Time-Dependent Profiles of Transcripts Encoding Lignocellulose-Modifying Enzymes of the White Rot Fungus *Phanerochaete carnosa* Grown on Multiple Wood Substrates

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The abundances of nine transcripts predicted to encode lignocellulose-modifying enzymes were measured over the course of *Phanerochaete carnosa* cultivation on four wood species. Profiles were consistent with sequential decay; transcripts encoding lignin-degrading peroxidases featured a significant substrate-dependent response. The chitin synthase gene was identified as the optimal internal reference gene for transcript quantification.

*P*hanerochaete carnosa is a white rot fungus that has been found growing almost exclusively on coniferous wood (softwood) (3) and so might employ strategies for degradation that could be developed to reduce the recalcitrance of softwood resources to bioprocessing for the production of liquid fuel and other renewable chemicals. For example, previous transcriptome sequencing of *P. carnosa* suggested a principal role for manganese peroxidase (MnP) activity in lignin degradation (9). Moreover, the abundance of transcripts encoding lignin-degrading activity were much higher than those encoding carbohydrate-active enzymes, which is consistent with the selective lignin decay elicited by *P. carnosa* grown on softwood (10) but opposite to previous transcriptomic analyses of *Phanerochaete chrysosporium* grown on hardwood (12, 20). However, the transcriptome study of *P. carnosa* analyzed a single time point, and it is known that enzyme and transcript expression in white-rot fungi growing on lignocellulosic substrates can vary over time (2, 16, 18). Therefore, in the present study, *P. carnosa* was grown on four wood species, and at regular time intervals, reverse-transcription quantitative PCR (RT-qPCR) was used to determine the abundance of transcripts predicted to encode two MnPs, two lignin peroxidases (LiP), a mannanase (Man) from glycoside hydrolase family 5 (GH5; www.cazy.org), a GH6 cellobiohydrolase (Cbh), a GH10 xylanase (Xyl), an acetylxylan esterase (AXE) from carbohydrate esterase family 1 (CE1), and a CE15 glucuronoyl esterase (GE).

P. carnosa strain HHB-10118-sp was grown on 4 g of ground and sifted wood samples prepared from the softwood species balsam fir (*Abies balsamea*), lodgepole pine (*Pinus contorta*), or white spruce (*Picea glauca*) or the hardwood species sugar maple (*Acer saccharum*), each with B3 buffer as previously described (9). Cultures were inoculated with 11-mm circular plugs from the growing edge of *P. carnosa* on solid nutrient medium and then incubated at 27°C under stationary conditions. The central 28 mm of fungal growth was harvested from triplicate cultivations at predefined growth points (GP): 4 days (GP1), a 4-cm colony diameter (GP2), and a 6-cm colony diameter (GP3). Wood species did not significantly affect the incubation time required to reach each GP (1-way analysis of variance [ANOVA]; $P > 0.1$) (see Fig. S1 in the supplemental material).

The RNeasy plant minikit (Qiagen Inc., Mississauga, ON, Canada) was used to isolate total RNA from frozen samples collected at each GP. Reverse transcription was performed using RevertAID H Minus Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas Canada Inc., Burlington, ON, Canada), $T_{25}VN$ primer, and 30 ng of total RNA in a volume of 50 μl, while real-time qPCR was performed in 25 μl with mixtures containing 2 μl of cDNA, SYBR green JumpStart *Taq* ReadyMix (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), and 1 μM gene-specific primers (see Table S1 in the supplemental material). Cycles were run as follows: 1 cycle of 94°C for 2 min and 40 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 30s, and plate read, with the exception of *lip-263501*, *lip-213241*, and *ge-247750* (transcript names are based on protein IDs at http://www.jgi.doe.gov/Pcarnosa), for which the annealing temperature was 63°C. Reaction products were quantified based on plasmid standard curves using the DNA Engine Opticon 2 detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada). Plasmid standards were constructed using primers that amplified the qPCR fragments and flanking cDNA sequence (see Table S2 in the supplemental material). Graphical representations of the data and associated statistical analyses were generated using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA).

Three transcript sequences were tested as internal reference genes for RT-qPCR. Transcripts for actin and GAPDH have traditionally been used as standards in studies of *P. chrysosporium* (6, 14, 17). However, previous transcriptomic analysis of *P. carnosa* suggests that the abundance of these transcripts varies during the growth of this organism on different substrates, and that the level of transcripts encoding a chitin synthase (Chs-257626) is more stable (Fig. 1a) (9). RT-qPCR was therefore used to amplify *P. carnosa* transcripts encoding actin-249164, GAPDH-261268, and Chs-257626. The standard deviation of transcript abundance for each gene product in all RNA

a)

Gene ID	Predicted activity	YMPG	Fir	Pine	Spruce	Maple
249164	actin	1700	570	780	1000	880
261268	GAPDH	4300	820	890	920	1100
257626	chitin synthase	150	150	160	160	160

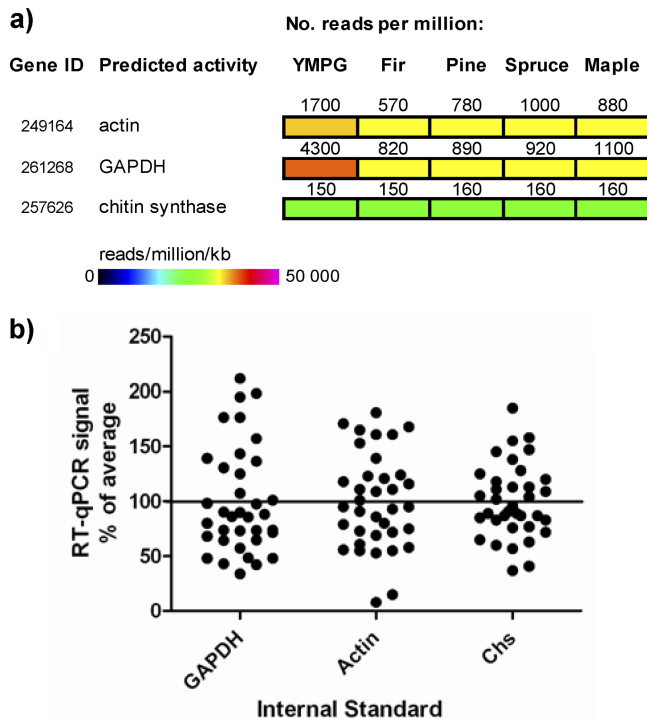No. reads per million:

reads/million/kb
0 — 50 000

b)

FIG 1 Abundance of transcripts encoding chitin synthase (Chs) is less variable than abundance of transcripts encoding actin or GAPDH in *P. carnosa*. (a) Heat map representation of *P. carnosa* transcript abundance during growth on nutrient medium (YMPG), fir, pine, spruce, and maple from mRNA-Seq analysis ($n = 1$) (9). Protein IDs are from the JGI genome portal (http://www.jgi.doe.gov/Pcarnosa). (b) RT-qPCR amplification of transcripts from *P. carnosa* grown on fir, pine, spruce, and maple over 3 time points, plotted as the percentage of the average value for each transcript sequence ($n = 36$). Standard deviations for transcripts encoding Chs, actin, and GAPDH were 33, 43, and 48, respectively.

preparations indicated that the abundance of *chs-257626* transcripts was less variable than that of transcripts encoding GAPDH-261268 or actin-249164 (Fig. 1b).

As determined by absolute quantification, the abundance of

all targeted gene transcripts except for *axe-248451* changed significantly over time ($P < 0.05$; 2-way ANOVA) (Table 1). In contrast, the source of wood fiber did not significantly affect transcript abundances ($P > 0.05$; 2-way ANOVA). Analyses that used *chs-257626* levels as an internal reference did not significantly differ from absolute quantifications, whereas analyses that used *gapdh-261268* or *actin-249164* omitted time as a significant factor for two and five of the eight target transcripts that showed significance under absolute quantification, respectively (Table 1). As is often noted, it is important to test several gene transcripts as internal references to correctly interpret the abundance of queried transcripts by RT-qPCR. In this study, transcript patterns obtained using absolute quantification were consistent with those normalized to transcripts encoding chitin synthase (Table 1; also, see Fig. S2 in the supplemental material).

The particular sequences used to monitor the expression of genes that encode lignocellulose-degrading activities were selected based on their being among the most abundant in *P. carnosa* during growth on wood substrates (9). Manganese peroxidase (MnP) and lignin peroxidase (LiP) are oxidative enzymes that promote the degradation of lignin. In the current study, the abundance of *mnp-256991*, *mnp-262882*, and *lip-263501* transcripts increased from GP1 to GP2 and then decreased at GP3 during growth on each of the wood substrates, whereas the abundance of *lip-213241* remained more similar between GP2 and GP3 (Fig. 2; also, see Data Set S1 in the supplemental material). Overall, the abundance of the *mnp* transcripts was greater than that of the *lip* transcripts at GP2 and GP3. In fact, at GP2 the average abundance of transcript *mnp-256991* during growth on all wood samples combined was significantly higher than that of any of the other target transcripts except for *mnp-262882* (Bonferroni's posttest following repeated-measures ANOVA) (see Table S3 in the supplemental material). At GP2, both *mnp* transcripts had highest abundance in *P. carnosa* grown on spruce, and the transcript abundance of *mnp-262882* was lowest in *P. carnosa* grown on maple (Fig. 2). The difference in *mnp-262882* abundance between growth on spruce and maple at GP2 was determined to be significant by

TABLE 1 Two-way ANOVA to determine the effects of time (growth point) and wood substrate on the abundance of each target transcript in *P. carnosa*

Target gene[a]	P value[b] (with reference gene) for:							
	Time[c]				Substrate[d]			
	None	chs	gapdh	actin	None	chs	gapdh	actin
mnp-256991	**0.0001**	**0.0001**	**0.0011**	**0.0198**	0.0869	0.0862	0.5712	0.4424
mnp-262882	**<0.0001**	**<0.0001**	**0.0029**	**0.0008**	0.2617	0.2594	0.2966	0.1134
lip-263501	**0.0251**	**0.0252**	0.1332	0.2758	0.0797	0.0800	0.1951	0.3114
lip-213241	**0.0445**	**0.0463**	0.0702	**0.0291**	0.8516	0.8373	0.8278	0.6076
man-248589	**0.0119**	**0.0120**	**0.0065**	0.2418	0.1939	0.1944	0.2070	0.4782
xyl-262694	**0.0026**	**0.0025**	**0.0018**	0.2022	0.6209	0.6098	0.9318	0.4926
cbh-264060	**0.0005**	**0.0005**	**0.0003**	0.2467	0.2163	0.2153	0.1453	0.4329
axe-248451	0.0885	0.0888	**0.0143**	0.1628	0.2295	0.2306	0.3021	0.5275
ge-247750	**0.0005**	**0.0005**	**0.0007**	0.2439	0.2445	0.2428	0.2664	0.4227

[a] Numbers correspond to protein IDs used by the JGI genome portal (http://www.jgi.doe.gov/Pcarnosa).
[b] P values are shown for absolute transcript abundances (no reference gene) and for transcript abundances normalized using reference genes (*chs*, *gapdh*, and *actin*). Values of <0.05 are in bold.
[c] Null hypothesis: the values of the means from each of the three time points (GPs), without considering the wood substrates, are the same.
[d] Null hypothesis: the values of the means from each of the four wood substrates, without considering the GP, are the same.
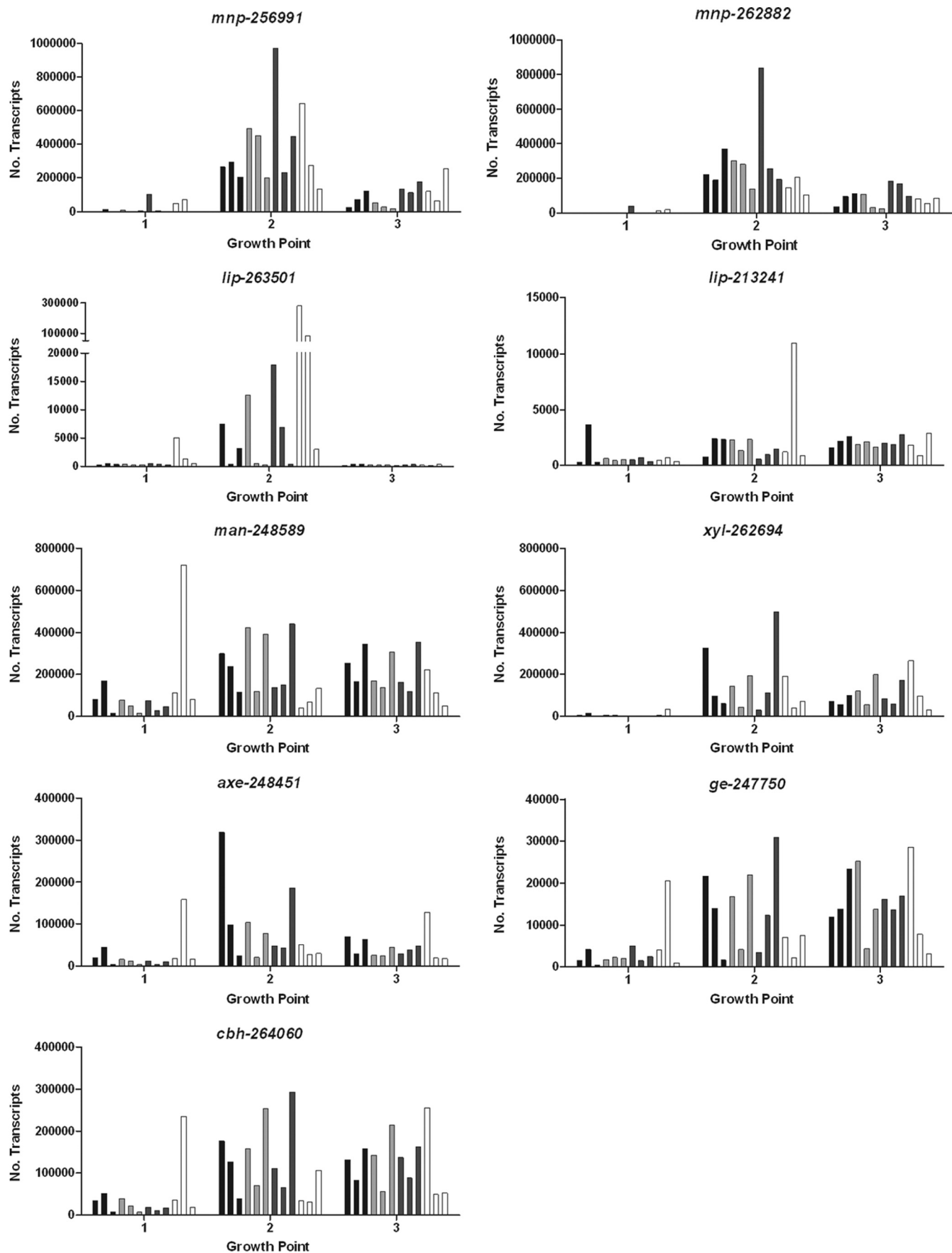
FIG 2 Time-dependent abundance of transcript sequences in triplicate cultivations of *P. carnosa* grown on fir (black), pine (light gray), spruce (dark gray), and maple (white). Absolute transcript abundances are indicated.

Bonferroni's multiple comparison posttest following 2-way ANOVA ($P < 0.05$). In contrast, at GP2 the abundance of *lip-263501* was significantly higher in *P. carnosa* grown on maple than in *P. carnosa* grown on each of the three softwoods ($P < 0.01$). Bonferroni's posttest is a conservative estimate of significance because it reduces the chance of obtaining a higher number of false positives as the number of comparisons is increased (13).

The pattern of *mnp* and *lip* transcript abundances in *P. carnosa* suggests that MnP might be particularly important for the degradation of softwood guaiacyl (G) lignin compared to the guaiacyl-syringyl (GS) lignin of hardwood. Notably, eucalyptus (hardwood) treated with MnP from *Ceriporiopsis subvermispora* shows a greater percent reduction in G than syringyl (S) lignin units (4), whereas *P. chrysosporium*, which produces more *lip* than *mnp* transcripts under ligninolytic conditions (5, 12, 19), depolymerizes natural and synthetic GS lignins more quickly than natural and synthetic G lignins (7, 11).

Mannanases (Man) and xylanases (Xyl) hydrolyze glycosidic bonds that link backbone sugars of the most abundant hemicelluloses in higher plants. In contrast to *mnp* and *lip* transcript abundances, which in most cases peaked at GP2, both *man-248589* and *xyl-262694* retained higher relative abundance through GP2 and GP3 (Fig. 2; also, see Data Set S1 in the supplemental material). When all wood species were considered together at GP3, the abundance of *man-248589* transcripts was significantly higher than the abundance of all other target transcripts, including *xyl-262694* (see Table S3 in the supplemental material). This observation is consistent with *P. carnosa* adaptation to hemicelluloses that predominate in coniferous wood fiber, where glucomannans are 2 to 4 times more abundant than xylan (15). Notably, when maple cultivations were assessed alone, the abundances of *man-248589* and *xyl-262694* transcripts were deemed more similar. This subtle difference in expression of predicted hemicellulases was not statistically significant but is consistent with the higher xylan content in the maple cultivations.

Cellobiohydrolases (Cbh) degrade cellulose by releasing cellobiose from either the reducing or nonreducing end of cellulose molecules. Overall, the abundance of the *cbh-264060* transcript increased from GP1 to GP2 and remained at similar levels from GP2 to GP3.

Acetylxylan esterase (AXE) and glucuronoyl esterase (GE) catalyze the hydrolysis of acetyl groups from acetylated hemicelluloses and ester linkages between hydroxyl groups of lignin and glucuronic acid residues of glucuronoxylans, respectively. Given the partial substitution of the backbone sugars, the levels of transcripts encoding AXE and GE were expected to be lower than those encoding the main-chain glycoside hydrolases. Indeed, at GP2 and GP3, the levels of *axe-248451* and *ge-247750* transcripts were lower than those of transcripts encoding mannanase and xylanase activities (Fig. 2; also, see Data Set S1 in the supplemental material). Similar to *man-248589* and *xyl-262694*, *axe-248451* and *ge-247750* had higher relative abundances at GP2 and GP3.

The higher abundance of transcripts encoding MnPs than carbohydrate-active enzymes at GP2, and the prolonged abundance of transcripts encoding carbohydrate-active enzymes through to GP3, is consistent with a sequential mode of decay where lignin is degraded to some extent before the carbohydrate components (1). Indeed, Fourier transform infrared spectroscopy (FT-IR) analysis of the wood substrates used in this study con-firmed that lignin degradation most clearly distinguished untreated and fungal treated wood fiber (10).

In summary, transcript profiles revealed comparatively high abundances of transcripts encoding MnP and LiP at early stages of cultivation followed by comparatively high abundances of carbohydrate-active enzyme transcripts at later stages of cultivation. Substrate-dependent patterns of transcript abundance were most evident for transcripts encoding lignin-degrading activity. The relative abundance of transcripts encoding hemicellulase activity at GP3 was mannanase > xylanase > glucuronoyl esterase, which reflects the typical abundance and composition of softwood hemicellulose. However, the *P. carnosa* genome contains at least two mannanase- and four xylanase-encoding genes (http://www.jgi.doe.gov/Pcarnosa), and only one of each was analyzed in the current study. Finally, an analysis of three internal standards for RT-qPCR revealed that transcript patterns obtained using absolute quantification were consistent with those normalized to transcripts encoding chitin synthase.

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