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Elucidation of aromatic catabolic pathways in white-rot fungi

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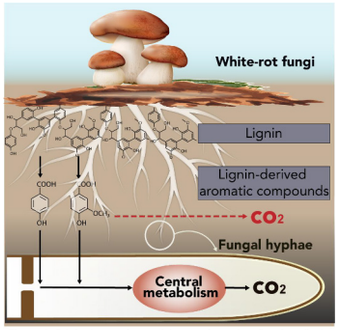

Figure 1. Brief scheme of potential routes for carbon flow from lignin to CO_2 during lignin decay by white-rot fungi.

Project Goals

This project aims to investigate the hypothesis that white-rot fungi can simultaneously depolymerize lignin extracellularly and catabolize depolymerization products intracellularly as carbon and energy sources (**Figure 1**). Evaluating this hypothesis will provide deeper understanding of the role of white-rot fungi in facilitating carbon sequestration in Nature. Additionally, identifying the most promising fungal strains for lignin turnover and catabolism will catalyze future efforts in genetic tool development to enable metabolic engineering in white-rot fungi for lignin bioconversion to bioproducts.

Background

Lignin is the second most abundant plant-based biopolymer on Earth and represents up to 40% of the energy density of lignocellulosic biomass. Even though lignin is a massive natural carbon and energy reservoir, only a small group of basidiomycete fungi, namely white-rot fungi (WRF), have evolved the ability to efficiently depolymerize and mineralize lignin to CO_2 and H_2O. Considerable research efforts have been undertaken to understand how WRF depolymerize lignin but the biochemical reactions that convert lignin into CO_2 have been largely neglected. In fact, it is unclear if WRF intracellularly catabolize lignin-derived aromatic compounds to utilize them as a carbon and energy source, or rather if lignin is depolymerized and mineralized extracellularly merely to facilitate access to cellulose and hemicellulose for use as a primary carbon source.

Results

To date, we have employed ^{13}C-isotope labeling, systems biology approaches, and *in vitro* enzyme assays to definitively demonstrate that two WRF, *Trametes versicolor* and *Gelatoporia (Ceriporiopsis) subvermispora*, funnel carbon from lignin-derived aromatic compounds into central carbon metabolism via intracellular catabolic pathways [1]. Specifically, ^{13}C-isotopic labeling approaches showed that these WRF utilize **lignin-derived aromatic compounds from poplar** (i.e. 4-hydroxybenzoic acid (4-HBA)) as a carbon source (**Figure 2**). *In silico* genome analysis led us to hypothesize a complete catabolic pathway for 4-HBA and identify multiple homologous sequences for enzymes with putative oxidative decarboxylase, hydroxylase, and ring-opening dioxygenase activities, which are among the main biochemical reactions acting on aromatic compounds. Spatial and differential metabolomic (**Figure 3**) analyses supported the proposed catabolic pathways and showed alternative catabolic steps in *T. versicolor* that were not present in *G. subvermispora*. Further, based on differential proteomics and transcriptomics results (**Figure 4**), we down-selected enzymes for further *in vitro* characterization, and we have assigned a function to six fungal enzymes (including oxidative decarboxylases, hydroxylases, and ring-opening dioxygenases) (**Figure 5**).

^{13}C-isotopic labeling approaches show that 4-HBA is utilized as a carbon source by *T. versicolor* and *G. subvermispora*

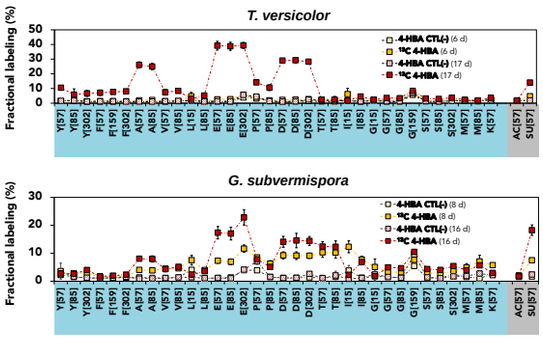

Figure 2. ^{13}C-labeling experiments demonstrate carbon flux from 4-hydroxybenzoic acid to proteinogenic amino acids in *T. versicolor* and *G. subvermispora*, which indicates that carbon is going through central carbon metabolism. The graphs show the fractional labeling (%) in intracellular proteinogenic amino acid fragments and other metabolites (acetate (AC) and succinate (SU)) in *T. versicolor* and *G. subvermispora* cultivations, when providing unlabeled 4-HBA (negative control, CTL(-)) and ^{13}C-ring-labeled 4-HBA at two different time points. Individual points are connected with discontinuous lines to facilitate visualization. All results are the average of biological triplicates and error bars represent the standard deviation.

Differential NMR metabolomic analyses provide clues on key metabolic intermediates for the conversion of 4-HBA towards central metabolism

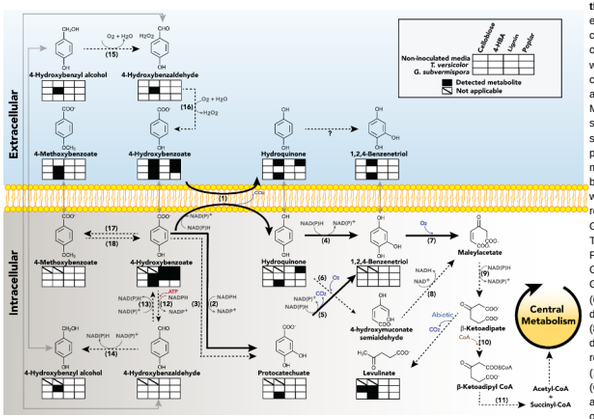

Figure 3. Proposed metabolic pathway in *T. versicolor* and *G. subvermispora* for the conversion of 4-HBA. Intracellular and extracellular metabolites detected in cellobiose, 4-HBA, lignin, and poplar-containing cultivations. Media not inoculated with fungi (non-inoculated) was also used as control for extracellular metabolomic analyses and intracellular when applicable. Molecules without boxes next to the structure do not have commercially available standards. Continuous grey arrows indicate potential transport through the cell membrane. Continuous and discontinuous black lines correspond to validated (in this work) and proposed enzymatic steps, respectively: (1) oxidative decarboxylases GS_120062, GS_90429, (2) hydroxylase TV_58730, (3) hydroxylation by cytochrome P450, (4) hydroxylases TV_58730 and GS_82057, (5) oxidative decarboxylases GS_120062 and TV_32834 and GS_90429, (6) dioxygenase, (7) ring-cleaving dioxygenases TV_28066 and GS_116134, (8) 4-hydroxymuconic semialdehyde dehydrogenase, (9) maleylacetate reductase, (10) ketoacid CoA transferase, (11) thiolase, (12) carboxylic acid reductase (CARs), (13) aldehyde dehydrogenase, (14) alcohol dehydrogenase, (15) alcohol oxidase, (16) aldehyde oxidase, (17) 4-O-methyl transferase, (18) demethylase.

Proteomics and transcriptomics enabled down-selection of putative enzymes for 4-HBA conversion

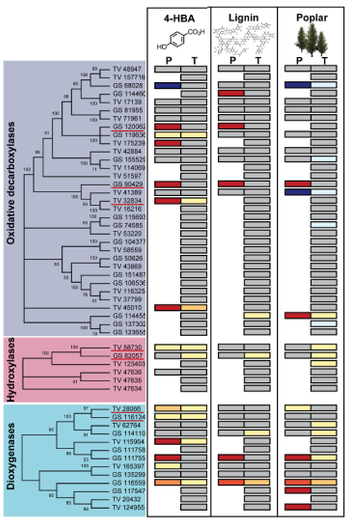

Figure 4. Proteomic and transcriptomic analyses. Phylogenetic relationships with putative oxidative decarboxylases, hydroxylases, and dioxygenases selected from *in silico* analyses in *T. versicolor* (TV) and *G. subvermispora* (GS). The heat map shows proteomic (P) and transcriptomic (T) results for protein expression and gene regulation levels, respectively, in each growth media compared to the inoculated control (cellobiose-containing media) from biological triplicates. NS = non-significant differential expression compared to the control; U = unique.

In vitro enzyme assays validated several of the down-selected oxidative decarboxylases, hydroxylases, and dioxygenases

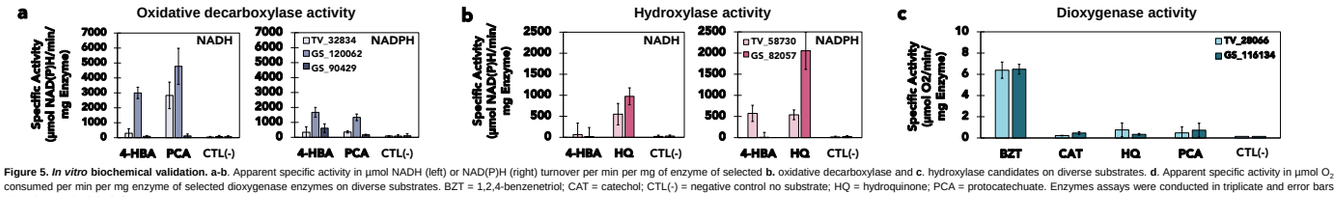

Figure 5. ***In vitro*** **biochemical validation. a-b**. Apparent specific activity in μmol NADH (left) or NAD(P)H (right) turnover per min per mg of enzyme of selected **b.** oxidative decarboxylase and **c**. hydroxylase candidates on diverse substrates. **d**. Apparent specific activity in μmol O_2 consumed per min per mg enzyme of selected dioxygenase enzymes on diverse substrates. BZT = 1,2,4-benzenetriol; CAT = catechol; CTL(-) = negative control no substrate; HQ = hydroquinone; PCA = protocatechuate. Enzymes assays were conducted in triplicate and error bars show the standard deviation.

Future work and conclusions

Going forward, further characterization of the selected and additional aromatic catabolic enzyme candidates will be a high priority for continued studies to validate different enzymatic steps. Additionally, WRF performance warrants further examination in modeled environmental conditions (e.g., solid-state cultivations instead of submerged cultivations) to better understand regulatory processes and rates for simultaneous lignin degradation and catabolism. Overall, the findings from this study imply that annotation, analysis, and inclusion of aromatic catabolic pathways in genomics and systems biology studies of lignin-degrading WRF is a worthwhile pursuit and forms the foundation of a new research area based on lignin catabolism by WRF, which could be further exploited to convert the undervalued biopolymer lignin into value-added compounds.

Reference

[1] Carlos del Cerro, Erika Erickson, Tao Dong, Allison R. Wong, Elizabeth K. Eder, Samuel O. Purvine, Hugh D. Mitchell, Karl K. Weitz, Lye Meng Markillie, Meagan C. Burnet, David W. Hoyt, Rosalie K. Chu, Jan-Fang Cheng, Kelsey J. Ramirez, Rui Katahira, Wei Xiong, Michael E. Himmel, Venkataramanan Subramanian, Jeffrey G. Linger, and Davinia Salvachúa. Intracellular pathways for lignin catabolism in white-rot fungi. ***PNAS***, in press. **2021**.

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