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Phanerochaete chrysosporium Cellobiohydrolase and Cellobiose Dehydrogenase Transcripts in Wood

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The transcripts of structurally related cellobiohydrolase genes in *Phanerochaete chrysosporium*-colonized wood chips were quantified. The transcript patterns obtained were dramatically different from the transcript patterns obtained previously in defined media. Cellobiose dehydrogenase transcripts were also detected, which is consistent with the hypothesis that such transcripts play an important role in cellulose degradation.

In submerged cultures, the white rot basidiomycete *Phanerochaete chrysosporium* secretes an array of hydrolytic cellulases, including multiple isozyme forms of endoglucanase (13), cellobiohydrolase (CBH) (14, 33), and β-glucosidase (11, 28) (for reviews see references 15 and 31).

On the basis of structural similarities to corresponding *Trichoderma reesei* cellulase genes, the genes for a single CBHII-like clone (32) and six CBHI-like clones (8–10, 26) were isolated from *P. chrysosporium*. Except for *cbh1-1*, all of these genes have the tripartite architecture common to microbial cellulase genes (i.e., catalytic and cellulose-binding domains separated by a glycosylated linker region) (reviewed in references 16 and 37). The *cbh1-1* gene lacks a binding domain and hinge region (9), and similarly truncated *cbh1* genes have now been identified in the non-wood-decaying fungi *Cochiobolus carbonum* and *Cryphonectria parasitica* (29, 35). No β-glucosidase or endoglucanase-like genes have been cloned from *P. chrysosporium*, and it has been suggested that *cbh1*-like genes may encode proteins with endoglucanase activity (27).

The multiple CBHI genes of *P. chrysosporium* are transcriptionally regulated. With cellulose induction, the levels of the dominant transcript, *cbh1-4*, exceed the levels of the closely related genes *cbh1-2* and *cbh1-1* by more than 1,000-fold (9, 34). These results are consistent with the results of protein purification, which identified the *cbh1-4* gene product as the dominant isozyme (33, 34). The transcript levels for *cbh1-1* and *cbh1-2* are relatively low in cellulose-supplemented medium. However, both *cbh1-1* and *cbh1-2* transcripts can be detected in minimal media containing glucose as the sole carbon source, while *cbh1-3*, *cbh1-4*, *cbh1-5*, and *cbh1-6* cannot be detected under these conditions (9). The precise roles and interactions of individual genes in cellulose degradation are unclear, particularly in complex substrates, such as wood.

In addition to the hydrolytic cellulases, extracellular oxidative enzymes apparently are involved in cellulose degradation by *P. chrysosporium* and, presumably, other white rot fungi (3, 15). Cellobiose dehydrogenase (CDH) is an extracellular enzyme containing two domains; one domain contains flavin adenine dinucleotide, and the other domain contains a heme (18, 19). CDH is produced by several fungi, including *P. chrysosporium*, and has been shown to oxidize cellobiose and various oligosaccharides. Production of CDH is stimulated by the presence of cellulose as a carbon source, and, like the cellulases, CDH strongly binds to the substrate. The exact role of CDH is uncertain, but removal of cellobiose by CDH removes a powerful inhibitor of CBHs (4, 36). Igarashi et al. (20) showed that CDH adsorbed to the surfaces of cellulose particles in stationary cultures and suggested that CDH plays a role in cellulose degradation in cooperation with the hydrolytic cellulases.

cDNAs encoding *P. chrysosporium* CDH have been characterized previously (24, 25). The flavin- and heme-binding domains were recognized in the predicted amino acid sequence, but no cellulase-like cellulose-binding domain was observed. The results of Southern blotting suggested that CDH is encoded by a single gene, and the results of Northern blotting identified CDH transcripts in cultures containing cellulose but not in glucose- or cellobiose-containing cultures (24). Like expression of the CBH genes, nothing is known about CDH gene expression in solid wood. To address this issue, magnetic capture and reverse transcriptase PCR (RT-PCR) techniques were used to quantify CBH and CDH transcripts in wood chips.

Approximately 2.5 kg of fresh aspen wood chips that were not amended with any nutrients were steam sterilized and inoculated by standard biopulping methods (1). Ten-gram samples were collected every 2 weeks, snap frozen in liquid nitrogen, and stored at −80°C. Frozen samples were ground

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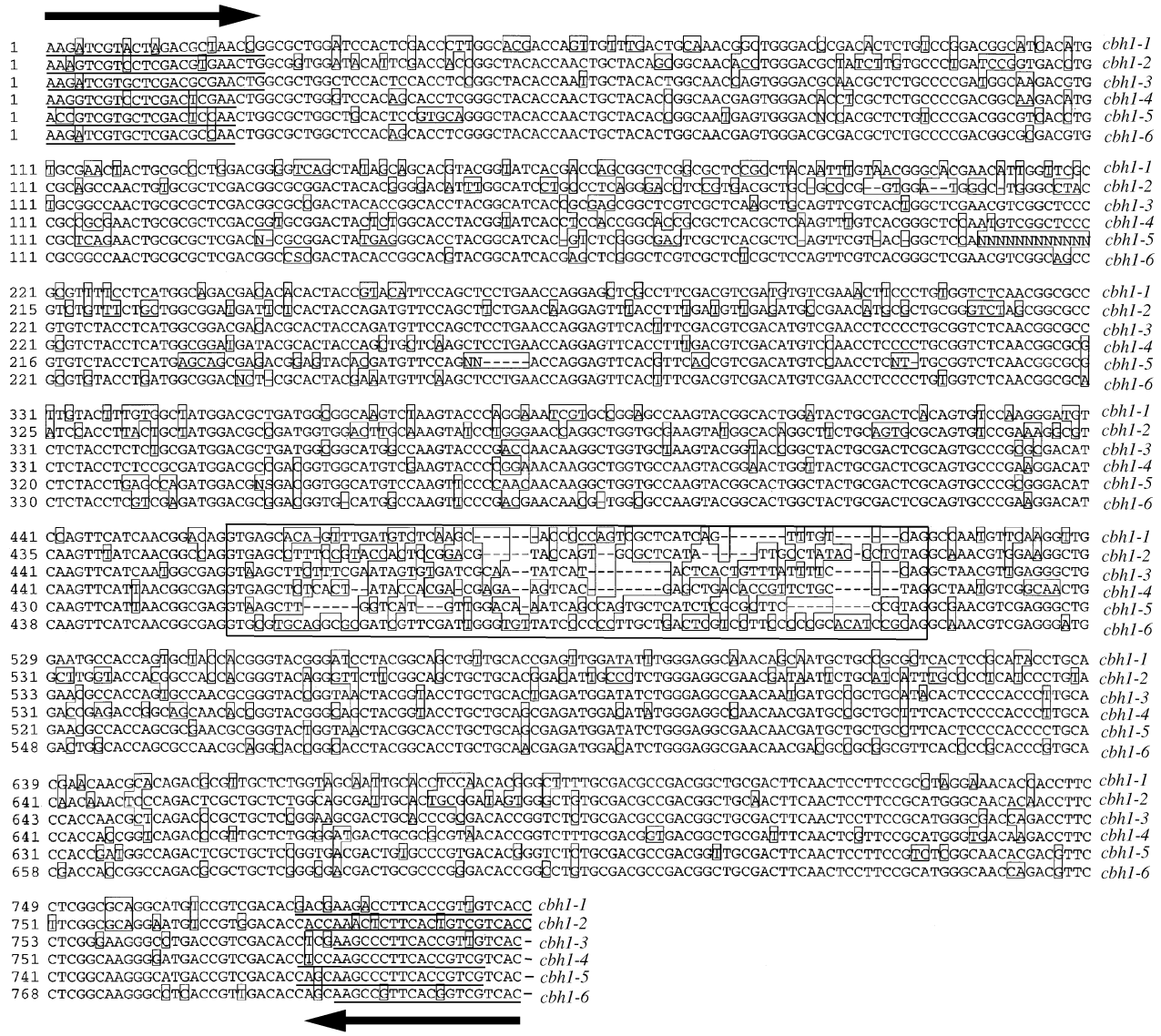

FIG. 1. Partial sequence alignment of the six known *cbh1* genes of *P. chrysosporium* BKM-F-1767. The positions of PCR primers are indicated by arrows and underlining. The conserved intron is enclosed in a box, as are individual residues which differ from the residues in the consensus sequence.

in a clean coffee grinder in the presence of dry ice. Each frozen powder sample was suspended in 20 ml of a solution containing 4 M guanidinium thiocyanate, 100 mM Tris-HCl (pH 8.0), 1% dithiothreitol, and 0.5% lauryl sarcosinate in a 50-ml conical tube. The contents of the tube were incubated on ice for 30 min and mixed by gently inverting the tube every 5 to 10 min. Following centrifugation at 2,000 × *g* for 10 min, the supernatant was filtered through a Miracloth

TABLE 1. Oligonucleotides used as primers for competitive RT-PCR

Gene	5′ Primer (5′→3′)	3′ Primer (5′→3′)
cbh1-1[a]	AAGATCGTACTAGACGCTAACCG	GGTGACAACGGTGAAGGTCTTCGTC
cbh1-2	AAAGTCGTCCTCGACGTGAACTG	GGTGACGACAGTGAAGAGTTTGGTG
cbh1-3	AAGATCGTGCTCGACGCGAA	GTGACAACGGTGAAGGGCTT
cbh1-4	AAGGTCGTCCTCGACTCGAA	CGACGGTGAAGGGCTTGGAG
cbh1-5	ACCGTCGTGCTCGACTCCAA	CGACGGTGAAGGGCTTGCTG
cbh1-6	AAGATCGTGCTCGACGCCAA	GTGACGACCGTGAACGGCTT
cbh2[b]	AGCGCCAACTACCAGAACTACCT	CTTCGGGGTCACGGGGATGAAAC
cdh[b]	CAGTGCGGTGGCATTGGCTG	CTGGTCGACGATGAAGGTCG

[a] The primers for *cbh1-3*, *cbh1-4*, *cbh1-5*, and *cbh1-6* have been described previously (33). The design and relative positions of all *cbh1* primers are shown in Fig. 1.
[b] The *cbh2* and *cdh* primers were based on previously published sequences (23, 32).

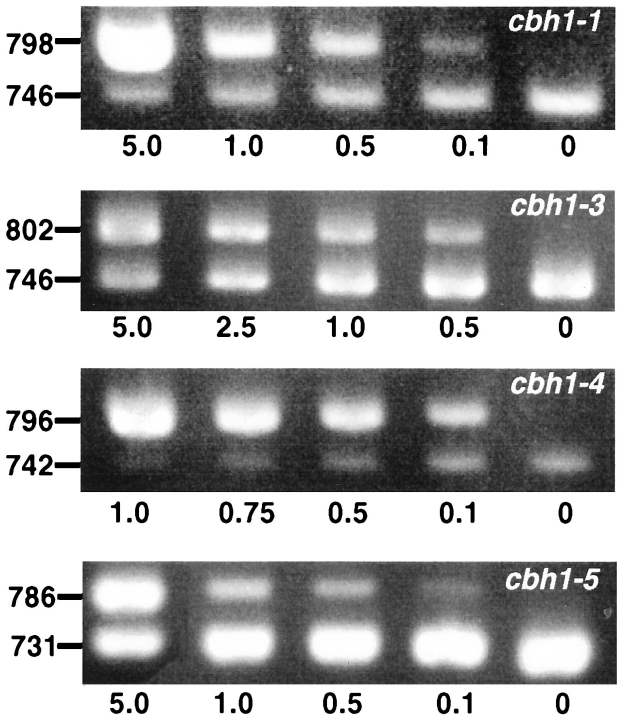

FIG. 2. Competitive PCRs for 4-week aspen chip samples comparing the detectable *cbh1* transcripts. The gene-specific PCR primers used are shown in Fig. 1 and Table 1. PCR mixtures contained the amounts of the competitive templates indicated below the gels in picograms of plasmid. As described previously (17), the levels of transcripts in samples were based on estimated equivalence points between competitive product and target cDNAs. The sizes of PCR products in base pairs are indicated on the left. Ethidium bromide-stained gels were photographed with a Foto/Analyst Visionary system (Fotodyne, Hartland, Wis.) and then scanned with a Microtek ScanMaker III and Adobe Photoshop 3.0.

filter (Calbiochem, San Diego, Calif.) and incubated on ice for 30 min with 1.5 mg of Dynabeads oligo$(dT)_{25}$ (Dynal, Inc., Great Neck, N.Y.). Dynabead-poly(A) hybrids were isolated with a model MPC-1 magnetic concentrator (Dynal, Inc.) and washed repeatedly in a 1.5-ml Eppendorf tube as previously described (6, 7). The eluted mRNA was stored as an ethanol precipitate at −20°C. Aliquots (2 μl) were dried under a vacuum, and reverse transcriptions were carried out in 20-μl reaction mixtures containing 50 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, Md.), 15 pmol of oligo $(dT)_{15}$, and 20 U of RNasin (Promega Biotech, Madison, Wis.). Reactions were performed at 23°C for 10 min, at 42°C for 45 min, and at 95°C for 5 min.

Competitive PCRs were performed as reported previously (6, 7, 17, 30) with 10^{-13} to 10^{-8} μg of genomic template. The competitive templates consisted of full-length genomic copies of the genes which had been PCR amplified and subcloned into pKSII (Stratagene, La Jolla, Calif.). Gene-specific primers were prepared based on multiple alignments (Fig. 1) and previously published sequences (24, 32) (Table 1). As described previously (6, 7), the reaction mixtures contained 21 pmol of each primer (Table 1).

The specificity of *cbh1* primer pairs (Fig. 1 and Table 1) was established experimentally by performing PCR amplifications with separate clones containing different *cbh1* genes. No cross amplification was observed for *cbh1-1*-, *cbh1-2*-, *cbh1-3*-, *cbh1-4*-, and *cbh1-6*-specific primers. Primers designed for *cbh1-5* sometimes yielded minor products with other plasmid templates, but direct sequencing of the RT-PCR products revealed only the *cbh1-5* sequence (data not shown).

The PCR products were size fractionated in 2% agarose containing 1% NuSieve (FMC) in 0.5× TBE buffer. Ethidium bromide-stained agarose gels were analyzed by using NIH Image 1.58 (National Institutes of Health). Image data was analyzed with Cricket Graphic (version 1.53).

The transcript patterns of *P. chrysosporium cbh1*-like genes on aspen wood chips differed markedly from the transcript patterns observed previously on defined media supplemented with cellulose or glucose. For example, after 4 weeks of incubation, the *cbh1-5* transcript levels were highest and the *cbh1-4* transcripts were barely detectable in wood chips (Fig. 2 and Table 2). In contrast, *cbh1-4* encodes the dominant transcript and isozyme found in submerged cultures (33, 34). Transcripts of *cbh1-6* and *cbh1-2* were not detected, although both transcripts are present in cellulose-amended cultures. These results provide a framework for strain improvement, particularly in biomechanical pulping processes in which active cellulase genes must be targeted (5, 12, 22).

The relative *cbh1* transcript levels were similar throughout the 10-week time course, and the levels of the *cbh1-3* and/or *cbh1-5* transcripts were consistently highest (Table 2). All cellulase transcript levels decreased at 6 weeks, although the reasons for this are unclear. The levels of lignin peroxidase transcripts were not similarly depressed (21), suggesting that variability in mRNA yields was not responsible.

In marked contrast to submerged cultures (9, 34), substantial levels of the transcript of the truncated CBH gene,

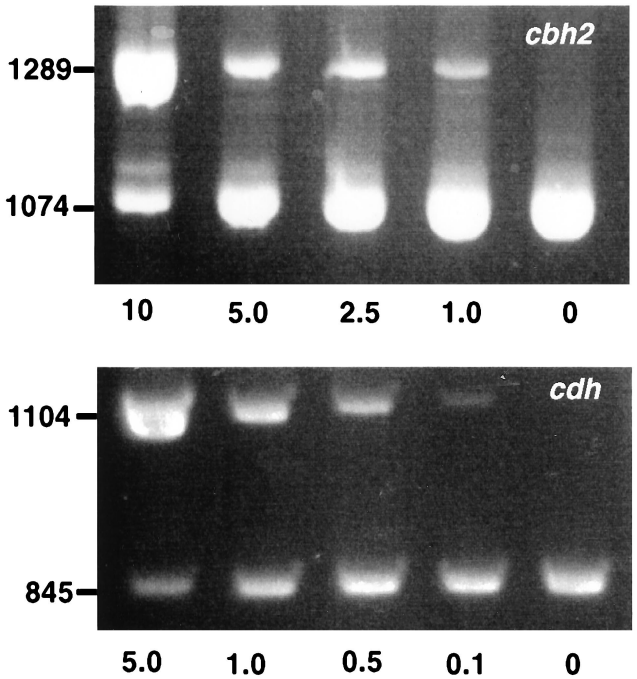

FIG. 3. Competitive PCRs for 4-week aspen chip samples showing amplification of *cbh2* and *cdh* with the gene-specific PCR primers listed in Table 1. The amounts of the competitive templates used are indicated below the gels in picograms. The sizes of PCR products in base pairs are indicated on the left.

TABLE 2. Transcript levels in aspen wood chips as estimated by competitive PCR analysis

Gene	cDNA concn (pg) after:				
	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
cbh1-1	0.36	0.47	0.14	0.32	0.12
cbh1-3	1.87	2.43	0.26	1.81	0.25
cbh1-4	ND[a]	0.10	ND	0.08	0.03
cbh1-5	0.44	3.30	0.33	2.35	1.61
cbh2	2.68	5.96	0.67	3.51	5.62
cdh	0.20	1.55	0.10	0.45	0.29

[a] ND, not detected.

cbh1-1, were detected in all wood samples. This result supports the hypothesis that *cbh1-1* contributes to the degradation of native cellulose. In submerged cultures, the levels of *cbh1-1* transcripts are exceedingly low (more than 1,000-fold less than the levels of *cbh1-3*, *cbh1-4*, *cbh1-5*, and *cbh1-6* transcripts) (9, 34). Similarly, the consistent presence of *cbh2* and *cdh* transcripts (Fig. 3 and Table 2) strongly supports the hypothesis that these transcripts play a role in cellulose degradation.

It is interesting that the same *P. chrysosporium*-colonized wood chips also contained transcripts of certain lignin peroxidase and manganese peroxidase genes (21). The appearance of *cdh* and peroxidase transcripts together in wood may support the hypothesis that there is a physiological connection (reviewed in reference 2) in which CDH regulates lignin depolymerization through reduction of peroxidase products (phenoxy radicals) and thereby prevents their repolymerization.

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