Mycoscience (2012) 53:194–202
DOI 10.1007/s10267-011-0153-0

FULL PAPER

Characterization of the glycoside hydrolase family 15 glucoamylase gene from the ectomycorrhizal basidiomycete *Tricholoma matsutake*

Jianing Wan · Yan Li · Ruirong Yi · Wiyada Mongkolthanaruk · Yukiko Kinjo · Takao Terashita · Katsuji Yamanaka · Norihiro Shimomura · Takeshi Yamaguchi · Tadanori Aimi

Received: 18 February 2011 / Accepted: 19 August 2011 / Published online: 17 September 2011

Abstract The gene encoding the glycoside hydrolase family 15 glucoamylase (*TmGlu1*) in the ectomycorrhizal fungus *Tricholoma matsutake* was cloned and characterized. After the culture of *T. matsutake* mycelia in media containing different forms of starch as a carbon source, increased extracellular glucoamylase activity in the culture medium and a correspondingly higher transcriptional level of *TmGlu1* in mycelia were detected, particularly in amylose-supplemented medium, when compared with those in the glucose medium. These results suggest that starch, especially amylose, affects the transcription of *TmGlu1* and downstream glucoamylase activity, which is directly related to starch utilization. Similar results were obtained when compound forms of starch were used to culture mycelia. Glucoamylase genes from saprophytic and ectomycorrhizal fungi formed a single clade. The observed inducibility of *TmGlu1* and lack of distinct phylogenetic differences among glucoamylase genes of saprophytic and ectomycorrhizal fungi suggest that glucoamylase may relate to some common functions in these two types of fungi.

Keywords Amylose · GH15 protein · Glucoamylase · Starch · *Tricholoma matsutake*

J. Wan · Y. Li
The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-cho, Minami, Tottori 680-8553, Japan

R. Yi · Y. Kinjo · N. Shimomura · T. Yamaguchi · T. Aimi (✉)
Faculty of Agriculture, Tottori University, 4-101 Koyama-cho, Minami, Tottori 680-8553, Japan
e-mail: taimi@muses.tottori-u.ac.jp

W. Mongkolthanaruk
Faculty of Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

T. Terashita
Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan

K. Yamanaka
Kyoto Mycological Institute, 1-55 Misasahirabayashi, Yamashina, Kyoto 607-8406, Japan

Introduction

The ectomycorrhizal fungus *Tricholoma matsutake* (S. Ito et Imai) Sing is one of the most prized edible mushrooms in Japan. The annual production of *T. matsutake* in Japan reportedly was 12,000 Mg in 1941, 211 Mg in 1995 (Yamada 2005), and decreased substantially to only 24 Mg in 2009. To date, attempts to cultivate *T. matsutake* using an artificial medium have been unsuccessful. However, mature fruiting bodies of the ectomycorrhizal fungus *Lyophyllum shimeji* (Kawamura) Hongo have been artificially cultivated in a cultivation medium containing barley grain without a host plant (Ohta 1994). The successful artificial cultivation of *L. shimeji* suggests that identifying a suitable and sufficient quantity of starch, such as barley grain, as a carbon source may permit the artificial cultivation of mature basidiocarps of other ectomycorrhizal fungi, including *T. matsutake*.

Numerous mycorrhizal fungi exhibit a wide range of starch-utilization abilities (Ohta 1997). Although several enzymes have been identified that allow these fungi to utilize starch as a growth substrate, the enzyme glucoamylase (glucan 1,4-α-glucosidase; EC 3.2.1.3) is particularly important as it catalyzes the release of β-D-glucose units from the non-reducing ends of starch, including amylose

and amylopectin, and other polysaccharides (Sakaguchi et al. 1992). Here, to clarify the direct relationship of the glucoamylase gene and starch utilization in *T. matsutake*, the gene encoding glucoamylase was cloned in *T. matsutake* NBRC 30773 and characterized in terms of its expression and enzyme activity in media containing different forms of starch.

Materials and methods

Fungal strains and culture conditions

Tricholoma matsutake NBRC 30773 was used in this study. The mycelia of *T. matsutake* NBRC 30773 were routinely cultured on modified Hamada's agar medium (0.5% KH_2PO_4, 0.2% yeast extract, 2% glucose, and 1.5% agar, pH 5.1) prepared with tap water (Hamada 1964). For the preparation of genomic DNA, three square agar blocks (5 × 5 × 5 mm) of fungal mycelia were cut and transferred into 20 ml modified Hamada's liquid medium in a 100-ml Erlenmeyer flask and were then incubated at 25°C for 30 days. Mycelia were then collected, lyophilized, and used for genomic DNA extraction.

Measurement of enzyme activity

Tricholoma matsutake mycelia were inoculated into modified Hamada's medium, which contained 1% (w/v) starch from different sources (pure starch: corn starch, soluble starch, wheat starch, potato starch, sweet potato starch, and amylose; and compound starch obtained from rice, wheat, barley, buckwheat, and corn) in place of 1% glucose. Following incubation of the cultures at 25°C for 30 days, the culture medium was filtered through filter paper (Toyo Roshi, Tokyo, Japan) to remove mycelia, and the culture filtrate was then lyophilized. The lyophilized powder was dissolved in 50 mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid, pH 5.5]. Glucoamylase activity was measured with a Glucoamylase and α-Glucosidase Assay kit (Kikkoman, Tokyo, Japan), and amylose was measured with an Amylose and Amylopectin Assay Procedure kit (Megazyme, Wicklow, Ireland). One unit of enzyme activity was defined as the activity that produces 1 μmol glucose per 1 min at 37°C. The total protein concentration present in the freeze-dried culture filtrate was measured by the Lowry method (Lowry et al. 1951).

Preparation of DNA and RNA

Genomic DNA was prepared from lyophilized mycelia of *T. matsutake* strain NBRC 30773 using a GENEALL Plant SV Mini kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. For RNA preparation, mycelia of strain NBRC 30773 were harvested by filtration, frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. Total RNA was extracted from the frozen mycelia powder using an RNeasy Plant Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. A 1:10 dilution of stock total RNA was used for real-time reverse transcription-polymerase chain reaction (PCR).

Amplification of the *T. matsutake* glucoamylase gene

Initially, fragments of genomic DNA encoding the putative glucoamylase protein (TmGlu1) were amplified by PCR with the degenerate oligonucleotide primer pair F15-GP2-AF and F15-GP2-BR (Table 1). The F15-GP2-AF and F15-GP2-BR primers were designed based on the amino acid sequences GLGEPKF and FDLWEEI, respectively, which are conserved in the glucoamylase proteins of *Lentinula edodes* and several other basidiomycetes (Zhao et al. 2000). PCR was performed in a 50-μl reaction containing 1× Ex *Taq* buffer (Takara Bio, Shiga, Japan), 50 ng genomic DNA, 50 pmol each primer, 0.2 mM each dNTP, and 1.25 U Ex *Taq* polymerase (Takara Bio). The PCR reaction was performed with a Takara PCR Thermal Cycler Personal (Takara Bio) and consisted of an initial denaturation for 3 min at 95°C, followed by 30 cycles of 30 s at 94°C, 2 min at 50°C, and 30 s at 72°C, and a final elongation step at 72°C for 10 min. The amplified PCR fragment (approximately 400 bp) was directly cloned into the pT7Blue (R) T-vector (Novagen, Madison, WI, USA), generating pTMGLU1. Partial sequences for *TmGlu1* were obtained from the amplified PCR fragment, and complete nucleotide sequencing of the coding region of *TmGlu1* was conducted using cassette amplification by PCR with two primer sets (30773-GP2-A1/30773-GP2-A2 and 30773-GP2-S1/30773-GP2-S2) (Table 1). The primer sets were designed based on the partial nucleotide sequences of *TmGlu1* obtained from the first PCR. Template DNAs for cassette PCR were prepared with a Takara LA PCR In Vitro Cloning kit (Takara Bio) according to the manufacturer's instructions. Genomic DNA from *T. matsutake* was digested with *Hin*dIII and *Sal*I; the fragments were ligated with nucleotide linkers and used as templates for PCR. For *TmGlu1*, an approximately 2.0-kbp PCR product from 30773-GP2-A2 to a *Hin*dIII site containing the 5′-untranslated region and a 2.0-kbp product from 30773-GP2-S2 to a *Sal*I site containing the 3′-untranslated region were cloned and sequenced. To amplify the entire genomic sequence of *TmGlu1*, oligonucleotide primers T-M30773GP2F and T-M30773GP2R were designed based on the nucleotide sequence of DNA fragment amplified by the cassette PCR method. PCR reactions were performed in a 100-μl mixture containing 1× Ex *Taq* buffer, 100 ng

Table 1 Primers used for the identification of *TmGlu1* sequences

Primer	Sequence	Remark
F15-GP2-AF	5′-GGNYTNGGNGARCCNAARTT-3′	Used for initial amplification
F15-GP2-BR	5′-ATYTCYTCCCANARRTCRAA-3′	
30773-GP2-S1	5′-CCTTCTCGCTCACAACAATGCTTC-3′	Used for cassette PCR
30773-GP2-S2	5′-TTGACTACGTGGCTTCGAACTGGA-3′	
30773-GP2-A1	5′-CGAGAAGGTAATTTGCCCAGGTGA-3′	
30773-GP2-A2	5′-CATATGCTAGGTTGAGCGTCCACT-3′	
CaPrC1	5′-TCGTTAGAACGCGTAATACGACTCA-3′	Used for cassette PCR
CaPrC2	5′-CGTAATACGACTCACTATAGGGAGA-3′	
T-M30773GP2F	5′-ATCTTCAAGCACACGCACGTAGTC-3′	Used for amplification of complete *TmGlu1* and the flanking region
T-M30773GP2R	5′-CATTTTCTGCCCTGCAACTTCACC-3′	
T-M30773GP2F2	5′-AACGGTATAGCTGCTGAA-3′	Used for direct sequencing
T-M30773GP2R2	5′-AGACGGGAAACAGAGACA-3′	
T-M30773GP2F3	5′-GTAGACATGCTCATGGACACGTCA-3′	
T-M30773GP2R3	5′-AGCGTAACTCCAGCTAAGGTCAAC-3′	
T-M30773GP2Fr1	5′-AGAACACGAACGGTCACATACAGG-3′	
T-M30773GP2Fr2	5′-TTGCTGGGCCAGAAAACATACCGA-3′	
T-M30773GP2Rr1	5′-TGTAAGTCTGACTGCGCAGCTTTG-3′	
T-M30773GP2F4	5′-TAGCCAAGGTCAACCTAC-3′	
T-M30773GP2R4	5′-CCCGAATTGATTGCATAG-3′	
T-M30773Gp2-5RACEZ1	5′-AGAAGGTAATTTGCCCAGGTG-3′	Used for 5′-RACE
T-M30773Gp2-5RACEZ2	5′-CAAGGACCCGTAAAGGCAGTT-3′	
T-M30773Gp2-5RACER1	5′-TACGTTACCAGCAGTCTTTG-3′	
T-M30773Gp2-5RACER2	5′-CGACCTTGACTACGTGGCTT-3′	
T-M30773Gp2-5RACE2p	5′-pCCTCCCAAAGATCAAATGTT-3′	
T-M30773Gp2-3RACE1	5′-ATTACAACGGCAACCCTTGGTACT-3′	Used for 3′-RACE
T-M-R1	5′-AAGGCTGACGGTAATGCTC -3′	Used for real-time RT-PCR
T-M-F1	5′-CGGTAAACTTCAATGTCCAGG-3′	
Actin-R1	5′-GGCGACAATCTTGACCTTCAT-3′	Used for real-time RT-PCR of actin
Actin-F1	5′-GCTGGCATCCACGAGACTAC-3′	
Univ Act F1	5′-CARGGTGTCMTGGTYGGWATGG-3′	Used for partial actin gene amplification
Univ Act R1	5′-CCGAYTCGTCGTAYTCYTGCTT-3′	

extracted genomic DNA, 100 pmol each primer, 0.2 mM each dNTP, and 2.5 U Ex *Taq* polymerase. PCR reactions were conducted using an initial denaturation at 94°C for 1 min, followed by 30 cycles of 30 s at 94°C and 5 min at 68°C. The amplified DNA fragments of *TmGlu1* were purified with a QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions and used as a DNA template for direct sequencing with oligonucleotide primers. DNA sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a BigDye Terminator Cycle Sequencing version 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. The nucleotide sequences of *TmGlu1* and its flanking regions from *T. matsutake* NBRC 30773 have been deposited in the DDBJ database under accession no. AB604354.

Analysis of nucleotide and protein sequences

Nucleotide and protein sequence data were analyzed using GENETYX 9.0 (Genetyx, Foster City, CA, USA). Protein motifs in the amino acid sequence of glucoamylase were identified using the web-based MOTIF Search program (http://motif.genome.jp/). Subcellular localization of glucoamylase was predicted by the PSORTII (Horton and Nakai 1997; http://psort.ims.u-tokyo.ac.jp/form2.html) and SOSUI programs (Hirokawa et al. 1998; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html).

Southern hybridization

Southern hybridization analysis of strain NBRC 30773 was performed to determine the copy number of the glucoamylase gene in *T. matsutake*. Genomic DNA (0.3–0.5 μg)

from NBRC 30773 strain was digested for 5 h at 37°C in a 500-μl reaction mixture containing 20 U of restriction enzymes *Bam*HI and *Pst*I in the buffer supplied by the manufacturer (Toyobo). The digested fragments were concentrated by ethanol precipitation, electrophoretically separated in a 1.0% agarose gel, and then blotted onto a nylon membrane (Hybond-N+; Amersham Biosciences, London, UK). A partial glucoamylase gene sequence was amplified using primers T-M30773GP2F4 and T-M30773Gp2-5RACEZ1 (see Table 1) and used as the DNA hybridization probe, which was labeled and detected using a Dig-High Prime DNA Labeling and Detection kit (Roche Diagnostics, Tokyo, Japan). Nested PCR was used to label the probe.

Reverse transcription-PCR (RT-PCR)

Total RNA from *T. matsutake* strain NBRC 30773 was used as a template for all RT-PCR reactions performed in this study. Amplification of full-length cDNA by RT-PCR and 3′-rapid amplification of cDNA ends (RACE)-PCR were performed using a Takara RNA LA PCR kit (AMV) (Version 1.1; Takara Bio). 5′-RACE PCR was performed using a 5′-Full RACE Core Set (Takara Bio). All reverse transcription reactions and PCR were performed according to the manufacturer's instructions. The amplified fragments generated by these methods were subcloned into the pT7Blue (R) T-vector and sequenced, as already described.

Real-time PCR assay

A real-time PCR assay was performed to monitor the expression of the *TmGlu1* gene using the actin gene as a housekeeping gene. A partial actin gene fragment from *T. matsutake* NBRC 30773 was amplified using the degenerate PCR primers UnivActF1 and UnivActR1 (see Table 1). Primers for *TmGlu1* and actin used in the real-time PCR assay were designed based on their cDNA sequences using GENETYX 9.0 (Genetyx). In addition, 3–6 bases of the 3′-primer were designed to overlap the intron–exon boundary of each target gene. All primers were tested to ensure amplification of a single band without the formation of primer dimers. The PCR products of the partial actin gene and *TmGlu1* were each ligated into pT7Blue (R) T-vector. Plasmid extraction was performed according to a modified method of Birnboim (1983). Four 10-fold dilutions of plasmid were made to construct standard curves. Real-time PCR was conducted using the one-step RNA-direct SYBR Green Real-time PCR Master Mix (Toyobo) and Linegene (BioFlux, Hangzhou, China). The cycling parameters were 90°C for 30 s to activate thermostable DNA polymerase, 61°C for 20 min for reverse transcription, 95°C for 30 s pre-denaturation, and then 35 cycles of 95°C for 15 s, 68°C for 15 s, and 74°C for 30 s. Each reaction was run twice using a LineGene Real-Time Thermal Cycler (BioFlux). Melting curves were determined according to the manufacturer's instructions. After real-time RT-PCR, samples were run on a 1.5% agarose gel to confirm amplification specificity. Relative gene expression was expressed as a ratio of transcript concentration of the target gene (*TmGlu1*) to that of the housekeeping gene (actin), and values are reported as the mean level of gene expression from at least two separate PCR experiments using the same preparation of RNA. The level of transcription was determined in triplicate for all tested media.

Results

Structure of the gene encoding glucoamylase (*TmGlu1*) in *T. matsutake*

To confirm the presence of a gene encoding glucoamylase in the *T. matsutake* genome, extracted genomic DNA was used as a template for PCR performed with degenerate primers based on known fungal glucoamylase sequences. A putative glucoamylase gene, designated *TmGlu1*, with a 2,186-bp coding region (from ATG to the stop codon), was successfully amplified and sequenced. The locations of exons and introns were determined from the nucleotide sequences of the PCR products amplified by 3′- and 5′-RACE PCR and RT-PCR. The coding region consisted of nine exons and eight introns. All of the introns started with the nucleotides GT and ended with AG. In the southern hybridization performed using a partial *TmGlu1* DNA fragment as a probe, one hybridization band was detected in the strain NBRC 30773 genome (Fig. 1), indicating that only a single copy of *TmGlu1* exists in the haploid genome of *T. matsutake* NBRC 30773.

Tricholoma matsutake strain NBRC 30773 was originally isolated by a fruiting-body tissue isolation method; there is no report whether *T. matsutake* isolated from the fruiting body is heterokaryotic or homokaryotic. Our Southern hybridization analysis using a partial *TmGlu1* DNA fragment as probe detected only one hybridization band in strain NBRC 30773. Thus, we cannot rule out the possible existence of an allelic copy of TmGlu1 in NBRC 30773 if this strain was heterokaryotic. In future experiments, we will attempt to isolate the allelic *TmGlu1* gene in strain NBRC 30773.

The identified *TmGlu1* gene was predicted to encode a glycoside hydrolase protein of 576 amino acids and contain 4 signatures of family 15 glycoside hydrolases and 2 starch-binding domains. The PSORT II program predicted that this protein is extracellular or anchored in the cell wall (probability = 55.6%). The first 15 amino acids in the N-terminal region functioning as a putative signal peptide

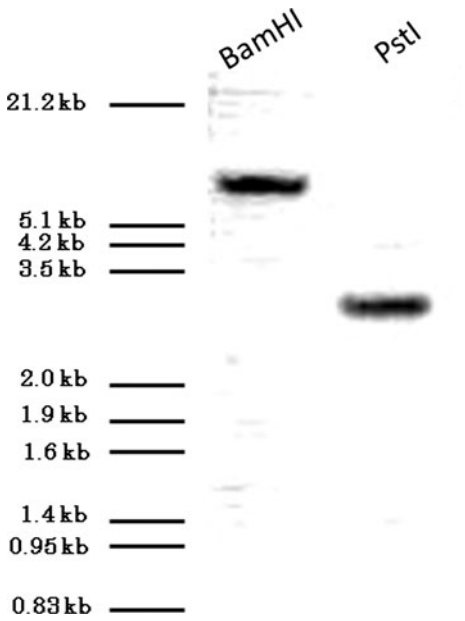

Fig. 1 Hybridization of a *TmGlu1* probe to *Tricholoma matsutake* genomic DNA. Genomic DNA (1 μg) from *T. matsutake* was digested with either *Bam*HI or *Pst*I, as indicated *above each lane*. The blot was probed with PCR products of *TmGlu1* labeled with digoxigenin. The marker sizes correspond to the fragments of λDNA digested with *Eco*RI and *Hin*dIII

was also predicted. Moreover, the SOSUI program estimated that the TmGlu1 protein is water soluble.

The deduced protein sequence of the strain NBRC 30773 glucoamylase had 69% identity and 83% similarity with the glycoside hydrolase family 15 protein of *Laccaria bicolor* (Maire) P.D. Orton (Martin et al. 2008) and 67% identity and 81% similarity with the glucoamylase protein of *L. edodes* (Zhao et al. 2000). The phylogenetic tree of fungal glucoamylases constructed by the unweighted pair group method with arithmetic mean (UPGMA) clustering method is shown in Fig. 2. The glucoamylase from the ectomycorrhizal basidiomycetes *T. matsutake* and *Laccaria bicolor* and several saprophytic basidiomycetes, including *Lentinula edodes* and *Athelia rolfsii*, formed a single clade, which was distinct from that formed by ascomycete fungi.

Effect of pure starch on glucoamylase production and gene expression

To investigate the ability of *T. matsutake* NBRC 30773 to utilized pure starch as a growth substrate, glucoamylase activity was measured after incubation of mycelia for 30 days in liquid media containing several different sole carbon sources. The results showed that the glucoamylase activity in medium corn starch (8.0 mU/ml), soluble starch (6.2 mU/ml), wheat starch (8.3 mU/ml), and amylose (16.1 mU/ml) was higher compared to that in media containing glucose (1.5 mU/ml) (Fig. 3). The total protein concentration in the culture broth after 30 days of incubation was also measured (Fig. 3). The highest protein concentration was observed in the amylose-supplemented medium (6.7 mg/ml).

To determine the reasons for the increased glucoamylase activity in the media containing pure starch as a carbon source, real-time PCR was used to monitor the expression of *TmGlu1* mRNA in mycelia cultured in the different media. When mycelia were cultured in medium supplemented with glucose for 30 days and then transferred into medium containing a different starch carbon source for 2 days, no obvious increases in the transcription level of *TmGlu1* were detected (data not shown).

In contrast, when mycelia were initially cultured in medium containing different types of starch for 30 days, higher relative gene transcripts (compared to actin) were detected in the medium supplemented with corn starch (relative gene expression was 0.005, 1.808 times that of glucose medium), soluble starch (0.006, 2.199), wheat starch (0.009, 3.129), and amylose (0.012, 4.216) (Fig. 4). These analyses revealed that both glucoamylase activity and the expression level of *TmGlu1* in *T. matsutake* NBRC 30773 were highest in medium containing amylose compared with media supplemented with the other examined starch compounds.

Effects of compound starch on glucoamylase activity and *TmGlu1* expression

Because of the high cost of preparing pure starch, compound starch sources such as rice, wheat, and barley grains represent cheap and potentially suitable carbon sources for the artificial cultivation of *T. matsutake*. Here, the glucoamylase activity and total protein amount in culture medium and the transcriptional level of *TmGlu1* in mycelia after incubation for 30 days in media containing several types of compound starch powder were also investigated. Compared with the glucose-supplemented medium, the total amount of extracellular protein in the media supplemented with the compound starch sources showed no marked differences; however, a higher level of glucoamylase activity was detected in the media of all examined compounds, particularly in the barley (4.6 mU/ml) medium (Fig. 3). In addition, increased transcription of *TmGlu1* was detected in the mycelia cultured using wheat (relative gene expression was 0.004, 1.429 times that of glucose medium), barley (0.006, 2.230), buckwheat (0.005, 1.847), and corn (0.005, 1.850) compared to that of rice (0.003, 1.101) and glucose (0.003) (Fig. 4). Although there was no significance between transcription levels in wheat and rice, there were significant differences between three compound starches (barley, buckwheat, corn) and glucose. These results suggest that the media containing barley, buckwheat, and corn affected glucoamylase production.

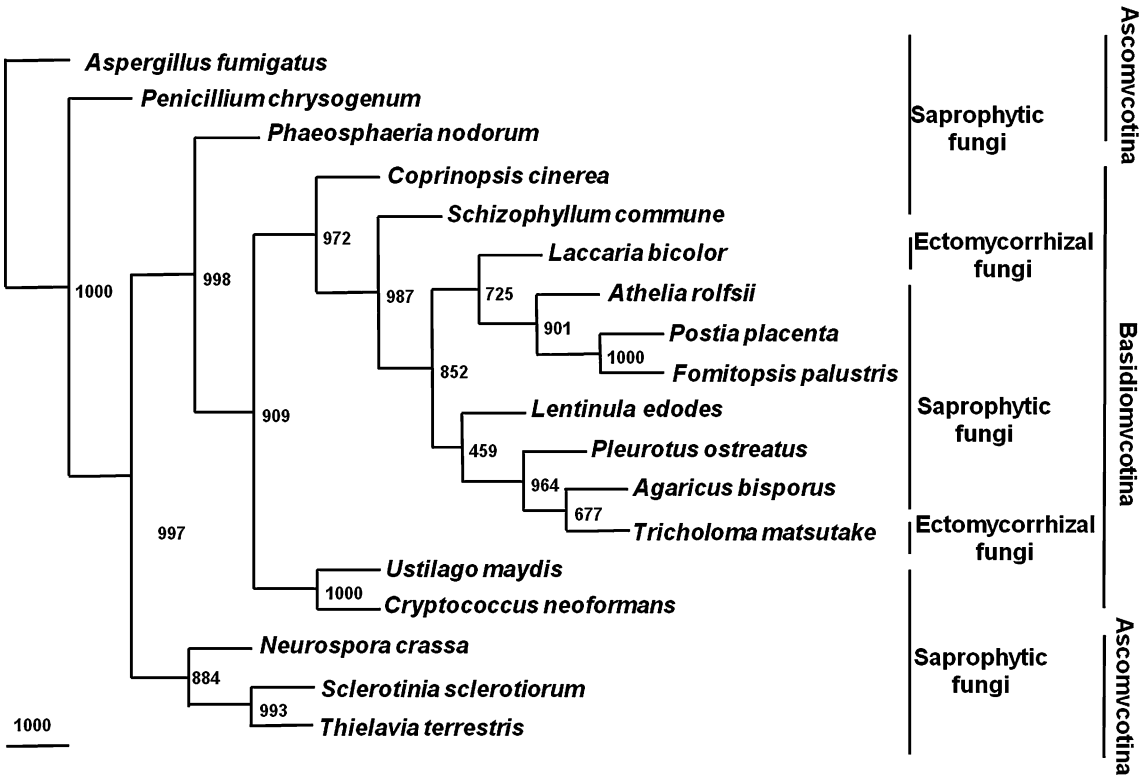

Fig. 2 Phylogenetic tree constructed by the UPGMA method based on the amino acid sequences of fungal glucoamylases showing the bootstrap values from 1,000 replicates. Protein sequences of glucoamylase proteins from *Lentinula edodes* (Zhao et al. 2000), *Laccaria bicolor* (Martin et al. 2008), *Coprinopsis cinerea*, *Athelia rolfsii* (Curzi) C.C. Tu & Kimbrough (Nagasaka et al. 1995), *Postia placenta* (Fr.) M. J. Larsen & Lombard (Martinez et al. 2009), *Neurospora crassa* Shear & B.O. Dodge (Stone et al. 1993), *Aspergillus fumigatus* Fresenius (Fedorova et al. 2008), *Sclerotinia sclerotiorum* (Libert) de Bary, *Schizophyllum commune* Fries, *Phaeosphaeria nodorum* (E. Muller) Hedjaroude, *Penicillium chrysogenum* Thom (Van den Berg et al. 2008), *Thielavia terrestris* (Apinis) Malloch & Cain (Rey et al. 2003), and *Fomitopsis palustris* (Berkeley & Curtis) Teixeira (Yoon et al. 2006) are listed in the DNA database under accession numbers Q9P4C5, B0CVJ1, A8NSG1, Q12596, B8PI57, P14804, EDP53734.1, XP001588171, XP003030591, XP001805434, XP002560481, AAE85601, and AB239766, respectively. Protein sequences of glucoamylase proteins from *Pleurotus ostreatus* (Jacquin: Fries) Kummer and *Agaricus bisporus* (Lange) Imbach are listed in the DOE Joint Genome Institute (JGI) database on the location scaffold_05:2170159-2172303 and scaffold_5:607527-609814. Protein sequences of glucoamylase proteins from *Cryptococcus neoformans* (Sanfelice) Vuillemin and *Ustilago maydis* (DeCandolle) Corda are listed in the broad institute database on the location CNAG_02283.2 and UM04064.1

Discussion

In the present study, a single copy of a gene encoding glucoamylase was identified and characterized in the *T. matsutake* NBRC 30773 genome and was found to be induced in the presence of starch.

In fungal fruiting bodies, carbohydrates are mainly present as polysaccharides and glycoproteins, ranging in content from 50% to 90%; the most abundant polysaccharides are glucans that are synthesized from glucose (Wasser 2002; Synytsya et al. 2008). Glucose can also be used as an energy source by fungal cells and is a common metabolic intermediate. To obtain glucose, fungi produce enzymes to degrade complex molecules such as cellulose and starch. In the course of cellulose degradation, fungi synthesize cellobiohydrolases, which are divided into two groups: glycoside hydrolase family 6 (GH6) and glycoside hydrolase family 7 (GH7) enzymes. During starch degradation, glucoamylase functions to directly break down polymeric starch to glucose (Phillips and Caldwell 1951). The genes encoding GH6 or GH7 enzymes have been identified and characterized in the saprophytic fungi *Coprinopsis cinerea* (Schaeff) (Yoshida et al. 2009; Stajich et al. 2010) and *L. edodes* (Lee et al. 2001). Moreover, glucoamylase genes have also been cloned and characterized in these two saprophytic fungal species (Stajich et al. 2010; Zhao et al. 2000). In the ectomycorrhizal fungus *Laccaria bicolor*, a gene for glucoamylase, but not cellobiohydrolase, has been identified (Martin et al. 2008). Also, in this study, the gene encoding glucoamylase was cloned in *T. matsutake* NBRC 30773 and induced by starch. Basis on phylogenetic tree results, the lack of distinct phylogenetic relationships in the predicted glucoamylase protein between saprophytic and ectomycorrhizal fungi indicate

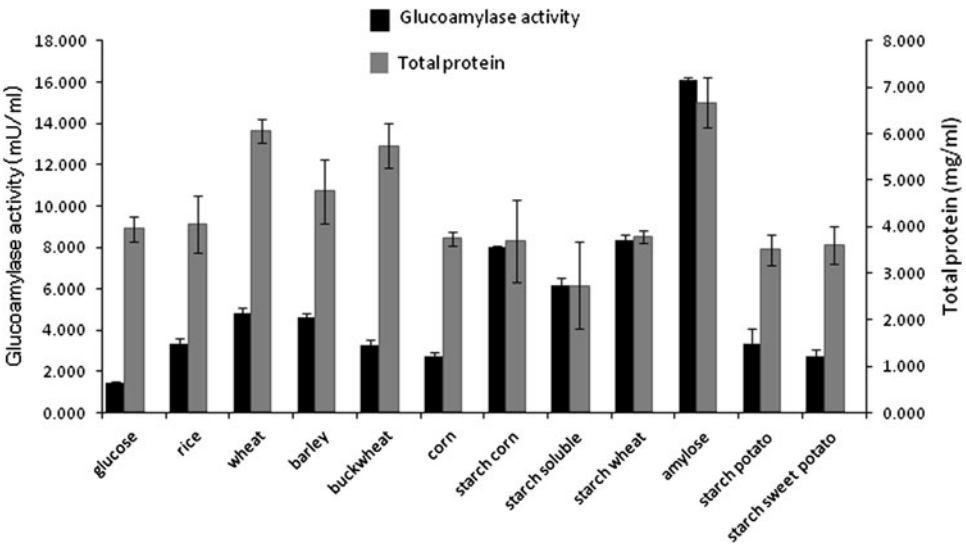

Fig. 3 Effects of different forms of starch on the production of glucoamylase. Mycelia of *Tricholoma matsutake* were cultured in medium supplemented with the indicated forms of starch for 30 days, and glucoamylase activity in the medium and protein content of the medium were then determined. *Error bars* indicate standard deviation of experiments performed in triplicate

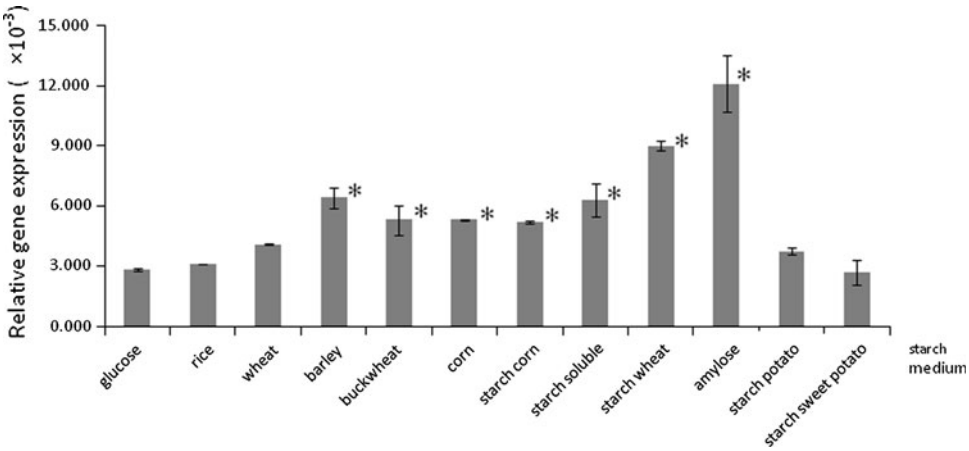

Fig. 4 Transcription levels of *TmGlu1* in *T. matsutake* mycelia when cultured using either glucose or different types of starch as the carbon source. The transcription levels are relative to the housekeeping gene actin. *Error bars* indicate standard deviation of experiments performed in triplicate; *asterisks* indicate difference of transcription level is significant between glucose and tested medium (t test, $P < 0.05$)

that the evolution of glucoamylase did not influence the relationships of saprophytic and ectomycorrhizal fungi. These findings also suggest that, in these two types of fungi, glucoamylase may relate to some common functions, for example, mycelium growth and fruit body formation. However, these details are not yet clear.

From a survey of much work on growth of ectomycorrhizae (ECM) fungi in artificial medium, Harley and Smith (1983) concluded that most ECM fungi have at most a limited ability to use lignin and cellulose as substrates for their growth. Later, Smith and Read (2008) concluded that monosaccharides such as glucose, mannose, and fructose are usually good carbon sources for growth, whereas pectic substances (polygalacturonic acid) can be used for growth by some ECM fungi but not others. The potential to use different sources of carbon clearly varies between fungal species and could possibly be related to fungal survival in soil (Smith and Read 2008). Also, in this study, we cloned the glucoamylase gene in *T. matsutake* NBRC 30773 and confirmed that the glucoamylase gene can be induced by starch. We speculated that in the natural habitat, host plants supply short-chain starch molecules to ectomycorrhizal fungi, and glucoamylase is therefore required to directly degrade this short-chain starch to glucose, which the fungi require for growth.

Here, we found that glucoamylase activity and expression of the *TmGlu1* gene in *T. matsutake* NBRC 30773 were highest in medium containing amylose compared with media supplemented with other forms of starch. This finding suggests that amylose may strongly induce the expression of *TmGlu1*, further increasing glucoamylase activity. Moreover, in the media containing different types of compound starch, glucoamylase activity was highest in medium supplemented with barely, which also had a higher content of amylose than that of rice, wheat, and buckwheat. A similar phenomenon was discovered in a study involving several species of ectomycorrhizal fungi cultured using starch and related substrates as carbon sources (Ohta 1997). Ohta suggested that amylose in barley grains may be preferentially decomposed by the ectomycorrhizal fungi. Thus, we also measured the content of amylose in the four

types of compound starch. Barley (0.107%) contained a higher content of amylose than that of rice (0.084%), wheat (0.095%), or buckwheat (0.097%). This finding may explain the higher extracellular glucoamylase activity and transcription level of *TmGlu1* in the barley-supplemented medium than that of wheat, rice, and buckwheat. Although corn contained the highest level of amylose (0.115%), only a slight increment of extracellular glucoamylase activity and no obvious increase of *TmGlu1* transcription by mycelia were detected in the corn-supplemented medium. It is possible that other components in the corn also affect the utilization of starch.

In the present study, we also detected no obvious increases in the transcription level of *TmGlu1* when mycelia were cultured in medium supplemented with glucose for 30 days and then transferred into medium containing a different starch carbon source for 2 days. The reason for this difference may be the indirect induction of glucoamylase gene expression by starch in *T. matsutake* mycelium. During mycelium growth, enzymes related to glucoamylase are secreted that convert a portion of the available starch to oligosaccharides, which can easily enter the cell and induce *TmGlu1* expression.

In several ectomycorrhizal fungi, such as *Lyophyllum shimeji*, artificial cultivation has been achieved using barley grain as a carbon source. However, the characteristics of glucoamylase activity and expression that affect the utilization of carbon source and the formation of fruiting bodies are unclear. In a future study, we will focus on the glucoamylase in *L. shimeji*, as it may provide further insight into successful artificial cultivation approaches for ectomycorrhizal fungi.

Acknowledgments This research was partially supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology via a Grant-in-Aid for Scientific Research (C), 20580175, 2008–2010. This work was also supported in part by the Global COE Program (Advanced Utilization of Fungus/Mushroom Resources for Sustainable Society in Harmony with Nature), MEXT, Japan.

References

Birnboim HC (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol 100:243–255

Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bowyer P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Haas BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, White OR, Whitty BR, Youngman P, Wolfe KH, Goldman GH, Wortman JR, Jiang B, Denning DW, Nierman WC (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PloS Genet 4:e1000046

Harley JL, Smith SE (1983) Mycorrhizal symbiosis, 2nd edn. Academic Press, London

Hirokawa T, Boon-Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14:378–379

Horton P, Nakai K (1997) Better prediction of protein cellular localization sites with the k nearest neighbors classifier. Proc Int Conf Intell Syst Mol Biol l5:147–152

Lee CC, Wong WD, Robertson GH (2001) Cloning and characterization of two cellulase genes from *Lentinula edodes*. FEMS Microbiol Lett 205:355–360

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Martin F, Aerts A, Ahren D, Brun A, Danchin EGJ, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, Salamov A, Shapiro HJ, Wuyts J, Blaudez D, Buee M, Brokstein P, Canbaeck B, Cohen D, Courty PE, Coutinho PM, Delaruelle C, Detter JC, Deveau A, DiFazio S, Duplessis S, Fraissinet-Tachet L, Lucic E, Frey-Klett P, Fourrey C, Feussner I, Gay G, Grimwood J, Hoegger PJ, Jain P, Kilaru S, Labbe J, Lin YC, Legue V, Le Tacon F, Marmeisse R, Melayah D, Montanini B, Muratet M, Nehls U, Niculita-Hirzel H, Oudot-Le Secq MP, Peter M, Quesneville H, Rajashekar B, Reich M, Rouhier N, Schmutz J, Yin T, Chalot M, Henrissat B, Kuees U, Lucas S, Van de Peer Y, Podila GK, Polle A, Pukkila PJ, Richardson PM, Rouze P, Sanders IR, Stajich JE, Tunlid A, Tuskan G, Grigoriev IV (2008) The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature (Lond) 452:88–92

Martinez D, Challacombe J, Morgenstern I, Hibbett D, Schmoll M, Kubicek CP, Ferreira P, Ruiz-Duenas FJ, Martinez AT, Kersten P, Hammel KE, Vanden Wymelenberg A, Gaskell J, Lindquist E, Sabat G, Bondurant SS, Larrondo LF, Canessa P, Vicuna R, Yadav J, Doddapaneni H, Subramanian V, Pisabarro AG, Lavin JL, Oguiza JA, Master E, Henrissat B, Coutinho PM, Harris P, Magnuson JK, Baker SE, Bruno K, Kenealy W, Hoegger PJ, Kües U, Ramaiya P, Lucas S, Salamov A, Shapiro H, Tu H, Chee CL, Misra M, Xie G, Teter S, Yaver D, James T, Mokrejs M, Pospisek M, Grigoriev IV, Brettin T, Rokhsar D, Berka R, Cullen D (2009) Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. Proc Natl Acad Sci USA 106:1954–1959

Nagasaka Y, Muraki N, Kimura A, Suto M, Yokota A, Tomita F (1995) Cloning of *Corticium rolfsii* glucoamylase cDNA and its expression in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 44:451–458

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

Phillips LL, Caldwell ML (1951) A study of the action of glucoamylase, a glucose-producing amylase, formed by the mold, *Rhizopus delemar*. J Am Chem Soc 73:3563–3568

Rey MW, Brown KM, Golightly EJ, Fuglasang CC, Nielsen BR, Hendriksen HV, Butterworth A, Xu F (2003) Cloning, heterologous expression, and characterization of *Thielavia terrestris* glucoamylase. Appl Biochem Biotechnol 111:153–166

Sakaguchi K, Takagi M, Horiuchi H, Gomi K (1992) Fungal enzymes used in oriental food and beverage industries. In: Kinghorn JR, Turner G (eds) Applied molecular genetics of filamentous fungi. Blackie Academic, London

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, London

Stajich JS, Wilke SK, Ahrén D, Au CH, Birren BW, Borodovsky M, Burns C, Canbäck B, Casselton LA, Cheng CK, Deng JX, Dietrich FS, Fargo DC, Farman ML, Gathman AC, Goldberg J, Guigó R, Hoegger PJ, Hooker JB, Huggins A, James TY,

Kamada T, Kilaru S, Kodira C, Kües U, Kupfer D, Kwan HS, Lomsadze A, Li WX, Lilly WW, Ma LJ, Mackey AJ, Manning G, Martin F, Muraguchi H, Natvig DO, Palmerini H, Ramesh MA, Rehmeyer CJ, Roe BA, Shenoy N, Stanke M, Ter-Hovhannisyan V, Tunlid A, Velagapudi R, Vision TJ, Zeng QD, Zolan ME, Pukkila PJ (2010) Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom *Coprinopsis cinerea* (*Coprinus cinereus*). Proc Natl Acad Sci USA 107:11889–11894

Stone PJ, Makoff AJ, Parish JH, Radford A (1993) Cloning and sequence analysis of the glucoamylase gene of *Neurospora crassa*. Curr Genet 24:205–211

Synytsya A, Míčková K, Jablonský I, Sluková M, Čopíková J (2008) Mushrooms of genus *Pleurotus* as a source of dietary fibers and glucans for food supplements. Czech J Food Sci 26:441–446

Van den Berg MA, Albang R, Albermann K, Badger JH, Daran JM, Driessen AJ, Garcia-Estrada C, Fedorova ND, Harris DM, Heijne WH, Joardar V, Kiel JA, Kovalchuk A, Martin JF, Nierman WC, Nijland JG, Pronk JT, Roubos JA, van der Klei IJ, van Peij NN, Veenhuis M, von Dohren H, Wagner C, Wortman J, Bovenberg RA (2008) Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. Nat Biotechnol 26:1161–1168

Wasser SP (2002) Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol 60:258–274

Yamada A (2005) To the artificial cultivation: the present situation and the approach on the developmental research. Technol Innov 15:24–28 (in Japanese)

Yoon JJ, Igarashi K, Kajisa T, Samejima M (2006) Purification, identification and molecular cloning of glycoside hydrolase family 15 glucoamylase from the brown-rot basidiomycete *Fomitopsis palustris*. FEMS Microbiol Lett 259:288–294

Yoshida M, Sato K, Kaneko S, Fukuda K (2009) Cloning and transcript analysis of multiple genes encoding the glycoside hydrolase family 6 enzyme from *Coprinopsis cinerea*. Biosci Biotechnol Biochem 73:67–73

Zhao J, Chen YH, Kwan HS (2000) Molecular cloning, characterization, and differential expression of a glucoamylase gene from the basidiomycetous fungus *Lentinula edodes*. Appl Environ Microbiol 66:2531–2535