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Gene Expression Patterns of Wood Decay Fungi *Postia placenta* and *Phanerochaete chrysosporium* Are Influenced by Wood Substrate Composition during Degradation

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ABSTRACT

Identification of the specific genes and enzymes involved in the fungal degradation of lignocellulosic biomass derived from feedstocks with various compositions is essential to the development of improved bioenergy processes. In order to elucidate the effect of substrate composition on gene expression in wood-rotting fungi, we employed microarrays based on the annotated genomes of the brown- and white-rot fungi, *Rhodonia placenta* (formerly *Postia placenta*) and *Phanerochaete chrysosporium*, respectively. We monitored the expression of genes involved in the enzymatic deconstruction of the cell walls of three 4-year-old *Populus trichocarpa* (poplar) trees of genotypes with distinct cell wall chemistries, selected from a population of several hundred trees grown in a common garden. The woody substrates were incubated with wood decay fungi for 10, 20, and 30 days. An analysis of transcript abundance in all pairwise comparisons highlighted 64 and 84 differentially expressed genes (>2-fold, $P < 0.05$) in *P. chrysosporium* and *P. placenta*, respectively. Cross-fungal comparisons also revealed an array of highly differentially expressed genes (>4-fold, $P < 0.01$) across different substrates and time points. These results clearly demonstrate that gene expression profiles of *P. chrysosporium* and *P. placenta* are influenced by wood substrate composition and the duration of incubation. Many of the significantly expressed genes encode "proteins of unknown function," and determining their role in lignocellulose degradation presents opportunities and challenges for future research.

IMPORTANCE

This study describes the variation in expression patterns of two wood-degrading fungi (brown- and white-rot fungi) during colonization and incubation on three different naturally occurring poplar substrates of differing chemical compositions, over time. The results clearly show that the two fungi respond differentially to their substrates and that several known and, more interestingly, currently unknown genes are highly misregulated in response to various substrate compositions. These findings highlight the need to characterize several unknown proteins for catalytic function but also as potential candidate proteins to improve the efficiency of enzymatic cocktails to degrade lignocellulosic substrates in industrial applications, such as in a biochemically based bioenergy platform.

In an attempt to reduce societal dependence on fossil fuels and consequently aid in the alleviation of associated economic and environmental concerns regarding the exploitation of petroleum reserves, biofuels derived from renewable and domestic sources have received extensive interest in recent years (1). The lignocellulosic biomass derived from plant cell walls is the most abundant global renewable carbon source (2), stemming from either agricultural or forestry residuals or from dedicated energy crops. While improvement in lignocellulosic feedstock for biofuel applications via advanced breeding or genetic engineering appears feasible and is critical for the future of this industry (3–5), another key technical parameter that has potential for additional optimization is the identification of new enzymes and/or cofactors that could improve the efficacy of lignocellulosic biochemical processing.

Ethanol generated via the biochemical processing of lignocellulosic biomass currently is a three-step process that requires pretreatment of lignocellulosics by acidolysis, organosolv treatment, steam explosion, or other methods (6) to deconstruct cell walls and liberate the cellulose and hemicellulose carbohydrate fractions. This is typically followed by enzymatic depolymerization of the polysaccharides (cellulose and xylan) to C_6 and C_5 monomers and, finally, the fermentation of the monomeric sugars to ethanol or other valued products by microorganisms (7–9).

Although commercial enzyme preparations are readily available, efforts to improve these preparations and to identify and introduce new enzyme components and/or cofactors to enhance the efficiency of enzymatic conversion are ongoing. Microorganisms such as white-, brown-, and soft-rot fungi are all capable of degrading and/or modifying lignocellulosic biomass to some extent (10). For example, white-rot fungi, such as *Phanerochaete*

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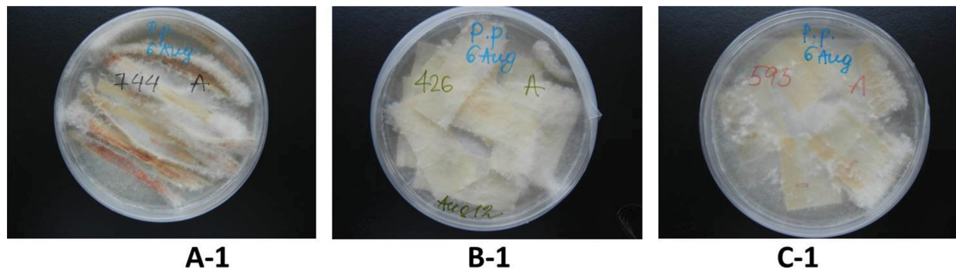

FIG 1 Poplar wood wafers of three different poplar substrates (A, B, and C) inoculated with *P. placenta* in petri dishes for 10 days of growth.

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chrysosporium, employ an array of hydrolases that attack cellulose, while simultaneously depolymerizing lignin by oxidative mechanisms (reviewed in reference 11). In contrast, brown-rot fungi employ a different approach—they modify lignin extensively and possess the capacity to rapidly depolymerize cellulose, causing significant loss of strength to the fibrous feedstock (11).

White-rot fungi produce complex lignolytic systems that are thought to depend on extracellular oxidative enzymes, especially peroxidases, laccases, and other oxidases (11). Moreover, *P. chrysosporium* has been shown to possess an extensive cytochrome P450 enzyme system that is thought to be responsible for the intracellular metabolism of lignin metabolites (12). White-rot fungi also secrete extracellular cellulase complexes, which include both endo- and exo-acting enzymes (see reference 13 and references therein), that synergistically act to degrade cellulose. These exo-cellobiohydrolases and endoglucanases often share architectures that include separate catalytic and cellulose binding domains. Beyond these well-known hydrolases, oxidative enzymes such as cellobiose dehydrogenase (CDH) and lytic polysaccharide monooxygenase (LPMO; formerly classified as a glycoside hydrolase [GH] family 61 member) have been implicated in cellulose attack (14–21).

The identification of specific genes and enzymes involved in the conversion of lignocellulosics originating from an expanding number of potential feedstocks is of growing interest to the emerging field of bioenergy process development (22). Substrate preference among certain brown-rot fungal species associated with gymnosperms is well known (10, 23). A better understanding of the enzymatic mechanisms underlying such selectivity could open the door to deploying enzymes optimized for deconstruction of chemically distinct wood substrates or the development of more generic enzyme cocktails for all substrates with equal efficiencies.

Although the tools of molecular biology and protein engineering have helped elucidate the roles of some of the enzymes involved in the synergistic degradation/modification of lignocellulosic substrates, a thorough understanding of basic mechanisms such as enzyme degradation, kinetics, and the true extent of interactions between enzymes is still lacking. As a result, an important aspect of the research to date has been to ascertain the limiting factors involved in decreased rates of hydrolysis over time. These factors have traditionally been ascribed to two key categories: those related to the differences in substrate structure and those related to the mechanisms and interactions of the enzymatic arsenal of various wood-degrading fungi (24, 25).

In this study, we exploited the genome sequences of a brown-rot fungus [*Postia placenta*, currently named *Rhodonia placenta* (Fr.) Niemelä, Larss. & Schigel; for comparative purposes with previously published work examining annotated gene expression patterns, we have elected to continue to use the original name, *P. placenta*, while recognizing the recent reclassification] and a white-rot fungus (*Phanerochaete chrysosporium*) that effectively deconstruct the major components of plant cell walls, including cellulose, hemicellulose, and the recalcitrant lignin. Using whole-genome microarrays based on the annotated genomes of these fungi, we monitored the changes in their transcriptomes relevant to cell wall degradation during growth on three chemically distinct *Populus trichocarpa* (poplar) wood substrates. Moreover, we com-

TABLE 1 Summary of chemical and physical traits of three different selected poplar wood substrates (A, B, and C)

Trait	Value for substrate:		
	A (high lignin, low glucose)	B (low lignin, high glucose)	C (approx avg lignin and glucose)
Klason lignin, %	24.82	18.19	19.57
Acid-soluble lignin, %	3.08	3.50	3.53
Total lignin, %	27.90	21.69	23.10
Arabinose, %	0.75	0.49	0.51
Rhamnose, %	0.40	0.38	0.43
Galactose, %	1.02	0.47	0.53
Glucose, %	44.01	53.80	49.67
Xylose, %	19.69	18.57	20.07
Mannose, %	2.42	2.78	2.82
Density, kg m^{-3}	571.9	492.17	532.99

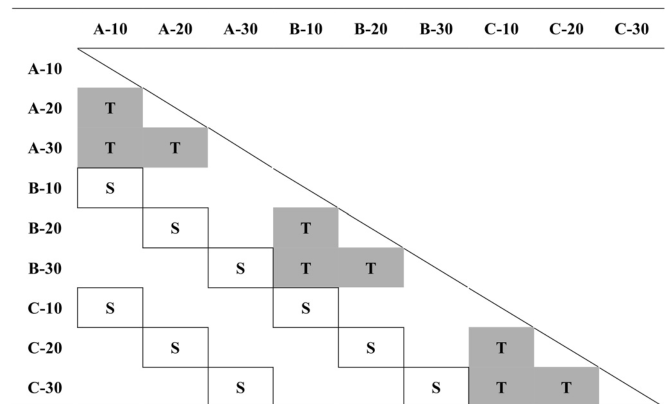

FIG 2 Pairwise comparisons for one wood decay fungus grown on three different poplar substrates (A, B, and C) for 10, 20, and 30 days (time points 10, 20, and 30). T, pairwise comparisons for time attribute; S, pairwise comparisons for substrate attribute.

TABLE 2 Differentially expressed *P. chrysosporium* genes that exhibited ≥2-fold differences ($P < 0.05$) in pairwise comparisons between three time points and when grown on three different poplar substrates[a]

	No. of DE genes[b]		
Substrate	D10 vs D20	D10 vs D30	D20 vs D30
A	0	0	0
B	1	5	0
C	0	4	3

[a] Time points of 10, 20, and 30 days and poplar substrates A, B, and C were used in the comparisons.
[b] DE, differentially expressed; D10, day 10; D20, day 20; D30, day 30.

TABLE 3 Differentially expressed *P. chrysosporium* genes that exhibited ≥2-fold differences ($P < 0.05$) in pairwise comparisons between different poplar substrates at three time points[a]

	No. of DE genes[b]		
Substrates	10 days	20 days	30 days
A vs B	24	15	0
B vs C	9	2	0
A vs C	34	33	0

[a] Time points of 10, 20, and 30 days and poplar substrates A, B, and C were used in the comparisons.
[b] DE, differentially expressed.

pared and contrasted *P. chrysosporium* and *P. placenta* by carrying out parallel transcript profiling experiments with the two fungi over time on similar starting feedstocks.

MATERIALS AND METHODS

Chemical analysis. Samples were ground in a Wiley mill to pass through a 40-mesh screen and Soxhlet extraction was performed overnight with hot acetone at 70°C to remove extractives. Lignin and carbohydrate contents were determined using a modified Klason procedure, where extracted ground stem tissue (0.2 g) was treated with 3 ml of 72% H_2SO_4 (26). The composition of neutral cell wall-associated carbohydrates (arabinose, rhamnose, galactose, glucose, mannose, and xylose) was determined using high-performance liquid chromatography (Dionex DX-600 system; Dionex, CA) equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector (ED 40) with a gold electrode, and a Spectra AS 3500 autoinjector (Spectra-Physics, CA). Aliquots (20 μl) were injected after passage through a 0.45-μm nylon syringe filter (Chromatographic Specialties Inc., Brockville, Ontario, Canada), and the column was eluted with distilled, deionized water at a flow rate of 1 ml min^{-1}. The optimization of baseline stability and detector sensitivity was achieved by post-column addition of 0.2 M NaOH at 0.5 ml min^{-1}. Acid-soluble lignin was determined by UV absorbance at 205 nm according to TAPPI standard Useful Method UM 250 (27), while insoluble lignin was determined gravimetrically using preweighed, medium-coarseness sintered glass crucibles.

Culture conditions and characterization. *P. chrysosporium* (strain MAD RP-78) and *P. placenta* (strain MAD 698-R) were obtained from the U.S. Department of Agriculture (USDA) Forest Products Laboratory (Madison, WI). Each fungal strain was cultured on 2% malt extract agar (MEA) (Oxoid, United Kingdom) for 10 days prior to inoculation into wood wafers.

Poplar wood stems were cut into 0.5-mm wafers on a microtome, sterilized for 20 min at 121°C, dried at 50°C overnight, and cooled to room temperature. Sterilized glass rods were then placed on the surface of the actively growing mycelia, and the wood specimens were then placed on top of the glass rods in the petri plates to avoid contact with the growth medium and yet to make contact with the actively growing mycelia (Fig. 1). Approximately 5 g of wood wafers was placed in each petri dish (exact weights were recorded), sealed with Parafilm, and incubated at 22°C and 70% ± 5% relative humidity for 10, 20, or 30 days (time points 10, 20, and 30, respectively). Following incubation, the wafers were removed from the petri dishes, immediately snap-frozen in liquid nitrogen, and stored at −80°C for later use. For substrates A and B, three replicates were used for each combination of substrate/fungus and incubation period. For substrate C, only two biological replicates were employed.

Expression microarrays. Frozen wood wafers with fungal mycelia were ground to a fine powder with liquid nitrogen in an acid-washed, prechilled mortar and pestle. The ground material was transferred to Falcon tubes (VWR International, West Chester, PA), extracted in freshly made buffer that was kept on ice and shaken immediately prior to use (the buffer preparation included 10 ml 690 mM sodium *para*-aminosalicylate [Sigma-Aldrich, St. Louis, MO] with 10 ml 56 mM sodium triisopropyl naphthalene sulfonic acid [sodium salt; Sigma-Aldrich, St. Louis, MO]), and placed on ice. To this was added 5 ml 5× RNB (1.0 M Tris, 1.25 M

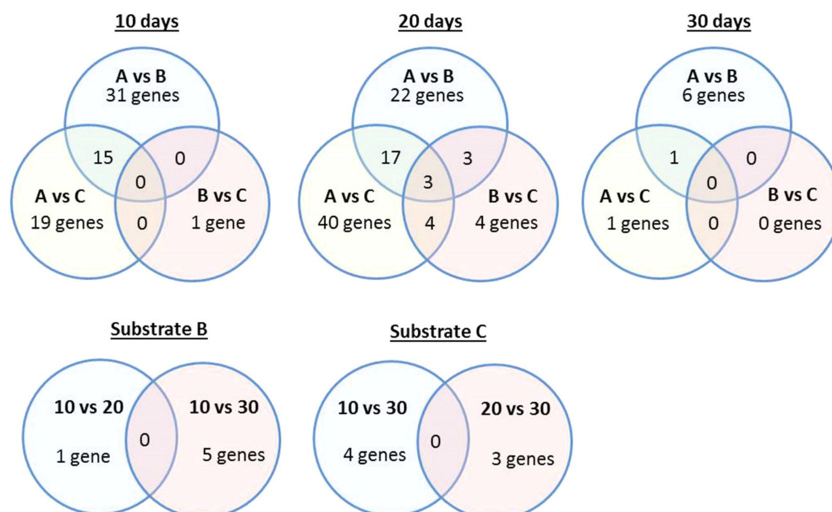

FIG 3 Venn diagrams illustrating the partitioning of *P. chrysosporium* misregulated genes displaying ≥2-fold change when grown on three different poplar substrates. Letters denote substrates (A, B, and C), and numbers refer to time points (10, 20, and 30 days).

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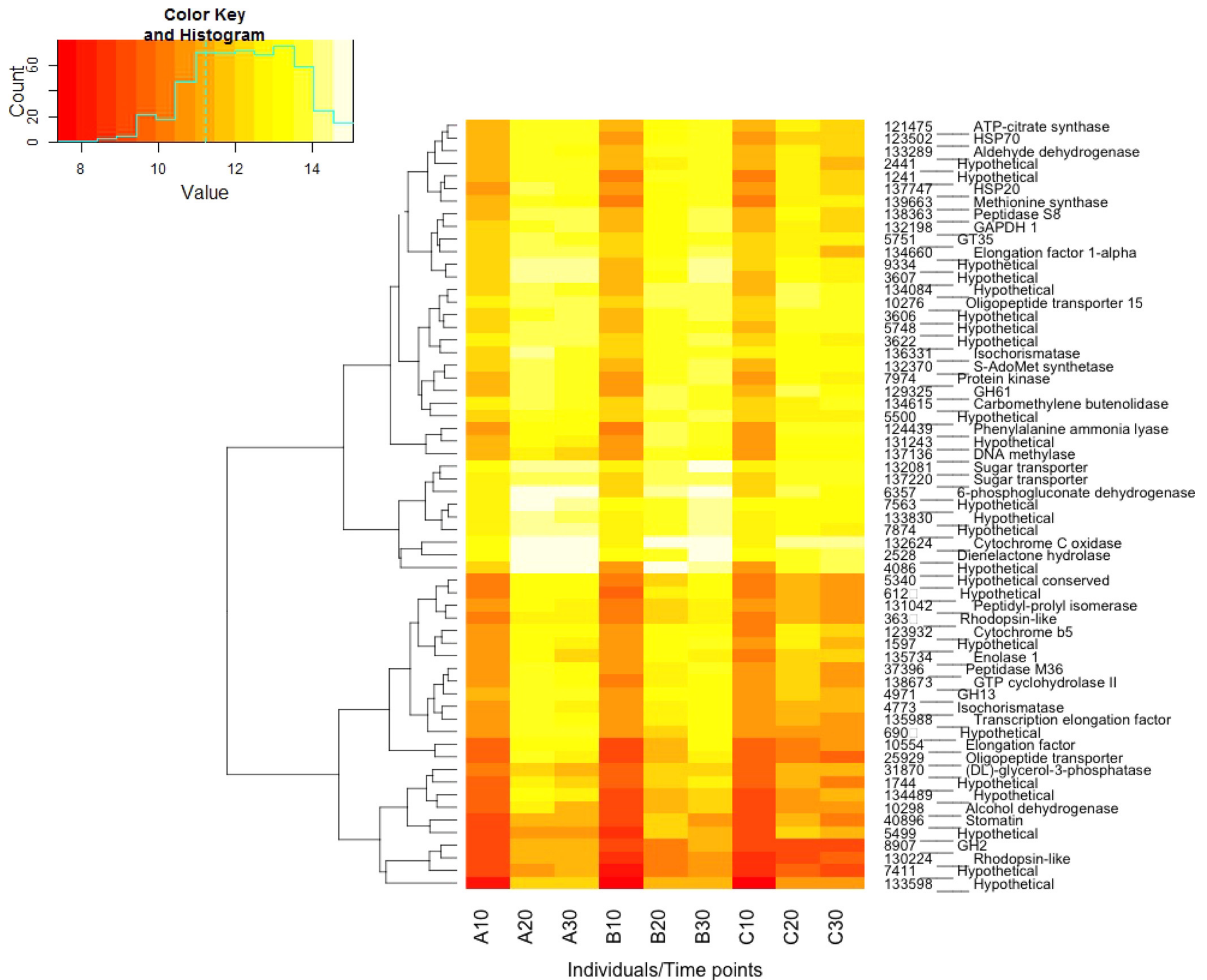

FIG 4 Heatmap showing hierarchical clustering of 61 *P. chrysosporium* genes with ≥2-fold ($P < 0.05$) transcript accumulation in pairwise comparisons between poplar substrates (A, B, and C) at different time points (10, 20, and 30 days). The scale above the map shows $\log_2$-based signals and their distribution. Protein IDs and putative functions are indicated on the right side of the heatmap.

NaCl, 0.25 M EGTA), with the pH adjusted to 8.5 with NaOH. The samples were vortexed vigorously and placed on ice until all samples were processed. One-half volume of Tris-EDTA-saturated phenol and one-quarter volume of chloroform (Sigma-Aldrich, St. Louis, MO) were added to each sample, followed by rigorous vortex mixing. Samples were centrifuged at 2,940 × *g* in a fixed-angle rotor for 5 min. The aqueous layer was removed to a new tube, and phenol-chloroform extractions were repeated until the interface between the aqueous and organic layers was clear. The final aqueous extractions were placed in sterile Falcon tubes, to which 0.1 volume of 3 M sodium acetate (pH 5.2; diethyl pyrocarbonate [DEPC] treated) was added with 2 volumes of absolute ethanol. The tubes were then shaken vigorously and stored overnight at −20°C.

The tubes were then centrifuged for 1 h at 2,940 × *g*, the supernatants were decanted, and the pellets were resuspended in 4 ml RNase-free H_2O. Total RNA was purified using the RNeasy Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNAs were eluted from RNeasy spin columns by using two spins for a final volume of 2 ml. The eluted RNAs were ethanol precipitated and stored overnight at −20°C. The RNAs were centrifuged for 1 h at 2,940 × *g*, washed once with 70% ethanol, and resuspended in 50 to 100 μl RNase-free water.

Total RNA was converted to Cy3-labeled cDNA, hybridized to microarrays, and scanned as described previously (28). The 24 arrays per fungal species were scanned and data were extracted using NimbleScan v.2.4. The raw data were loaded into GeneSpring, where the intensities were converted to $\log_2$ and quantile normalized, and the numbers of all probes per gene were averaged. These data were then exported and further analyzed in R. The data were again quantile normalized, and average intensities per group were calculated. All comparisons and their graphical representations were done using the Bioconductor package for R.

Expression levels are presented as $\log_2$ signals, and significant differences in expression were determined using a moderated two-sided *t* test (variances were not assumed to be equal) with a false discovery rate (FDR) threshold set at a *P* value of <0.05.

P. chrysosporium and *P. placenta* Roche NimbleGen array designs are available under platforms GPL8022 and GPL7187, respectively, within the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/).

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TABLE 4 Transcripts of *P. chrysosporium* genes of known functions with ≥2-fold ($P < 0.05$) accumulation resulting from pairwise comparisons between three substrates and three incubation times[a]

P. chrysosporium gene ID	Putative function	Transcript ratio (log_2)[b]								
		10 days			20 days			30 days		
		A vs B	A vs C	B vs C	A vs B	A vs C	B vs C	A vs B	A vs C	B vs C
139663	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	0.2	0.2	—	—	0.21	—	—	—	—
6357	6-Phosphogluconate dehydrogenase	—	—	—	0.22	0.18	—	—	—	—
133289	Aldehyde dehydrogenase	—	—	—	0.19	—	—	—	—	—
121475	ATP-citrate synthase	0.19	0.19	—	0.25	0.17	—	—	—	—
134615	Carbomethylene butenolidase	—	—	—	—	0.19	—	—	—	—
132624	Cytochrome *c* oxidase	—	—	—	—	0.25	—	—	—	—
2528	Dienelactone hydrolase	0.13	0.14	—	—	0.12	—	—	—	—
10554	Elongation factor	0.18	0.19	—	0.22	0.17	—	—	—	—
134660	Elongation factor 1-alpha	—	—	—	0.19	0.17	—	0.24	—	—
135734	Enolase 1	0.24	—	—	—	—	—	—	—	—
132198	GAPDH 1	0.21	—	—	0.18	0.14	—	—	—	—
4971	GH13, alpha-amylase	0.22	0.25	—	—	0.23	—	—	—	—
129325	AA9	—	—	—	—	0.23	—	—	—	—
5751	GT35	—	0.24	—	—	—	—	—	—	—
123502	Peptidase HSP70	0.23	0.22	—	0.15	0.13	—	—	—	—
25929	Oligopeptide transporter	0.21	0.23	—	—	0.24	—	—	—	—
10276	Oligopeptide transporter 15	0.18	0.2	—	—	0.17	—	—	—	—
131042	Peptidyl-prolyl *cis-trans* isomerase	—	—	—	0.23	0.23	—	—	—	—
124439	Phenylalanine ammonia lyase	—	0.25	—	—	—	—	—	—	—
7974	Protein kinase	0.25	—	—	—	—	—	—	—	—
130224	Rhodopsin-like	0.19	—	—	—	0.14	—	—	—	—
363	Rhodopsin-like	—	—	—	—	0.2	—	—	—	—
135988	Transcription elongation factor S-II	—	—	—	0.05	0.05	—	0.1	—	—
137220	Transporter group 1 high affinity glucose	0.23	—	—	—	—	—	—	—	—

[a] Time points of 10, 20, and 30 days and poplar substrates A, B, and C were used in the comparisons.
[b] —, no regulation.

Microarray data accession number. The MIAME-compliant (29) microarray expression data were deposited in NCBI's GEO database under accession number GSE69012.

RESULTS

Wood substrate selection. Three poplar wood substrates with distinct cell wall chemical properties were selected from several hundred 4-year-old *Populus trichocarpa* (Torr. & A. Gray) trees grown in a common garden field trial at the University of British Columbia (Canada). The genotypes employed were derived from *P. trichocarpa* genotypes that represent individuals that span the natural range of the species (30, 31). We selected genotypes based on cell wall chemical and physical traits and performed wet chemistry analysis on harvested material to confirm the substrate chemical compositions (Table 1). Substrate A corresponds to a genotype with a higher than average lignin content and a lower than average glucose content; substrate B corresponds to a genotype with a lower than average lignin content and a higher than average glucose content; substrate C corresponds to a genotype with lignin and glucose contents near the population averages (Table 1).

Overview of gene expression analysis. To analyze the transcript profiles of *P. placenta*, a brown-rot fungus, and *P. chrysosporium*, a white-rot fungus, over the course of growth on poplar wood samples, we employed Roche NimbleGen microarrays whose design was based on the annotated whole-genome sequences of the two fungal species (12, 17) (see Materials and Methods). The two species were grown on wood wafers of the three different poplar substrates (A, B, and C) for 10, 20, and 30 days (time points 10, 20, and 30) (Fig. 1). For each fungal species, we used the microarray data to generate nine pairwise comparisons for substrate and nine pairwise comparisons for incubation time (Fig. 2). Furthermore, for each substrate-time combination, we performed a cross-fungal pairwise comparison; *P. placenta* versus *P. chrysosporium* (A10, A20, A30, B10, B20, B30, C10, C20, C30).

White-rot fungus (*P. chrysosporium*) expression profiling. Of the 10,004 genes represented on the *P. chrysosporium* array, 64 genes were differentially expressed among the three poplar substrates and three incubation periods. No genes on the arrays exhibited ≥2-fold difference ($P < 0.05$) in transcript abundance in the pairwise comparisons of different times of incubation on substrate A (Table 2). Five genes were expressed at relatively high levels and were substantially upregulated (transcripts accumulated ≥2-fold) on substrate B after 10 days relative to 30 days of incubation, while 4 genes were upregulated on substrate C after 10 days versus 30 days of incubation and 3 genes were upregulated after 30 days versus 20 days (Fig. 3; Table 2). Genes encoding dienelactone hydrolase (Pchr2528; where Pchr represents *P. chrysosporium* and the number is the gene identification [ID]) and haloacid dehalogenase-like hydrolase (Pchr31870) were identified among significantly upregulated genes, while the other differentially expressed genes were annotated as either hypothetical (Pchr5500, Pchr132177) or of unknown function (Pchr7411,

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Pchr8907, Pchr6139, Pchr8722). A complete listing of all *P. chrysosporium* genes, their putative functions, average signal strength, and accounts of significant regulation can be found in Tables S1 and S2 in the supplemental material.

Substrate-dependent differential gene expression. We next analyzed the data for *P. chrysosporium* genes that were differentially expressed when the fungus was grown on chemically distinct wood substrates. Of the 10,004 genes represented on the microarrays, transcripts of 31 genes exhibited ≥2-fold differences ($P < 0.05$) in abundance in the pairwise comparisons of substrates A (high lignin and low glucose) and B (low lignin and high glucose) after 10 days (Table 3). After 20 days of incubation, only 8 genes remained differentially expressed in substrate A relative to substrate B; they were complemented by 13 new differentially expressed genes that were downregulated when *P. chrysosporium* was growing on lignin-rich substrate A relative to glucose-rich substrate B. By the end of the incubation trial (30 days), only 6 genes remained differentially expressed when grown on substrate A relative to substrate B.

Pairwise comparisons of transcripts that accumulated in *P. chrysosporium* grown on substrates B and C revealed only 4 differentially expressed genes, none of which, however, are of known function (see Table S1 in the supplemental material). On the other hand, 19 genes were differentially expressed when *P. chrysosporium* was grown for 10 days on substrate A relative to substrate C. Of those, 14 genes remained highly expressed after 20 days of incubation on substrate A relative to substrate B, with 26 additional new genes appearing at this time, for a total of 40 downregulated genes in substrate A relative to substrate C after 20 days (Fig. 4). However, by 30 days of incubation, these differences had disappeared and only one hypothetical heat shock protein (Pchr133830) was upregulated in substrate A relative to substrate C.

Differentially expressed *P. chrysosporium* genes. Of the 64 differentially regulated *P. chrysosporium* genes, only 9 were significantly differentially regulated over the entire incubation time course, and most differentially regulated genes were observed near the end of the incubation trial. Among these genes, only two, the dienelactone hydrolase gene (Pchr2528) and a rhodopsin-like gene (Pchr130224), were of known functions. However, among the 64 genes, expression variation in 61 of them was associated with the growth on the three different wood substrates and 5 could be assigned to the carbohydrate active class of enzymes (CAZy; http://www.cazy.org). Pchr4971, encoding a glycoside hydrolase GH13 enzyme, was preferentially expressed in fungi grown on a glucose-rich substrate B (Fig. 4). In addition, expression of Pchr5751 (encoding a glycoside transferase GT35 enzyme) and Pchr124439 (encoding phenylalanine ammonia lyase) varied significantly according to the substrate. The latter gene is likely involved in the biosynthesis of veratryl alcohol, a possible diffusible oxidant implicated in lignin degradation (reviewed in reference 32). Interestingly, 4 transporter proteins and 3 elongation factors (Pchr10554, Pchr134660, Pch135988) were also differentially expressed in response to different starting substrates (Table 4). Among the transporters, the gene encoding oligopeptide transporter (Pchr10276) was previously shown to be significantly upregulated in media containing milled aspen relative to pine (33).

Brown-rot fungus (*P. placenta*) expression profiling. The *P. placenta* expression arrays identified 84 genes whose transcript abundance differed substantially among the three poplar substrates and three incubation periods. Time course comparisons revealed 30 differentially expressed genes, 23 of which showed significant sequence similarity to known proteins (Table 5). Transcripts corresponding to 27 *P. placenta* gene models (hypothetical models derived largely from expressed sequence tags [EST] of genes that have not been identified biochemically) accumulated ≥2-fold during incubation on substrate C after 30 days relative their accumulation with other treatments. Of these, 25 were upregulated (accumulated ≥2-fold) after 10 days relative to 20 days (Table 5). Only 3 genes were significantly ($P < 0.05$) upregulated on substrate B after 10 days relative to the 30-day incubation period. Specifically, these genes were those encoding alcohol oxidase (Ppl118723; where Ppl represent *P. placenta* and the number is the gene ID), glycolate oxidase (Ppl121561), and a hypothetical protein Ppl91204 (Table 5). A complete listing of all *P. placenta* genes, their putative functions, average signal strength, and accounts of significant regulation can be found in Tables S3 and S4 in the supplemental material.

Among the 25 upregulated genes, five encode putative glycoside hydrolases: GH2 beta-mannosidase (Ppl57564), GH51 alpha-*N*-arabinofuranosidase (Ppl100251), GH10 endo-1,4-beta-xylanase (Ppl105534), GH79 glycosidase (Ppl126692), and GH43 galactan 1,3-beta-galactosidase (Ppl110809). Genes encoding

TABLE 5 Transcripts of *P. placenta* genes of known functions with ≥2-fold accumulation ($P < 0.05$) resulting from pairwise comparisons between two substrates and three incubation times[a]

P. postia gene ID	Putative function	Transcript ratio ($\log_2$)[b]	
		Substrate B, D10 vs D30	Substrate C, D10 vs D20
64080	Adenosylhomocysteinase	—	4.15
118926	Aldehyde dehydrogenase	—	4.86
122109	Carbonic anhydrase	—	4.11
43588	Carboxylesterase	—	7.98
119730	Formate dehydrogenase	—	6.64
105534	GH10 endo-1,4-beta xylanase	—	4.18
57564	GH2 beta-mannosidase	—	6.59
110809	GH43 galactan 1,3-beta-galactosidase	—	4
100251	GH51 alpha-*N*-arabinofuranosidase	—	4.29
126692	GH79	—	5.5
112172	**Heat shock protein**	—	**0.16**
110682	Lipolytic enzyme	—	4.78
125801	Lipolytic GDSL	—	4.69
52153	Peptidase A1A	—	6.19
58246	Peptidase G1	—	16.87
50115	Peptidase S53	—	5.56
58105	Peptidase S53	—	7.08
126233	Reductoisomerase	—	4.1
62157	UDP-glucose 4-epimerase	—	7.01
104872	UDP-glucose 4-epimerase	—	10.3
48204	Hypothetical	—	4.4
128151	Hypothetical	—	5.8
128848	Hypothetical	—	6.78
118723	Alcohol oxidase	5.63	—
121561	Glycolate oxidase	4.34	—
91204	Hypothetical	5.56	—

[a] Time points of 10, 20, and 30 days and poplar substrates B and C were used in the comparisons.

[b] —, no regulation. Data for only two pairs are listed, since no other pairwise comparisons flagged any significant genes. Bold indicates the only downregulated protein. D10, day 10; D20, day 20; D30, day 30.

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TABLE 6 Transcripts of *P. placenta* genes of known functions with ≥2-fold ($P < 0.05$) accumulation resulting from pairwise comparisons between three substrates and two incubation times[a]

P. postia gene ID	Putative function	Transcript ratio (log_2)[b]					
		10 days			20 days		
		A vs B	A vs C	B vs C	A vs B	A vs C	B vs C
64080	Adenosylhomocysteinase	—	0.22	—	—	—	—
118723	Alcohol oxidase	0.16	—	—	—	—	—
126217	Alcohol oxidase	0.17	0.15	—	—	—	—
118926	Aldehyde dehydrogenase	—	0.24	—	—	—	—
114720	Catalase	—	0.24	—	—	—	—
130305	CRO2	—	—	—	0.24	0.21	—
115505	Dihydroxyacetone kinase	—	—	—	—	0.21	—
47184	Elongation factor	0.14	0.12	0.12	—	—	—
116830	Enolase	—	—	—	—	0.21	—
107376	EXPN	—	—	—	—	0.20	—
119730	Formate dehydrogenase	—	—	—	—	—	—
117665	GAPDH	—	—	—	—	0.19	—
105534	GH10 endo-1,4-beta xylanase	—	0.24	—	—	—	—
125346	GH16 glycosidase	—	—	—	0.08	0.10	—
113926	GH16 glycosidase	—	—	—	0.10	0.12	—
119525	GH18 chitinase	—	—	—	0.24	—	—
57564	GH2 beta-mannosidase	—	—	0.25	—	—	—
129476	GH2 beta-mannosidase	—	0.19	0.19	—	—	—
117860	GH72 1,3-beta-glucanosyltransferase (GAS-like)	0.22	0.20	—	0.14	0.13	—
126692	GH79	—	—	—	—	—	—
128976	GLP-like	0.16	0.15	—	—	0.19	—
128371	GLP-like	—	0.25	0.25	—	—	—
47889	Glutamine synthetase	—	0.25	—	—	—	—
120620	Glycerol dehydrogenase	—	—	—	0.17	—	—
121561	Glycolate oxidase	0.20	—	—	—	—	—
112172	Heat shock protein	—	—	—	—	0.14	—
128541	Histidine kinase involved in signal transduction	0.17	0.18	—	—	—	—
95467	Lipolytic enzyme	0.17	0.15	—	—	—	—
43912	Oxalate decarboxylase	—	0.24	—	—	—	—
124972	Oxidoreductase	0.22	0.18	—	—	—	—
50115	Peptidase S53	—	0.20	—	—	—	—
58105	Peptidase S53	—	0.18	—	—	—	—
111839	Peroxidase	0.23	0.19	—	—	—	—
127047	Phosphodiesterase	—	—	—	—	0.10	—
36953	Protein kinase	0.20	0.18	0.18	—	0.23	—
118336	Pyruvate decarboxylase	0.24	0.24	—	—	0.08	—
107796	Pyruvate decarboxylase	—	—	—	—	0.08	—
128157	RNA binding protein	—	—	—	0.22	0.24	—
38710	Transcription factor	—	—	—	—	0.20	—
62157	UDP-glucose 4-epimerase	—	—	0.25	—	—	—
104872	UDP-glucose 4-epimerase	—	—	0.15	—	—	—

[a] Time points of 10 and 20 days and poplar substrates A, B, and C were used in the comparisons.

[b] —, no regulation. Only six pairs are listed, since comparisons after 30 days did not flag any significant genes.

enzymes of the following families were differentially regulated when the fungus was grown on substrate C for 10 days, relative to 20 days: two dehydrogenases, namely, aldehyde dehydrogenase (Ppl118926) and formate dehydrogenase (Ppl119730), and four peptidases, namely, peptidases S53 (Ppl0115 and Ppl58105), A1A (Ppl52153), and G1 (Ppl58246) together with carboxylesterase (Ppl43588), and two UDP-glucose 4-epimerases (Ppl62157 and Ppl104872), adenosylhomocysteinase (Ppl64080), carboxylesterase (Ppl43558), carbonic anhydrase (Ppl122109), lipolytic GDSL (Ppl125801; a lipase/esterase hydrolytic enzyme with multifunctional properties that contains a Gly-Asp-Ser-Leu amino acid sequence motif), and reductoisomerase (Ppl126233) (Table 5).

Substrate-dependent differential gene expression. Differentially expressed transcripts corresponding to 68 *P. placenta* gene models were identified by the pairwise comparisons between three poplar substrates over the three incubation times (Table 6). Of these, 24 were upregulated (accumulated ≥2-fold) in mycelium grown on lignin-rich substrate A, relative to substrate B, after 10 days of incubation (Fig. 5A). After 20 days of incubation, only 2 genes of the 24 remained substantially upregulated on substrate A relative to substrate B. Moreover, a suite of 13 new upregulated genes was detected on substrate A relative to substrate B. By the end of the incubation period (30 days), there were no differentially regulated genes identified from fungi

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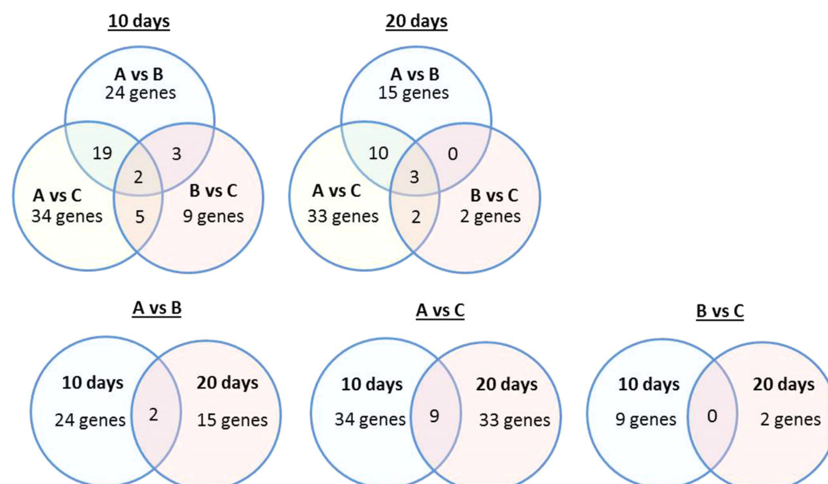

FIG 5 Venn diagrams illustrating the partitioning of *P. placenta* misregulated genes displaying ≥2-fold change when grown on three different poplar substrates and for two incubation times. Only two time points (10 and 20 days) are presented, as no genes were significantly regulated at time point 30 (30 days).

grown on substrate A or B. The trend of decreasing numbers of downregulated genes was mirrored for pairwise comparisons on the following substrates: B versus C and A versus C. In the latter case, of the 34 substantially upregulated genes on substrate C relative to substrate A after 10 days, 9 genes remained upregulated and 25 new genes were differentially expressed after 20 days of incubation (Fig. 5B). Transcript abundance and a complete listing of all 68 differentially expressed *P. placenta* protein models together with their putative functions are depicted in Fig. 6 as a heatmap.

Time-wise comparisons did not reveal any differentially expressed genes on lignin-rich substrate A. Three genes were downregulated on glucose-rich substrate B after 10 days, relative to 30 days, of incubation. Substrate C induced a significant accumulation of 27 genes transcripts after 10 days, relative to 20 days, of incubation.

Differentially expressed *P. placenta* genes. Among the 84 *P. placenta* genes that were differentially expressed, 68 could be associated with the differences in wood substrate. Genes encoding 10 glycoside hydrolases (Table 6) were upregulated when the fungus was grown on the glucose-rich substrate (B) or on substrate C, relative to substrate A (high lignin). Transcripts corresponding to the copper radical oxidase gene *CRO2* (Ppl130305) accumulated in both substrates B and C after 20 days of incubation, relative to substrate A. Among others, genes encoding the following proteins were preferentially expressed on substrate C after 10 days: alcohol oxidase (Ppl126217), aldehyde dehydrogenase (Ppl118926), catalase (Ppl114720), a histidine kinase involved in signal transduction (Ppl128541), lipolytic enzyme (Ppl95467), oxalate decarboxylase (Ppl43912), oxidoreductase (Ppl124972), two peptidases S53 (Ppl50115 and Ppl58105), peroxidase (Ppl111839), protein pyruvate (Ppl36953), and decarboxylase kinase (Ppl118336).

Cross-fungal comparisons. In order to compare gene expression patterns between the two fungi, we used a previously reported database of matched gene models (33). Of the 12,438 *P. placenta* gene models represented on the microarray, 8,871 were matched using BLASTP to *P. chrysosporium* proteins with pairwise identities ranging from 28% to 100%. The total number of unique *P. chrysosporium* gene models matching the *P. placenta* data set was 5,538, which is approximately 55% of the total *P. chrysosporium* gene models.

Effect of the substrate. After the first 10 days of incubation, *P. placenta* and *P. chrysosporium* shared 65 differentially expressed genes when grown on all three substrates (Fig. 7A). As the fungal growth continued, the number of shared genes increased to 73 (Fig. 7B and C), suggesting that both white- and brown-rot fungi became less "sensitive" to differences between substrates. Substrate A supported the highest number of unique genes (19) sharply regulated by either *P. placenta* or *P. chrysosporium*.

Effect of the incubation time. (i) Substrate A. Transcripts from 110 matched models were shown to differentially accumulate during incubation with the lignin-rich substrate A after 10 days of growth. Among these, 47 were downregulated and 63 were upregulated in *P. placenta* relative to *P. chrysosporium* (Table 7). After 20 days of incubation on substrate A, 50 genes were downregulated and 70 were upregulated in *P. placenta* relative to *P. chrysosporium*, while after 30 days, out of 111 differentially expressed genes, 49 were downregulated and 62 were upregulated in the brown-rot fungus relative to the white-rot fungus. Of all the differentially regulated genes, 97 were common to both fungi at different incubation times on substrate A (Fig. 8A).

(ii) Substrate B. When grown on the glucose-rich substrate B for 10, 20, or 30 days, both brown- and white-rot organisms shared 90 differentially regulated genes (Fig. 8B). Of 115 matched models, 47 genes were downregulated and 68 were upregulated in *P. placenta* relative to *P. chrysosporium* after 10 days and 48 genes were downregulated and 62 were upregulated in *P. placenta* relative to *P. chrysosporium* after 20 days (Table 7). Finally, out of 112 genes, 48 were downregulated and 64 were upregulated in *P. placenta* relative to *P. chrysosporium* after 30 days of incubation.

(iii) Substrate C. Of the 119 genes matched by BLASTP that accumulated on substrate C, 71 genes were ubiquitous between all incubation time points (Fig. 8C). After 10 days, 36 genes were downregulated, whereas 56 were upregulated. By the end of second incubation period, 34 genes were downregulated and 66 were

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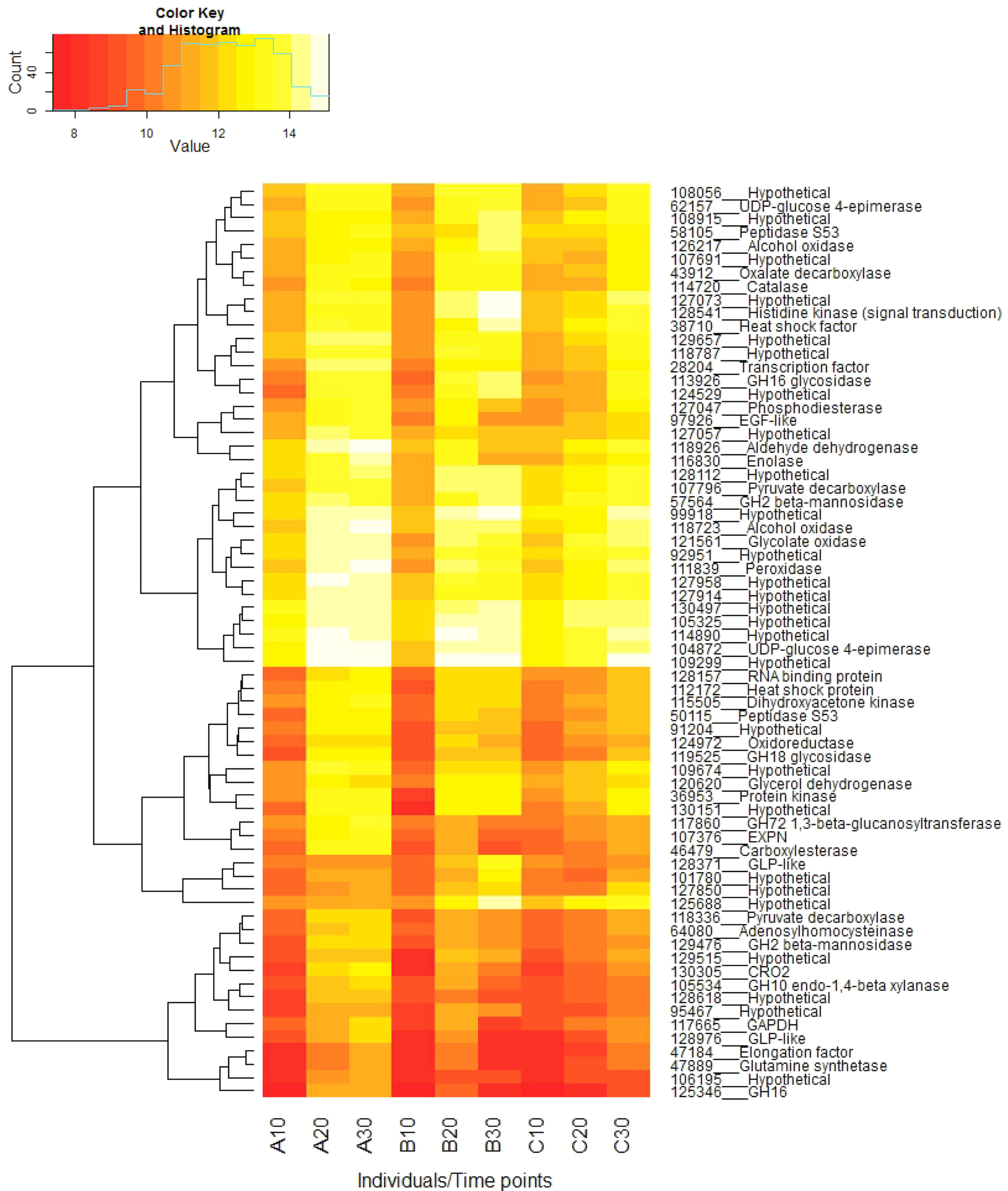

FIG 6 Heatmap showing hierarchical clustering of 68 *P. placenta* genes with ≥2-fold ($P < 0.05$) transcript accumulation in pairwise comparisons between poplar substrates (A, B, and C) at different time points (10, 20, and 30 days). The scale above the map shows $\log_2$-based signals and their distribution. Protein IDs and putative functions are indicated on the right side of the heatmap.

upregulated. Finally, at the end of the incubation, 36 genes were downregulated and 53 were upregulated in *P. placenta* relative to *P. chrysosporium* (Table 7).

Despite a seemingly high number of highly differentially expressed genes (fold change of ≥4), only 30 of 170 were annotated and had known or hypothetical functions (Table 8). Glycoside hydrolase GH 18 (Ppl47920 and Pchr129436) was highly upregulated (up to 30-fold) in all substrate and time-wise comparisons. For the complete listing of other proteins, see Table S5 in the supplemental material.

DISCUSSION

To date, genome-wide transcriptome studies of gene expression in *P. chrysosporium* and *P. placenta* have been conducted only after incubation for a single, predetermined growth period in submerged cultures supplemented with ground wood substrate, glu-

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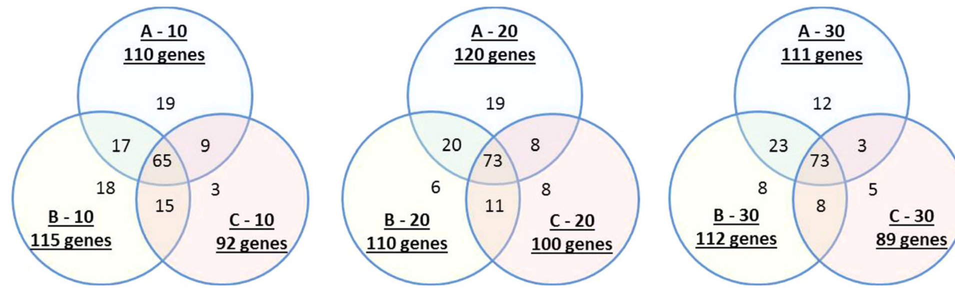

FIG 7 Venn diagrams illustrating the substrate partitioning of significantly ($P < 0.05$) misregulated genes with transcript levels that display a ≥4-fold change in cross-fungal comparisons of *P. placenta* and *P. chrysosporium* grown on three different poplar substrates (A, B, and C) and for three incubation times (10, 20, and 30 days).

TABLE 7 Regulation and number of differentially expressed and common matched genes of *P. chrysosporium* and *P. placenta* in cross-fungal pairwise comparisons[a]

	Total no. of DE genes at incubation time of[b]:		
Substrate	10 days	20 days	30 days
A	110 (↓ 47 + ↑ 63)	120 (↓ 50 + ↑ 70)	111 (↓ 49 + ↑ 62)
B	115 (↓ 47 + ↑ 68)	110 (↓ 48 + ↑ 62)	112 (↓ 48 + ↑ 64)
C	92 (↓ 36 + ↑ 56)	100 (↓ 34 + ↑ 66)	89 (↓ 36 + ↑ 53)

[a] Transcript accumulation fold change cutoff, >4; significance threshold, $P < 0.05$.
[b] DE, differentially expressed. For each given comparison, ↓ indicates downregulation and ↑ indicates upregulation.

cose, or microcrystalline cellulose (17, 28, 33–37). Although these studies have contributed to our understanding of the transcriptional regulation and modulation of gene expression on woody substrates, they bear little resemblance to solid substrates, where accessibility to cell wall polymers likely influences transcript levels. In this study, we employed solid wood samples inoculated with fungal cultures to mimic, as closely as possible, natural conditions, in which wood-rotting fungi gradually degrade the wood substrate over time.

Our results show that gene expression profiles of *P. placenta* and *P. chrysosporium* are indeed influenced by the wood substrate and the length of incubation. For most differentially expressed *P. placenta* genes, we observed an increase in transcript accumulation with an increase in the length of incubation time (Fig. 6). The overall pattern of *P. placenta* transcript accumulation suggests that during incipient growth, differences in substrate chemistry were not recognized by the organism (Fig. 6). However, as incubation proceeded and enzymes commenced the decomposition of substrate, the transcript abundance of genes changed. It is also likely that the low lignin content of substrate B might have facilitated degradation by *P. placenta*, which targets mainly hemicelluloses and cellulose. Among the 68 differentially expressed genes, the increase in transcript accumulation is more pronounced on substrates A and B than on substrate C. Therefore, substrates A and B seem to be more similar for *P. placenta* (Fig. 6). The genome of the brown-rot basidiomycete *P. placenta* encodes 153 putative glycoside hydrolases (GH) (17), of which only 10 were differentially expressed in the current experiment (Table 6). This pattern and specific predicted functions of the differentially regulated *P. placenta* GH genes confirm and highlight the importance of hemicellulose hydrolysis on all substrates at different time points, as an endo-1,4-beta-xylanase (GH10), two glycosidases (GH16 and GH79), chitinase (GH18), β-mannosidase (GH2), arabinofuranosidase (GH51), and a galactosidase (GH43) were among the gene products induced by the substrate (Fig. 9). Differential expression of genes encoding proteins involved in hemicellulose degradation on substrate A versus substrate B would imply a mechanism of increasing substrate availability (porosity and accessibility), relative to cellulose and lignin, especially early in the decay process.

The genes encoding the proteins copper radical oxidase (CRO2; Ppl130305) and aldehyde dehydrogenase (Ppl118926) are involved in extracellular H_2O_2 generation. Both were upregulated on the lignin-rich substrate A relative to substrate C. Catalytically distinct from CROs, glucose-methanol-choline (GMC) oxidoreductases include various alcohol and sugar oxidases. Among the former, protein models Ppl118723 and Ppl126217 (alcohol oxidases) show significant transcript accumulation on the higher-glucose substrates B and C relative to that on the low-glucose, high-lignin substrate A. Alcohol oxidases may contribute to the extracellular

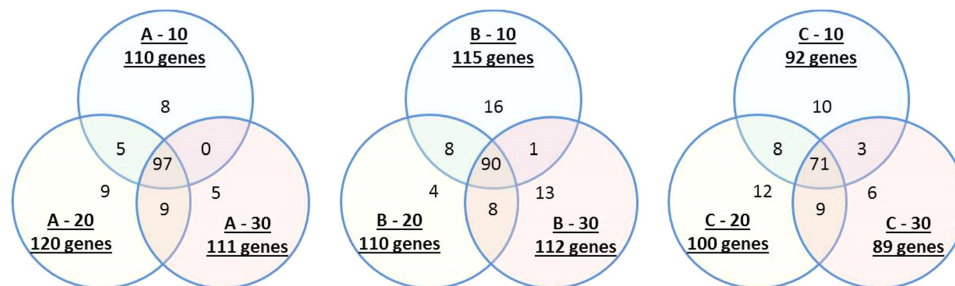

FIG 8 Venn diagrams illustrating the incubation period partitioning of significantly ($P < 0.05$) misregulated genes with transcript levels that display a ≥4-fold change in cross-fungal comparisons of *P. placenta* and *P. chrysosporium* grown on three different poplar substrates (A, B, and C) and for three incubation times (10, 20, and 30 days).

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TABLE 8 Transcripts of *P. placenta* and corresponding *P. chrysosporium* genes of known and hypothetical functions with ≥4-fold ($P < 0.05$) accumulation resulting from pairwise comparisons between three substrates and three incubation times[a]

P. placenta gene ID	Putative function	*P. chrysosporium* gene ID	Putative function	*P. placenta*/*P. chrysosporium* transcript ratio (log_2)[b]								
				10 days			20 days			30 days		
				A	B	C	A	B	C	A	B	C
96593	ATP-dependent RNA helicase	130400	ATP-dependent RNA helicase	0.033	0.03	0.032	0.033	0.029	0.033	0.028	0.035	0.033
47184	Elongation factor	134660	Elongation factor 1-alpha	—	—	—	—	—	0.048	—	0.061	—
47920	GH18 chitinase	129436	GH18 chitinase	**31.7**	**17.706**	**29.859**	**24.403**	**19.336**	**21.262**	**25.587**	**27.33**	**30.111**
124964	Glutathione *S*-transferase	7971	Glutathione *S*-transferase	**17.368**	**20.485**	**21.005**	**17.718**	**19.319**	**21.142**	**18.493**	**22.524**	**22.217**
47306	Heat shock protein	38176	Hypothetical	0.01	0.014	0.013	0.008	0.009	0.012	0.008	0.009	0.009
33879	HMG-coenzyme A lyase	121359	Hypothetical	—	0.061	—	—	0.057	0.057	—	—	0.057
27005	Hypothetical	3737	Hypothetical	0.028	0.036	0.024	0.039	0.039	0.036	0.037	0.034	0.028
92157	Hypothetical	197	Hypothetical	0.052	0.041	0.047	0.042	0.046	0.035	0.05	0.051	0.041
97315	Hypothetical	5411	Hypothetical	0.059	0.041	0.048	0.049	0.047	0.038	0.047	0.054	0.056
116986	Hypothetical	6975	Hypothetical	0.015	0.019	0.019	0.016	0.018	0.022	0.016	0.018	0.013
92829	Hypothetical	5225	Hypothetical	0.030	0.054	—	0.035	0.049	—	0.036	0.035	—
99898	Hypothetical	7062	Hypothetical proline-rich	—	—	—	0.044	—	—	0.046	0.053	0.062
103452	Hypothetical	136398	Hypothetical	—	—	—	0.061	—	0.049	—	—	0.051
91947	Hypothetical	132827	Hypothetical	—	0.061	0.062	—	—	—	—	—	—
53535	Hypothetical	138049	Hypothetical	—	—	—	0.060	—	—	—	—	—
91397	Hypothetical	4329	Hypothetical	0.060	—	—	—	—	—	—	—	—
128179	Hypothetical	5517	Hypothetical	—	—	—	—	—	—	16.379	—	—
129525	Hypothetical	325	Hypothetical	—	—	—	—	—	0.050	—	—	—
125158	Importin beta-4-subunit	2024	Hypothetical	—	**19.135**	—	—	**16.249**	—	—	**16.505**	—
33906	Lipase	132378	Hypothetical	0.056	—	—	0.056	—	—	0.052	0.052	—
93518	Hypothetical	3619	Hypothetical conserved	0.022	0.023	0.019	0.018	0.019	0.018	0.019	0.021	0.019
125963	Hypothetical	7222	Hypothetical	**19.364**	—	—	**20.45**	**17.825**	**19.524**	**21.546**	**21.050**	**23.596**
104795	Hypothetical	1619	Hypothetical (similar to small *Laccaria* secreted, GPI anchored)	—	0.034	0.035	—	0.037	0.042	—	0.057	0.052
128095	Hypothetical	7222	Hypothetical	**19.441**	**23.1**	—	**18.109**	**22.506**	—	**22.404**	**27.677**	—
95446	Hypothetical	125165	Epoxide hydrolase	—	0.048	—	—	0.042			0.046	—
128626	Hypothetical	135988	Transcription elongation factor S-II	—	—	—	**29.252**	—	—	**18.517**	—	—
105130	Hypothetical	7396	Hypothetical conserved group 12	—	—	0.051	—	—	—	—	—	—
28683	Nitropropane dioxygenase	127345	Hypothetical	0.061	—	—	—	0.058	—	—	—	—
101092	Protein disulfide isomerase	131571	Protein disulfide isomerase	—	0.044	0.04	—	0.049	0.059	—	—	0.061
128228	Vegetative incompatibility protein	130066	Hypothetical	**20.031**	**16.865**	—	**18.979**	**20.642**	**18.486**	**18.401**	**18.838**	**16.398**

[a] Time points of 10, 20, and 30 days and poplar substrates A, B, and C were used in the comparisons.

[b] —, no regulation. Bold indicates values for transcripts that were upregulated in *P. placenta* relative to *P. chrysosporium*.

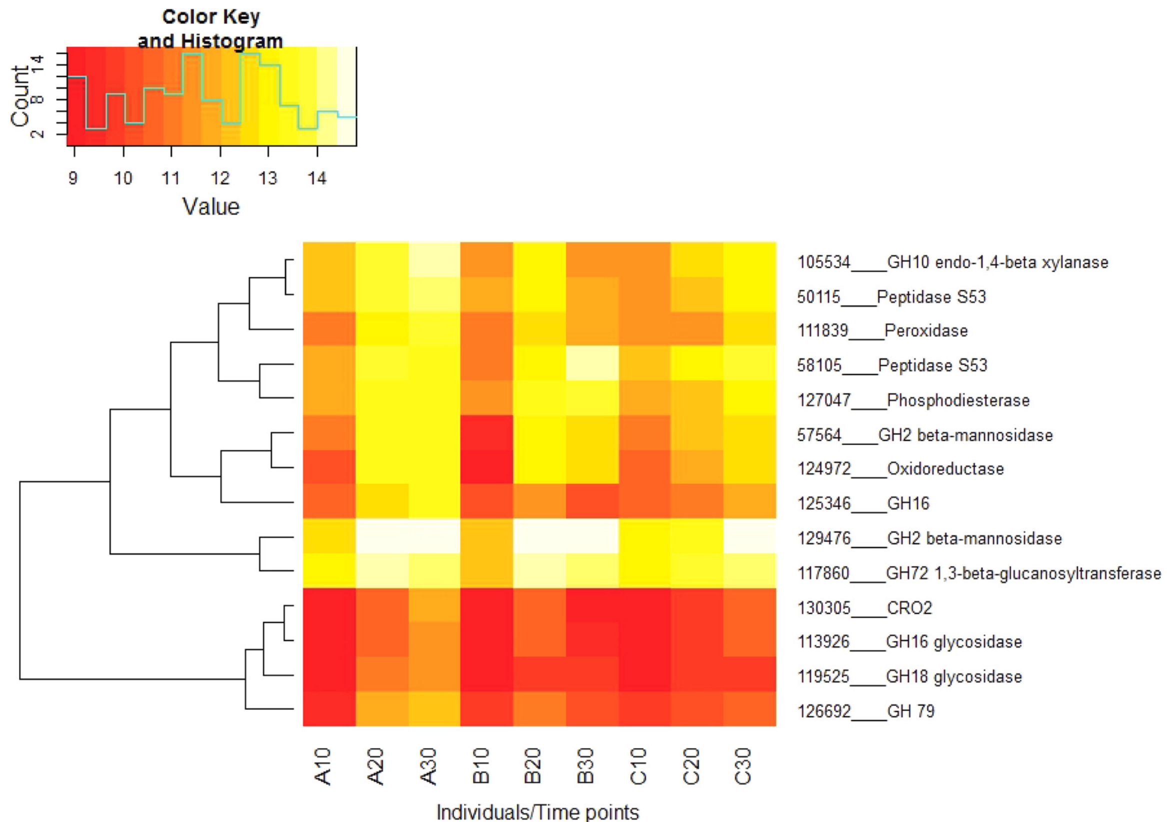

FIG 9 Heatmap showing hierarchical clustering of selected *P. placenta* genes (peptidases, GHs, CRO2, and peroxidase) with ≥2-fold ($P < 0.05$) transcript accumulation in pairwise comparisons between poplar substrates at different time points. The scale above the map shows $\log_2$-based signals and their distributions. Protein IDs and their putative functions are indicated on the right side of the heatmap.

production of hydrogen peroxide, since it has a preference for methanol, which potentially is available from the demethylation of lignin. Consistent with our findings, Martinez at al. (17) also observed high levels and a sharp increase in abundance of Ppl118723 transcripts in cellulose-grown cultures relative to those grown in a glucose medium. Extracellular peroxide production in brown-rot fungi such as *P. placenta* may play a critical role in the generation of reactive hydroxyl radicals that are responsible for cellulose depolymerization (reviewed in references 11 and 38).

Based on the *P. chrysosporium* transcription profile analysis, AA9 protein was downregulated on low-glucose substrate A. Formerly classified as members of the glycoside hydrolase family 61 (GH61) (20), the copper-dependent LPMOs (lytic polysaccharide monooxygenases) such as AA9 protein require molecular oxygen and an external electron donor to function properly (39). It has been shown that in numerous white-rot fungi (13), LPMOs act on recalcitrant polysaccharides by combining hydrolytic and oxidative functions, which generates oxidized and nonoxidized chain ends and acts synergistically with cellobiose dehydrogenase (CDH) (18, 19, 40). LPMOs boost the performance of commercial cellulases (41) and are, therefore, of increasing biotechnological interest for conversions of lignocellulosic biomass to fermentation feedstocks and other high-value chemicals (42). Certainly, the fact that not all organisms have genes encoding CDH in their genomes (even brown-rot fungi have LPMOs, albeit at lower numbers) (43) suggests that their cooperative activity is not mandatory for proper function. However, another member of the GH family, GH13 (Pchr4971), possesses alpha-amylase catalytic function and, similar to AA9, was also downregulated on low-glucose substrate A relative to both substrates B and C. It has been previously reported that some of the AA9 enzymes are expressed solely during incipient growth, only the first few days following fungal incubation (44), and therefore it is possible that a number of early responding genes/enzymes were not detected as a consequence of our experimental design (with the first sampling point at day 10).

Cross-fungal comparisons highlighted only one CAZy chitinase gene, GH18 (Ppl47920, Pchr12946), which showed an astonishing 30-fold upregulation in *P. placenta* (Table 8) relative to *P. chrysosporium* on all substrates throughout the duration of the experiment. Chitin is a major component of the cell walls of yeasts and other fungi; therefore, chitinase is not directly involved in woody substrate degradation. Chitinases (GH18) and α-trehalases (GH37), together with glycosidases, have been suggested to be involved in cell wall morphogenesis and, according to recent reports, have a potential application in the biocontrol of fungal phytopathogens (45).

A significant number of proteins of unknown function were identified in the cross-fungal comparisons, and their number can surge to a thousand if the significance threshold applied in our study were reduced (see Table S5 in the supplemental material). The most chemically distinct substrates, A and B, showed the highest number of differentially expressed genes. Assessing the role of hypothetical proteins remains problematic and especially challenging for *P. placenta*, which has a significantly higher num-

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ber of hypothetical proteins expressed on all substrates relative to *P. chrysosporium*. Further determination of the precise biochemical function of the many hypothetical proteins revealed in this study and elucidation of their roles in lignocellulose degradation remain a key task for future research.

Conclusions. Our results clearly show that gene expression profiles of *P. chrysosporium* and *P. placenta* were influenced by poplar wood substrate and incubation time. An analysis of transcript abundance in all 18 pairwise comparisons showed 64 and 84 differentially regulated genes in *P. chrysosporium* and *P. placenta*, respectively, when the fungi were cultured on chemically different substrates over time. Many of the significantly expressed proteins are proteins of unknown function, and determining the precise role of the corresponding genes in lignocellulose degradation presents a major challenge for future research, although perhaps a less daunting one than determining the role of the many interesting hypothetical proteins. These findings pave the path for identifying new and novel enzymes, mediators, cofactors, or biomimetic degradation mechanisms and targets for lignocellulosic feedstock.

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We declare we have no competing interests.

O.S., D.C., C.J.D., and S.D.M. designed the study, O.S. conducted the research, O.S. and D.C. performed the gene expression analyses, and O.S., D.C., C.J.D., and S.D.M. wrote the manuscript. All authors read and approved the manuscript.

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REFERENCES

1. **Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD.** 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science **315**:804–807. http://dx.doi.org/10.1126/science.1137016.
2. **Galbe M, Zacchi G.** 2002. A review of the production of ethanol from softwood. Appl Microbiol Biotechnol **59**:618–628. http://dx.doi.org/10.1007/s00253-002-1058-9.
3. **Feltus FA, Vandenbrink JP.** 2012. Bioenergy grass feedstock: current options and prospects for trait improvement using emerging genetic, genomic, and systems biology toolkits. Biotechnol Biofuels **5**:80. http://dx.doi.org/10.1186/1754-6834-5-80.
4. **Singh AP, Singh T.** 2014. Biotechnological applications of wood-rotting fungi: a review. Biomass Bioenergy **62**:198–206. http://dx.doi.org/10.1016/j.biombioe.2013.12.013.
5. **Porth I, El-Kassaby YA.** 2015. Using Populus as a lignocellulosic feedstock for bioethanol. Biotechnol J **10**:510–524. http://dx.doi.org/10.1002/biot.201400194.
6. **Conde-Mejíaa C, Jiménez-Gutiérreza A, El-Halwagib M.** 2012. A comparison of pretreatment methods for bioethanol production from lignocellulosic materials. Process Saf Environ Prot **90**:189–202. http://dx.doi.org/10.1016/j.psep.2011.08.004.
7. **Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R.** 2010. Hemicelluloses for fuel ethanol: a review. Bioresour Technol **101**:4775–4800. http://dx.doi.org/10.1016/j.biortech.2010.01.088.
8. **Dale BE, Ong RG.** 2012. Energy, wealth, and human development: why and how biomass pretreatment research must improve. Biotechnol Prog **28**:893–898. http://dx.doi.org/10.1002/btpr.1575.
9. **Podkaminer KK, Guss AM, Trajano HL, Hogsett DA, Lynd LR.** 2012. Characterization of xylan utilization and discovery of a new endoxylanase in *Thermoanaerobacterium saccharolyticum* through targeted gene deletions. Appl Environ Microbiol **78**:8441–8447. http://dx.doi.org/10.1128/AEM.02130-12.
10. **Eriksson K-E, Blanchette RA, Ander P.** 1990. Microbial and enzymatic degradation of wood and wood components. Springer, Berlin, Germany.
11. **Hatakka A, Hammel KE.** 2011. Fungal biodegradation of lignocellulosics, p 319–340. *In* Hofrichter M (ed), Industrial applications, the Mycota, vol 10. Springer-Verlag, Heidelberg, Germany.
12. **Martinez D, Larrondo LF, Putnam N, Sollewijn Gelpke MD, Huang K, Chapman J, Helfenbein KG, Ramaiya P, Detter JC, Larimer F, Coutinho PM, Henrissat B, Berka R, Cullen D, Rokhsar D.** 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat Biotechnol **22**:695–700. http://dx.doi.org/10.1038/nbt967.
13. **Hori C, Gaskell J, Igarashi K, Samejima M, Hibbett D, Henrissat B, Cullen D.** 2013. Genomewide analysis of polysaccharides degrading enzymes in 11 white- and brown-rot Polyporales provides insight into mechanisms of wood decay. Mycologia **105**:1412–1427. http://dx.doi.org/10.3852/13-072.
14. **Harris PV, Welner D, McFarland KC, Re E, Navarro Poulsen J-C, Brown K, Salbo R, Ding H, Vlasenko E, Merino S, Xu F, Cherry J, Larsen S, Lo Leggio L.** 2010. Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry **49**:3305–3316. http://dx.doi.org/10.1021/bi100009p.
15. **Langston JA, Shaghasi T, Abbate E, Xu F, Vlasenko E, Sweeney MD.** 2011. Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. Appl Environ Microbiol **77**:7007–7015. http://dx.doi.org/10.1128/AEM.05815-11.
16. **Carrard G, Koivula A, Söderlund H, Béguin P.** 2000. Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. Proc Natl Acad Sci U S A **97**:10342–10347. http://dx.doi.org/10.1073/pnas.160216697.
17. **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, Splinter BonDurant S, 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 U S A **106**:1954–1959. http://dx.doi.org/10.1073/pnas.0809575106.
18. **Quinlan RJ, Sweeney MD, Lo Leggio L, Otten H, Poulsen J-CN, Johansen KS, Krogh KBRM, Jørgensen CI, Tovborg M, Anthonsen A, Tryfona T, Walter CP, Dupree P, Xu F, Davies GJ, Walton PH.** 2011. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci U S A **108**:15079–15084. http://dx.doi.org/10.1073/pnas.1105776108.
19. **Westereng B, Ishida T, Vaaje-Kolstad G, Wu M, Eijsink VGH, Igarashi K, Samejima M, Ståhlberg J, Horn SJ, Sandgren M.** 2011. The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. PLoS One **6**:e27807. http://dx.doi.org/10.1371/journal.pone.0027807.
20. **Levasseur A, Drula E, Lombard V, Coutinho P, Henrissat B.** 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels **6**:41. http://dx.doi.org/10.1186/1754-6834-6-41.
21. **Mansfield SD, De Jong E, Saddler JN.** 1997. Cellobiose dehydrogenase, an active agent in cellulose depolymerization. Appl Environ Microbiol **63**:3804–3809.
22. **Mansfield SD.** 2009. Solutions for dissolution—engineering cell walls for deconstruction. Curr Opin Biotechnol **20**:286–294. http://dx.doi.org/10.1016/j.copbio.2009.05.001.
23. **Worrall JJ, Anagnost SE, Zabel RA.** 1997. Comparison of wood decay among diverse lignicolous fungi. Mycologia **89**:199–219. http://dx.doi.org/10.2307/3761073.
24. **Wong KKY, Richardson JD, Mansfield SD.** 2000. Enzymatic treatment

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of mechanical pulp fibres for improved papermaking properties. Biotechnol Prog **16**:1025–1029. http://dx.doi.org/10.1021/bp000064d.
25. **Mansfield SD, Mooney C, Saddler JN.** 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol Prog **15**:804–816. http://dx.doi.org/10.1021/bp9900864.
26. **Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD.** 2003. Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agric Food Chem **51**:6178–6183. http://dx.doi.org/10.1021/jf034320o.
27. **TAPPI.** 1985. UM 250. Acid soluble lignin in wood and pulp. TAPPI Press, Atlanta, GA.
28. **Vanden Wymelenberg A, Gaskell J, Mozuch M, Sabat G, Ralph J, Skyba O, Mansfield SD, Blanchette RA, Martinez D, Grigoriev I, Kersten PJ, Cullen D.** 2010. Comparative transcriptome and secretome analysis of wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium*. Appl Environ Microbiol **76**:3599–3610. http://dx.doi.org/10.1128/AEM.00058-10.
29. **Brazma A, Hingamp P, Hingamp P, Quackenbush J, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M.** 2001. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet **29**:365–371. http://dx.doi.org/10.1038/ng1201-365.
30. **Porth I, Klápště J, Skyba O, Lai BSK, Geraldes A, Muchero W, Tuskan GA, Douglas CJ, El-Kassaby YA, Mansfield SD.** 2013. *Populus trichocarpa* cell wall chemistry and ultrastructure trait variation, genetic control and genetic correlations. New Phytol **197**:777–790. http://dx.doi.org/10.1111/nph.12014.
31. **Geraldes A, Pang J, Thiessen N, Cezard T, Moore R, Zhao Y, Tam A, Wang S, Friedmann M, Birol I, Jones SJM, Cronk QCB, Douglas CJ.** 2011. SNP discovery in black cottonwood (*Populus trichocarpa*) by population transcriptome resequencing. Mol Ecol Resour **11**:81–92. http://dx.doi.org/10.1111/j.1755-0998.2010.02960.x.
32. **Hammel KE, Cullen D.** 2008. Role of fungal peroxidases in biological ligninolysis. Curr Opin Plant Biol **11**:349–355. http://dx.doi.org/10.1016/j.pbi.2008.02.003.
33. **Vanden Wymelenberg A, Gaskell J, Mozuch M, Splinter BonDurant S, Sabat G, Ralph J, Skyba O, Mansfield SD, Blanchette RA, Grigoriev IV, Kersten PJ, Cullen D.** 2011. Significant alteration of gene expression in wood decay fungi *Postia placenta* and *Phanerochaete chrysosporium* by plant species. Appl Environ Microbiol **77**:4499–4507. http://dx.doi.org/10.1128/AEM.00508-11.
34. **Vanden Wymelenberg A, Gaskell J, Mozuch M, Kersten P, Sabat G, Martinez D, Cullen D.** 2009. Transcriptome and secretome analyses of *Phanerochaete chrysosporium* reveal complex patterns of gene expression. Appl Environ Microbiol **75**:4058–4068. http://dx.doi.org/10.1128/AEM.00314-09.
35. **Gaskell J, Marty A, Mozuch M, Kersten PJ, Splinter BonDurant S, Sabat G, Azarpira A, Ralph J, Skyba O, Mansfield SD, Blanchette RA, Cullen D.** 2014. Influence of *Populus* genotype on gene expression by the wood decay fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol **80**:5828–5835. http://dx.doi.org/10.1128/AEM.01604-14.
36. **Fernandez-Fueyo E, Ruiz-Dueñas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P, Larrondo LF, James TY, Seelenfreund D, Lobos S, Polanco R, Tello M, Honda Y, Watanabe T, Watanabe T, Ryu JS, Kubicek CP, Schmoll M, Gaskell J, Hammel KE, St John FJ, Vanden Wymelenberg A, Sabat G, Splinter BonDurant S, Syed K, Yadav JS, Doddapaneni H, Subramanian V, Lavín JL, Oguiza JA, Perez G, Pisabarro AG, Ramirez L, Santoyo F, Master E, Coutinho PM, Henrissat B, Lombard V, Magnuson JK, Kües U, Hori C, Igarashi K, Samejima M, Held BW, Barry KW, LaButti KM, Lapidus A, Lindquist EA, Lucas SM, Riley R, Salamov AA, Hoffmeister D, Schwenk D, Hadar Y, Yarden O, de Vries RP, Wiebenga A, Stenlid J, Eastwood D, Grigoriev IV, Berka RM, Blanchette RA, Kersten P, Martinez AT, Vicuna R, Cullen D.** 2012. Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc Natl Acad Sci U S A **109**:5458–5463. http://dx.doi.org/10.1073/pnas.1119912109.
37. **Sato S, Feltus FA, Iyer P, Tien M.** 2009. The first genome-level transcriptome of the wood-degrading fungus *Phanerochaete chrysosporium* grown on red oak. Curr Genet **55**:273–286. http://dx.doi.org/10.1007/s00294-009-0243-0.
38. **Cullen D.** 2013. Wood decay, p 41–62. *In* Martin F (ed), Ecological genomics of fungi. Wiley-Blackwell, New York, NY.
39. **Phillips CM, Beeson WT, Cate JH, Marletta MA.** 2011. Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. ACS Chem Biol **6**:1399–1406. http://dx.doi.org/10.1021/cb200351y.
40. **Bey M, Zhou S, Poidevin L, Henrissat B, Coutinho PM, Berrin J-G, Sigoillot J-C.** 2013. Cello-oligosaccharide oxidation reveals differences between two lytic polysaccharide monooxygenases (family GH61) from *Podospora anserina*. Appl Environ Microbiol **79**:488–496. http://dx.doi.org/10.1128/AEM.02942-12.
41. **Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H, Sørlie M, Eijsink VGH.** 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science **330**:219–222. http://dx.doi.org/10.1126/science.1192231.
42. **Dimarogona M, Topakas E, Christakopoulos P.** 2013. Recalcitrant polysaccharide degradation by novel oxidative biocatalysts. Appl Microbiol Biotechnol **97**:8455–8465. http://dx.doi.org/10.1007/s00253-013-5197-y.
43. **Riley R, Salamov AA, Brown DW, Nagy LG, Floudas D, Held BW, Levasseur A, Lombard V, Morin E, Otillar R, Lindquist EA, Sun H, LaButti KM, Schmutz J, Jabbour D, Luo H, Baker SE, Pisabarro AG, Walton JD, Blanchette RA, Henrissat B, Martin F, Cullen D, Hibbett DS, Grigoriev IV.** 2014. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. Proc Natl Acad Sci U S A **111**:9923–9928. http://dx.doi.org/10.1073/pnas.1400592111.
44. **Bennati-Granier C, Garajova S, Champion C, Grisel S, Haon M, Zhou S, Fanuel F, Ropartz D, Rogniaux H, Gimbert I, Record E, Berrin J-G.** 2015. Substrate specificity and regioselectivity of fungal AA9 lytic polysaccharide monooxygenases secreted by *Podospora anserine*. Biotechnol Biofuels **8**:90. http://dx.doi.org/10.1186/s13068-015-0274-3.
45. **Hjort K, Prest I, Elvang A, Marinell F, Sjoling S.** 2014. Bacterial chitinase with phytopathogen control capacity from suppressive soil revealed by functional metagenomics. Appl Microbiol Biotechnol **98**:2819–2828. http://dx.doi.org/10.1007/s00253-013-5287-x.

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