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Functional Analysis of a Gene Encoding Endoglucanase that Belongs to Glycosyl Hydrolase Family 12 from the Brown-Rot Basidiomycete *Fomitopsis palustris*

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The brown-rot basidiomycete *Fomitopsis palustris* is known to degrade crystalline cellulose (Avicel) and produce three major cellulases, exoglucanases, endoglucanases, and β-glucosidases. A gene encoding endoglucanase, designated as *cel12*, was cloned from total RNA prepared from *F. palustris* grown at the expense of Avicel. The gene encoding Cel12 has an open reading frame of 732 bp, encoding a putative protein of 244 amino acid residues with a putative signal peptide residing at the first 18 amino acid residues of the N-terminus of the protein. Sequence analysis of Cel12 identified three consensus regions, which are highly conserved among fungal cellulases belonging to GH family 12. However, a cellulose-binding domain was not found in Cel12, like other GH family 12 fungal cellulases. Northern blot analysis showed a dramatic increase of *cel12* mRNA levels in *F. palustris* cells cultivated on Avicel from the early to late stages of growth and the maintenance of a high level of expression in the late stage, suggesting that Cel12 takes a significant part in endoglucanase activity throughout the growth of *F. palustris*. Adventitious expression of *cel12* in the yeast *Pichia pastoris* successfully produced the recombinant protein that exhibited endoglucanase activity with carboxymethyl cellulose, but not with crystalline cellulose, suggesting that the enzyme is not a processive endoglucanase unlike two other endoglucanases previously identified in *F. palustris*.

Keywords: Cellulase, endoglucanase, *Fomitopsis palustris*, glycosyl hydrolase family 12, *Pichia pastoris*

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Cellulose, a linear polymer of glucose units linked by β-1,4-D-glucosidic bonds, is the main constituent of woody biomass. Cellulolytic microorganisms, which have the ability to survive at the expense of crystalline cellulose, produce a comprehensive cellulase system consisting of three classes of enzymes: endoglucanases (E.C. 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91), and β-glucosidases (E.C. 3.2.1.21) [13]. Synergistic action of all these classes is required for complete hydrolysis of crystalline cellulose to smaller oligosaccharides and finally to glucose [7]. A number of microorganisms have been reported to produce various kinds of cellulases [11, 12, 16, 20, 21, 24]. A general feature of most cellulases is a modular structure including catalytic and carbohydrate-binding modules [13], and classification of cellulases is currently grouped into 15 of more than 80 known glycosyl hydrolase (GH) families according to the structural features of their catalytic domains [2, 5, 8, 17].

Brown-rot basidiomycetes cause the most destructive type of wood decay and are important contributors to biomass recycling [10]. These basidiomycetes are unusual since they rapidly depolymerize the cellulose in wood without removing the surrounding lignin that normally prevents microbial attack [9]. The brown-rot basidiomycete *Fomitopsis palustris* is known to degrade crystalline cellulose (Avicel) and produce three major cellulases [22]. Yoon *et al.* [23] recently reported that *F. palustris* possesses two processive endoglucanases capable of degrading crystalline cellulose, one of which appears to belong to glycosyl hydrolase family 5 according to the analysis of partial amino acid sequences.

In this paper, we report the expression pattern and enzymatic properties of a previously uncharacterized endoglucanase in *F. palustris* that belongs to GH family 12.

MATERIALS AND METHODS

Strains and Culture Conditions

The brown-rot fungus *Fomitopsis palustris* FFPRI 0507 (Berkeley *et* Curtis) Murill was used in this study. The fungus was precultured on PDA (Potato Dextrose Agar; Conda, U.S.A.) plates at 28°C for 7 days. To prepare the precultures, 10 agar-mycelium plugs with 5-mm diameter were punched out and inoculated into 100 ml of PDB (Potato Dextrose Broth; Conda, U.S.A.) media at 28°C for 7 days on a rotary shaker at 150 rpm. Fifteen ml of *F. palustris* preculture was inoculated into 100 ml of fresh liquid media (*Fomitopsis* Avicel Medium, FAM) containing 2% (w/v) Avicel (Fluka, Switzerland) as a carbon source, 0.8% (w/v) peptone, 0.5% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, 0.3% (w/v) $MgSO_4 \cdot 7H_2O$, 0.2% (w/v) yeast extract, and 5 ppm thiamine-HCl. The cultures were then incubated at 28°C for up to 28 days on a rotary shaker at 150 rpm. *Pichia pastoris* GS115 carrying recombinant plasmids was precultured in BMGY medium consisting of 0.5% (w/v) yeast extract, 1% (w/v) peptone, 1% (v/v) glycerol, 1.34% (w/v) YNB (Yeast Nitrogen Base with amino acids), and 0.00004% (w/v) biotin in 100 mM potassium phosphate buffer (pH 6.0) and transferred to BMMY consisting of the same composition as BMGY medium, except that 0.5% (v/v) methanol was added instead of glycerol for induction.

RNA Extraction and cDNA Synthesis

F. palustris was grown as described above and the mycelia recovered by filtration were crushed in liquid nitrogen. Total RNA was isolated from mycelia using a total RNA extraction kit (RNA-spin; iNtRON Biotechnology, Korea). Polyadenylated RNA was isolated from total RNA using an mRNA purification kit (Oligotex mRNA Mini Kit; Qiagen, Germany). cDNA was synthesized using Maxime RT-premix (iNtRON Biotechnology, Korea) from polyadenylated RNA.

PCR Amplification and Cloning

PCR amplification of cDNA fragments encoding a putative endoglucanase was carried out using a PalmCycler (Corbett, Australia). Degenerate oligonucleotide primers were designed based on the deduced amino acid sequence in the NCBI database (BAF49602): forward, 5'-ATGCARCTSCGBACBTCNTTYGT-3'; reverse 5'-GTRTTNACNGTNACNGARTARTC-3' (B, N, R, S, and Y represent G/C/T, A/G/C/T, A/G, C/G, and C/T, respectively). Reaction conditions for PCR amplification were initial denaturation at 94°C for 10 min, 30 cycles of extension at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 1 min, and final extension at 72°C for 10 min. The amplified DNA fragment was analyzed by gel electrophoresis and purified by a gene clean kit (QIAquick Gel Extraction Kit; Qiagen, Germany). The purified DNA fragment was cloned into a T&A cloning vector (RBC Bioscience, Taiwan) and transformed into *E. coli* DH5α. The nucleotide sequence of the cloned DNA fragment was analyzed by Macrogen (Korea).

Northern Blot Analysis

Total RNA (30 μg) was denatured in 50% formamide containing 6.5% formaldehyde and electrophoresed in a 1.2% agarose gel containing 0.6 M formaldehyde. Separated RNAs were blotted onto a charged nylon membrane and hybridized with a ^{32}P-labeled probe synthesized using a random primed DNA labeling kit (Roche, U.S.A.). The primers used for synthesizing template DNA were EG12-F (5'-ATGCAGCTTCGGACCTCGTTC-3') and EG12-R (5'-TGTGTTTACGGTGACAGAGTAGTCG-3'). Hybridization was carried out using Amersharm rapid-hyb buffer (GE Healthcare, U.S.A.) according to the manufacturer's recommendations. The radioactivity of each band was detected using a phosphorimager and measured by OptiQuant image software.

Expression of *cel12* in *P. pastoris*

The full-length cDNA fragment cloned in T&A cloning vector was digested with EcoRI and XbaI restriction enzymes and subcloned into the same restriction sites of shuttle vector pPICZαC (Invitrogen, U.S.A.). The resulting plasmid pPICZαC-cel12 was linearized by digesting with PmeI and transformed into *P. pastoris* GS115 by electroporation. Yeast cells transformed with plasmid were screened on plates containing zeocin (30 μg/ml). A single colony harboring pPICZαC-cel12 was inoculated into a BMGY medium and the culture was incubated at 30°C with shaking at 200 rpm. The culture was harvested by centrifugation at 5,000 ×*g* for 10 min and the cell pellet was resuspended in a fresh BMMY medium. For induction of *cel12* expression, methanol (100%) was added every 24 h to a final concentration of 0.5% (v/v).

Enzyme Assays and Protein Analysis

Endoglucanase activity of culture media was assayed in 100 mM sodium acetate (pH 5.0) using Ostazin Brilliant Red H-3B hydroxyethyl cellulose (OBR-HEC) (Sigma, U.S.A.) as described by Biely *et al.* [1]. The enzyme solution was incubated with 120 μl of 2.5 mg/ml OBR-HEC in a final volume of 300 μl for 15 min at 40°C. The enzymatic reaction was stopped by adding 900 μl of ethanol and the solution was further incubated for 5 min at room temperature. After the solution was centrifuged for 5 min at 15,000 ×*g*, the absorption of the supernatant was measured at 550 nm. The enzyme activity was expressed as the increase in ΔA_{550} per min.

Cellulase activity (CMCase) against carboxymethylcellulose (CMC) was assayed in a 50-μl reaction mixture containing 1.0% (w/v) CMC (Sigma, U.S.A.) in 100 mM sodium acetate buffer (pH 5.0) at 50°C. Reducing sugars were determined by the Somogyi-Nelson methods [14, 19]. One unit (U) of CMCase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min. Cellulase activity (Avicelase) against Avicel was determined in a 50-μl reaction mixture that contained 1.0% (w/v) Avicel in 100 mM sodium acetate buffer (pH 5.0). The reaction mixtures were incubated in a rotary shaker at 50°C for 21 h. Reducing sugars released from Avicel were determined as described above.

Protein concentration in the enzyme solution was measured with a Protein Assay kit (Bio-Rad Laboratories, U.S.A.) based on Bradford's method [3].

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of the *cel12* Gene from *F. palustris*

The DNA fragment encoding a previously uncharacterized endoglucanase in *F. palustris* was amplified by PCR using

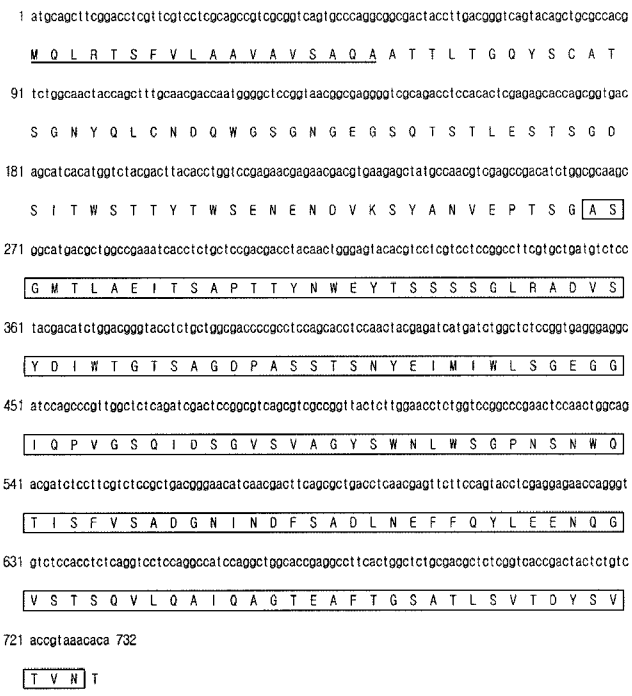

Fig. 1. The deduced amino acid sequence of Cel12.
The amino acid residues corresponding to a signal peptide are underlined. The catalytic domain is boxed.

degenerate oligonucleotide primers designed based on the amino acid sequence in the NCBI database (BAF49602). The amino acid sequence deduced from the nucleotide sequence of the amplified DNA fragment matched exactly with that from the NCBI database. The full-length cDNA consists of 732 bp nucleotides, indicating an open reading frame of 244 amino acid residues, and this gene was designated as *cel12*. Analysis of the amino acid sequence of the protein using the online program SignalP (http://www.cbs.dtu.dk/services/SignalP/) indicated that the first 18 amino acid residues in the N-terminus were assigned as a signal peptide (Fig. 1). The catalytic domain of endoglucanase was observed but a cellulose-binding domain (CBD) was not found in the protein (Fig. 1), like other GH family 12 fungal cellulases [6].

To compare the deduced amino acid sequence of Cel12 with those of other proteins, the BLASTX database was searched. The *F. palustris* endoglucanase sequence showed high sequence similarities with those of *Polyporus arcularius* (59%), *Phanerochaete chrysosporium* (56%), *Aspergillus aculeatus* (43%), *Neosartorya fischeri* (40%), and *Humicola grisea* (35%). Fig. 2 shows multiple alignments of the *F. palustris* sequence with those from other fungal endoglucanases belonging to GH family 12, revealing three highly conserved regions among this group of proteins [6]. Phylogenetic analysis indicated that Cel12 has high homology with enzymes that belong to glycosyl hydrolase (GH) subfamily 12-2 (Fig. 3).

Measurement of *cel12* mRNA Abundance in *F. palustris*

Transcript levels of *cel12* were determined by Northern blot analysis with total RNA isolated from the *F. palustris* mycelia cultured in FAM up to 28 days. The results showed that the abundance of *cel12* mRNA was the highest in the mycelia harvested at 21 days of incubation (Fig. 4). The mRNA level increased dramatically from 14 days of incubation and the high level of expression was maintained until 28 days, suggesting that the enzyme might play an important role in the later stage of growth. The endoglucanase activity of culture supernatant obtained from cells grown on 2% Avicel increased gradually from 0 to 8 days of cultivation, with the highest level at 14 days (Fig. 5). These results indicate that Cel12 takes a significant

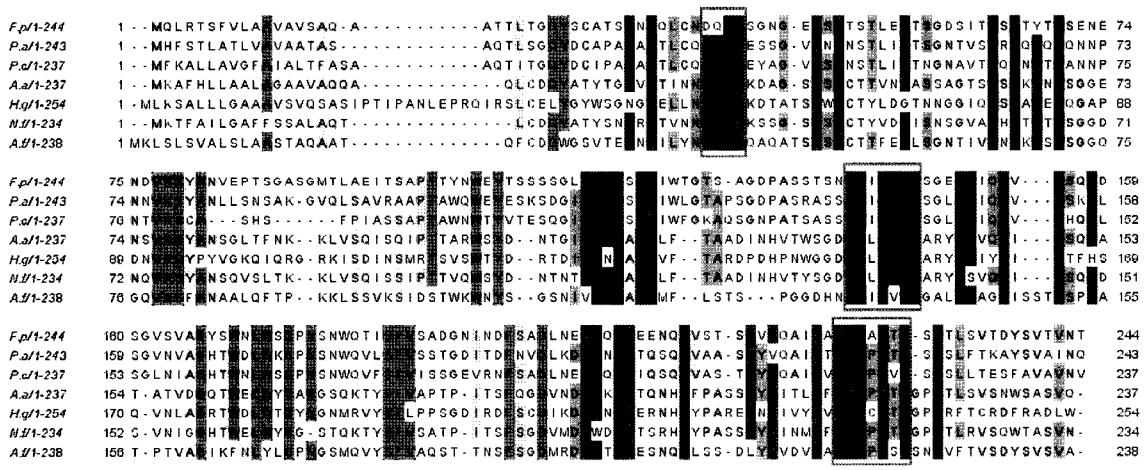

Fig. 2. Comparison of the amino acid sequence of *F. palustris* endogulcanase with those of other fungal enzymes.
Multiple alignments of the sequences were carried out using ClustalW method. The residues conserved in at least three sequences are shaded. Consensus sequences among the fungal enzymes are boxed. Data for other fungi were obtained from sequence databases (DDBJ/EMBL/GenBank). Abbreviations: P.a, *Polyporus arcularius* (BAD98315); P.c, *Phanerochaete chrysosporium* (AAU12276); A.a, *Aspergillus aculeatus* (BAA00435); H.g, *Humicola grisea* (AF435071); N.f, *Neosartorya fischeri* (EAW19666); A.f, *Aspergillus fumigatus* (EAL88184).

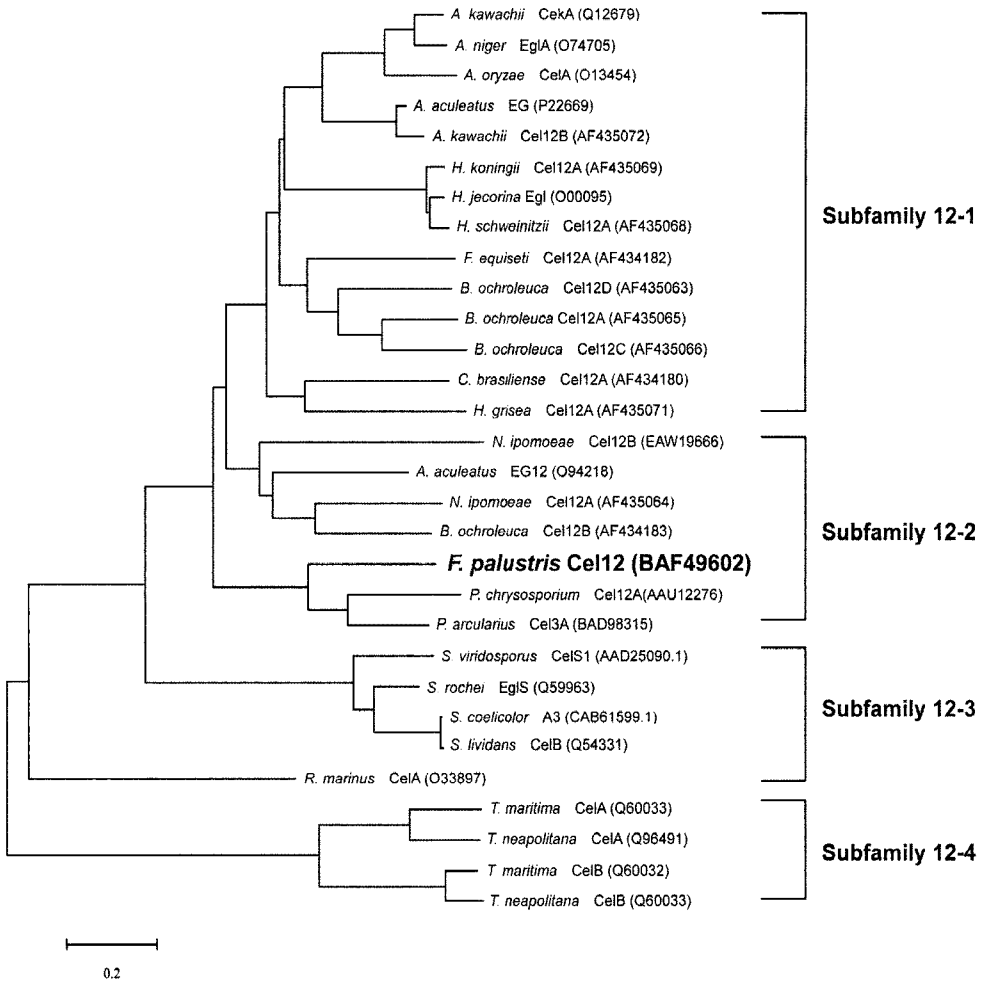

Fig. 3. Phylogenetic tree of known GH family 12 endoglucanases.
Cel12 from *F. palustris* is shown in bold. The dendrogram was constructed from the matrix of correlation distances using the program Mega 4 by the neighbor-joining method.

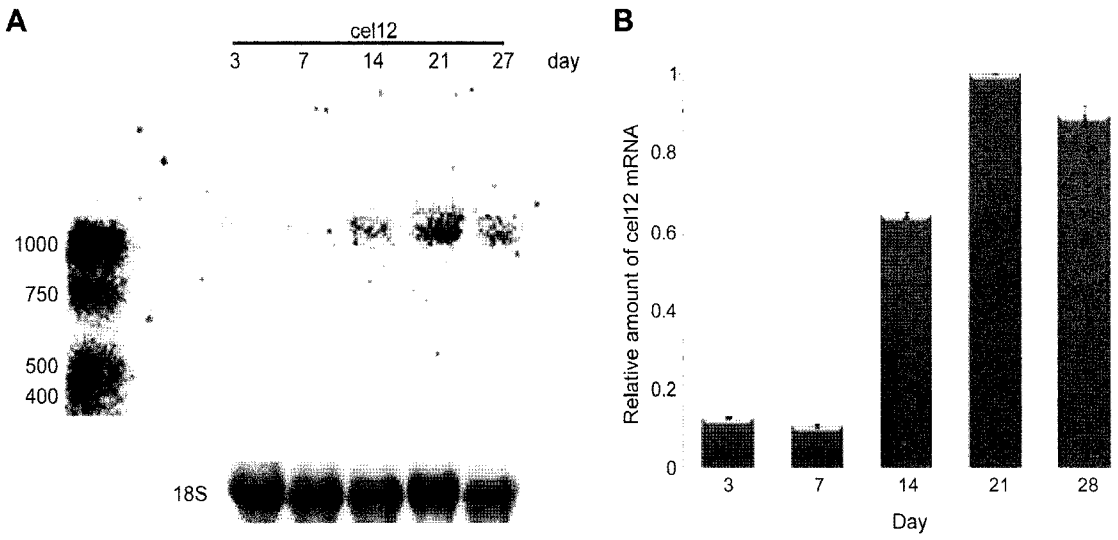

Fig. 4. Abundance of *cel12* mRNA during the growth of *F. palustris*.
Total RNA was isolated from *F. palustris* mycelia cultured in FAM for 3, 7, 14, 21, and 28 days, and the abundance of *cel12* mRNA was measured by Northern blot analysis as described in Materials and Methods. The agarose gel was stained with ethidium bromide before separated RNAs were transferred onto a charged nylon membrane and 18S rRNA bands were visualized.

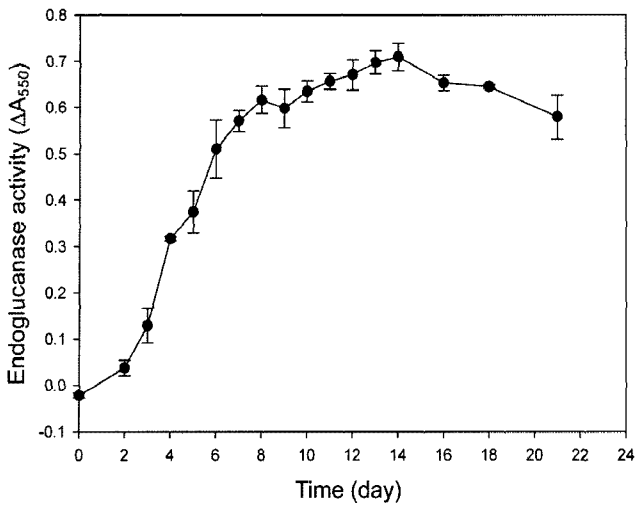

Fig. 5. Endoglucanase activity of culture supernatant during the growth of *F. palustris* on Avicel (2%).
The activity was expressed as ΔA_{550} per min. Values represent the mean of three replicate determinations.

part in endoglucanase activity throughout the growth of *F. palustris* cells.

Functional Expression of *cel12* Gene in *P. pastoris*

Although the amino acid sequence of Cel12 is highly homologous with those of other endoglucanases, whether the amplified DNA fragment encodes an endoglucanase remained to be shown. The *cel12* gene from *F. palustris* was amplified by PCR and inserted into vector pPICZαC to generate an in-frame fusion of the leader peptide of the yeast α-mating factor and Cel12. The resulting recombinant plasmid was transformed into *P. pastoris* and induced for expression of Cel12. The endoglucanase activity of recombinant protein expressed in *P. pastoris* showed that the Cel12 protein was functionally expressed in the yeast (Table 1).

Previous studies revealed that *F. palustris* possesses two processive endoglucanases (EG47 and EG35) that are able to hydrolyze crystalline cellulose [23]. Processive endoglucanases usually display high activity toward the β-1,4-glucanase substrates such as CMC and Avicel since they have cellobiohydrolase activity [4, 23]. A peptide sequence [T(A) XLTGQYSXATTGN] of a previously reported endoglucanase (EG35) from *F. palustris* significantly

Table 1. Endoglucanase activity of the recombinant protein expressed in *P. pastoris*.

Crude protein	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Relative activity (%)
pPICZαC	17.05	15.26	0.89	100
pPICZαC-cel12	6.85	118.21	17.50	1,966

[a]Activity was assayed using CMC as substrate. Avicelase activity was not detected. One unit (U) of CMCase activity is defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min.

Table 2. Substrate specificity of the recombinant enzyme of Cel12.

Substrate	Specific activity (U/mg of protein)[b]
CMC	17.19±1.14
Xylan	2.90±0.31
*p*NPG[a]	4.49±0.01
Avicel	0

[a]*p*NPG, *p*-nitrophenyl-β-glucopyranose.
[b]The presented values are averages (±SD) of duplicate experiments.

matched with the amino acid sequence of Cel12 (data not shown), suggesting a possibility that the *cel12* gene encodes EG35. However, the substrate specificity of the recombinant enzyme of Cel12 was not in accordance with that of the EG35. The Cel12 exhibited endoglucanase activities with CMC, xylan, and *p*-nitrophenyl-β-glucopyranose, but did not have Avicelase activity (Table 2), like other fungal GH family 12 enzymes [6]. Therefore, the sequence analysis and the substrate specificity of Cel12 clearly indicate that Cel12 is different from those previously known in *F. palustris*. Fungi such as *Humicola insolens* [18] and *Trichoderma reesei* [4, 15] have been known to produce at least seven cellulases that belong to five to six enzyme families. So far, *F. palustris* has been known to produce at least three different endoglucanases, including each of GH families 5 and 12 [23].

Our results show that *F. palustris* produces an endoglucanase (Cel12) belonging to GH family 12, which does not have Avicelase activity, unlike previously known endoglucanases from this fungus. This endoglucanase was shown to be maximally expressed from 14 to 28 days of cultivation, suggesting that it might play a distinct role in the metabolism of cellulose in the later growth period. The gene was also functionally expressed in *P. pastoris*. Further studies on the characterization of the recombinant enzyme will be required for a better understanding of the endoglucanase for its potential use in bioethanol production.

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References

1. Biely, P., O. Markovic, and D. Mislovicova. 1985. Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. *Anal. Biochem.* **144:** 147–151.
2. Bourne, Y. and B. Henrissat. 2001. Glycoside hydrolases and glycosyltransferases families and functional modules. *Curr. Opin. Struct. Biol.* **11:** 593–600.

3. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
4. Cohen, R., M. Suzuki, and K. E. Hammel. 2005. Processive endoglucanase active in crystalline cellulose hydrolysis by the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.* **71:** 2412–2417.
5. Gilkes, N. R., B. Henrissat, and D. G. Kilburn. 1991. Domains in microbial beta-1,4-glycanases: Sequence conservation, function, and enzyme families. *Microbiol. Rev.* **55:** 303–315.
6. Goedegebuur, F., T. Fowler, J. Phillips, P. van der Kley, P. van Solingen, L. Dankmeyer, and S. D. Power. 2002. Cloning and relational analysis of 15 novel fungal endoglucanases from family 12 glycosyl hydrolase. *Curr. Genet.* **41:** 89–98.
7. Henrissat, B., H. Driguez, C. Viet, and M. Schulein. 1985. Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. *Biotechnology* **3:** 722–726.
8. Henrissat, B., M. Claeyssens, P. Tomme, L. Lemesle, and J. P. Mornon. 1989. Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81:** 83–95.
9. Highley, T. L. 1973. Influence of carbon source on cellulose activity of white-rot and brown-rot fungi. *Wood Fiber* **5:** 50–58.
10. Kerem, Z., K. A. Jensen, and K. E. Hammel. 1999. Biodegradative mechanism of the brown rot basidiomycete *Gleophyllum trabeum*: Evidence for an extracellular hydroquinone-driven Fenton reaction. *FEBS Lett.* **446:** 49–54.
11. Lee, T.-H., P.-O. Lim, and Y.-E. Lee. 2007. Cloning, characterization, and expression of a xylanase gene from *Paenibacillus* sp. DG-22 in *Escherichia coli*. *J. Microbiol. Biotechnol.* **17:** 29–36.
12. Lim, W. J., S. K. Ryu, S. R. Park, M. K. Kim, C. L. An, S. Y. Hong, E. C. Shin, J. Y. Lee, Y. P. Lim, and H. D. Yun. 2005. Cloning of *celC*, third cellulase gene, from *Pectobacterium carotovorum* subsp. *carotovorum* LY34 and its comparison to those of *Pectobacterium* sp. *J. Microbiol. Biotechnol.* **15:** 302–309.
13. Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* **66:** 506–577.
14. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **153:** 375–380.
15. Nidetzky, B., W. Steiner, and M. Claeyssens. 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: Adsorptions of two cellobiohydrolases, two endocellulases and their core proteins on filter paper and their relation to hydrolysis. *Biochem. J.* **303:** 817–823.
16. Ouyang, J., M. Yan, D. Kong, and L. Xu. 2006. A complete protein pattern of cellulase and hemicellulase genes in the filamentous fungus *Trichoderma reesei*. *Biotechnol. J.* **1:** 1266–1274.
17. Rabinovich, M. L., M. S. Melnick and A. V. Bolobova. 2002. The structure and mechanism of action of cellulolytic enzymes. *Biochemistry (Moscow)* **67:** 1026–1050.
18. Schülein, M. 1997. Enzymatic properties of cellulases from *Humicola insolens*. *J. Biotechnol.* **57:** 71–81.
19. Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* **195:** 19–23.
20. Vanden Wymelenberg, A., P. Minges, G. Sabat, D. Martinez, A. Aerts, A. Salamov, I. Grigoriev, H. Shapiro, N. Putnam, P. Belinky, C. Dosoretz, J. Gaskell, P. Kersten, and D. Cullen. 2006. Computational analysis of the *Phanerochaete chrysosporium* v2.0 genome database and mass spectrometry identification of peptides in ligninolytic cultures reveal complex mixtures of secreted proteins. *Fungal Genet. Biol.* **43:** 343–356.
21. Yang, P., P. Shi, Y. Wang, Y. Bai, K. Meng, H. Luo, T. Yuan, and B. Yao. 2007. Cloning and overexpression of a *Paenibacillus* β-glucanase in *Pichia pastoris*: Purification and characterization of the recombinant enzyme. *J. Microbiol. Biotechnol.* **17:** 58–66.
22. Yoon, J.-J. and Y.-K. Kim. 2005. Degradation of crystalline cellulose by the brown-rot basidiomycete *Fomitopsis palustris*. *J. Microbiol.* **43:** 487–492.
23. Yoon, J.-J., C.-J. Cha, Y.-S. Kim, D.-W. Son, and Y.-K. Kim. 2007. The brown-rot basidiomycete *Fomitopsis palustris* has the endo-glucanases capable of degrading microcrystalline cellulose. *J. Microbiol. Biotechnol.* **17:** 800–805.
24. Yoon, M.-H. and W.-Y. Choi. 2007. Characterization and action patterns of two β-1,4-glucanases purified from *Cellulomonas uda* CS1-1. *J. Microbiol. Biotechnol.* **17:** 1291–1299.