Cloning and Transcript Analysis of Multiple Genes Encoding the Glycoside Hydrolase Family 6 Enzyme from *Coprinopsis cinerea*

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We searched the genome database of the basidiomycete *Coprinopsis cinerea* (*Coprinus cinereus*) and found five genes encoding the glycoside hydrolase family 6 (GH6) enzyme, *CcCel6A*, *CcCel6B*, *CcCel6C*, *CcCel6D*, and *CcCel6E*, designated in order of increasing locus number (CC1G_01107.1, CC1G_04166.1, CC1G_08276.1, CC1G_08277.1, CC1G_10605.1). The amino acid sequence of *CcCel6A* suggests a two-domain structure consisting of an N-terminal family 1 carbohydrate-binding module (CBM1) and a GH6 catalytic domain, while the other genes lack CBM1. The transcripts of *CcCel6A* were observed at the active growth stage in cellulose culture, whereas they were absent from glucose culture. Cellobiose strongly induced transcription of *CcCel6A*. On the other hand, transcripts of *CcCel6B*, *CcCel6D*, and *CcCel6E* were detected in both glucose and cellulose cultures, and transcription of them was induced weakly by cellobiose. The transcript level of *CcCel6C* was not influenced by glucose or cellobiose.

**Key words:** *Coprinus cinereus*; glycoside hydrolase family 6; cellulose degradation; basidiomycete

Cellulose, the most abundant biomass resource on earth, is a linear polymer consisting of glucose units linked by β-1,4-glucosidic bonds. Cellulolytic microorganisms secrete various cellulases, which are traditionally classified into two groups: the endoglucanase (EG; EC 3.2.1.4) and the cellobiohydrolase (CBH; EC 3.2.1.91). EGs cleave the internal β-1,4-glucosidic bond of cellulose at random, while CBHs act primarily on the reducing end of the cellulose chain. CBHs from the ascomycete *Hypocrea jecorina* (imperfect stage *Trichoderma reesei*), the best-studied cellulolytic organism, are further divided into two groups, CBH1 and CBHII. The former acts on the reducing end of the cellulose chain while the latter prefers the non-reducing end.1–4 Together, they are highly efficient in the degradation of crystalline cellulose.5 The three-dimensional structures of CBHs show a tunnel-enclosed active site.6,7 This site permits cleavage of several sequential bonds of the substrate by trapping the cellulose chain in the tunnel, which delays the dissociation of enzyme and substrate, and hence, these enzymes are considered to be processive. The amino acid sequences of the enzymes reveal a two-domain structure8–10 CBH1 consists of a catalytic domain belonging to glycoside hydrolase family 7 (GH7) and a C-terminal family 1 carbohydrate-binding module (CBM1), while CBHII has a GH6 catalytic domain with a CBM1 at the N terminus of the mature protein.

In the plant cell wall, cellulose is normally found in association with hemicellulose and lignin. White rot fungi, a group of basidiomycetes, achieve lignin degradation through reactions of extracellular oxidative enzymes such as lignin peroxidases, manganese peroxidases, versatile peroxidases, and laccases.11,12 In the course of cellulose degradation, the fungi also produce GH7 and GH6 enzymes, which are similar to CBH1 and CBHII, respectively, from ascomycetes,13,14 and the corresponding genes have been cloned from many basidiomycetes. It has been reported that several basidiomycetes, such as *Phanerochaete chrysosporium*15–17 and *Volvariella volvacea*,18 possess multiple genes encoding GH7. Conversely, no basidiomycete with multiple genes encoding GH6 has been found.

The basidiomycete *Coprinopsis cinerea* (*Coprinus cinereus*) produces an extracellular peroxidase19 and multiple laccases.20 In contrast to phenol oxidases, only limited information is available concerning the cellulolytic enzyme produced by this fungus. To identify the genes encoding cellulase, we searched the genome database of *C. cinerea* strain Okayama 7 (#130), provided by the Broad Institute (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html), and found five regions homologous for the gene encoding GH6. We cloned the cDNAs and used quantitative real-time polymerase chain reaction (PCR) to estimate the transcript levels of the genes in cultures containing various carbon sources.

**Materials and Methods**

*Strain.* *C. cinerea* strain 533821 was kindly provided by Dr. Yasuhiro Ito (National Food Research Institute, Ibaraki, Japan).

**Cloning procedure.** Total RNA was extracted from *C. cinerea* grown in Kremer and Wood medium22 containing 2% Avicel (Merck,
Whitehouse Station, NJ) for 12 d using the RNase Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) using an oligo(dT) primer (Takara Bio, Shiga, Japan). The primers shown in Table 1 were used in PCR with KOD plus version 2 DNA polymerase (Toyobo, Osaka, Japan) with synthesized first-strand cDNA as a template. The PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI), followed by sequencing with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sequence analysis. The C. cinerea genome database was searched with the amino acid sequence of CcCel6A from P. chrysosporium (accession no. AAB32942) using the TBLASTN algorithm. The search was carried out with standard settings and the BLOSUM 62 matrix. The amino acid sequences of the candidate genes were scanned for the presence of signal peptides using SignalP version 3.0 software at the Center for Biological Sequence Analysis (http:////www.cbs.dtu.dk/services/SignalP/). Multiple alignment analysis was done with ClustalW at GenomeNet (http://align.genome.jp/). A phylogenetic tree was generated from ClustalW by the neighbor-joining (NJ) method.

Cultivation for time-course experiment. C. cinerea strain 5338 was cultivated for 14 d at 26 °C on MYG agar (glucose, 4 g/l; malt extract, 10 g/l; yeast extract, 4 g/l; agar, 15 g/l). Five pieces of mycelium (diameter 7 mm) were punched out from the agar and inoculated into 200 ml of Kremer and Wood medium containing 2% glucose or 2% Avicel in a 500-ml Erlenmeyer flask. The culture was incubated at 26 °C with rotary shaking (180 rpm, diameter 20 mm). The mycelia were harvested at the appropriate time, immediately frozen in liquid nitrogen, and stored at −80 °C.

Cultivation for induction experiment. The fungus was first cultivated for 7 d in 200 ml of Kremer and Wood medium containing 2% glucose. The mycelia were harvested, washed with medium containing no carbon source, and transferred to a medium containing 10 mM glucose, 10 mM cellobiose, or no carbon source. After incubation for 3 h, the mycelia were frozen in liquid nitrogen, and stored at −80 °C.

Quantification of CcCel6 transcripts. Total RNA was extracted from the mycelia and used in first-strand cDNA synthesis, as described above. The primers for quantitative analysis are given in Table 1. To ensure the specificity of the primers, PCR was carried out with each pair of primers using the synthesized first-strand cDNA as template, and the PCR products were sequenced. Real-time quantitative PCR was performed with an iCycler iQ real-time detection system (Bio-Rad Laboratories, Hercules, CA), as follows: cDNA (100 ng) was used as a template in 25 μl of solution containing PCR buffer, 0.2 mM each dNTP, 0.5 μM each primer, 0.5 × SYBR green master mix (Bio Whittaker Molecular Applications, Rockland, ME), and 0.625 U of Takara Taq Hot Start Version (Takara Bio, Tsu, Japan). The mixtures were incubated at 98 °C for 3 min, followed by amplification for up to 40 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. SYBR green fluorescence was monitored at the end of each cycle to determine the amount of PCR product formed, and the melting curve profiles were recorded after each run. The standard curve for each product followed the calculation of the respective gene transcript. pGEM-T Easy/ CcCel6A cDNA, pGEM-T Easy/CcCel6B cDNA, pGEM-T Easy/ CcCel6C cDNA, pGEM-T Easy/CcCel6D cDNA, and pGEM-T Easy/ CcCel6E cDNA were used in the calibration of the copy numbers of the transcripts. The transcript level of the actin gene (accession no. XM_001839313) was confirmed as a housekeeping target.

Accession numbers of the sequences of CcCel6s. The nucleotide sequences of the cDNAs encoding CcCel6A, CcCel6B, CcCel6C, CcCel6D, and CcCel6E have been submitted to the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases under accession nos. AB433537, AB433538, AB433539, AB433540, and AB433541 respectively.

Table 1. Nucleotide Sequences of Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>For cloning</td>
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<tr>
<td>CcCel6A-F</td>
<td>5'-GCTCATCCGGTTAACAATATACACC-3'</td>
</tr>
<tr>
<td>CcCel6A-R</td>
<td>5'-CAGGATGTGATATAGTGAACGCCGA-3'</td>
</tr>
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<td>CcCel6B-F</td>
<td>5'-TAGGATCATCCTCTGCGCAAC-3'</td>
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<td>5'-ACAATAGCAGTACACAAATAAGATACCACG-3'</td>
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<tr>
<td>CcCel6C-F</td>
<td>5'-CCACCCTCTACAACCCCAA-3'</td>
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<tr>
<td>CcCel6C-R</td>
<td>5'-CAGAAATTAATCATATCTCCTCCATC-3'</td>
</tr>
<tr>
<td>CcCel6D-F</td>
<td>5'-CATGGCTATTCTTACACCTACAGCTCCTT-3'</td>
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<td>5'-ACTCTGCACCATGCGACC-3'</td>
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<tr>
<td>CcCel6E-F</td>
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<tr>
<td>Actin1-F</td>
<td>5'-CCAGAAGACTCTTATATGTCGTTGATGA-3'</td>
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<tr>
<td>Actin1-R</td>
<td>5'-GCTCTGATGCCGGCGTGAAG-3'</td>
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Results

Nucleotide sequences of CcCel6s

We found five genes encoding the enzyme with the GH6 signature in the genome database of C. cinerea strain Okayama 7 (#130) (locus nos. CC1G_01107.1, CC1G_04166.1, CC1G_08276.1, CC1G_08277.1, and CC1G_10605.1), and designated them CcCel6A, CcCel6B, CcCel6C, CcCel6D, and CcCel6E in order of increasing locus number. CcCel6A, -B, and -E were individually distinct on the genome, while CcCel6C and -D were separated by only 367 bp. On the basis of the sequences in the genome database, we cloned the cDNAs using total RNA of C. cinerea strain 5338. When the cloned cDNA sequences were compared with those of the corresponding genes in the genome database, three nucleotides in CcCel6E were different, but no difference of the amino acid residue was observed (data not shown).
Amino acid sequences of CcCel6s

The open reading frames of CcCel6A, CcCel6B, CcCel6C, CcCel6D, and CcCel6E encode 454, 400, 403, 404, and 400 amino acid residues respectively. The amino acid sequences implied a signal peptide in all CcCel6s, suggesting that Coprinopsis cinerea produces at least five extracellular GH6 enzymes. The mature CcCel6A protein has a CBM1 at the N-terminal end, whereas the other CcCel6s lack this module. Multiple alignment analysis using the amino acid sequences of the catalytic domain of the GH6 enzyme from C. cinerea and other filamentous fungi showed that aspartic acid, predicted to be the catalytic acid, was conserved (Fig. 1). All the CcCel6s showed amino acid sequences consisting of loops enclosing the active site and conserved cysteines forming disulfide bonds. Moreover, all residues predicted to be involved in ligand interaction in the subsites of Cel6As from Hypocrea jecorina and Humicola insolens were conserved in CcCel6A. On the other hand, two amino acids, which correspond to the residues in the −2 subsite of ascomycete Cel6As, were changed in CcCel6B-E. Asparagine 193 in CcCel6B was found instead of histidine in the +2 subsite of ascomycete Cel6As. In the evolutionary tree for the catalytic domains of GH6 enzymes, CcCel6A was placed into the group that contains all known CBHIs from filamentous fungi, whereas the other CcCel6s are located near HiCel6B (Fig. 2).

Transcription of CcCel6s at the active growth stage

Coprinopsis cinerea was cultivated with rotary shaking at 26 °C in a medium containing 2% glucose or 2% Avicel as the sole source of carbon, and time-course change of the growth was monitored by measuring the fungal volume per 5 ml of culture solution. Active growth was observed between days 4 and 8 in both cultures (data not shown).

To determine the transcript number of CcCel6s at the active growth stage, total RNA was extracted from the cultures on days 4 and 8. The transcript number of each CcCel6s was determined by quantitative real-time PCR.

Fig. 1. Multiple Alignments of the Catalytic Domains of CcCel6s and Other GH6 Enzymes.

Aspartic acid as the catalytic acid is indicated by the solid box. The amino acid residues of active-site-enclosing loops are boxed. Arrowheads indicate the conserved cysteines that form a disulfide bond at the N-terminal loop. Open arrowheads indicate cysteines used for disulfide bond formation at the C-terminal loop. Boxes with a dark background indicate the amino acid residues related to ligand interaction in the subsites of HjCel6A and HiCel6A. The catalytic domain of the GH6 enzyme from Phanerochaete chrysosporium (S76141); HjCel6A, Cel6A from Hypocrea jecorina (M16190); HiCel6A, Cel6A from Humicola insolens (AB048710); HiCel6B, Cel6B from H. insolens (Q7SIG5).
mycelia in glucose and cellulose cultures after cultivation for 8 d, and this was used in real-time PCR analysis (Fig. 3). In the cellulose culture, CcCel6A was the most abundant of the CcCel6s transcripts, and the transcript numbers were 4.9-, 47.6-, 5.4-, and 2.7-fold higher than CcCel6s (Fig. 3). In the cellulose culture, transcription of CcCel6A, -B, and -D was also induced by cellobiose, but the numbers of CcCel6s were detected in the glucose culture; their transcript numbers were 1/6.4, 1/1.6, 1/3.4, and 1/7.6, respectively, of those in the cellulose culture.

Effects of glucose and cellobiose on transcription of CcCel6s

The mycelia grown in glucose culture were washed with a medium containing no carbon source, and transferred to the medium containing 10 mM glucose, 10 mM cellobiose, or no carbon source. After incubation for 3 h, the transcripts of CcCel6s were determined by real-time quantitative PCR (Fig. 4). In the culture containing no carbon source CcCel6A transcripts were undetectable, whereas significant transcript levels of the other CcCel6s were observed, indicating high basal transcription of CcCel6B, -C, -D, and -E. The transcript levels of all the genes in the glucose culture were almost the same as or were higher than that in the culture containing no carbon source. Hence, transcription of CcCel6s might not be regulated by glucose catabolite repression or higher glucose concentrations might be required for transcriptional repression of them.

The transcript level of CcCel6A was much higher in the cellulbiose culture than in the other cultures, indicating clearly that cellobiose is a strong inducer of CcCel6A transcription. Transcription of CcCel6B, -D, and -E was also induced by cellobiose, but the number of transcripts in the cellulbiose culture was only 2–3 times greater than that in the culture containing no carbon source. In the case of CcCel6C, no difference was observed in transcript level among the cultures, and the transcript levels were of the same order of magnitude as those at the active growth stage.

Discussion

Several basidiomycetes are known to have genes encoding CBH isozymes. Among these, P. chrysosporium strain BKM-1767, one of the best studied lignocellulolytic basidiomycetes, possesses at least seven genes encoding the GH7 enzyme (PcCel7A-G). On the basis of three-dimensional structure, homology model structure, and amino acid sequence, PcCel7C-F should be the enzyme corresponding to CBH II from H. jecorina. On the other hand, PcCel7A and PcCel7B were predicted to be enzymes with activity profiles different from that of typical CBH II. In addition, transcript analysis demonstrated the different transcript patterns of the genes in cultures containing glucose or cellulose and on aspen wood chips. The facts indicate that the genes are transcriptionally regulated by the carbon source, and that their roles in cellulose and wood degradation might be different. In contrast to the diversity of GH7 enzymes, no basidiomycete having genes encoding multiple GH6 isozymes has been found. The GH6 enzymes produced by basidiomycetes show significant similarity to Cel6A (CBH II) from H. jecorina, suggesting that basidiomycetes have only one gene encoding the GH6 enzyme, which corresponds to CBH II. In the present study, however, we found that C. cinereus has five genes encoding the GH6 enzyme. As
Multiple Genes Encoding GH6 Enzyme from Coprinopsis cinerea

Fig. 4. Effects of Carbon Sources on CcCel6A (a), CcCel6B (b), CcCel6C (c), CcCel6D (d), and CcCel6E (e) Transcription.

The fungus was incubated in a medium containing 2% glucose for 7 d, and then the mycelia were harvested, washed, and transferred to a culture medium containing 10 mM glucose, 10 mM cellobiose, or no carbon source. After incubation for a further 3 h, total RNA (100 ng) extracted from the mycelium was subjected to real-time quantitative RT-PCR. Error bars show the standard errors in triplicate tests for the sample.

far as we know, this is first example of a basidiomycete that has multiple genes encoding the GH6 enzyme.

The evolutionary tree analysis using amino acid sequences of catalytic domains indicated that CcCel6A belongs to the branch containing known CBHII of filamentous fungi, whereas the other CcCel6s are located near HiCel6B, previously called EGVI of filamentous fungi, whereas the other CcCel6s are belongs to the branch containing known CBHII of sequences of catalytic domains indicated that CcCel6A that has multiple genes encoding the GH6 enzyme.

As well as CcCel6A, significant transcripts of CcCel6B, -D, and -E were detected at the active growth stage in the presence of cellulose, suggesting an involvement in cellulose degradation, but induction of transcription by cellobiose was not strong in comparison with that of CcCel6A. Moreover, their transcripts were observed when the mycelium was transferred to cultures containing glucose or no carbon source, whereas the transcript of CcCel6A was slightly or unobserved under the same conditions. The similarity of transcript patterns in CcCel6B, -D, and -E suggests that the enzymes work together in the degradation of cellulose. In the case of CcCel6C, the transcript level was very low at the active growth stage in the cellulose culture, and almost the same transcript level was detected at the active growth stage in the glucose culture. Moreover, the transcript level did not change when the mycelia were transferred to a medium containing glucose, cellobiose, or no carbon source. These results indicate that the gene is constitutively transcribed rather than changing depending on the presence of a sugar.

H. jecorina conidia have been found to contain cellulases, mainly CBHII, on the surface, and surface-displayed cellulases are perhaps responsible for the initial release of an inducer of cellulase from cellulose by conidia, leading to growth on cellulose. In addition, it has been reported that basal expression of CBHII is necessary for the formation of the inducer of the cellulase gene at the growth stage of mycelia on cellulose cultures. These facts indicate that CBHII has a role not only in cellulose hydrolysis but also in the formation of the inducer of cellulases. Recently, Suzuki et al. reported a significant transcript level of the CBHII gene from P. chrysosporium, which has a single-copy gene of the GH6 enzyme on its genome, in a culture containing no carbon source. Therefore, basal...
expression of CBHII of basidiomycetes might also have a role, as predicted for H. jecorina. In the case of C. cinereus, CcCel6A, which corresponds to the CBHII gene of H. jecorina, is considered to be an essentially inducible gene, because the transcripts were absent from the culture containing no carbon source, but were significantly detected in cellobiose culture. Hence, it should be unsuited for a role in the formation of the inducer of cellulase genes. On the other hand, high basal transcript levels of CcCel6B, -C, -D, and -E are suggestive of a role in the formation of the inducer of cellulase genes, although it is impossible to conclude what role is from the results obtained in the present study.

In conclusion, we found five genes encoding the GH6 enzyme in the genome database of C. cinerea, and we cloned the cDNA. On the basis of the amino acid sequence, CcCel6A was predicted to be the enzyme corresponding to known CBHII, and the other CcCel6s demonstrated different features. In addition, transcript analysis indicated that transcription of CcCel6s was regulated differentially by the carbon source. These results indicate the diversity of GH6 enzymes in basidiomycetes. The existence of multiple GH6 enzymes provides novel insight into cellulase degradation by basidiomycetes.

Acknowledgments

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