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NOTE

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The genes encoding glycoside hydrolase family 6 and 7 cellulases from the brown-rot fungus *Coniophora puteana*

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Abstract Four genes encoding glycoside hydrolase (GH) family 6 and 7 cellulases (*cel6A*, *cel6B*, *cel7A*, and *cel7B*) were obtained from the brown-rot fungus *Coniophora puteana* by genomic polymerase chain reaction (PCR) using consensus degenerate hybrid oligonucleotide primers (CODEHOPs) designated from the amino acid sequence of cellobiohydrolases (CBHs) from white-rot fungi. The nucleotide sequences of four genes showed high homology with basidiomycetes CBHs, suggesting the first cloning of the genes encoding Cel6 and Cel7 from brown-rot fungi. PCR using CODEHOP pairs at the catalytic domain successfully amplified both *cel6A* and *cel6B*, whereas only *cel6A* fragment was obtained using the primers including the carbohydrate-binding modules (CBMs), suggesting lack of CBM in Cel6B. Moreover, both *cel7A* and *cel7B* were amplified by the PCR using CODEHOP pairs at the catalytic domain, but not by those including CBM, suggesting the absence of Cel7 with CBM in the fungus. From these results, three of four cellulases from *C. puteana* may not carry CBM, which has an important role for the degradation of crystalline cellulose.

Key words Cellulase · Glycoside hydrolase family 6 and 7 · Carbohydrate binding modules · Consensus degenerate hybrid oligonucleotide primer (CODEHOP) · Brown-rot fungus

Introduction

Wood-rotting basidiomycetes have been divided into the two categories of white-rot and brown-rot fungi according to the color of the decayed wood. The major difference between these fungi is the ability to degrade lignin; white-rot fungi completely mineralize lignin whereas brown-rot fungi only modify lignin, which then remains as an insoluble form.[1–3] In addition, the abilities of these fungi to degrade cellulose are known to be different, mainly with respect to degradation of crystalline cellulose. Brown-rot fungi depolymerize the amorphous region of cellulose during the early stages of wood decay, whereas white-rot fungi hydrolyze both the crystalline and amorphous regions of cellulose without a significant decrease in the degree of polymerization (DP) of cellulose.[4–6] The different mode of cellulose degradation is explained by different extracellular enzymes in white-rot and brown-rot fungi, in particular the existence of cellobiohydrolases (CBHs; EC: 3. 2. 1. 91), which is a key for the enzymatic degradation of crystalline cellulose.[7,8]

In wood-rotting basidiomycetes, CBHs from the white-rot fungus *Phanerochaete chrysosporium* have been studied from physiological, biochemical, and molecular biological viewpoints, and cellulases belonging to glycoside hydrolase (GH) family 6 (Cel6) and 7 (Cel7) are involved in degradation of crystalline cellulose.[9] In contrast, information on the cellulolytic enzymes from brown-rot fungi is limited, but generally brown-rot fungi have been known not to produce CBH except for *Coniophora puteana*.[10–12] Two enzymes from *C. puteana* have been isolated from the cellulolytic culture and reported to have hydrolyzing activity for *p*-nitrophenyl-*β*-D-lactoside, which is commonly used for the assay of Cel7. This indicates the possible existence of CBHs, but no molecular biological aspect has been obtained for these enzymes. In the present study, we attempted to obtain the genes encoding Cel6 and Cel7 by genomic polymerase chain reaction (PCR) using consensus degenerated hybrid oligonucleotide primers (CODEHOPs), which has the advantage of standard degenerated PCR in terms of homolog gene detection.[13–15]

Materials and methods

Fungal strain

Coniophora puteana WD-758 was used for the experiment, and was obtained from the National Institute of Agricul-

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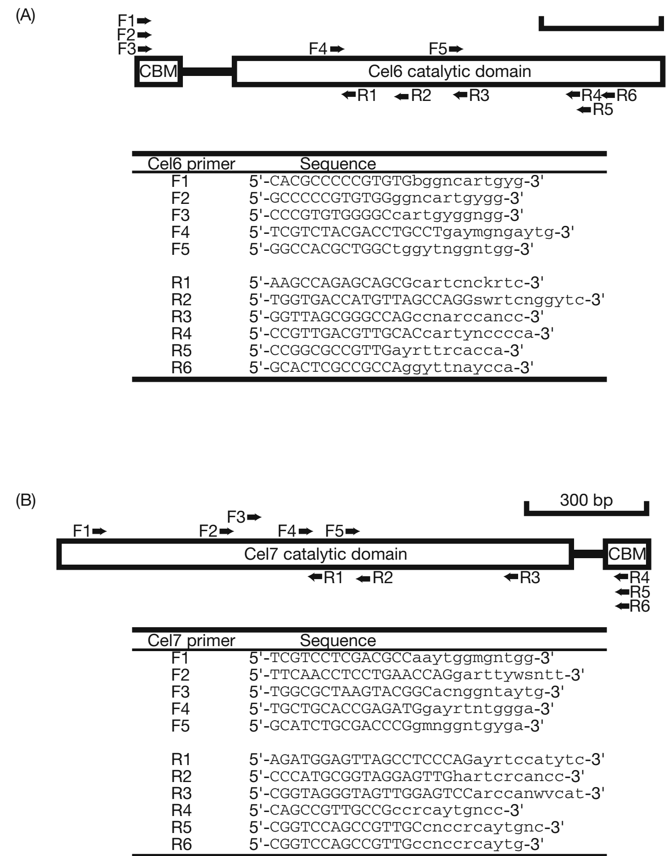
tural Science (NIAS). The mycelia of *C. puteana* were grown on potato dextrose agar (PDA; Nihon Pharmaceutical, Tokyo, Japan) plates at 26.5°C for 7 days.

Preparation of genomic DNA from mycelium

The mycelia of *C. puteana* were punched out and inoculated into 200 ml of potato dextrose broth (PDB; Sigma-Aldrich, MO, USA) liquid medium in a 500-ml Erlenmeyer flask. After 3 days of incubation at 26.5°C with rotary shaking (150 rpm, diameter 30 mm), the mycelia were harvested and frozen in liquid nitrogen. After the mycelia were crushed in liquid nitrogen using a mortar and pestle, genomic DNA was isolated using DNeasy plant maxi kit (Qiagen, Hilden, Germany). The concentration of genomic DNA was evaluated spectrophotometrically from the values at 260 nm and the quality was examined by the ratio at 260/280 nm by Gene Spec I (Naka Instruments, Japan).

Amplification of *cel6* and *cel7* genes from genomic DNA of *C. puteana* by CODEHOP PCR

Primers for amplifying *cel6* and *cel7* genes were designated according to the CODEHOP strategy.[13–15] Amino acid sequences of the enzymes belonging to GH family 6 and 7 from basidiomycetes and ascomycetes were aligned by the EBI-ClustalW server (http://www.ebi.ac.uk/Tools/clustalw/index.html).[16] The resultant alignment data were further uploaded to Blocks Multiple Alignment Processor (http://blocks.fhcrc.org/blocks/process_blocks.html) to make amino acid sequence segments without gaps that represented highly conserved regions of homologous proteins. The primers were constructed by submission to the CODEHOP program (http://bioinformatics.weizmann.ac.il/blocks/codehop.html). Locations and nucleotide sequences of each primer are shown in Fig. 1. All PCR reaction mixtures contained 20 ng of total DNA template extracted as described above, 200 μM of each deoxynucleotide, 300 pM of each primer, 1.5 mM $MgSO_4$, 0.5 U of KOD-plus ver.2 DNA polymerase (Toyobo, Japan) and 1x buffer for KOD plus-ver. 2 in a final volume of 25 μl. DNA amplification was performed with a PCR thermal cycler Dice model TP600 (Takara Bio, Otsu, Japan). PCR was carried out by the touch-down (A) and the standard three-step PCR (B) protocols as follows: initial denaturation at 94°C for 2 min, then (A) 15 cycles of 10 s at 98°C, 30 s at 65°C (–0.5°C/cycle), and 1–1.5 min at 68°C; then (B) 20 cycles of 10 s at 98°C, 30 s at 58°C, and 1–1.5 min at 68°C, and finally 2 min at 68°C extension. Elongation time was adapted to the largest expected fragment in each PCR reaction. All PCR products were inserted into a Zero Blunts TOPO PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and sequenced using a Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare, Giles, UK) with a DNA sequencer SQ5500E (Hitachi Electronics Engineering, Japan). The determined nucleotide sequences of fragments were searched using BLASTX with the nonredundant protein sequences (nr) database at NCBI.



Cel6 primer	Sequence
F1	5'-CACGCCCCCGTGTGbggncartgyg-3'
F2	5'-GCCCCCGTGTGGggncartgygg-3'
F3	5'-CCCGTGTGGGGCcartgyggngg-3'
F4	5'-TCGTCTACGACCTGCCTgaymgngaytg-3'
F5	5'-GGCCACGCTGGCtggytnggntgg-3'
R1	5'-AAGCCAGAGCAGCGcartcnckrtc-3'
R2	5'-TGGTGACCATGTTAGCCAGGswrtcnggytc-3'
R3	5'-GGTTAGCGGGCCAGccnarccancc-3'
R4	5'-CCGTTGACGTTGCACcartynccccca-3'
R5	5'-CCGGCGCCGTTGayrttrcacca-3'
R6	5'-GCACTCGCCGCCAggyttnaycca-3'

Cel7 primer	Sequence
F1	5'-TCGTCCTCGACGCCaaytggmgntgg-3'
F2	5'-TTCAACCTCCTGAACCAGgarttywsntt-3'
F3	5'-TGGCGCTAAGTACGGCacnggntaytg-3'
F4	5'-TGCTGCACCGAGATGgayrtntggga-3'
F5	5'-GCATCTGCGACCCGgmnggntgyga-3'
R1	5'-AGATGGAGTTAGCCTCCCAGayrtccatytc-3'
R2	5'-CCCATGCGGTAGGAGTTGhartcrcancc-3'
R3	5'-CGGTAGGGTAGTTGGAGTCCarccanwvcat-3'
R4	5'-CAGCCGTTGCCGccrcaytgncc-3'
R5	5'-CGGTCCAGCCGTTGCcnccrcaytgnc-3'
R6	5'-CGGTCCAGCCGTTGccnccrcaytg-3'

Fig. 1A, B. Primer locations and nucleotide sequences of consensus degenerate hybrid oligonucleotide primers (CODEHOP) for the detection of **A** *cel6* gene, and **B** *cel7* gene. *CBM*, Carbohydrate-binding module

Results

Genomic PCR was carried out for amplifying *cel6 and cel7* using CODEHOP and genomic DNA of *Coniophora puteana* as shown in Fig. 2. Two major bands were obtained by amplification using CODEHOP pairs for the catalytic domain (F4-F5 and R2-R6), whereas only one fragment was amplified by forward primers for the carbohydrate-binding modules (CBMs) region (F1-F3) and reverse primers for the catalytic domain (R1-R6). The nucleotide sequences of two fragments for catalytic domain showed high homology with basidiomycetes CBHs belonging to GH family 6 in BLASTX analysis. As shown in Table 1, a 1412-bp fragment of *cel6A* obtained by the primer pair of F1 and R6 showed 75% homology with *Phanerochaete chrysosporium* CBHII (Cel6A) and *Irpex lacteus* CBHII belonging to GH family 6. A fragment of *cel6B* amplified by F4 and R6 primers (890 bp) was apparently different from *cel6A* and showed 82% homology with *Agaricus bisporus* CBH belonging to GH family 6. Multiple bands that appeared in PCR using primer pairs of F1-R2, F2-R2, F3-R3, and F2-R5 were false-positive by the sequence analysis.

As shown in Fig. 3, PCR fragments are amplified only by the primer pairs for catalytic domain of Cel7 (F1-F5 and

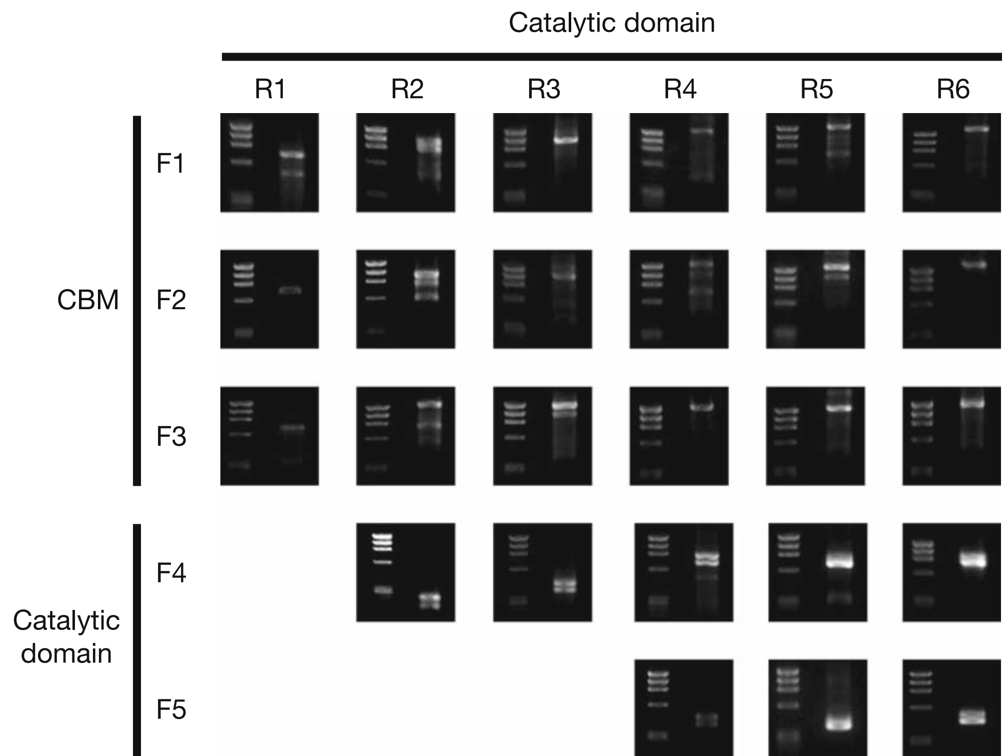


Fig. 2. Polymerase chain reaction (PCR) amplicons for detection of *cel6* gene from *Coniophora puteana* genomic DNA. Twenty nanograms of genomic DNA was used in PCR as a template. *Left lane* of each figure shows molecular weight size marker, ϕX174-*Hae*III-digest

Table 1. Homology of deduced *Coniophora puteana cel6A* and *cel6B* fragments with Cel6 proteins from other fungi

cel6A-F1 and R6–1412 bp fragment			*cel6B*-F4 and R6–890 bp fragment		
Strain/protein	Genebank accession no.	Homology (%)	Strain/protein	Genebank accession no.	Homology (%)
Phanerochaete chrysosporium/CBHII	AAB32942.1	75	*Agaricus bisporus*/CBH	AAA50608.1	82
Irpex lacteus/CBHII	BAG48183.1	75	*Lentinula edodes*/CEL6B	AAK95564.1	80
Polyporus arcularius/CBHII	BAF80327.1	73	*Pleurotus sajor-caju*/CBHII	AAL15037.1	79
Lentinula edodes/CEL6B	AAK95564.1	71	*Polyporus arcularius*/CBHII	BAF80327.1	78
Volvariella volvacea/CBHII-1	AAT64008.1	69	*Phanerochaete chrysosporium*/CBHII	AAB32942.1	75
Agaricus bisporus/CBH	AAA50608.1	68	*Volvariella volvacea*/CBHII-1	AAT64008.1	75
Coriolus versicolor/CBH	AAF35251.1	68	*Irpex lacteus*/CBHII	BAG48183.1	75
Pleurotus sajor-caju/CBHII	AAL15037.1	66	*Coriolus versicolor*/CBH	AAF35251.1	75
Talaromyces stipitatus/CBH	EED17133.1	61	*Talaromyces emersonii*/CBHII	AAL33604.4	71
Acremonium cellulolyticus/CBHII	BAA74458.1	61	*Acremonium cellulolyticus*/CBHII	BAA74458.1	68

The determined nucleotide sequences of fragments were searched using BLASTX with nonredundant protein sequences database at NCBI

R1-R3) when genomic DNA of *C. puteana* was used as template. Two different nucleotide sequences (*cel7A* and *cel7B*) showed high homology with CBH belonging to GH family 7 from basidiomycetes in BLASTX analysis. BLASTX results for each fragment are shown in Table 2. Both a 1081-bp fragment of *cel7A* and a 1060-bp fragment of *cel7B* obtained by primer pair F1 and R3 showed higher than 70% homology with basidiomycetes Cel7s from *P. chrysosporium*, *I. lacteus*, *Volvariella volvacea*, and *Talaromyces emersonii*. However, PCR using forward primers for catalytic domain (F1-F5) and reverse primers for CBM (R4-R6) failed to amplify the target gene encoding Cel7.

Discussion

As reported previously, extracellular enzymes in cellulolytic culture of *Coniophora puteana* are less active toward crystalline cellulose as is the case for many brown-rot fungi,[17,18] possibly because of a lack of CBH responsible for degradation of crystalline cellulose. However, the results obtained in the present study clearly indicate that the fungus has the genes encoding cellulases in GH families 6 and 7, where many fungal CBHs and endoglucanases (EGs; EC: 3.2.1.4) are categorized. Moreover, one of these genes

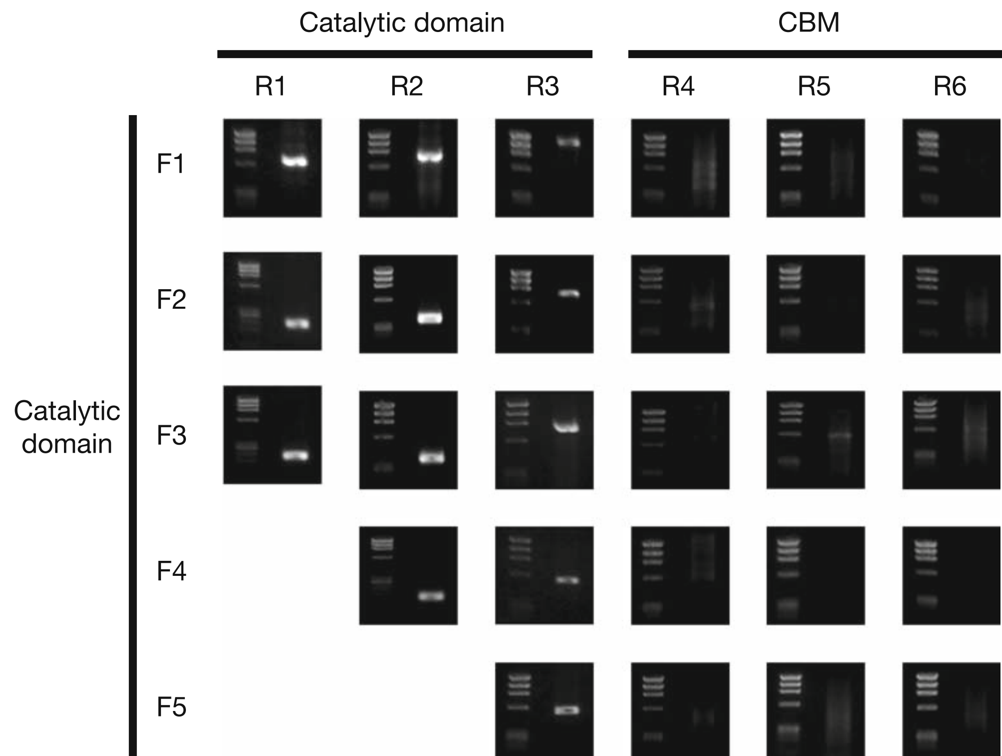


Fig. 3. PCR amplicons for detection of *cel7* gene from *C. puteana* genomic DNA. Twenty nanograms of genomic DNA was used in PCR as a template. *Left lane* of each figure shows molecular weight size marker, ϕX174-*Hae*III-digest

Table 2. Homology of deduced *Coniophora puteana cel7A* and *cel7B* fragments with Cel7 proteins from other fungi

cel7A-F1 and R3–1081 bp fragment			*cel7B*-F1 and R3–1060 bp fragment		
Strain/protein	Genebank accession no.	Homology (%)	Strain/protein	Genebank accession no.	Homology (%)
Phanerochaete chrysosporium/CBHI	AAB46373.1	80	*Phanerochaete chrysosporium*/CBHI	AAB46373.1	80
Phanerochaete chrysosporium/CBHI	AAA19802.1	80	*Volvariella volvacea*/CBHI-I	AAT64006.1	79
Irpex lacteus/CBH	BAD16575.1	80	*Irpex lacteus*/exocellulase	BAA76364.1	79
Irpex lacteus/exocellulase	BAA76364.1	80	*Irpex lacteus*/CBH	BAD16575.1	79
Volvariella volvacea/CBHI-I	AAT64006.1	80	*Volvariella volvacea*/CBHI-II	AAT64007.1	78
Talaromyces emersonii/CBHI	AAL89553.1	80	*Irpex lacteus*/cellulase	BAA76365.1	78
Irpex lacteus/cellulase	BAA76365.1	79	*Lentinula edodes*/CEL7A	AAK95563.1	77
Volvariella volvacea/CBHI-II	AAT64007.1	78	*Schizophyllum commune*/CBH	AAX55505.1	77
Lentinula edodes/CEL7A	AAK95563.1	77	*Polyporus arcularius*/CBHI	BAF80326.1	77
Schizophyllum commune/CBH	AAX55505.1	77	*Irpex lacteus*/cellulase	BAA76363.1	77

The determined nucleotide sequences of fragments were searched using BLASTX with nonredundant protein sequences database at NCBI

(*cel6A*) may code not only the catalytic domain but also CBMs, which function on the adsorption of solid substrate and are essential for degradation of crystalline cellulose.[19] We have also succeeded in isolating cDNA of *cel6A* and *cel6B*, and the deduced amino acid sequence of *cel6A* proved the existence of CBM in the N-terminal region, while that of *cel6B* did not. The nucleotide sequences of the cDNAs encoding *C. puteana* Cel6A and Cel6B have been deposited to the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases under accession numbers AB501099, and AB501100, respectively. Quite recently, total genome sequencing of the brown-rot fungus *Postia placenta* was disclosed (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html), but no putative genes encoding GH family 6 or 7 cellulase were found in the sequence. This suggests that at the genomic level, *C. puteana* and *P. placenta* have different cellulolytic systems, although both fungi are categorized as brown-rot fungi.

There have been two enzymes with *p*-nitrophenyl-β-D-lactoside-hydrolyzing activity isolated as the major extracellular proteins from cellulolytic culture of *C. puteana*, and they were called CBHs as reported by Schmidhalter and Canevascini.[11,12] Because both CBH-type and EG-type enzymes belong in GH family 7 and both of them hydrolyze this substrate, it is quite ambiguous that the fungus really produces CBH responsible for degradation of crystalline cellulose. In the present study, we could not amplify the genes of *cel7* when CODEHOP designated from CBM were used for PCR, whereas fragments having high homology to basidiomycetes Cel7s could be obtained using primer pairs for catalytic domain. We have also succeeded in isolating cDNA of *cel7A* and *cel7B*, and they did not have CBM-

coding regions at both 5' and 3' ends. The nucleotide sequences of the cDNAs encoding *C. puteana* Cel7A and Cel7B have been deposited the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases under accession numbers AB501101 and AB501102, respectively. Considering the molecular weight of the two proteins previously isolated (52 kDa and 50 kDa), they seem to lack CBM, which is essential for degradation of crystalline cellulose.

Consequently, we have succeeded in cloning Cel6 and Cel7 genes from the brown-rot fungus *C. puteana*. The information obtained in this work will contribute to a better understanding of the cellulolytic system of brown-rot fungi.

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