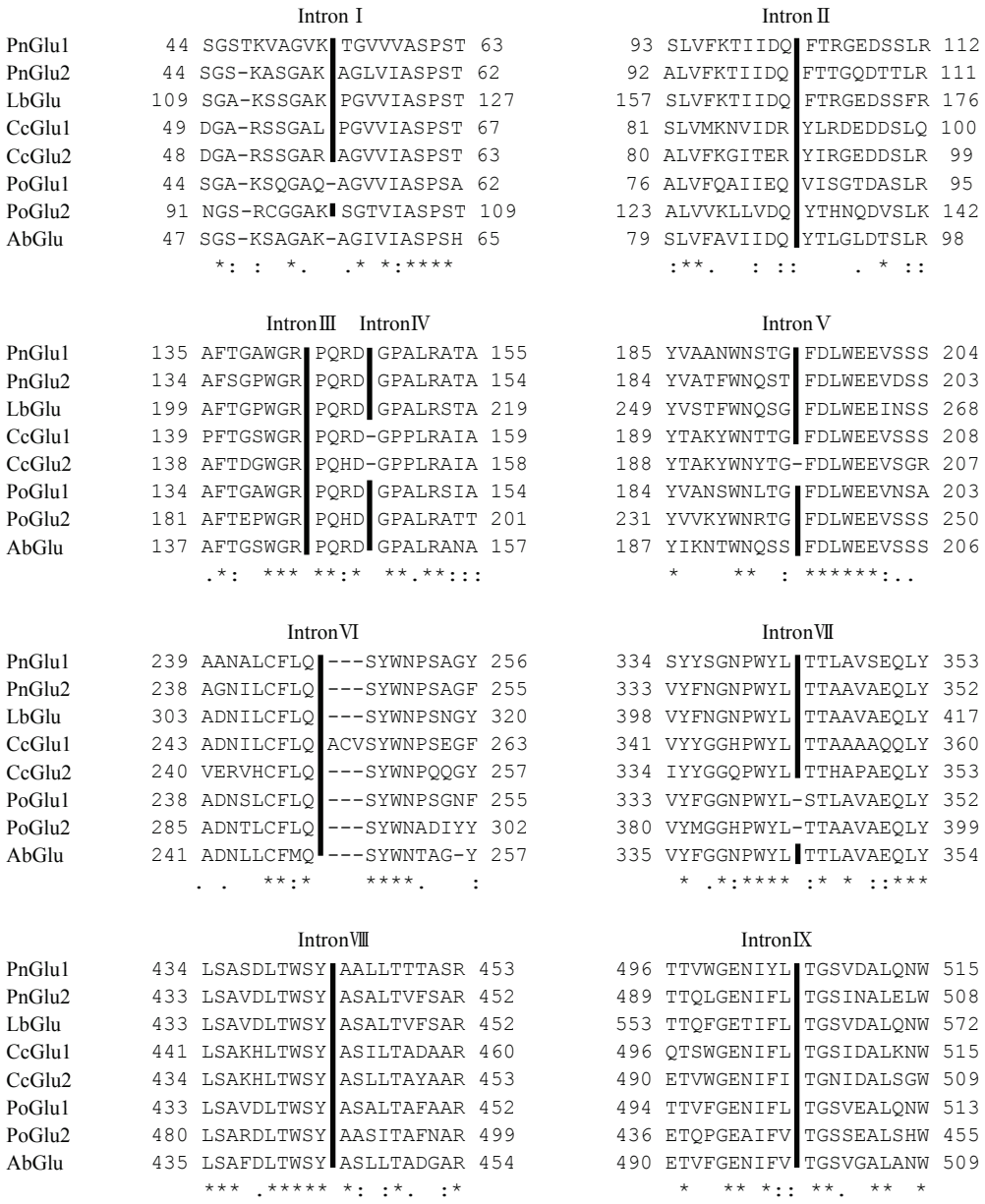


Fig. 2-2 Comparison of the glucoamylase amino acid sequence of *P. microspora* with corresponding sequences of other known species from the Agaricomycotina. Black bars indicate intron positions. Glucoamylase genes sequences are listed in the Joint Genome Institute (JGI) database with their position on the genome: *Agaricus bisporus* var. *bisporus* (H97) v2.0 (AbGlu scaffold_5: 607527-609814), *Coprinopsis cinerea* (*CcGlu1* Chr_8: 1774188-1776516, *CcGlu2* Chr_8: 1149866- 1151990), *Laccaria bicolor* v2.0 (LbGlu LG_4: 456677-459063), *Pleurotus ostreatus* PC15 v2.0 (*PoGlu1* scaffold_05: 2170159-2172303, *PoGlu2* scaffold_07: 2285935-2288248).

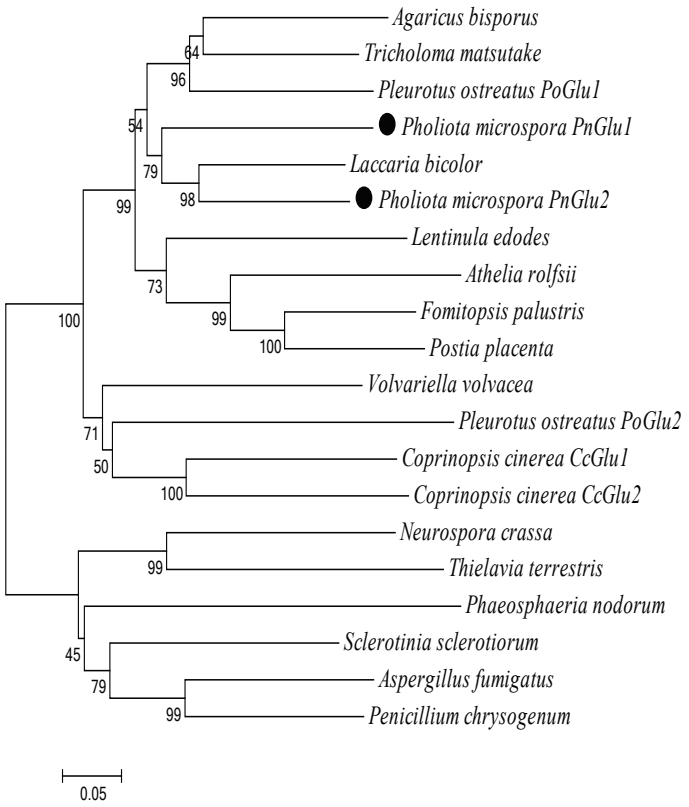


Fig. 2-3 Phylogenetic tree of the deduced amino acid sequences of *P. microspora* glucoamylases. The tree is calculated with *p*-distances using Mega ver. 5.05 software, based on a ClustalX alignment. The *scale bar* indicates a distance equivalent to 0.1 amino acid substitutions per site. Species and strains (with GenBank accession numbers) are: *Aspergillus fumigates* (EDP53734.1), *Athelia rolfsii* (Q12596), *Fomitopsis palustris* (AB239766), *Lentinula edodes* (Q9P4C5), *Neurospora crassa* (P14804), *Penicillium chrysogenum* (XP002560481), *Phaeosphaeria nodorum* (XP001805434), *Postia placenta* (B8PI57), *Sclerotinia sclerotiorum* (XP001588171), *Thielavia terrestris* (AAE85601), and *Tricholoma matsutake* (AB604354). The glucoamylase genes sequences are listed in the Joint Genome Institute (JGI) database with their position on genome: *Volvariella volvacea* V23 (VVO_00001: 1357756-1360059). The others species genes sequences were referred to Fig. 2-2 illustration.

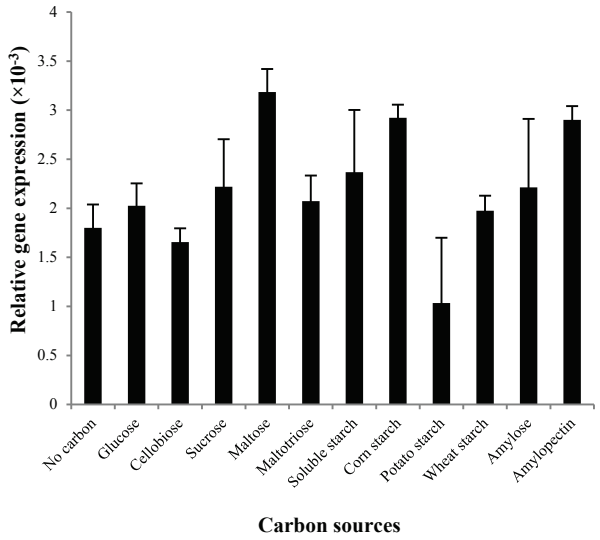


Fig. 2-4 *PnGlu1* expression levels in *P. microspora* grown in minimal media containing different carbon sources. Total RNA was extracted from mycelia after 24 h incubation.

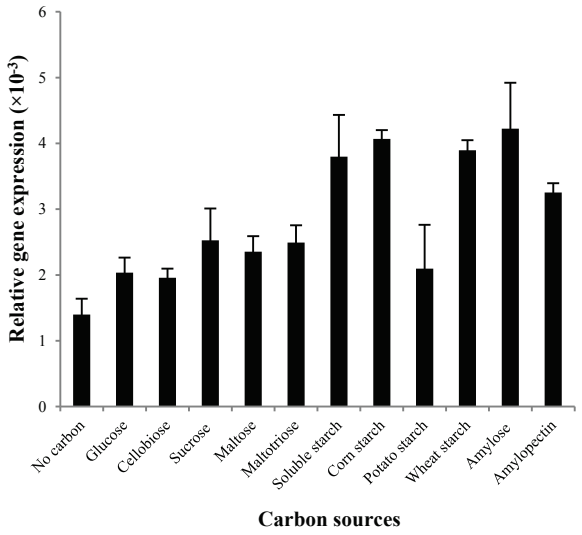


Fig. 2-5 *PnGlu2* expression levels in *P. microspora* grown in minimal media containing different carbon sources. Total RNA was extracted from mycelia after 24 h incubation.

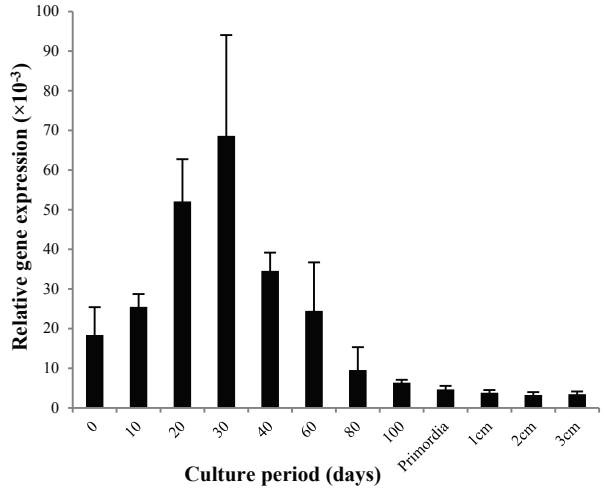


Fig. 2-6 *PnGlu1* expression levels in *P. microspora* grown in sawdust medium at different developmental stages. Total RNA was extracted from mycelia, primordia and fruiting bodies.

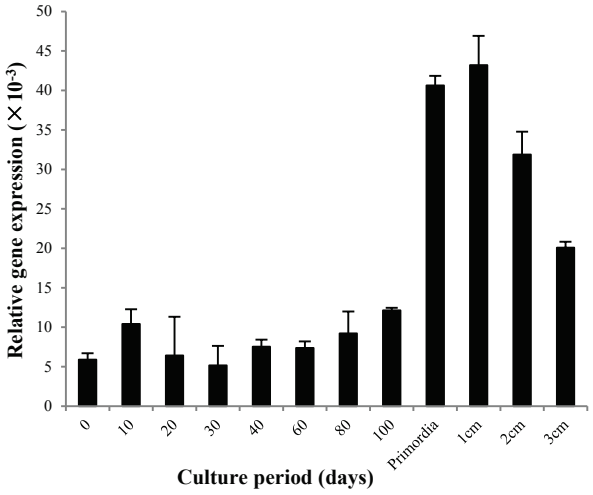


Fig. 2-7 *PnGlu2* expression levels in *P. microspora* grown in sawdust medium at different developmental stages. Total RNA was extracted from mycelia, primordia and fruiting bodies.

# Chapter 3

# Differential expression of three α-amylase genes from the basidiomycetous fungus *Pholiota microspora*

## 3-1 Abstract

The *Pholiota microspora* genome encodes three α-amylase genes (*PnAmy1, PnAmy2* and *PnAmy3*), which were identified and characterized in the current study. PnAmy1, PnAmy2 and PnAmy3 are members of glycoside hydrolase family 13, belonging to subfamily 32, 5 and 1, respectively. Transcription analysis was used to illuminate the physiological role of these α-amylases in *P. microspora*. Expression of *PnAmy1* and *PnAmy3* were regulated by various carbon sources in minimal media, which suggests that PnAmy1 and PnAmy3 play a role in starch degradation. In sawdust medium, PnAmy1 and PnAmy2 were closely correlated with fruiting body development, while PnAmy3 seems to play a role during vegetative mycelial growth.

## 3-2 Introduction

Starch is the second most abundant carbohydrate in nature (Buléon et al. 1998), and widely presents in plant storage organs (Tetlow et al. 2004). Because of the complex structure of starch, combinations of enzymes are required to degrade starch. These enzymes can be commonly divided into two groups, endoamylases and exoamylases (Chen et al. 2012).

Endoamylases such as α-amylases (EC 3.2.1.1) belong to the glycoside hydrolase (GH) 13 family, which randomly acts on α-1, 4-glycosidic linkages in starch and related polysaccharides to produce shorter maltooligosaccharides and α-limit dextrins (Pandey et al. 2000). GH13 is the largest GH family and groups together a number of enzymes with different activities and substrate specificities that act on α-glycosidic bonds. It has been divided into 40 official subfamilies by the CAZy (Carbohydrate-Active enZYmes) database curators. Within the GH13 family, α-amylase specificity is present in several subfamilies, such as GH13_1, 5, 6, 7, 15, 19, 24, 27, 28, 32, 36, and possibly a few more not yet defined (Stam et al. 2006). Exoamylases include glucoamylase (EC 3.2.1.3), α-glucosidase (EC 3.2.1.20) and β-amylase (EC 3.2.1.2). β-Amylases are typically of plant origin; fungi generally produce two types of exoamylase, glucoamylase

and α-glucosidase (Bertoldo and Antranikian 2002).

The basidiomycetous fungus *P. microspora* (Berk.) Sacc. [*P. nameko* (T. Ito) S. Ito & S. Imai] is an economically important edible mushroom in Japan. The identification and means of regulation of the α-amylase gene in *P. microspora* have not yet been reported. In this study, we cloned three α-amylase genes from *P. microspora* and examined their expression in minimal media supplemented with different carbon sources and in sawdust medium during the development cycle using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Our findings should clarify what role these genes play in *P. microspora*.

## 3-3 Materials and methods

### 3-3-1 Fungal strains

Monokaryotic strains of *P. microspora* NGW19-6 (*A4*, *pdx1*), an auxotrophic mutant for pyridoxine, and NGW12-163 (*A3*, *arg4*), an auxotrophic mutant for arginine, were used in this study. A dikaryotic strain obtained by crossing NGW19-6 and NGW12-163 was referred to as NGW19-6/12-163 (Masuda et al. 1995; Yi et al. 2009).

### 3-3-2 Genome sequencing and annotation

Genomic DNA was isolated from *P. microspora* NGW19-6 using the method of Dellaporta et al. (1983). The complete nucleotide sequence of the genomic DNA of monokaryotic NGW19-6 was determined using Illumina HiSeq 2000 paired-end technology provided by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). This sequencing run yielded 30,935,254 high-quality filtered reads with 101 bp paired-end sequencing. The genomic sequence was assembled using velvet assembler version 1.1.02 (hash length 85 bp) (Zerbino and Birney 2008). The final assembly contained 4,770 contigs of total length 33,400,256 bp, with an n50 length of 72,431 bp. Prediction of protein-coding sequences and annotation were performed by the Microbial Genome Annotation Pipeline (http://www.migap.org), which utilizes the MetaGeneAnnotator (Noguchi et al. 2008), RNAmmer (Lagesen et al. 2007), tRNAScan-SE (Lowe et al. 1997), and BLAST algorithms (Altschul et al. 1990).

### 3-3-3 RNA preparation

To prepare total RNA from *P. microspora* NGW19-6/12-163, the mycelium was grown on potato dextrose

agar at 25°C for 1 wk, and then 5 circular agar blocks (5 mm diameter) of mycelia were transferred into 10 ml minimal medium (1.5 g/l $(NH_4)_2HPO_4$, 1 g/l $KH_2PO_4$, 20 g/l glucose, 25 mg/ml thiamine hydrochloride, pH 5.5) in an Erlenmeyer flask and grown at 25 °C for 5 d. The mycelia were collected by filtration and washed three times with minimal medium containing no carbon source. The washed mycelia were suspended in 10 ml minimal medium containing 20 g/l glucose, 20 g/l cellobiose, 20 g/l sucrose, 20 g/l maltose, 20 g/l maltotriose, 20 g/l soluble starch, 20 g/l corn starch, 20 g/l amylose, 20 g/l amylopectin, or no carbon source. After 24 h of incubation, mycelia were harvested by filtration for RNA extraction.

To prepare total RNA from fruiting bodies, the cultivation of fruiting bodies of *P. microspora* NGW19-6/12-163 was carried out on a sawdust substrate. The substrate was prepared by mixing beech sawdust and rice bran at a gravimetric ratio of 5:1 and adjusting the moisture content to 65% using tap water. A 50 g substrate was placed into a 100 ml Erlenmeyer flask and autoclaved at 121 °C for 60 min. After cooling the flask in air, the circular agar blocks of NGW19-6/12-163 mycelia (5 mm diameter) were inoculated on the surface of the sawdust substrate and incubated at 25 °C in the dark. After completion of the spawn running (about 40 d after inoculation), the surface layer of the mycelia was scraped off by a spatula and then 50 ml sterilized distilled water was poured into the flask. The water was removed after the flasks had been incubated at 15 °C overnight, and then cultivation was continued at 15 °C under continuous fluorescent light at 200 lux to initiate fruiting bodies. The RNA from mycelia in sawdust substrate was isolated at 0 d, 10 d, 20 d, 30 d, 40 d, 60 d, 80 d and 100 d after removing water. We also isolated the RNA from primordia (about 120 d after removing water) and three stages of fruiting bodies (1 cm, 2 cm and 3 cm) in sawdust substrate. The RNA was extracted using a MagExtractor Kit (Toyobo, Osaka, Japan) according to the manufacturer′s instructions.

### 3-3-4 RT-PCR

Amplification of full-length cDNA by RT-PCR was carried out with a ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo) using total RNA as template. Amplification of 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE were performed with a Takara RNA PCR (AMV) version 3.0 kit (Takara Bio, Shiga, Japan) and a 5′-Full RACE Core Set (Takara Bio), respectively. PCRs were performed with a Takara PCR Thermal Cycler (Takara Bio). The PCR primers used in this study are shown in Table 1. The amplified fragments were subcloned into pMD20 T-vector (Takara Bio) and

sequenced.

### 3-3-5 Analysis of nucleotide and protein sequences

Nucleotide and protein sequence data were analyzed using GENETYX ver. 10.0.3 software (GENETYX, Tokyo, Japan). Protein motifs in the α-amylase and α-glucosidase amino acid sequence were identified using the web-based MOTIF search program (http://motif.genome.jp). The signal peptide position in the enzyme was predicted by the SignalP 4.1 server (http://www.cbs.dtu.dk/ services/SignalP/). A phylogenetic tree was constructed by MEGA 5.05 software (Tamura et al. 2011) using the neighbor-joining method with a bootstrap value of 1,000 replicates.

### 3-3-6 qRT-PCR assays

The actin gene (*act2* LC121583) was used as a reference gene. The primer pairs for amplification of cDNA of *PnAmy1, PnAmy2, PnAmy3* and *act2* were designed based on their cDNA sequences using GENETYX software. Amplification of genomic DNA was prevented by designing primers for exon-exon junctions. All primers were tested to ensure that they amplified a single band with no primer-dimers, as shown in Table 1. Plasmids with the inserted target gene (*PnAmy1, PnAmy2* or *PnAmy3*) or the housekeeping gene (*act2*) were extracted according to the method described by Birnboim (1983). Standard curves were constructed using five ten-fold dilutions of plasmid. Real-time PCR was performed using the KOD SYBR qPCR Mix kit (Toyobo). Thermocycling was carried out using a LineGene Real-Time Thermal Cycler (BioFlux, Tokyo, Japan), with an initial incubation for 2 min at 98°C, followed by 40 cycles of 98°C for 10 s, 60°C for 10 s and 68°C for 1 min. Each run was concluded with a melting curve analysis to confirm the specificity of amplification and absence of primer-dimers. Data analysis was performed according to the manufacturer′s instructions.

## 3-4 Results

### 3-4-1 Nucleotide sequence of α-amylase genes from *P. microspora*

The genomic DNA sequences of *PnAmy1*, *PnAmy2* and *PnAmy3* were identified by a tblastn search against the draft genome database of *P. microspora* using the α-amylase protein sequences of *Phanerochaete chrysosporium* (GH13_1 ABO42285.1), *Cryptococcus neoformans* (GH13_5 AFR96372.2), and *Phlebia radiata* (GH13_32 AFS18518.1).

The *PnAmy1*, *PnAmy2* and *PnAmy3* nucleotide sequences have been submitted to the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database under accession numbers LC074714, LC094144 and LC094145, respectively. The open reading frame region, from ATG to the stop codon, is 1767 bp in *PnAmy1*, 1560 bp in *PnAmy2*, and 1608 bp in *PnAmy3,* and encodes proteins of 589 amino acids in PnAmy1, 520 amino acids in PnAmy2, and 536 amino acids in PnAmy3. The locations of the initiation codon, stop codon and exons and introns were determined from the nucleotide sequences of the PCR products amplified by 3′- and 5′-RACE and RT-PCR. All of the introns started with GT and ended with AG. The coding region of *PnAmy1*, *PnAmy2*, and *PnAmy3* was interrupted by 16, 19 and 13 introns, respectively.

### 3-4-2 Characterization of protein sequence of α-amylases from *P. microspora*

SignalP 4.1 predicted the first 19 N-terminal amino acid residues of PnAmy1 and PnAmy3 might be a signal peptide sequence, suggesting that they are probably extracellular enzymes. PnAmy2 did not have any signal peptide sequences predicted by SignalP 4.1, indicating that it is an intracellular enzyme.

The amino acid sequences of PnAmy1, PnAmy2 and PnAmy3 were analyzed using MOTIF (Marchler-Bauer et al. 2012; Finn et al. 2013; Sigrist et al. 2013). PnAmy1 possesses an α-amylase catalytic domain (positioned at amino acids 70–342) and a C-terminal all-beta domain of α-amylase (amino acids 402–479). The starch-binding domain, belonging to the carbohydrate binding module 20 family, was found at amino acids 488–589, located at the C-terminus of PnAmy1. PnAmy2 and PnAmy3 also possess an α-amylase catalytic domain (amino acids 59–407 and 59–361, respectively), but they don't contain a starch-binding domain, indicating that PnAmy2 and PnAaamy3 lack the ability to attach to and degrade raw starch (Machovič and Janeček 2006). PnAmy1 has 18% and 20% identity with PnAmy2 and PnAmy3*,* respectively, and PnAmy2 has 15% identity with PnAmy3. The three α-amylases share very limited sequence identity. The notable amino acid sequence divergence among the three enzymes implies that they perform different functions in *P. microspora*.

### 3-4-3 Phylogenetic analysis of α-amylases from *P. microspora*

Fig. 3-1 shows a phylogenetic tree of α-amylases, constructed using representatives from each of the α-amylase subfamilies as well as PnAmy1, PnAmy2 and PnAmy3. The phylogenetic tree clearly shows that PnAmy1, PnAmy2 and PnAmy3 belong to α-amylase subfamily GH13_32, GH13_5 and GH13_1,

respectively. GH13_1 subfamily members are extracellular enzymes, and includes eukaryotic α-amylases from fungi and yeast only. GH13_5 are intracellular enzymes with amino acid sequences having high similarity to bacterial α-amylase. GH13_32 are extracellular enzymes, containing bacterial α-amylases mostly from actinomycetes (Stam et al. 2006). This evolutionary study suggests that the α-amylase in GH13_32 from basidiomycetes may have originated from Actinobacteria. Horizontal gene transfer has occurred from an Actinobacterium to an ancestor of the Agaricomycotina (Lage et al. 2013). These evolutionarily distinct α-amylase genes from three different subfamilies in *P. microspora* suggests that they may fulfill a variety of targeted functions for obtaining nutrients for their saprophytic lifestyle. In other species from the Agaricomycotina, α-amylases also appear in the same three subfamilies: GH13_1, GH13_5 and GH13_32.

### 3-4-4 Transcription analysis of α-amylase genes from *P. microspora*

To investigate the effect of carbon source on α-amylase gene expression, *P. microspora* was cultured in minimal medium containing glucose, cellobiose, sucrose, maltose, maltotriose, soluble starch, corn starch, amylose, amylopectin or no carbon source. To analyze the regulation of α-amylase biosynthesis on sawdust medium, the expression of *PnAmy1, PnAmy2* and *PnAmy3* was measured at three stages, during vegetative mycelial growth, at appearance of primordia and during fruiting body formation. Based on qRT-PCR, transcription of *PnAmy1, PnAmy2* and *PnAmy3* in minimal media and at various stages of the developmental cycle on sawdust medium is shown in Fig. 3-2A, Fig. 3-2B, and Fig. 3-2C, respectively.

The highest level of *PnAmy1* expression was observed when amylopectin was used as the sole carbon source in minimal medium after incubation for 24 h. In sawdust medium, the *PnAmy1* transcript level then increased rapidly the first 10 d and reached a peak at 30 d. Expression of *PnAmy1* decreased after 40 d until primordia developed. In the fruiting body formation stage, the expression of *PnAmy1* dramatically increased. The highest *PnAmy1* expression in the entire *P. microspora* development cycle appeared at the 2 cm fruiting body stage.

*PnAmy2* expression was barely induced by soluble starch other than glucose in minimal medium. In sawdust medium, *PnAmy2* expression increased at 10 d in the mycelial growth stage, then decreased rapidly and dropped to its lowest level at 40 d, and then increased beginning at 60 d until the primordia developed.

*PnAmy3* expression was higher in minimal medium supplemented with amylose than with other carbon sources. In sawdust medium, the highest *PnAmy3* expression in the entire development cycle was at 40 d.

*PnAmy1* expression at the mycelia stage in sawdust medium was similar to that in minimal medium containing different carbon sources, but it expression at the fruiting body stage in sawdust medium was dramatically higher. We inferred that pure starch is not the only inducer of its expression; other inducible factors at the fruiting body stage are required for the strikingly high expression of *PnAmy1*.

## 3-5 Discussion

In the present study, three α-amylase genes from *P. microspora* were identified and characterized. The presence of multiplex α-amylase genes in the *P. microspora* genome suggests that starch degradation is a very important feature of the saprophytic lifestyle in this mushroom. Phylogenetic analyses showed that *P. microspora* α-amylases PnAmy1, PnAmy2, and PnAmy3 might have originated from three sources, and that enzymes might have different functions. Transcription profiling of α-amylase genes may provide useful evidence for illustrating the physiological role of these three different types of α-amylase in *P. microspora*.

PnAmy1, which is closely related to bacterial α-amylases, belongs to GH13_32 (Janeček et al. 2014). The expression of PnAmy1 during fruiting body development was higher than in mycelia in sawdust medium. Abundant expression of PnAmy1 in the fruiting body stage suggests that it is important for mature fruiting body formation.

PnAmy2 is an intracellular enzyme belonging to GH13_5. The enzymes from this subfamily in other fungi are involved in the synthesis or modification of α-glucan in the fungal cell wall, rather than in starch degradation (Janeček et al. 2014). The α-amylase in this subfamily from dimorphic fungus *Histoplasma capsulatum* seems to participate in biosynthesis of α-(1, 3)-glucan, which is related to virulence of the strain (Marion et al. 2006). Starch and maltose did not induce *PnAmy2* expression in minimal medium, and the highest expression appeared at the primordia stage in sawdust medium, which implies that PnAmy2 may participate in α-glucan synthesis for the cell wall of primordia from *P. microspora* instead of in starch decomposition.

PnAmy3 is a member of GH13_1. The representative enzyme in GH13_1 from the fungus *Aspergillus oryzae* is used for the production of industrial α-amylase (Agger et al. 2001). We hypothesized that

PnAmy3 may be a key α-amylase decomposing starch in *P. microspora*. The highest *PnAmy3* expression appeared in minimal medium containing amylose, and expression in mycelia at 20, 30, 40, and 100d was higher than at the primordia or fruiting body stages in sawdust medium, which supports this conjecture.

The three α-amylases were constitutively expressed in mycelia in sawdust medium, presumably gradually decomposing the starch from rice bran into maltooligosaccharide or maltose. When *P. microspora* forms primordia and fruiting bodies, large amounts of mycelia may be needed. *PnAmy1* and *PnAmy2* expression dramatically increased at this time. Since synthesis of β-glucan, a major component of the cell wall of the primordia and fruiting body of *P. microspora*, requires glucose, and α-glucosidase degrades maltose into glucose, this suggests that an α-glucosidase gene may also be present in the *P. microspora* genome. Further research is aimed at cloning and characterizing this gene.

In this study, we performed a transcriptional analysis of *P. microspora* α-amylase gene expression, focusing on inducibility by different carbon sources in minimal media and investigating the function of these genes at different developmental stages. The puzzle of α-amylase gene functions in *P. microspora* will be clarified in future studies using genetic manipulation, such as gene silencing or overexpression *in vivo*.

Table 3-1 PCR primers used in this study.

| Primer | Sequence | Use |
|---|---|---|
| 3RAmy1 | 5'-CTGTTTCTTTGCGAATAAAC-3' | 3′-RACE of *PnAmy1* |
| 5RAmy1P | 5'-pGATATGTTTTGC-3' | 5′-RACE of *PnAmy1* |
| 5RA1Amy1 | 5'-TATTCAGTCTCTGTCGCCAG-3' | |
| 5RA2Amy1 | 5'-TAAGCTCGCAAGTCTGGACT-3' | |
| 5RS1Amy1 | 5'-TGCATATGGAAACGACCTGC-3' | |
| 5RS2Amy1 | 5'-GGTGTTGATGGATTCCGTCT-3' | |
| qAmy1F | 5'-ATCACTTCACGCCTCACCAC-3' | Real-time PCR for amplification of *PnAmy1* |
| qAmy1R | 5'-TTCCCGATACCCATCCTTG -3' | |
| 3RAmy2 | 5'-GTATGGGGCAACTGAAGATTAC-3' | 3′-RACE of *PnAmy2* |
| 5RAmy2P | 5'-pACAGCATCTATCAG-3' | 5′-RACE of *PnAmy2* |
| 5RA1Amy2 | 5'-TGTAGGCAATCCCAGGTGAAG-3' | |
| 5RA2Amy2 | 5'-GGGTTGTCATTGCTTCCTTC-3' | |
| 5RS1Amy2 | 5'-GCCGAGTTAGGAGTCACTCAAG-3' | |
| 5RS2Amy2 | 5'-TGGGGCACTCGAGAAGAATTAC-3' | |
| qAmy2F | 5'-CGACCAAAAGGGAAGCATTAAAAC-3' | Real-time PCR for amplification of *PnAmy2* |
| qAmy2F | 5'-TGTTGGCGAAGCAGGAAAATATAC-3' | |
| 3RAmy3 | 5'-TATCTCCATTGTCACCAACATTG-3' | 3′-RACE of *PnAmy3* |
| 5RAmy3P | 5'-pTGATTCACAGGAC-3' | 5′-RACE of *PnAmy3* |
| 5RA1Amy3 | 5'-GGGATCCGTTCCGTTAGGAG-3' | |
| 5RA2Amy3 | 5'-CTCGCCATTGATCTGCAGTG-3' | |
| 5RS1Amy3 | 5'-GAACCTGGTGTGGTGGTAGTTG-3' | |
| 5RS2Amy3 | 5'-CGCGAGAATCTCGATTACGTC-3' | |
| qAmy3F | 5'-TGTCTATTATGGGCAAGAGC-3' | Real-time PCR for amplification of *PnAmy3* |
| qAmy3R | 5'-GTGGAAGGCAAGTATGGTG-3' | |
| qActinF | 5'-CTTCACCACCACCGCCGA-3' | Real-time PCR for amplification of *Actin* |
| qActinR | 5'-CTTCAGGAGCACGGAATCGC-3' | |

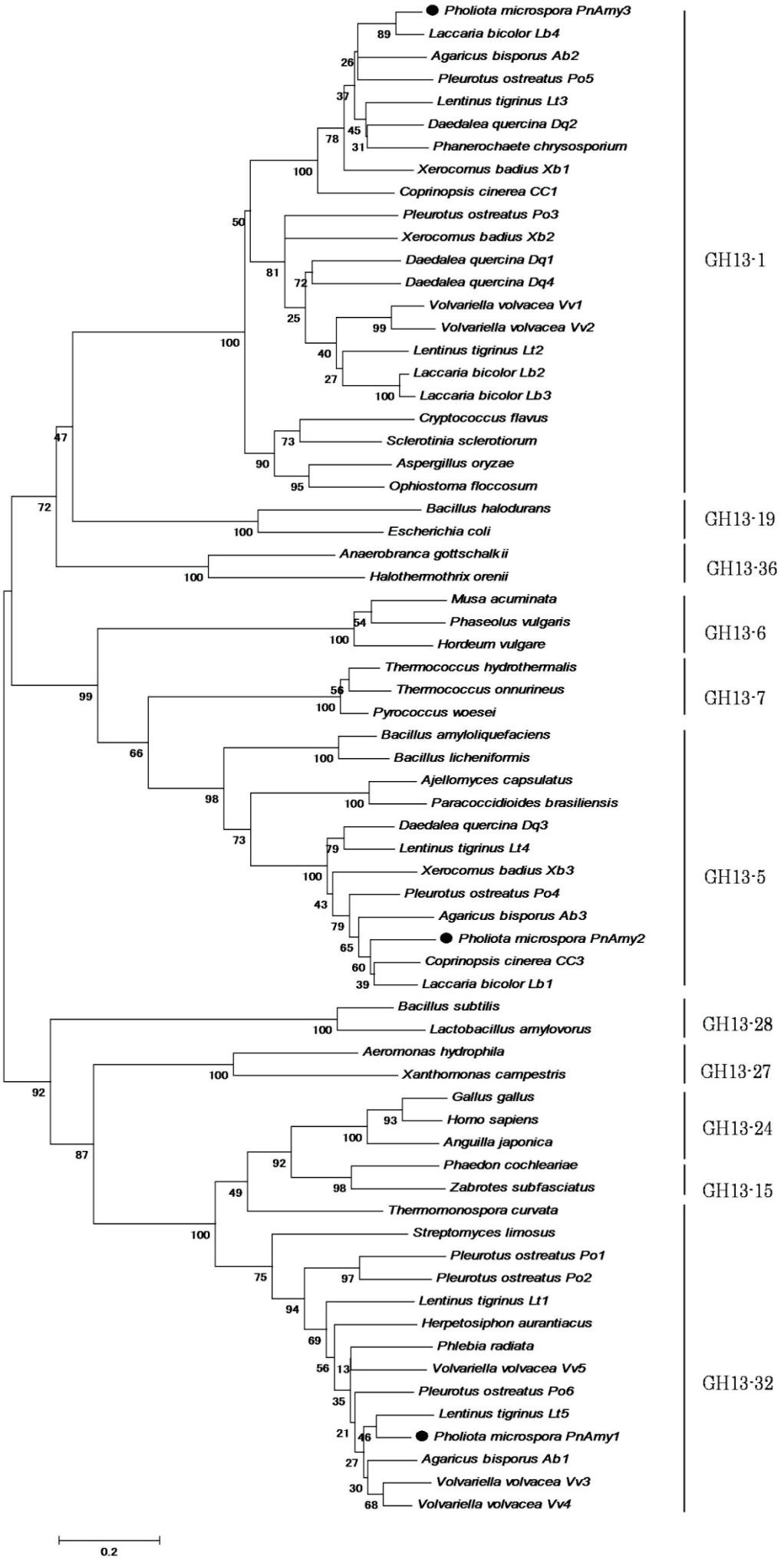
Pholiota microspora PnAmy3
Laccaria bicolor Lb4
Agaricus bisporus Ab2
Pleurotus ostreatus Po5
Lentinus tigrinus Lt3
Daedalea quercina Dq2
Phanerochaete chrysosporium
Xerocomus badius Xb1
Coprinopsis cinerea CC1
Pleurotus ostreatus Po3
Xerocomus badius Xb2
Daedalea quercina Dq1
Daedalea quercina Dq4
Volvariella volvacea Vv1
Volvariella volvacea Vv2
Lentinus tigrinus Lt2
Laccaria bicolor Lb2
Laccaria bicolor Lb3
Cryptococcus flavus
Sclerotinia sclerotiorum
Aspergillus oryzae
Ophiostoma floccosum
Bacillus halodurans
Escherichia coli
Anaerobranca gottschalkii
Halothermothrix orenii
Musa acuminata
Phaseolus vulgaris
Hordeum vulgare
Thermococcus hydrothermalis
Thermococcus onnurineus
Pyrococcus woesei
Bacillus amyloliquefaciens
Bacillus licheniformis
Ajellomyces capsulatus
Paracoccidioides brasiliensis
Daedalea quercina Dq3
Lentinus tigrinus Lt4
Xerocomus badius Xb3
Pleurotus ostreatus Po4
Agaricus bisporus Ab3
Pholiota microspora PnAmy2
Coprinopsis cinerea CC3
Laccaria bicolor Lb1
Bacillus subtilis
Lactobacillus amylovorus
Aeromonas hydrophila
Xanthomonas campestris
Gallus gallus
Homo sapiens
Anguilla japonica
Phaedon cochleariae
Zabrotes subfasciatus
Thermomonospora curvata
Streptomyces limosus
Pleurotus ostreatus Po1
Pleurotus ostreatus Po2
Lentinus tigrinus Lt1
Herpetosiphon aurantiacus
Phlebia radiata
Volvariella volvacea Vv5
Pleurotus ostreatus Po6
Lentinus tigrinus Lt5
Pholiota microspora PnAmy1
Agaricus bisporus Ab1
Volvariella volvacea Vv3
Volvariella volvacea Vv4
GH13-1
GH13-19
GH13-36
GH13-6
GH13-7
GH13-5
GH13-28
GH13-27
GH13-24
GH13-15
GH13-32
0.2

Fig. 3-1. Phylogenetic tree of the deduced amino acid sequences of *Pholiota microspora* α-amylase. The tree was calculated with *p*-distances using Mega ver. 5.05, based on a ClustalX alignment. The *scale bar* indicates a distance equivalent to 0.2 amino acid substitutions per site. Species and strains (with Taxonomy, GH subfamily and UniProtKB accession numbers) are: *Aspergillus oryzae* (Fungi 1 P0C1B3), *Cryptococcus flavus* (Fungi 1 A7LGW4), *Ophiostoma floccosum* (Fungi 1 Q06SN2), *Sclerotinia sclerotiorum* (Fungi 1 C0LW29), *Ajellomyces capsulatum* (Fungi 5 A0T074), *Paracoccidioides brasiliensis* (Fungi 5 A7L832), *Bacillus amyloliquefaciens* (Bacteria 5 P00692), *Bacillus licheniformis* (Bacteria 5 P06278), *Hordeum vulgare* (Eukaryota 6 P00693), *Musa acuminata* (Eukaryota 6 AEO09337), *Phaseolus vulgaris* (Eukaryota 6 Q9ZP43), *Pyrococcus woesei* (Archaea 7 Q7LYT7), *Thermococcus hydrothermalis* (Archaea 7 O93647), *Thermococcus onnurineus* (Archaea 7 B6YUG5), *Phaedon cochleariae* (Eukaryota 15 O97396), *Zabrotes subfasciatus* (Eukaryota 15 Q9N2P9), *Bacillus halodurans* (Bacteria 19 Q5GIA5), *Escherichia coli* (Bacteria 19 P25718), *Anguilla japonica* (Eukaryota 24 Q8QGW2), *Gallus gallus* (Eukaryota 24 Q98942), *Homo sapiens* (Eukaryota 24 P04746), *Aeromonas hydrophila* (Bacteria 27 P22630), *Xanthomonas campestris* (Bacteria 27 Q56791), *Bacillus subtilis* (Bacteria 28 P00691), *Lactobacillus amylovorus* (Bacteria 28 Q48502), *Streptomyces limosus* (Bacteria 32 P09794), *Thermomonospora curvata* (Bacteria 32 P29750), *Herpetosiphon aurantiacus* (Fungi 32 CCA69081), *Phledia radiata* (Fungi 32 J9WQR4), *Anaerobranca gottschalkii* (Bacteria 36 Q5I942), and *Halothermothrix orenii* (Bacteria 36 Q8GPL8). The other α-amylase gene sequences from the Agaricomycotina are listed in the Joint Genome Institute (JGI) database with their position on the genome: *Agaricus bisporus* var. *bisporus* (H97) v2.0 (*AbAmy1* scaffold_13: 170570-172513, *AbAmy2* scaffold_2: 1562453-1564986, *AbAmy3* scaffold_3: 1973395-1976053), *Coprinopsis cinerea* (*CcAmy1* Chr_2: 1978356-1980938, *CcAmy2* Chr_4: 2366073-2368907), *Daedalea quercina* v1.0 (*DqAmy1* scaffold_62: 89961-92692, *DqAmy2* scaffold_3: 700238- 702811, *DqAmy3* scaffold_5: 593479-596231, *DqAmy4* scaffold_62: 73259-75682), *Laccaria bicolor* v2.0 (*LbAmy1* LG_3: 3953860-3956394, *LbAmy2* LG_6: 2839663-2841986, *LbAmy3* LG_6: 2848039-2850368, *LbAmy4* scaffold_11: 2063663-2066025), *Lentinus tigrinus* v1.0 (*LtAmy1* scaffold_28: 105439-107396, *LtAmy2* scaffold_31: 187104-189961, *LtAmy3* scaffold_4: 230296-232654, *LtAmy4* scaffold_7: 968220-971047, *LtAmy5* scaffold_3: 1582177-1584723), *Pleurotus ostreatus* PC15 v2.0 (*PoAmy1* scaffold_01: 39235-41323, *PoAmy2* scaffold_01: 42172-44215, *PoAmy3* scaffold_03: 814385-817278, *PoAmy4* scaffold_08: 1943413-1945993, *PoAmy5* scaffold_01: 3373909-3376452, *PoAmy6* scaffold_03: 302013-304549), *Volvariella volvacea* V23 (*VvAmy1* VVO_00038: 36242-38444, *VvAmy2* VVO_00001: 261512-263809, *VvAmy3* VVO_00033: 94922-97382, *VvAmy4* VVO_00001: 238547- 241199, *VvAmy5* VVO_00001: 1325519-1328391), and *Xerocomus badius* 84.06 v1.0 (*XbAmy1* scaffold_7: 42186-44812, *XbAmy2* scaffold_243: 7130-9134, *XbAmy3* scaffold_60: 26653-28917).

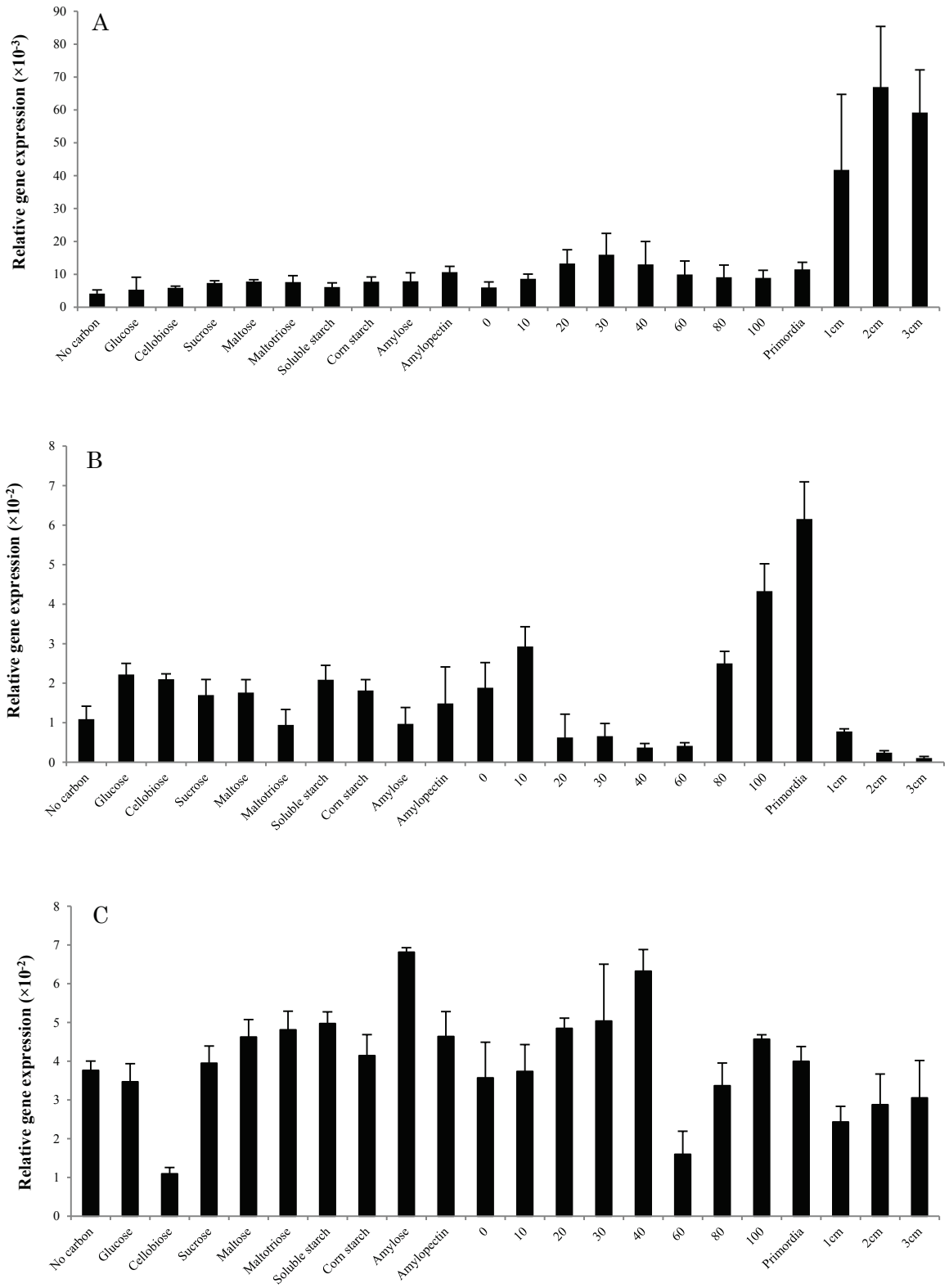


Fig. 3-2. Expression of three *Pholiota microspora* α-amylase genes in minimal media containing various carbon sources and at different developmental stages in sawdust medium. A, *PnAmy1*; B, *PnAmy2*; C, *PnAmy3*.

# Chapter 4

# Expression of α-glucosidase during morphological differentiation in the basidiomycetous fungus *Pholiota microspora*

## 4-1 Abstract

The α-glucosidase gene from *Pholiota microspora*, designated PnGcs, was amplified and characterized. The open reading frame region of PnGcs, from ATG to the stop codon, is 2937 bp and encodes a protein of 979 amino acids with a signal peptide of 20 amino acids at the N-terminus. The predicted amino acid sequence of PnGcs indicated that it is a glycoside hydrolase family 31 protein. Quantitative reverse transcription PCR was used to investigate PnGcs expression in mycelia cultured in minimal medium containing various carbon sources, as well as in tissue during different stages of development of fruiting bodies. When *P. microspora* was grown in minimal medium supplemented with different carbon sources, PnGcs expression was highest when induced by maltose. During cultivation on sawdust medium, PnGcs expression increased dramatically at the fruiting body formation stage compared with the mycelial growth stage, which implied that PnGcs is closely associated with fruiting body development.

## 4-2 Introduction

α-Glucosidases (EC 3.2.1.20; α-D-glucoside glucohydrolase) are a group of exo-acting enzymes that catalyze the liberation of α-D-glucose from the non-reducing terminus of substrates (Chiba 1988). Various types of α-glucosidases from mammals, insects, plants, fungi and bacteria have been purified and the nucleotide sequences of their genes have been identified (Yamamoto et al. 2004). They were suggested to be grouped into two major families based on their primary structure, glycoside hydrolase families 13 and 31. Most α-glucosidases from bacteria and insects belong to GH13 and those from plants, animals, and fungi belong to GH31. According to their substrate specificity, these enzymes are conventionally classified into three types. Type I hydrolyze heterogeneous substrates, more efficiently those with sucrose and *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) than homogeneous substrates such as maltose. Type II enzymes are more specific for maltose and have low activity on heterogeneous substrates. Type III enzymes resemble type II, but hydrolyze oligosaccharides and starch at similar rates. Family 13 includes

enzymes designated as type I. The α-glucosidase types II and III are classified as family 31 (Henrissat 1991; Henrissat and Bairoch 1993; Chiba 1997).

The basidiomycetous fungus *P. microspora* (T. Ito) S. Ito & S. Imai is an economically important edible mushroom in Japan. Following sequencing of its genome, we identified and characterized three α-amylase genes (*PnAmy1*, *PnAmy2* and *PnAmy3*) in previous work, which encode proteins involved in starch degradation that randomly act on α-1, 4-glycosidic linkages in starch to produce shorter maltooligosaccharides and α-limit dextrins (Pandey et al. 2000). When mushrooms form fruiting bodies, a large amount of glucose is needed as an energy source (Hirato and Kitamoto 1995), so more detailed information about α-glucosidases is essential to elucidate the starch degradation process and its role in this mushroom. The identification and regulation of the α-glucosidase gene in *P. microspora* has not yet been reported. In the present work, we cloned the α-glucosidase gene from *P. microspora* and examined its expression in minimal media supplemented with different carbon sources. We also measured α-glucosidase activity and gene expression during the developmental cycle of *P. microspora* in sawdust media.

## 4-3 Materials and methods

### 4-3-1 Fungal strains and culture conditions

Monokaryotic strains of *P. microspora*, NGW19-6 (*A4*, *pdx1*), an auxotrophic mutant for pyridoxine, and NGW12-163 (*A3*, *arg4*), an auxotrophic mutant for arginine, were used in this study (Masuda et al. 1995; Yi et al. 2009). A dikaryotic strain obtained by crossing NGW19-6 and NGW12-163 was referred to as NGW19-6/12-163.

To analyze the effect of carbon source on gene expression, strain NGW19-6/12-163 was grown on potato dextrose agar at 25°C for 1 week, and then 5 circular agar blocks (5 mm diameter) of mycelia were transferred into 10 ml minimal medium (1.5 g/l $(NH_4)_2HPO_4$, 1 g/l $KH_2PO_4$, 20 g/l glucose, 25 mg/ml thiamine hydrochloride, pH 5.5) in an Erlenmeyer flask and grown at 25°C for 5 days. The mycelia were collected by filtration and washed three times with minimal medium containing no carbon source. The washed mycelia were suspended in 10 ml minimal medium containing 20 g/l glucose, 20 g/l cellobiose, 20 g/l sucrose, 20 g/l maltose, 20 g/l soluble starch, 20 g/l corn starch, 20 g/l potato starch, 20 g/l wheat starch, 20 g/l amylose, 20 g/l amylopectin, or no carbon source. After 24 h of incubation, mycelia were harvested by filtration for RNA extraction.

Fruiting bodies of *P. microspora* were cultivated on a sawdust substrate. The sawdust substrate was prepared as follows: beech sawdust was mixed with rice bran at a gravimetric ratio of 5: 1 and adjusted to 65% moisture using tap water, and the medium was put into a 100 mL Erlenmeyer flask and autoclaved at 121°C for 60 min. After cooling the medium, it was inoculated with circular agar blocks of NGW19-6/12-163 mycelia (5 mm diameter) and incubated at 25°C. When the mycelia had colonized the substrate (about 40 days after inoculation), the surface layer of the mycelia was scratched by a spatula and then 50 ml sterilized distilled water was poured into the flask. The water was removed after the flasks had been incubated at 15°C overnight, and then cultivation was continued at 15°C until the fruiting body developed. We defined the day after removing the water as day 0. The RNA from mycelia in sawdust substrate was isolated at 0 d, 10 d, 20 d, 30 d, 40 d, 60 d, 80 d and 100 d. We also isolated the RNA from primordia (about 120 d after removing water) and three stages of fruiting bodies (total length included cap and stipe is 1 cm, 2 cm and 3 cm, respectively) in sawdust substrate.

### 4-3-2 Genome sequencing and annotation

The complete nucleotide sequence of the genomic DNA of monokaryotic NGW19-6 was determined using Illumina HiSeq 2000 paired-end technology provided by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). This sequencing run yielded 30,935,254 high-quality filtered reads with 101 bp paired-end sequencing. The genomic sequence was assembled using velvet assembler version 1.1.02 (hash length 85 bp) (Zerbino and Birney, 2008). The final assembly contained 4,770 contigs of total length 33,400,256 bp, with an n50 length of 72,431 bp. The prediction of protein-coding sequences and annotation was performed by the Microbial Genome Annotation Pipeline (http://www.migap.org/), which utilizes the MetaGeneAnnotator (Noguchi et al. 2008), RNAmmer (Lagesen et al. 2007), tRNAScan-SE (Lowe and Eddy, 1997), and BLAST algorithms (Altschul et al. 1990).

### 4-3-3 DNA and RNA preparation and cDNA synthesis

Genomic DNA extraction was followed the method of Dellaporta（Dellaporta et al. 1983）. The RNA was extracted using a MagExtractor Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The cDNA was synthesized using total RNA as template by ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo).

Amplification of cDNA fragments and 3′-rapid amplification of cDNA ends (RACE) were performed

with a Takara RNA PCR (AMV) version 3.0 kit (Takara Bio, Shiga, Japan) and 5′-RACE with a 5′-Full RACE Core Set (Takara Bio). PCR was carried out according to the manufacturer's instructions using the oligonucleotide primers listed in Table 4-1. The amplified fragments were subcloned into pMD20 T-vector (Takara Bio) and sequenced.

### 4-3-4 Analysis of nucleotide and protein sequences

Nucleotide and protein sequence data were analyzed using GENETYX ver. 10.0.3 software (GENETYX, Tokyo, Japan). Protein motifs in the α-amylase and α-glucosidase amino acid sequence were identified using the web-based MOTIF search program (http://motif.genome.jp). The signal peptide position and subcellular location of enzymes was predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and TargetP 1.1 (http://www.cbs.dtu.dk/services/ TargetP/) software, respectively. A phylogenetic tree was constructed by MEGA 5.05 software using the neighbor-joining method with a bootstrap value of 1,000 replicates (Tamura et al. 2011).

### 4-3-5 Quantitative reverse transcription-PCR assays

The actin gene (*act2* LC121583) was used as a reference gene. The primer pairs for amplification of cDNA of *PnGcs* and *act2* were designed based on their cDNA sequences using GENETYX software. Amplification of genomic DNA was prevented by designing primers for exon-exon junctions. All primers were tested to ensure that they amplified a single band with no primer-dimers, as shown in Table 1. Plasmids with the inserted target gene (*PnGcs*) or the housekeeping gene (*act2*) were extracted according to the method described by Birnboim, 1983. Standard curves were constructed using five ten-fold dilutions of plasmid. Real-time PCR was performed using the KOD SYBR qPCR Mix kit (Toyobo). Thermocycling was carried out using a LineGene Real-Time Thermal Cycler (BioFlux, Tokyo, Japan), with an initial incubation for 2 min at 98°C, followed by 40 cycles of 98°C for 10 s, 60°C for 10 s and 68°C for 1 min. Each run was completed with a melting curve analysis to confirm the specificity of amplification and absence of primer-dimers. Data analysis was performed according to the manufacturer′s instructions.

### 4-3-6 Measurement of α-glucosidase activity

For the measurement of changes in α-glucosidase activity of *P. microspora* in sawdust medium, mycelium with sawdust or whole fruiting body tissue was frozen in liquid nitrogen, ground in a mortar and pestle to a

fine powder, and then transferred into 0.1 M sodium acetate buffer, pH 5.0. Crude enzyme solution was centrifuged (8000 x *g*, 10 min). α-Glucosidase activity was measured with a Glucoamylase and α-Glucosidase Assay Kit (Kikkoman, Tokyo, Japan). Protein concentration was determined by the Bradford assay (Bradford, 1976). The relative enzyme activity is presented as the ratio of enzyme activity to protein concentration.

## 4-4 Results

### 4-4-1 Nucleotide sequence of *PnGcs* from *P. microspora*

The *PnGcs* nucleotide sequence has been submitted to the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database under accession number LC074713. The open reading frame region, from ATG to the stop codon, is 2937 bp and encodes a protein of 979 amino acids. The location of the initiation and stop codons, and the exons and introns of the gene were determined from the nucleotide sequences of the PCR products amplified by 3′- and 5′-RACE PCR and RT-PCR products. All of the introns started with GT and ended with AG. The coding region of *PnGcs* gene was interrupted by 7 introns.

The nucleotide sequence of the 5′-flanking region of the *PnGcs* gene is shown in Fig. 4-1. The 1999 bp upstream of the ATG start codon in *PnGcs* were analyzed. There were three CAAT boxes at positions -1237, -757 and -743, and one GC box at position -17 in the promoter region of *PnGcs*. The stress responsive element STRE (5′-AGGGG-3′), which has been suggested to function in osmotic stress induction (Moskvina et al. 1998), was found in the *PnGcs* promoter region at positions -1430 and -764. The carbon catabolite repressor (CREA) binding site (5′-SYGGRG-3′), identified as involved in glucose repression (Dowzer and Kelly, 1991), was identified in *PnGcs* at position -435, suggesting that transcription of the α-glucosidase gene may be repressed by glucose.

### 4-4-2 Characterization of protein sequence of PnGcs of *P. microspora*

The first 20 N-terminal amino acid residues of PnGcs may be a signal peptide sequence, as predicted by SignalP 4.1. TargetP 1.1 predicts that PnGcs are extracellular enzymes. The putative mature protein has a molecular mass of 107.81 kDa after removal of the signal peptide.

The amino acid sequence of PnGcs was analyzed using MOTIF. The protein PnGcs possesses a α-1, 4-glycosyltransferase conserved region (amino acids 358 to 813). PnGcs has the highest amino acid

identity (86%) with the glycoside hydrolase family 31 protein from *Heterobasidion irregular* and 75% identity with the α-glucosidase from *Coprinopsis cinerea*. Thus the PnGcs protein appears to be a α-glucosidase and a member of glycoside hydrolase family 31 (GH31).

A phylogenetic tree of α-glucosidases, constructed using GH 31 α- glucosidases from other known species, is shown in Fig. 4-2. PnGcs clusters with the *C. cinerea* enzyme, and forms a single clade.

### 4-4-3 Constitutive expression of *PnGcs* in *P. microspora*

To analyze the regulation of α-glucosidase biosynthesis, *P. microspora* was cultivated on different carbon sources in minimal medium. After 24 h of incubation, relative differences in gene expression on media supplemented with different carbon sources were detectable (Fig. 4-3). After 24 h of incubation, expression of the α-glucosidase gene in minimal medium containing maltose was the highest of the tested carbon sources in this study, which also included amylopectin, amylose, wheat starch, potato starch, corn starch, soluble starch, sucrose, cellobiose and glucose, as well as in the absence of a carbon source. The lowest *PnGcs* expression appeared when cellobiose was added to the minimal medium. *PnGcs* was equally expressed when cultured both in the presence and absence of glucose, and expression in the culture medium containing maltose was approximately two times higher in the presence or absence of glucose. Thus, *PnGcs* appears to be constitutively transcribed by *P. microspora*, but maltose promotes its expression.

There is no direct evidence that starch or starch hydrolysates mediate the promotion of *PnGcs* expression like maltose did in this study, so we tested this by examining the time course of *PnGcs* expression in minimal medium containing soluble starch or maltose (Fig. 4-4). The maximum amount of *PnGcs* transcript was detected after 6 h when maltose was used as the sole carbon source. This promotion of *PnGcs* transcription with maltose was more rapid than promotion with soluble starch when used as a sole carbon source. The soluble starch did not affect the transcription of *PnGcs* after 6 h; at least 12 h were required for *P. microspora* cells to induce the transcription of *PnGcs* in response to soluble starch. Therefore, soluble starch hydrolysate components, such as maltooligosaccharides, might be required for the promotion of α-glucosidase transcription in *P. microspora* cells.

### 4-4-4 PnGcs production and gene expression during growth and fruiting of *P.*

## *microspora* on sawdust medium

To investigate α-glucosidase production on sawdust medium, α-glucosidase activity was measured at three stages: during vegetative mycelial growth, at appearance of primordia, and during fruiting body formation. Fig. 4-5 shows that the α-glucosidase activity at the primordia and fruiting body formation stages dramatically increased over the mycelial growth stage. The highest enzyme activity was observed in the primordia throughout the whole *P. microspora* development cycle.

To determine the reasons for the increased α-glucosidase activity on sawdust medium, quantitative reverse transcription-PCR was used to study gene expression. The transcription of α-glucosidase in the mycelial stage and at various developmental stages when *P. microspora* was grown on sawdust medium is shown in Fig. 4-6. In the mycelial stage, the number of transcripts of *PnGcs* decreased rapidly, dropping to the lowest levels at 40 days, and then increased from 60 days until the primordia had developed. α-Glucosidase gene expression at the primordia and fruiting body formation stages also dramatically increased relative to the mycelia growth stage. The highest *PnGcs* expression during the entire *P. microspora* development cycle appeared in 1 cm fruiting bodies, and then decreased.

## 4-5 Discussion

In the present study, an α-glucosidase gene, *PnGcs*, was identified and characterized from the *P. microspora* genome. PnGcs was identified as a GH31 family protein based on amino acid sequence similarity. The lowest gene expression appeared when cellobiose, the smallest cellooligosaccharide with a β-1, 4-linkage, was added to minimal medium, which implies that PnGcs is a specific α-glucosidase that hydrolyzes α-1, 4-bonds. One CREA binding site appeared in the *PnGcs* promoter region, but *PnGcs* was equally expressed in culture both in the presence and absence of glucose. We inferred that the glucose concentration did not affect *PnGcs* expression, or that the glucose concentration used in this study was below a critical concentration that represses *PnGcs* expression, since *PnGcs* might be expressed to accumulate glucose, and thus a low concentration of glucose should not affect its expression.

In *Aspergillus oryzae*, metabolism and regulation of maltose requires a functional *MAL* locus, which is composed of a cluster of three genes: *MALT* encoding maltose permease that is a maltose transmembrane transporter protein, *MALS* encoding maltase (EC 3.2.1.20) and *MALR*, encoding a transcriptional activator

specifically activating expression of the *MALT* and *MALS* genes (Vongsangnak et al. 2009). The *MALR* transcription factor induces maltose permeases to transport extracellular maltose into the cell and *MALR* also induces maltase, which hydrolyses intracellular maltose into glucose (Vongsangnak et al. 2009). In *Aspergillus niger*, *MAL* regulons are not present, so it exists one other regulatory system that involves the AmyR regulator for maltose metabolism (Yuan et al. 2008). AmyR activates genes encoding known extracellular starch-degrading enzymes, such as glucoamylase, α-amylase and α-glucosidase from *A. niger* (Yuan et al. 2008). In *P. microspora*, we did not identify the *MAL* gene cluster in genome using the gene structure of the *MAL* locus of *A. oryzae* as a model. Only a maltase gene (*PnMal*) was identified in the genome of *P. microspora,* while *MALR* and *MALT* are absent. PnMal, belongs to GH 13, is an intracellular enzyme, whose amino acid sequences shared 53% identities with maltase MalT from *Rhizoctonia solani.* The qRT-PCR results show that there is no significant change in *PnMal* when maltose was used as a sole carbon source in minimal medium after 24 h incubation when compared with glucose (Fig. 4-7). In sawdust medium, the *PnMal* expression in primordia and fruiting body stage far lower than that in mycelia stage, which is inferred that PnMal is not related with fruiting body formation (Fig. 4-8). We therefore propose that maltose utilization in *P. microspora* does not involve a *MAL* regulon, the PnGcs may be a key enzyme of maltose metabolism system in *P. microspora*. Our findings therefore support the conclusions that *P. microspora* utilizes maltose by means of extracellular hydrolysis by PnGcs.

The enzyme activity and expression levels of PnGcs at the fruiting body developmental stage were higher than in mycelium when grown on sawdust. Abundant expression of PnGcs in the fruiting body stage suggests that it is important for mature fruiting body development. The properties of the cell wall in α-glucosidase-lacking mutants of *Saccharomyces cerevisiae* are reportedly altered, with a decreased β-1, 6-glucan content, indicating that α-glucosidase activity is somehow important for normal β-1, 6-glucan biosynthesis and/or for β-1, 6-glucan insertion into the cell wall (Herscovics, 1999). Therefore, the α-glucosidase from *P. microspora* may participate in β-1, 6-glucan synthesis, which is a major component of its cell wall.

We suspect that double enzyme systems are present in *P. microspora* to hydrolyze starch into glucose. One is glucoamylase, which converts the starch into β-D-glucose. The other is a combination of α-amylase and α-glucosidase (Kusuda et al. 2008). Due to starch indirectly inducing *PnGcs* expression in minimal

medium, and maltose rapidly and strongly inducing *PnGcs* expression in minimal medium, our hypothesis is that the α-amylase from *P. microspora* is constitutively expressed in the mycelial stage in sawdust medium to gradually decompose the starch from rice bran into maltooligosaccharide or maltose, accompanied by the accumulation of maltooligosaccharide or maltose, which strongly induces α-glucosidase gene expression in the primordia and fruiting body formation stages. The α-glucosidase degrades the maltose into α-D-glucose, supplying glucose, and participates in β-glucan synthesis, which is a major component of the cell wall of the primordia and fruiting body of *P. microspora*.

We observed strong α-glucosidase gene expression in the fruiting stage, indicating that glucose production is one of the most important requirements for the morphogenesis of this basidiomycetous fungus.

Table 4-1 PCR primers used in this study

| Primer | Sequence | Use |
|---|---|---|
| 3RGcs | 5'-GTCACAGAAAAAGACGCCAGAG -3' | 3´RACE of *PnGcs* |
| 5RGcsP | 5'-GGTGGTGGATGGAAG-3' | 5´RACE of *PnGcs* |
| 5RA1Gcs | 5'-ACTTAATGCTGTTGCTGATCTTC-3' | |
| 5RA2Gcs | 5'-GTTTTGAAATCGTGCGCTTTG-3' | |
| 5RS1Gcs | 5'-AGAAGAAGGACACCGAAGTTG-3' | |
| 5RS2Gcs | 5'-GAACATTTCCGTACAAAGGAATCAG-3' | |
| qGcsF | 5'-ACTGAAGGCAAATGGTCGTGG-3' | Real-time PCR for amplification of *PnGcs* |
| qGcsR | 5'-TACCACCTCACAAGCATCTCCG-3' | |
| qActinF | 5'-CTTCACCACCACCGCCGA-3' | Real-time PCR for amplification of *Actin* |
| qActinR | 5'-CTTCAGGAGCACGGAATCGC-3' | |
| qMalF | 5'-AAACTTTCCGAGGTCATGG-3' | Real-time PCR for amplification of *Maltase* |
| qMalR | 5'-ACGTCCTTCATAACCACGC-3' | |

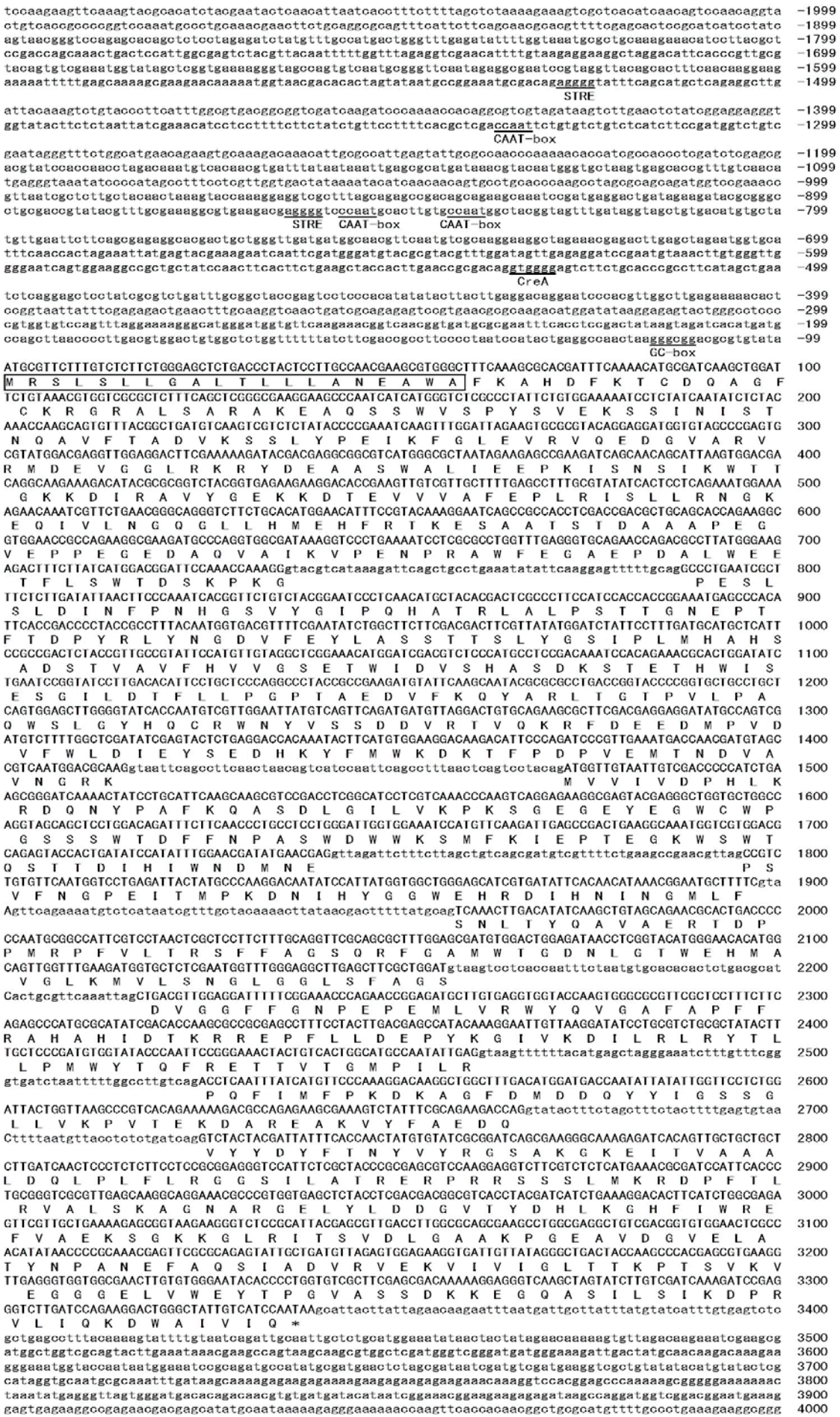


Fig. 4-1 Nucleotide and amino acid sequences of the *PnGcs* gene from *P. microspora*. Capital and lowercase letters indicate the exons and introns, respectively. The stop codon is indicated by an asterisk. The putative signal peptide sequence is underlined. The putative responsive elements in the promoter region are also underlined and indicated by annotations.

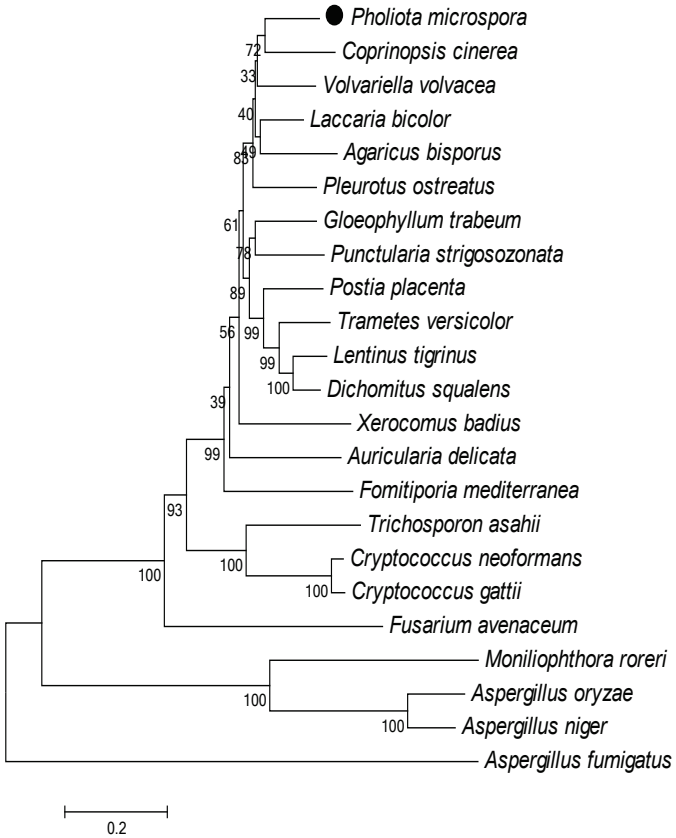


Fig. 4-2. Placement in phylogenetic tree of the deduced amino acid sequence of *P. microspora* α-glucosidase. The tree was calculated with *p*-distances using Mega ver. 5.05, based on a ClustalX alignment. The *scale bar* indicates a distance equivalent to 0.2 amino acid substitutions per site. Species and strains for α-glucosidases (with NCBI accession numbers) are: *Aspergillus fumigatus var.* RP-2014 (KEY81962), *Aspergillus niger* (BAA23616), *Aspergillus oryzae* (BAA95702), *Auricularia delicata* TFB-10046 SS5 (XP_007344921.1), *Coprinopsis cinerea* okayama 7#130 (XP_001830083.1), *Cryptococcus neoformans var. neoformans* JEC21 (XP_569264), *Cryptococcus gattii* WM276 (XP_003191128), *Dichomitus squalens* LYAD-421 SS1 (XP_007370079.1), *Fomitiporia mediterranea* MF3/22 (XP_007262137.1), *Fusarium avenaceum* (KIL91366), *Gloeophyllum trabeum* ATCC 11539 (XP_007866465.1), *Moniliophthora roreri MCA 2997* (XP_007843715), *Postia placenta* Mad-698-R (XP_002472172.1), *Punctularia strigosozonata* HHB-11173 SS5 (XP_007386154.1), *Trametes versicolor* FP-101664 SS1 (XP_008042972.1), *Trichosporon asahii var. asahii* CBS 8904 (EKD02384). The other α-glucosidase gene sequences from Agaricomycotina are listed in the Joint Genome Institute (JGI) database with their position on genome: *Agaricus bisporus* var. *bisporus* (H97) v2.0 (scaffold_5: 1987324-1990616), *Laccaria bicolor* v2.0 (LG_9: 850347-853637), *Lentinus tigrinus* v1.0 (scaffold_84: 30456-33866), *Pleurotus ostreatus* PC15 v2.0 (scaffold_05: 3188128-3191503), *Volvariella volvacea* V23 (VVO_00006: 381620-384975), *Xerocomus badius* 84.06 v1.0 (scaffold_94: 68137-71674).

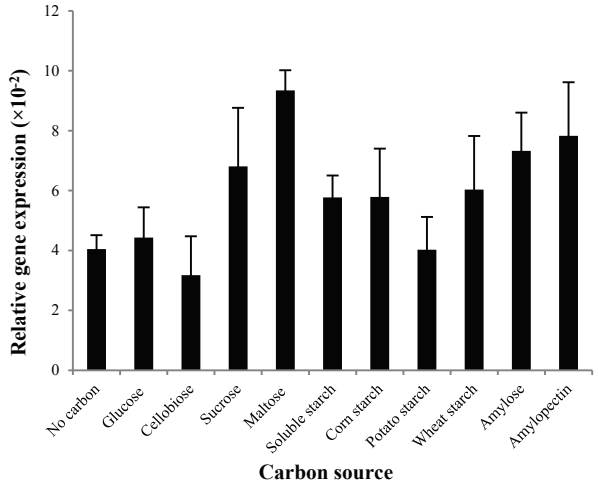


Fig. 4-3 *PnGcs* expression in *P. microspora* cultured in minimal media containing different carbon sources.

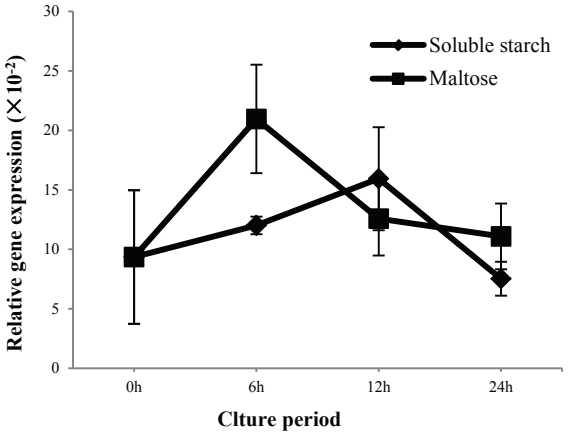


Fig. 4-4 Relative gene expression of *PnGcs* during cultivation in media containing soluble starch or maltose as the sole carbon source.

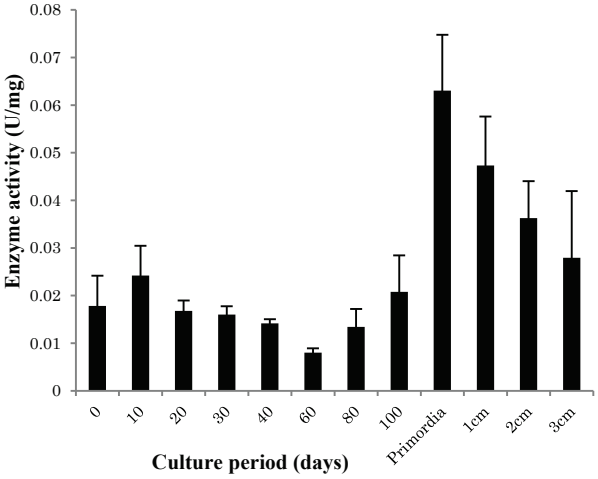


Fig. 4-5 PnGcs activity at different developmental stage of *P. microspora* in sawdust medium.

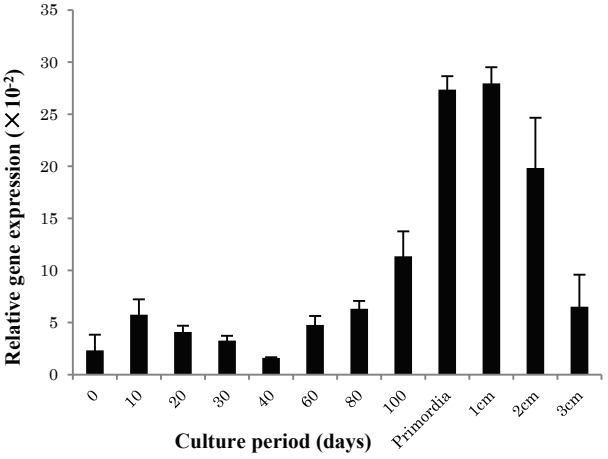


Fig. 4-6 *PnGcs* expression at different developmental stages of *P. microspora* in sawdust medium.

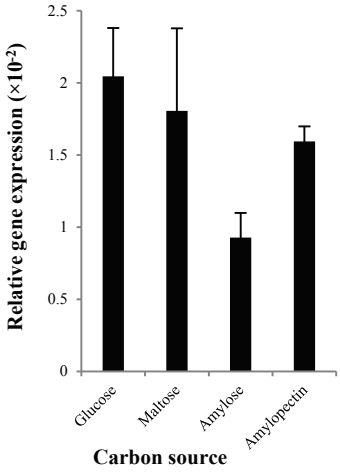


Fig. 4-7 *PnMal* expression in *P. microspora* cultured in minimal media containing different carbon sources.

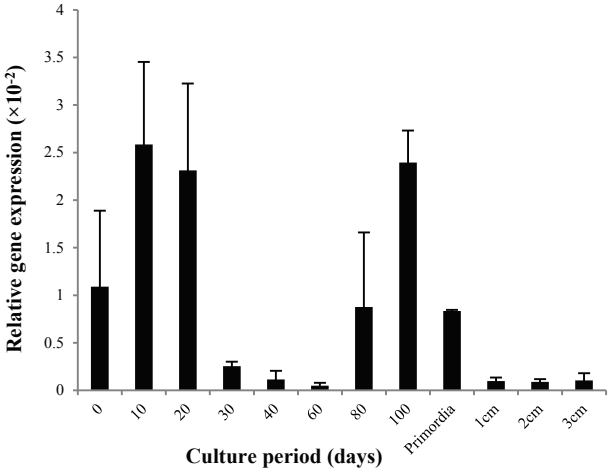


Fig. 4-8 *PnMal* expression at different developmental stages of *P. microspora* in sawdust medium.

# Chapter 5

# Discussions

Starch is of economic relevance of mushrooms production. The saprophytic fungus *P. microspora* was used to investigate starch metabolism and regulation in vegetable mycelium growth and fruiting body formation in sawdust medium. For helping to advance the understanding of starch degradation system, genome sequencing of this mushroom was carried out. The genes encoding several groups of starch degrading enzymes, such as glucoamylase, α-amylase and α-glucosidase, had been identified in the *P. microspora* genome, suggesting that starch degradation is a very important feature of the saprophytic lifestyle in this mushroom. We have combined transcriptional analysis and more detailed phylogenetic analysis to further understand genes functions.

Two GH15 glucoamylase genes *PnGlu1* and *PnGlu2* are located in the *P. microspora* genome. Based on intron position and amino acid sequence identity, *PnGlu1* and *PnGlu2* are likely duplicated genes that originated from a common ancestor. *P. microspora* have three α-amylases PnAmy1, PnAmy2, and PnAmy3, belong to GH13_32, GH13_5, and GH13_1, respectively. We also have identified α-glucosidase PnGcs within the GH31 family that had not been described before in *P. microspora*. We considered that these enzymes are associated with nutritional decomposition and feed the mycelial growth and fruiting bodies formation of *P. microspora*.

The *PnGlu1*, *PnGlu2* and *PnGcs* genes expression in minimal media contained glucose was higher than that potato starch (We did not use potato starch as a carbon source in minimal medium for α-amylase genes induction). Most starch digesting enzymes reported to date hardly digest potato starch because of the larger size of granules (Nidhi Goyal et al. 2005). There may be an indirect induction of these genes expression by starch in *P. microspora*. During mycelium growth, enzymes are secreted that convert a portion of the available starch to oligosaccharides, which can easily enter the cell and induce genes expression.

The transcriptional levels of *PnGlu1* and *PnGcs* in minimal medium contained maltose were higher than the other carbon sources, while amylose strongly induced *PnGlu2* and *PnAmy3* gene expression in minimal medium. Short-chain starch molecules may be easily recognized by fungi and enzymes are therefore required to directly degrade this short-chain starch to glucose, which fungi require for growth.

In sawdust medium, the transcriptional levels of *PnGlu2*, *PnAmy1*, *PnAmy2* and *PnGcs* during fruiting body development was higher than in mycelia growth stage. Abundant expression of these enzymes in the fruiting body stage suggests that they are important for mature fruiting body formation. PnGlu1 and PnAmy3 may be the key enzymes for decomposing starch in vegetable mycelium growth stage. *PnGlu1* expression increased rapidly for the first 10 days and reached a peak at 30 days in the mycelial growth stage and then decreased gradually to very low levels in the remaining mycelial growth and fruiting body development stages. The *PnAmy3* expression in mycelial stage at 20, 30, 40, and 100d were higher than that at the primordia or fruiting body stages in sawdust medium.

We observed strong glucoamylase, α-amylase and α-glucosidase gene expression in the fruiting stage in sawdust medium, indicating that glucose production is one of the most important requirements for the morphogenesis of this basidiomycetous fungus. The double enzyme systems are present in *P. microspora* to hydrolyze starch into glucose. One is glucoamylase, which directly converts the starch into β-D-glucose. The other is a combination of α-amylase and α-glucosidase (Kusuda et al. 2008). α-Amylase from *P. microspora* is constitutively expressed in the mycelial stage in sawdust medium to gradually decompose the α-1, 4-glucosidic linkage of starch into maltooligosaccharide or maltose. There also have two regulatory systems for maltose utilization in fungi, one is a functional *MAL* locus, and another one is AmyR regulator. In *P. microspora*, we did not identify the *MAL* gene cluster in genome. Therefore, we propose that the catabolism of maltose takes place mainly by the action of α-glucosidase in *P. microspora*.

The glucoamylase also have the ability to decompose the glycogen which is a homogeneous water-soluble polysaccharide whose branching points are uniformly distributed within the polymer. In *C. cinereus*, glycogen is accumulated in bulbous cells of the submerged mycelium at the early stages of vegetative growth. Glycogen accumulated in vegetative cells of the dikaryotic mycelium is lost when fruit bodies are formed, and losses of mycelial mass are correlated very closely with gain in mass of fruit bodies produced by the mycelium. The implication seems to be that glycogen formed by the vegetative mycelia is translocated towards sites at which multicellular structures are formed and that it accumulates in those structures to provide for their development (Moore 2003). In *P. microspora*, fruiting body formation may be accompanied by the glucoamylase degradation of cellular glycogen (Leatham 1985; Zhang et al. 1998). We inferred that the PnGlu1 catalyzes starch into glucose and supplies glucose for production of the

glycogen in vegetative mycelia growth stage. When primordia and fruiting bodies developed, the glycogen was hydrolyzed into glucose by PnGlu2.

In Agarics, the enlargement of fruiting body is based on hyphal inflation, not on proliferation of hyphae. Elongation of the stem in *C. cinereus* is dependent on an enormous increase in the volumes of the constituent cells which is accompanied by very active metabolism of wall polysaccharides which show rapid fluxes during elongation. The wall remains unthickened and most of the cell interior is occupied by vacuoles, implying that the stem is supported by a hydrostatic skeleton. The ingress of water into the stem during its development is quite dramatic. The turgor pressure of stem cells remains almost constant throughout the period of stem elongation so an appropriate osmotically active solute must be formed and accumulated in the stem cells in parallel with the absorption of water and synthesis of wall. The simple carbohydrates serve an osmoregulatory function in the developing stem of *C. cinereus*, no one sugar can be identified as being of prime importance; it seems more likely that a diverse metabolism contributes several components to an osmoregulatory compound (Moore 2003). We supposed that the hydrostatic pressure of *P. microspora* cell enhanced with the accumulation of glucose and maltose which were from starch hydrolysis. The considerable water uptake was needed, driving the cell expansion, stipe elongation and cap enlargement accompany by rapid cell wall polysaccharide metabolism such as β-glucan synthesis which is a major component of fungal cell wall. During this process, the amylase assisted glucan to obtain exclusively β-1, 3-linkages. In fruiting body stage, the PnAmy1, PnGcs and PnGlu2 continuously increased to produce the glucose. The starch hydrolysate, such as glucose and maltose, may be regulate osmotic pressure and participate in β-glucan synthesis.

This study presents a molecular analysis of starch utilization processes involved in growth and fruiting of edible mushroom *P. microspora*. The isolation of genes encoding starch acting enzymes that are dramatically expressed at fruiting body formation stage will provide valuable research tools to study the genetics involved in this process.

# Abstract


## Study of regulation of the starch degradation enzyme during fruiting in edible mushroom *Pholiota microspora*

*Pholiota microspora* is an economically important edible mushroom in Japan. Substrate for cultivation of this mushroom is containing sawdust and a suitable amount of rice bran. The major carbohydrates present in rice bran are cellulose, hemicellulose, and starch. Starch, which is a polymer of glucose units linked by glycosidic bonds, is the second most abundant carbohydrate in nature. The degradation of starch is performed by glucoamylase, α-amylase, and α-glucosidase.

The glucoamylase gene (*PnGlu1*) has been previously cloned and characterized. Following sequencing of *P. microspora* genome, we identified and characterized a second glucoamylase gene (*PnGlu2*), three α-amylase genes (*PnAmy1*, *PnAmy2* and *PnAmy3*), and an α-glucosidase gene (*PnGcs*) in current work. PnGlu1 and PnGlu2 belong to glycoside hydrolase family 15. PnAmy1, PnAmy2 and PnAmy3 are members of glycoside hydrolase family 13, belonging to subfamily 32, 5 and 1, respectively. The predicted amino acid sequence of PnGcs indicated that it is a glycoside hydrolase family 31 protein.

Quantitative reverse transcription-PCR was used to investigate these genes expression in the growth of mycelia when the fungus was cultured in minimal medium containing various carbon sources, as well as the growth of mycelia and tissue development in fruiting bodies at different stages in sawdust medium.

*PnGlu1* and *PnGlu2* expression in minimal media containing different carbon sources are far lower than that in sawdust medium. In minimal media, the highest level of *PnGlu1* expression was observed when maltose was used as the sole carbon source, while that of *PnGlu2* was much higher in the amylose culture than other culture media. In sawdust medium, transcription level of PnGlu1 is high in vegetative dikaryotic mycelia and that of PnGlu2 is high in primordia and at fruiting body stage. These data suggested that *PnGlu1* and *PnGlu2* may differentially regulate in each developmental stages such as mycelial growth and fruiting body formation.

Expression of *PnAmy1* and *PnAmy3* were regulated by various carbon sources in minimal media, which suggests that PnAmy1 and PnAmy3 play a role in starch degradation. The highest level of *PnAmy1* and *PnAmy3* expression was observed when amylopectin and amylose was used as the sole carbon source

in minimal medium, respectively. *PnAmy2* expression was barely induced by various starches other than glucose in minimal medium. PnAmy1 and PnAmy2 were closely correlated with fruiting body development in sawdust medium, while PnAmy3 seems to play a role during vegetative mycelial growth.

When *P. microspora* was grown in minimal medium supplemented with different carbon sources, *PnGcs* expression was highest when induced by maltose. During cultivation on sawdust medium, *PnGcs* expression increased dramatically at the fruiting body formation stage compared with the mycelial growth stage, which implied that *PnGcs* is closely associated with fruiting body development.

The cloning of genes encoding starch degradation enzymes combined with genes expression in sawdust medium established a solid base for explaining *P. microspora* starch utilization during fruiting body formation. To understand the whole process completely, much work should be done in the future.

# 和文摘要

## 食用きのこナメコにおける子実体形成過程におけるデンプン分解酵素の発現制御に関する研究

ナメコは，日本において，重要な食用きのこのひとつである。このきのこを栽培するための培地は，鋸屑に適量の米ヌカを加えて調製する。米糠中の主な炭水化物は，セルロース，ヘミセルロース，そして，デンプンである。デンプンは，グルコースがグリコシド結合で繋がったポリマーで，自然界で，2 番目に豊富な炭水化物であり，グルコアミラーゼ，または，α-アミラーゼ及びα-グルコシダーゼにより，グルコースまで分解される。

本研究では，これまでの研究で，明らかにされていたグルコアミラーゼ 1 遺伝子（*PnGlu1*）の他に，ナメコのゲノムの配列中に，第二のグルコアミラーゼ遺伝子（*PnGlu2*），3 つのα-アミラーゼ遺伝子（*PnAmy1*，*PnAmy2* と *PnAmy3*），α－グルコシダーゼ遺伝子（*PnGcs*）及びマルターゼ遺伝子(*PnMal*)を見出した。PnGlu1 と PnGlu2 は，糖質加水分解酵素ファミリー15 ，PnAmy1，PnAmy2 と PnAmy3 及び PnMal は，糖質加水分解酵素ファミリー13 に属し，それぞれサブファミリー32，サブファミリー5 とサブファミリー1 に属していた。また，PnGcs の推定アミノ酸配列は，糖質加水分解酵素ファミリー31 タンパク質であった。

次に，様々な炭素源を含む最少培地及び，鋸屑培地で栽培した際のこれらの遺伝子の菌糸体および原基，子実体などの組織における発現を定量的逆転写 PCR により調べた。その結果，異なる炭素源を含有する最少培地中の *PnGlu1* と *PnGlu2* 発現は，鋸屑培地に比べてはるかに低かった。マルトースを唯一の炭素源として用いた場合，*PnGlu1* 発現の菌糸体での最高レベルが観察された。*PnGlu2* の転写レベルは，他の炭素源よりもアミロース含有培地において高かった。鋸屑培地における *PnGlu1* の転写レベルは，二核性菌糸体において高く，*PnGlu2* の発現は，原基および子実体の段階で高かった。本研究では，このように，2 つのグルコアミラーゼ遺伝子 *PnGlu1* と *PnGlu2* の発現の差を見出し，このような菌糸の成長と子実体の形成などの各発達段階におい

て調節されていることを示した。

また, *PnAmy1* と *PnAmy3* の発現は, 最少培地中で, 様々な炭素源によって調節されており, デンプン分解における重要な役割を果たしていることを示唆していた。*PnAmy1* と *PnAmy3* の最高レベルの発現は，アミロペクチン及びアミロースを唯一の炭素源として使用した場合に観察された。一方，*PnAmy2* 発現は，最少培地中のグルコース以外の種々のデンプンによりわずかに誘導された。*PnAmy3* は，栄養菌糸成長中に発現している一方で，*PnAmy1* と *PnAmy2* 発現は鋸屑培地中の子実体の発達と密接に相関していた。

ナメコは，異なる炭素源を添加した最少培地中で増殖させたときに，*PnGcs* の発現は，マルトースによって高レベルで誘導された。鋸屑培地上で培養した際の *PnGcs* の発現は，菌糸成長時と比べ，子実体形成段階で劇的に増加したことから，子実体形成に密接に関連していることが，示唆された。一方，同じ酵素活性をもつと思われる，*PnMal* は，菌糸体で発現が高かった。

完全に子実体形成プロセス全体を理解するために、多くの仕事は、将来的に行われる必要があるが，本研究によるデンプン分解酵素をコードする遺伝子のクローニングと鋸屑培地における遺伝子発現と組み合わせた解析により，子実体の形成段階におけるナメコのデンプンの利用を説明するための強固な基盤が確立できた。

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# Literature cites


Agger, T., Spohr, A. B., Nielsen, J., 2001. Amylase production in high cell density submerged cultivation of *Aspergillus oryzae* and *A. nidulans*. Applied Microbiology Biotechnology, **55**, 81–84.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J., 1990. Basic local alignment search tool. Journal of Molecular Biology, **215**, 403–410.

Arai, Y., Takao, M., Sakamoto, R., Yoshikawa, K., Terashita, T., 2003. Promotive effect of the hot water soluble fraction from corn fiber on vegetative mycelial growth in edible mushrooms. Journal Wood of Science, **49**, 437–443. doi: 10.1007/s10086-002-0491-9.

Balint, S., Farkas, V., Bauer, S., 1976. Biosynthesis of β-glucans catalyzed by a particulate enzyme preparation from yeast. FEBS Letters, **64**, 44–47.

Bertoldo, C., Antranikian, G., 2001. Amylolytic enzymes from hyperthermophiles. Methods in Enzymology, **330**, 269–289. doi: 10.1016/ S0076-6879(01)30382-8.

Bertoldo, C., Antranikian, G., 2002. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. Chemical Biology, **6**, 151–160. doi: 10.1016/S1367-5931 (02)00311-3.

Birnboim, H. C., 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods in Enzymology, **100**, 243–255.

Bradford, M. M., 1976. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, **72**, 248–254.

Buléon, A., Colonna, P., Planchot, V., Ball, S., 1998. Starch granules: structure and biosynthesis. International Journal of Biological Macromolecules, **23**, 85–112. doi: 10.1016/S0141-8130(98) 00040-3.

Camelini, C. M., Maraschin, M., Matos de Mendonc, M., Zucco, C., Ferreira, A. G., Tavares, L. A., 2005. Structural characterization of β-glucans of *Agaricus brasiliensis* in different stages of fruiting body maturity and their use in nutraceutical products. Biotechnology Letters, **27**, 1295–1299.

Chang, S. T., Hayes W. A., 1978. The Biology and Cultivation of Edible Mushrooms, Academic Press, Inc.

Chen, W., Xie, T., Shao, Y., Chen, F., 2012. Phylogenomic relationships between amylolytic enzymes

from 85 strains of fungi. PLoS ONE **7**: e49679. doi: 10.1371/journal.pone.0049679.

Chiba, S., 1988. α-Glucosidase, Handbook of Amylases and Related Enzymes. In: The Amylase Research Society of Japan (Ed.), Pergamon, Oxford, pp. 104–116.

Chiba, S., 1997. Molecular mechanism in α-glucosidase and glucoamylase. Bioscience Biotechnology and Biochemistry, **61**, 1233–1239.

Dellaporta, S. L., Wood, J., Hicks, J. B., 1983. A plant DNA mini preparation: version II. Plant Molecular Biology Reporter, **1**, 19–21.

Dowzer C. E. A., Kelly J., M., 1991. Analysis of the creA gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. Molecular and Cellular Biology, **11**, 5701–5709.

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E.L., Tate, J., Punta, M., 2013. Pfam: the protein families database. Nucleic Acids Research, **42**, D222–D230. doi: 10.1093/nar/gkt1223.

Gruen H. E.,Wong W. M., 1982. Distribution of cellular amino acids, protein, and total organic nitrogen during fruitbody development in *Flammulina velutipes*. II. Growth on potato-glucose solution. Canadian Journal of Botany, **60**, 1342–1351.

Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochemical Journal, **280**, 309–316.

Henrissat, B., Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochemical Journal, **293**, 781–788.

Herscovics, A., 1999. Processing glycosidases of *Saccharomyces cerevisiae.* Biochimica et Biophysica Acta, **1426**, 275–285.

Hirato, H., Kitamoto, Y., 1995. Effect of physico-chemical characteristics of the culture substrate and nutritional conditions on mycelial growth in *Tricholoma matsutake* (in Japanese). Mushroom Science and Biotechnology, **2**, 17–22.

Ishida, H., Hata, Y., Kawato, A., Abe, Y., Suginami, K., Imayasu, S., 1999. Identification of functional elements that regulate the glucoamylase- encoding gene (*glaB*) expressed in solid-state culture of *Aspergillus oryzae*, Current Genetics, **37**, 373–379.

Janeček, Š., Svensson, B., MacGregor, E.A., 2014. α-Amylase: an enzyme specificity found in various

families of glycoside hydrolases. Cellular and Molecular Life Sciences, **71**, 1149–1170. doi: 10.1007/s00018-013-1388-z.

Ji, J., Moore, D., 1993. Glycogen metabolism in relation to fruit body maturation in *Coprinus cinereus*. Mycological Research, **97**, 283–289.

Kitamoto Y., Gruen H. E., 1976. Distribution of cellular carbohydrates during development of the mycelium and fruitbodies of *Flammulina velutipes*. Plant Physiology, **58**, 485–491.

Kusuda, M., Ueda, M., Miyatake, K., and Terashita, T., 2008. Characterization of the carbohydrase productions of an ectomycorrhizal fungus, *Tricholoma matsutake*. Mycoscience, **49**, 291–297. doi: 10.1007/S10267-008-0423-7.

Lage, J. L. D., Binder, M., Van, A. H., Janeček, Š., Casane, D., 2013. Gene make-up: rapid and massive intron gains after horizontal transfer of a bacterial α-amylase gene to Basidiomycetes. BMC Evolutionary Biology, **13**, 40–53. doi: 10.1186/1471- 2148-13-40.

Lagesen, K., Hallin, P., Rødland, E. A., Staerfeldt, H. H., Rognes, T., Ussery, D. W., 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Research, **35**, 3100–3108. doi: 10.1093/nar/gkm160.

Leatham, G. F., 1985. Extracellular enzymes produced by the cultivated mushroom *Lentinus edodes* during degradation of a lignocellulosic medium. Applied and Environmental Microbiology, **50**, 859–867.

Li, Y., Kinjo, Y., Kawai, Y., Wan, J. N., Shimomura, N., Yamaguchi, T., Aimi, T., 2012. *PnGlu1* (Glycoside Hydrolase Family 15) in *Pholiota microspora* is highly expressed in dikaryotic mycelia and fruiting body, Mushroom Science and Biotechnology, **20**, 135–140.

Liu Y., Srivilai P., Loos S., Aebi M., Kües, U., 2006. An Essential Gene for Fruiting Body Initiation in the Basidiomycete *Coprinopsis cinerea* Is Homologous to Bacterial Cyclopropane Fatty Acid Synthase Genes. Genetics, **172**, 873-884.

Lopez-Romero, E., Ruiz-Herrera, J., 1977. Biosynthesis of β-glucan by cell free extract from Saccharomyces cerevisiae. Biochimica et Biophysica Acta, **500**, 372–384.

Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research, **25**, 955–964. doi: 10.1093/nar/25.5.0955.

Mach, R. L., Zeilinger, S., 2003. Regulation of gene expression in industrial fungi: *Trichoderma*. Applied Microbiology and Biotechnology, **60**, 515–522.

Machovič, M., Janeček, Š., 2006. The evolution of putative starch-binding domains. FEBS Letttters, **580**, 6349–6356.

Manzi P., Pizzoferrato L., 2000. Beta-glucans in edible mushrooms. Food Chemistry, **68**, 315-318.

Masuda, P., Yamanaka, K., Sato, Y., Kitamoto, Y. 1995. Nuclear selection in monokaryoization of dikaryotic mycelia of *Pholiota nameko* as described by leading and following nuclei. Mycoscience, **36**, 413–420.

Mattila P., Suonpaa K., Piironen V., 2000. Functional properties of edible mushrooms. Nutrition Reviews, **16**, 694–696.

Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., Hurwitz, D. I., Lanczycki, C. J., Lu, F., Lu, S., Marchler, G. H., Song, J. S., Thanki, N., Yamashita, R. A., Zhang, D., Bryant, S. H., 2012. CDD: conserved domains and protein three-dimensional structure. Nucleic Acids Research, **41**, D348–D352. doi: 10.1093/nar/ gks1243.

Marion, C. L., Rappleye, C. A., Engle, J. T., Goldman, W. E., 2006. An α-(1, 4)-amylase is essential for α-(1, 3)-glucan production and virulence in *Histoplasma capsulatum.* Molecular Microbiology, **62**, 970–983. doi: 10.1111/j.1365-2958. 2006.05436.x.

Marshall, W. E., Wadsworth, J. I., 1993. Rice Science and Technology, CRC Press, 486.

Moore, D., 2003. Fungal Morphogenesis. Cambridge University Press, 154.

Moskvina, E., Schuller, C., Maurer C. T., Mager W. H., Ruis, H., 1998. A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. Yeast, **14**, 1041–1050.

Nidhi Goyal, Gupta, J. K., Soni, S. K., 2005. A novel raw starch digesting thermostable a-amylase from *Bacillus* sp.I-3 and its use in the direct hydrolysis of raw potato starch. Enzyme and Microbial Technology, **37**, 723–734.

Noguchi, H., Taniguchi, T., and Itoh, T., 2008. MetaGeneAnnotator: detecting species-specific patterns of ribosomal binding site for precise gene prediction in anonymous prokaryotic and phage genomes. DNA Research, **15**, 387–396. doi: 10.1093/dnares/dsn027.

Norouzian, D., Akbarzadeh, A., Scharer, J.M., Young, M.M., 2006. Fungal glucoamylases. Biotechnology

Advances, **24**, 80–85. doi: 10.1016/j.biotechadv. 2005.06.003.

Ohta, A., 1994. Production of fruit-bodies of a mycorrhizal fungus, *Lyophyllum shimeji*, in pure culture. Mycoscience, **35**, 147–151.

Ohta A., 1997. Ability of ectomycorrhizal fungi to utilize starch and related substrates. Mycoscience, **38**, 403–408.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.Y., Singh, D., Mohan, R., 2000. Advances in microbial amylases. Biotechnology and Applied Biochemistry, **31**, 135–152.

Petersen, K. L., Lehmbeck, J., Christensen, T., 1999. A new transcriptional activator for amylase genes in *Aspergillus*. Molecular and General Genetics, **262**, 668–676.

Robert, J. C., 1977. Fruiting of *Coprinus congregatus*: relationship to biochemical changes in the whole culture.Transactions of the British Mycological Society, **68**, 389–395.

Rossi, I. H., Monteiro, A. C., Machado, J. O., Andrioli, J. L., Barbosa, J. C., 2003. Shiitake *Lentinula edodes* production on a sterilized bagasse substrate enriched with rice bran and sugarcane molasses. Brazilian Journal of Microbiology, **34**, 66-71.

Sigrist, C. J. A., de Castro, E., Cerutti, L., Cuche, B.A., Hulo, N., Bridge, A., Bougueleret, L., Xenarios, I., 2013. New and continuing developments at PROSITE. Nucleic Acids Research, **41**, D344–D347. doi: 10.1093/nar/ gks1067.

Stajich, J. E., Wilke, S. K., Ahrén, D., Au, C.H., Birren, B. W., Borodovsky, M., Burns, C., Canbäck, B., Casselton, L. A., Cheng, C. K., Deng, J., Dietrich, F. S., Fargo, D. C., Farman, M.L., Gathman, A. C., Goldberg, J., Guigó, R., Hoegger, P. J., Hooker, J. B., Huggins, A., James, T.Y., Kamada, T., Kilaru, S., Kodira, C., Kües, U., Kupfer, D., Kwan, H. S., Lomsadze, A., Li, W., Lilly, W. W., Ma, L. J., Mackey, A. J., Manning, G., Martin, F., Muraguchi, H,, Natvig, D. O., Palmerini, H., Ramesh, M. A., Rehmeyer, C. J., Roe, B. A., Shenoy, N., Stanke, M., Ter-Hovhannisyan, V., Tunlid, A., Velagapudi, R., Vision, T. J., Zeng, Q., Zolan, M. E., Pukkila, P. J., 2010. Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). Proceeding of the National Academy of Sciences of the United States of America, **107**, 11889–11894. doi: 10.1073/pnas.1003391107.

Stam, M. R., Danchin, E. G., Rancurel, C., Coutinho, P. M., Henrissat, B., 2006. Dividing the large

glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of α-amylase-related proteins. Protein Engineering Design and Selection, **19**, 555–562. doi: 10.1093/protein/gz l044.

Stringer, M. A., Timberlake, W. E., 1995. *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. Molecular Microbiology, **16**, 33–44.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution, **28**, 2731–2739. doi: 10.1093/molbev/msr121.

Tetlow, I. J., Morell, M. K., and Emes, M. J. 2004. Recent developments in understanding the regulation of starch metabolism in higher plants. Journal of Experimental Botany, **55**, 2131–2145. doi: 10.1093/jxb/erh248.

Vongsangnak, W., Salazar, M., Hansen, K., Nielsen, J., 2009. Genome-wide analysis of maltose utilization and regulation in *aspergilli.* Microbiology, **155**, 3893–3902.

Wessels, J. G. H., 1994. Developmental regulation of fungal cell wall formation. Annual Review Phytopathology, **32**, 413–437.

Wösten, H. A. B., Wessels, J. G. H., 2005. The Emergence of Fruiting Bodies in Basidiomycetes. Growth, Differentiation and Sexuality, Mycota, **1**, 393-414.

Yamamoto, T., Unno, T., Watanabe, Y., Yamamoto, M., Okuyama, M., Mori, H., Chiba, S., Kimura, A., 2004. Purification and characterization of *Acremonium implicatum* α-glucosidase having regioselectivity for α-1, 3-glucosidic linkage. Biochimica et Biophysica Acta, **1700**, 189–198.

Yi, R., Tachikawa, T., Mukaiyama, H., Mochida, Y., Ishikawa, M., Aimi, T., 2009. DNA-mediated transformation system in a bipolar basidiomycete, *Pholiota microspora* (*P. nameko*). Mycoscience, **50**, 123–129.

Yi, R., Mukaiyama, H., Tachikawa, T., Shimomura, N., Aimi, T., 2010. *A* -Mating-Type gene expression can drive clamp formation in the bipolar mushroom *Pholiota microspora* (*Pholiota nameko*), Eukaryotic Cell, **9**, 1109–1119

Yoon, J. J., Igarashi, K., Kajisa1, T., and Samejima, M. 2006. Purification, identification and molecular

cloning of glycoside hydrolase family15 glucoamylase from the brown-rot basidiomycete *Fomitopsis palustris*. FEMS Microbiology Letters, **259**, 288–294. doi: 10.1111/j.1574-6968. 2006. 00279.x.

Yuan, X. L., van der Kaaij, R. M., van den Hondel, C., Punt, P. J., vander Maarel, M., Dijkhuizen, L., Ram, A. F. J., 2008. *Aspergillus niger* genome-wide analysis reveals a large number of novel alpha-glucan acting enzymes with unexpected expression profiles. Molecular Genetics and Genomics, **279**, 545–561.

Zerbino, D. R., Birney, E., 2008. Velvet: algorithms for *de novo* short read assembly using de Bruijn graphs. Genome Research, **18**, 821–829. doi:10.1101/gr.074492. 107.

Zhang, M., Xie, W., Leung, G. S. W., Deane, E. E., Kwan, H. S., 1998. Cloning and characterization of the gene encoding beta subunit of mitochondrial processing peptidase from the basidiomycete *Lentinula edodes*. Gene, **206**, 23–27.

Zhao, J., Chen, Y. H., Kwan, H. S., 2000. Molecular cloning, characterization, and differential expression of a glucoamylase gene from the basidiomycetous fungus *Lentinula edodes*. Applied and Environmental Microbiology, **66**, 2531–2535. doi: 10.1128/AEM.66.6.2531-2535.2000.

Zhu, F. M., Dua, B., Bian, Z. X., Xu, B. J., 2015. Beta-glucans from edible and medicinal mushrooms: Characteristics, physicochemical and biological activities. Journal of Food Composition and Analysis, **41**, 165–173.

# List of publications

Gang Zhu, Mirai Hayashi, Norihiro Shimomura, Takeshi Yamaguchi, Tadanori Aimi. Expression of α-glucosidase during morphological differentiation in the basidiomycetous fungus *Pholiota microspora.* Journal of Basic Microbiology. Doi: 10.1002/jobm.201500752. (The corresponding content is in chapter 4)

Gang ZHU, Jianing WAN, Mirai HAYASHI, Norihiro SHIMOMURA, Takeshi YAMAGUCHI, Tadanori AIMI. Identification, characterization and expression of the second glucoamylase gene from *Pholiota microspora.* Mushroom Science and Biotechnology, 2016, Vo 24, No 2. (The corresponding content is in chapter 2)