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Gene Expression Analysis of Copper Tolerance and Wood Decay in the Brown Rot Fungus *Fibroporia radiculosa*

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High-throughput transcriptomics was used to identify *Fibroporia radiculosa* genes that were differentially regulated during colonization of wood treated with a copper-based preservative. The transcriptome was profiled at two time points while the fungus was growing on wood treated with micronized copper quat (MCQ). A total of 917 transcripts were differentially expressed. Fifty-eight of these genes were more highly expressed when the MCQ was protecting the wood from strength loss and had putative functions related to oxalate production/degradation, laccase activity, quinone biosynthesis, pectin degradation, ATP production, cytochrome P450 activity, signal transduction, and transcriptional regulation. Sixty-one genes were more highly expressed when the MCQ lost its effectiveness (>50% strength loss) and had functions related to oxalate degradation; cytochrome P450 activity; H₂O₂ production and degradation; degradation of cellulose, hemicellulose, and pectin; hexose transport; membrane glycerophospholipid metabolism; and cell wall chemistry. Ten of these differentially regulated genes were quantified by reverse transcriptase PCR for a more in-depth study (4 time points on wood with or without MCQ treatment). Our results showed that MCQ induced higher than normal levels of expression for four genes (putative annotations for isocitrate lyase, glyoxylate dehydrogenase, laccase, and oxalate decarboxylase 1), while four other genes (putative annotations for oxalate decarboxylase 2, aryl alcohol oxidase, glycoside hydrolase 5, and glycoside hydrolase 10) were repressed. The significance of these results is that we have identified several genes that appear to be coregulated, with putative functions related to copper tolerance and/or wood decay.

Copper has a long history of use for preventing fungal infestations. Its general efficacy and relatively low toxicity continue to be critical for the wood protection industry, where it is the principal active ingredient for controlling wood decay fungi (1). Several species of brown rot fungi, however, exhibit extremely high levels of tolerance for copper when exposed to copper sulfate (2) or copper-based preservatives in laboratory assays (3–6). These tolerant species include *Fibroporia* (= *Antrodia*) *vaillantii*, *Fibroporia radiculosa*, *Fomitopsis* (= *Tyromyces*) *palustris*, *Laetiporus sulphureus*, *Meruliporia incrassata*, *Postia* (= *Poria*) *placenta*, *Serpula lacrymans*, and *Wolfiporia cocos*.

Oxalic acid is a common metabolite of wood decay fungi (7) that has been implicated in metal tolerance (8). In the presence of high concentrations of zinc, calcium, cobalt, or copper, many species of fungi transform the metal into a metal-oxalate crystal (9). When *W. cocos* was grown on wood treated with ammoniacal copper citrate (CC), an accumulation of insoluble copper oxalate crystals was observed (4). Copper from different preservative formulations can also induce an increase in oxalic acid production (5, 10). In a time course analysis, 11 brown rot fungi were denoted copper tolerant and 4 copper sensitive based on their abilities to degrade CC-treated wood (5). After 2 weeks, 2 to 17 times more oxalic acid was present in treated blocks than untreated controls for the copper-tolerant fungi, while the copper-sensitive fungi produced low levels of oxalic acid regardless of block treatment (5). A similar trend was observed for copper-tolerant species tested against other copper-based preservatives, like ammoniacal copper quat types B and D and chromated copper arsenate type C (10). In all cases, the fungal bioprocess for copper tolerance seemed to involve immobilization of the toxic metal in the form of an insoluble oxalate crystal, effectively reducing its bioavailability.

Oxalate, however, exhibits a complex binding dynamic with

metal. It can bind iron irreversibly or reversibly, depending upon the pH and the oxalate/metal ratio (11, 12). During brown rot decay, the fungal bioprocess may also involve oxalate, but its role may be not to precipitate iron, but rather to increase iron bioavailability. Increased bioavailability means that Fe³⁺ can participate in redox reactions, be transported through the lignocellulose matrix, and be transferred to new binding sites (12). In this capacity, oxalate is believed to act as one of several low-molecular-weight mediators that contribute to the production of hydroxyl free radicals by the Fenton reaction, which has been proposed as the oxidative mechanism that brown rot fungi use to initiate decay (Fe²⁺ + H₂O₂ → Fe³⁺ + ·OH + OH[−]) (13, 14). Genes that regulate oxalate metabolism, though, are unknown.

Other potential mediators of the Fenton reaction include hydroquinones (11, 15–17), phenolate chelators (18, 19), and a low-molecular-weight peptide isolated from *Gloeophyllum trabeum* (20, 21). Many of these mediators have been detected in *G. trabeum* and have the ability to reduce Fe³⁺ to Fe²⁺. Sources of H₂O₂ have also been identified. H₂O₂ could be generated from within the Fenton system itself (11, 16, 17) or enzymatically from various

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flavin adenine dinucleotide (FAD)-binding oxidases, e.g., alcohol oxidase, methanol oxidase, and pyranose-2-oxidase (22–27) or copper radical oxidase (24, 25, 28). However, strong temporal correlations between periods of Fenton activity and H_2O_2 -producing oxidases have yet to be demonstrated.

Laccase, which is a polyphenol oxidase, appears to be another component of the Fenton system for high-oxalate-producing brown rot fungi (29). The ability of hydroquinones to bind and reduce iron directly occurs only in the absence of oxalate or when oxalate concentrations are low (11, 12, 15, 16). In the presence of higher oxalate concentrations, hydroquinones cannot reduce iron (11, 12, 29) unless laccase is present. Laccase acts on the hydroquinone substrate, performing a one-electron oxidation to form the corresponding semiquinone. The latter can reduce iron directly in the presence of high oxalate or indirectly by reducing O_2 to produce a perhydroxyl radical (29). Laccase-produced semiquinones were shown to reduce iron during wood decay by *P. placenta* and were present during decay initiation when both oxalate and methoxyhydroquinone concentrations were high (29).

Descriptions of brown rot decay demonstrate that it is a highly complex continuum of changing fungal bioprocesses that produce chemical, physical, and structural changes to the wood (30). The fungal hyphae ramify through the lumina of the wood cells (31, 32), causing notable changes to the wood with respect to pH, wood strength, wood cell wall pore size, and selective disappearance of the S2 layer of the wood secondary cell walls. The reduction in pH to <4.0 has been attributed to increases in oxalate secretion (33–35), which leads to an accumulation of calcium oxalate crystals (36). The rapid loss of wood mechanical strength is pervasive, occurs before weight loss (29, 37), and has been related to loss of birefringence of the wood cell wall (38, 39).

The consensus is that the causative agents of these physical changes to the wood cell wall are due to oxidative attack by hydroxyl free radicals. Exposure of wood or cellulose to artificial Fenton systems produces fragmentation and solubilization of cellulose and hemicellulose, as well as oxidative modification of lignin (13, 40–42). Because enzymes are too large to penetrate the cell wall (43), the selective disappearance of the S2 layer of the wood secondary cell wall (32, 39, 44) suggests that the low-molecular-weight mediators involved in the Fenton reaction are the only molecules small enough to diffuse through the S3 and reach the S2 layer. The net effect of oxidative attack is an increase in pore size of the wood cell walls (43, 45). This sets the stage for the ensuing hydrolytic attack because it gives the extracellular hemicellulases and cellulases direct access to their respective substrates. These hydrolases, which are diverse in function and specificity, cleave the fragmented polysaccharides down to their composite sugars (46, 47). As the sugars are depleted, the percent weight loss increases (37), and the wood develops a dark-brown, checked appearance, which is the stable residue of modified lignin that is left behind by the brown rot fungus (30).

The objective of this study was to improve our understanding of copper tolerance and wood decay by analyzing the gene expression of *F. radiculosa* while the fungus was growing on wood treated with micronized copper quat (MCQ). We used high-throughput RNA sequencing (RNA-Seq) as our initial discovery tool, as there are more than 9,000 genes in the genome of *F. radiculosa*, many of which are members of large gene families with the same functional annotation (48). We then selected 10 of the differentially regulated genes and examined their expression by quantitative reverse

transcriptase PCR (qRT-PCR) at 4 time points while the fungus was growing on wood in the presence or absence of MCQ. The putative annotations of the genes analyzed were isocitrate lyase (ICL) and glyoxylate dehydrogenase (GDH), with possible roles in oxalate production; two oxalate decarboxylases (ODC1 and ODC2), with possible roles in oxalate degradation; laccase (LCC), with a possible role as a mediator of decay initiation; glycoside hydrolase families 5 (GH5) and 10 (GH10), with possible roles in cellulose and hemicellulose degradation, respectively; aryl alcohol oxidase (AAOX) and catalase (CAT), with possible roles in hydrogen peroxide production and degradation, respectively; and copper resistance P-type ATPase pump (COP), with a possible role in copper homeostasis. Other observations made during the decay test included wood compression strength loss, type of crystal formed, and fungal phenotype.

MATERIALS AND METHODS

Fungus. *F. radiculosa* strain TFFH 294 was generously donated by Carol Clausen, U.S. Department of Agriculture (USDA) Forest Service, Forest Products Laboratory, Madison, WI. It was isolated in 1971 from wooden stakes treated with chromated copper arsenate and acid copper chromate that showed premature failure in field tests done at the Harrison National Experimental Forest, Saucier, MS (49).

Decay tests. The accelerated soil block test was set up according to American Wood Protection Association Standard E22-09 (50), with minor modifications. Southern yellow pine sapwood wafers (54 by 18 by 5 mm) were pressure treated with 0.8% (wt/wt) MCQ to an average retention ($\pm$ standard deviation [SD]) of 5.47 ± 0.12 kg MCQ/m³. The MCQ was a 2:1 (wt/wt) formulation of copper carbonate ($CuCO_3$)-dimethyldidecyl ammonium carbonate (DDAC). The percentages of the active ingredient in the stock solutions were 41 and 47%, respectively. The test containers held two sapwood feeder strips that were in contact with a bed of moist, field-collected soil. The feeder strips were inoculated with the fungus (6 5-mm-diameter plugs per container) and allowed to grow at 28°C until the strip was fully colonized (about 2 weeks). Two wafers (one treated and one untreated) were put into each test container so that they were 2 cm apart. The soil and materials for the decay tests were autoclaved prior to use. Untreated wafers were removed at days 4, 6, 8, 10, and 12. Treated wafers were removed at days 25, 31, 46, 70, and 154. Exposure times were different because decay progressed more slowly on the MCQ-treated wood. One end of the wafer was rasped (0.2 g rasped wood per tube; 5 tubes per sample), flash frozen in liquid N_2 , and stored at $-80^\circ C$ for RNA extraction. The other end (18 by 18 by 5 mm) was used for compression strength testing and saved for microscopic observations.

Wood compression strength. Compression strength was determined using a custom built apparatus that was capable of measuring the maximum load required to reach 5% compression across the radial surface of the wood wafer (5 wafer replicates per treatment and time combination). The percent compression strength loss was calculated relative to unexposed (treated and untreated) wafers cut from the same billet. For southern yellow pine, compression strength loss typically reaches a plateau at 60 to 70% strength loss.

RNA extraction. Each RNA sample was isolated from one test wafer, and RNA was isolated from all time points except untreated day 4, which did not appear to have enough hyphae. Extractions were performed following the Ambion RNaqueous Kit instructions (Ambion, Austin, TX). After addition of the lysis buffer (1 ml/0.2 g wood), RNA was released from the sample by beating it twice (3 min with a 3-min rest on ice) in a Minibeadbeater 16 (Bio Spec Products, Bartlesville, OK). The sample aliquots were recombined on a silica gel spin column after removing the wood particles by centrifugation. DNase I digestion was done on the silica gel spin column (51). Spectrophotometric determination (Nanodrop 1000; Thermo Fisher Scientific, Pittsburgh, PA) showed that RNA yields ranged from 2.2 to 18.3 $\mu g/g$ rasped wood, with longer growth periods

yielding more RNA. The quality of RNA was examined by Experion chip electrophoresis (RNA StdSens Analysis Kit; Bio-Rad, Hercules, CA). A high-molecular-weight band of genomic DNA was not detected, and only minimal RNA degradation was evident.

RNA-Seq. An RNA-Seq library was prepared from each RNA sample taken at days 31 (3 wafer replicates) and 154 (4 wafer replicates), when the wood showed 0 and 52% compression strength loss, respectively. RNA (5 µg) was used to generate libraries according to the protocol provided with the Illumina mRNA-Seq Sample Prep Kit (Illumina, San Diego, CA). Short-read sequencing was done on one single-end flow cell run on an Illumina GAIIX instrument (76-nucleotide [nt] read length; 1 library per lane). Sequence data were processed using Firecrest (image analysis) and Bustard (base-calling) as part of the Illumina GA Pipeline v1.4.0. The data files were converted to FASTQ format and then trimmed and filtered to remove poor-quality sequences (bases with “B” quality scores were trimmed, reads that had <38 bases left after trimming were discarded, and any read that began with an “N” was discarded). After filtering, the number of reads per sample ranged from 28 to 33 million.

The Bowtie alignment tool v0.12.7 (52) was used to map the filtered data set against the predicted CDS (coding-region sequence) generated from our genomics analysis of *F. radiculosa* (9,262 genes, of which 5,407 had an assigned gene ontology [GO] and 1,037 had no gene product match against the National Center for Biotechnology Information [NCBI] nr database) (48). The Bowtie options were set to output unique alignments with a maximum of two mismatches allowed in the first 28 bases of the read. The used reads (61 to 64%) aligned to only one location in the reference CDS database. The unused reads had more than one alignment to the CDS (0.14 to 0.27%) or failed to align (36 to 39%). Tallies of the alignments showed that 8,801 genes had digital count data for at least one of the RNA samples.

Differential gene expression of the Bowtie count data was determined using the edgeR package (53, 54) in Bioconductor. Genes that had count sums (<10) across all samples were removed. Composition normalization was performed to adjust for differences in library size and composition of the RNA being sampled. The absence of outlier replicates was confirmed by a multidimensional scaling plot. MA plots of each pairwise sample combination verified successful normalization (M is the log of the concentration ratio, A is the average of the two log concentrations, and concentration is an estimate of the transcript concentration in the sample). edgeR estimates a common dispersion parameter using a quantile-to-quantile method for a negative binomial distribution to compensate for small and unequal sample sizes. Differentially expressed genes were identified by the exactTest function with correction for multiple-hypothesis testing (false-discovery rate [FDR] $\leq 1E-4$).

A differential GO analysis (Blast2GO suite v 2.4.9) (55) was done to determine if there were significant differences in the GO term representation among the 917 differentially expressed genes. The enrichment analysis used Fisher's exact test with correction for multiple-hypothesis testing (FDR < 0.05) (56). The importance of a metabolic process, molecular function, or cellular component for copper tolerance and wood decay was substantiated if we detected multiple genes with similar fold change directions and if the predicted gene products had related functions and localization motifs. We also searched for related genes that may not have been detected by the GO analysis but were present in the edgeR analysis.

In this paper, we refer to genes by their locus tag numbers. The corresponding accession numbers for the predicted proteins of the differentially regulated genes are found in Table S2 in the supplemental material. Table S2 also lists the proteins from other species (accession numbers and associated similarity statistics) that had the highest sequence similarity to the proteins predicted by the *F. radiculosa* genes. The target motifs of the predicted proteins were determined by the genomic analysis, and all annotations at this point are putative (48).

qRT-PCR. Ten of the differentially regulated genes, which had putative functions involved in copper tolerance and wood decay, as well as exhibiting high fold changes, were selected from the RNA-Seq results for

qRT-PCR analysis. Forward and reverse primers were designed to target the gene-specific transcripts and our internal reference, 18S rRNA (see Table S1 in the supplemental material). For the transcripts, one primer of each pair spanned an exon-exon boundary, and the region between the primers crossed at least one intron of >50 bp. Amplicon lengths ranged from 182 to 207 bp for the transcripts and the length was 152 bp for 18S rRNA. A preliminary analysis was performed by electrophoresing the RT-PCR products from representative samples and negative controls (no-template and reverse-transcriptase-minus controls) on a 1.5% agarose gel in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.6). Target bands were absent from controls and present in samples, and bands from genomic DNA amplification were not conspicuous. The primer specificity was confirmed by sequencing the amplicon of RT-PCR. The target band was gel purified, the product was cloned into the pGEM T-easy vector (Promega, Madison, WI), and the amplicon sequence was confirmed by sequencing at least three individual recombinant colonies using a Beckman Coulter (Brea, CA) CEQ8000 DNA sequencer.

First-strand cDNA was produced from RNA (0.5 µg per 20-µl reaction mixture) using random hexadeoxynucleotide primers according to the protocol provided with SuperScript II reverse transcriptase (Promega, Madison, WI). qRT-PCR was performed on an iQ5 thermal cycler (Bio-Rad, Hercules, CA) using the Bio-Rad SYBR green Supermix kit and instructions. The PCR mixture (20 µl) contained 0.2 µM (each) forward and reverse primers and 1 µl of cDNA. The cDNA was diluted 20× for amplification of 18S rRNA; diluted 4× for genes 415, 1257, and 7157 (annotations, ICL, GDH, and ODC1, respectively); and undiluted for genes 4739, 6399, 2726, 509, 8299, 6682, and 1430 (annotations, LCC, ODC2, GH10, GH5, AAOX, CAT, and COP). The thermal program was 95°C for 3 min, 35 cycles of three steps (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s), and a final extension of 72°C for 3 min and ended with a melt curve program, 0.5°C increase every 15 s from 60°C to 95°C. Conditions 1 to 4 corresponded to MCQ days 25, 46, 70, and 154, and conditions 5 to 8 referred to untreated days 6, 8, 10, and 12, respectively. Each plate contained one biological replicate of all eight conditions tested against the internal reference gene and 2 target genes plus a no-template control (3 technical replicates per sample). A total of four biological replicates were run per condition. Primer specificity and the formation of primer dimers were verified by analyzing the melt curve of each reaction.

The relative expression values were exported from the iQ5 software (v2.1.97.1001). The calculations were based on the $\Delta\Delta C_T$ method (57), assuming 100% amplification efficiency for all genes. If a gene did not reach a C_T value within 35 cycles of amplification, then the C_T was set to 35. Expression was calculated relative to condition 5 (untreated day 6) or condition 1 (MCQ day 25) for genes with annotations for ICL, GDH, LCC, ODC1, CAT, and COP or GH10, GH5, AAOX, and ODC2, respectively. All expression values were normalized against 18S rRNA. Significant differences in relative expression values were determined using non-parametric statistical methods, because neither the data nor transformed data were normally distributed. The SAS procedure NPAR1WAY was used to test for significant differences between the two treatments (MCQ and untreated) in a 2-sample Wilcoxon test (58).

Microscopy. MCQ-treated (exposed to fungus for 31 and 154 days), untreated (exposed to fungus for 6 and 12 days), and unexposed control wafers were retained for microscopic analysis. The wafers were mounted on aluminum stubs with carbon paste. A conductive coating (~11 nm thick) was applied to the mounted samples using a Polaron SC7640 high-resolution scanning electron microscope (SEM) sputter coater with a gold/palladium target. The coated wafers were viewed with a Zeiss EVO50VP variable-pressure SEM attached to a Bruker Quantax 200 XFlash energy-dispersive X-ray (EDX) spectrometer. Identification of the metal component of the crystal was determined by spectral-point analysis with a JEOL JSM-6500F field emission SEM linked to an INCA X-sight EDX spectrometer from Oxford.

Accession numbers. Files generated from the RNA-Seq analysis (count files and SCARF files) were deposited in the NCBI Gene Expression

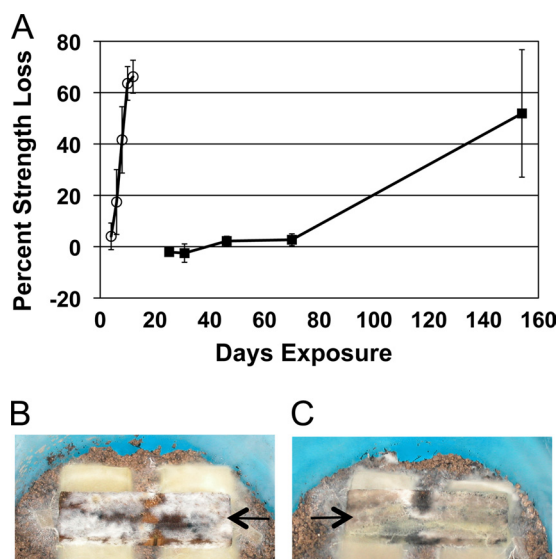


FIG 1 (A) Percent compression strength loss ($\pm$ SD) of untreated (open circles) and MCQ-treated (filled squares) wafers after exposure to *F. radiculosa*. (B and C) Phenotypes of the fungus growing on MCQ-treated wafers before (B) and after (C) the wood showed high strength loss (day 31 and day 154, respectively). The arrows point to the test wafer, which is resting on the feeder strips.

Omnibus under accession number [GSE35333](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35333) (release date, 26 November 2012). The genome and the predicted gene sequences have also been deposited in NCBI (accession numbers [PRJEA72357](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=PRJEA72357) and [CCL98008](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=CCL98008) to [CCM07269](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=CCM07269), respectively).

RESULTS

Wood compression strength and fungal morphology. During the first 70 days of exposure to fungus, wood wafers treated with MCQ showed no appreciable loss of compression strength, indicating that the MCQ was still protecting the wood (Fig. 1A). The mycelia during this period had white, highly branched hyphae that exhibited a dense, radial growth pattern (Fig. 1B). After 154 days of exposure, the treated wafers showed 52% loss of compression strength, indicating that the MCQ was no longer effective (Fig. 1A). The hyphae became yellow and less branched and displayed a more diffuse, linear growth pattern (Fig. 1C). Untreated wafers showed a rapid, linear increase in strength loss, quickly reaching plateau values of 60 to 70% at 10 days of exposure. The fungi growing on untreated wood for 12 days resembled the hyphae growing on MCQ-treated wood once the MCQ had lost its effectiveness but were more yellow, similar to the hyphae growing on the untreated feeder strips (Fig. 1B).

RNA-Seq. A plot of log fold change (logFC) versus log concentration showed the distribution of the 8,801 transcripts being compared before and after the MCQ lost its effectiveness, at days 31 and 154, respectively (Fig. 2). There were 917 genes that showed significant differences in their expression levels (Fig. 2, red points; $FDR = 1E-4$). Among these, 463 genes were expressed more at day 31 (negative logFC values; average, 4.3 $\times$; maximum, 58 $\times$; minimum, 2.3 $\times$). The remaining 454 genes were expressed more at day 154 (positive logFC values; average, 5.7 $\times$; maximum, 62 $\times$; minimum, 2.4 $\times$). Table S2 in the supplemental material lists the locus tags, accession numbers, annotations, nucleotide sequences, and associated statistics for the 917 differentially expressed genes.

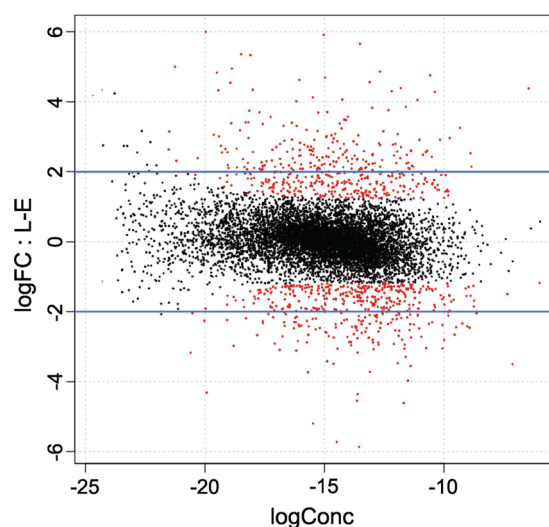


FIG 2 Plot of log fold change (logFC) versus log concentration (logConc) for the RNA-Seq analysis. Genes that were more highly expressed before (E, early, day 31) and after (L, late, day 154) the MCQ-treated wood showed high strength loss had negative and positive logFC values, respectively. Points above and below the blue lines were transcripts with greater than 4-fold changes. Red, significant ($FDR < 1E-4$); black, not significant; orange, zero counts artificially represented at the left edge of the graph.

Bar charts of the differential GO term distribution are shown in Fig. 3. For molecular function (Fig. 3A), there were significantly fewer genes with the term hydrolase activity at day 31 (early; 14%) than at day 154 (late; 27%) ($FDR = 0.002$). The term binding, however, had greater representation among the genes at day 31 (62%) than at day 154 (48%) ($FDR = 0.03$). For the biological process (Fig. 3B), the term lipid metabolic process was less abundant among the genes at day 31 (1%) than at day 154 (7%) ($FDR = 0.02$). The term cellular metabolic process, on the other hand, was observed more often among the genes at day 31 (14%) than at day 154 (6%) ($FDR = 0.04$). For the cellular component (Fig. 3C), there were significantly more genes with the term intracellular membrane-bounded organelle at day 31 (12%) than at day 154 (4%) ($FDR = 0.004$). The term cytoplasmic part was also more common at day 31 (9%) than at day 154 (3%) ($FDR = 0.04$). The genes detected by the GO analysis and their associated annotations and statistics are listed in Table S3 in the supplemental material.

Genes showing differential regulation on MCQ-treated wood. Table S4 in the supplemental material lists the genes that showed significant differences in expression between days 31 and 154. Eight of these genes ([locus tag, annotation] 415, ICL; 1257, GDH; 6889, citrate synthase; 5088, succinate/fumarate antiporter; 7902, aconitate hydratase; 4245, 2-oxoglutarate dehydrogenase e1; 7157, ODC1; and 6399, ODC2) had annotations related to oxalate metabolism. A proposed mechanism of genetic regulation of oxalate metabolism was obtained by mapping these genes onto established pathways (59, 60) (Fig. 4). Six of the genes showed increased expression at day 31 and had annotations related to oxalate biosynthesis. The annotations and fold changes in gene expression were as follows: citrate synthase, 4.3 $\times$; aconitate hydratase, 2.3 $\times$; 2-oxoglutarate dehydrogenase e1, 2.3 $\times$; a succinate/fumarate antiporter, 4.0 $\times$; ICL, 8.2 $\times$; and GDH, 4.9 $\times$. Of these, the predicted proteins for ICL and GDH had targeting sig-

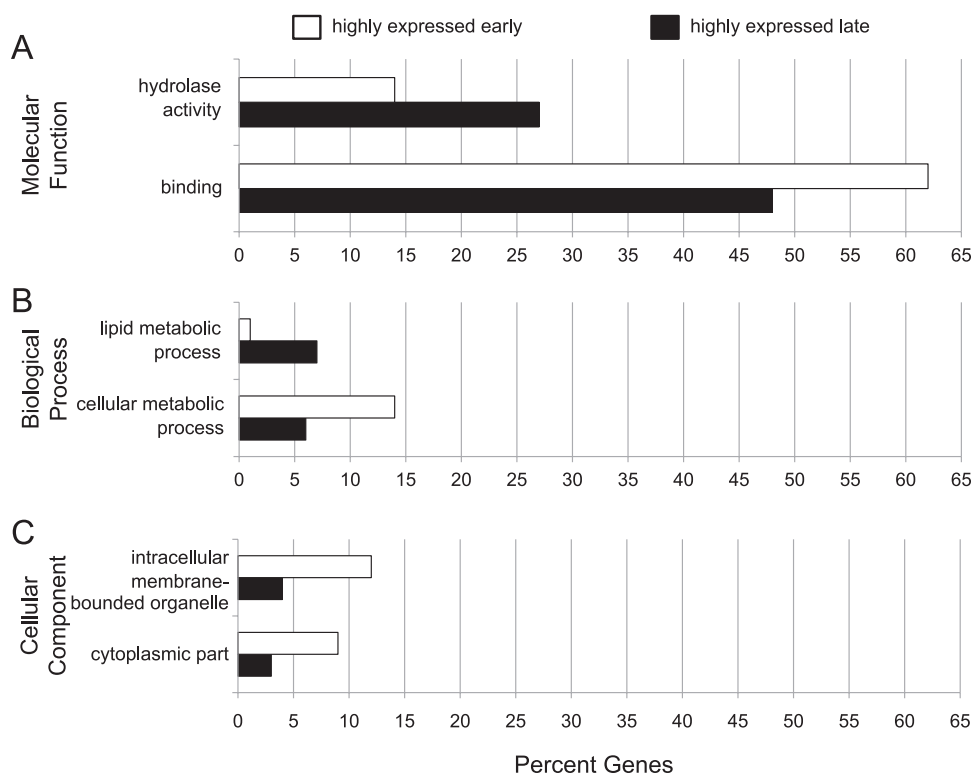


FIG 3 Differential representation of GO terms found in the RNA-Seq analysis. The three domains (and their terms) were molecular function (hydrolase activity and binding) (A), biological process (lipid metabolic process and cellular metabolic process) (B), and cellular component (intracellular membrane-bounded organelle and cytoplasmic part) (C). All terms showed significant differences ($FDR < 0.05$) in GO term abundance between the genes that were more highly expressed early (day 31, no wood strength loss) or late (day 154, high wood strength loss) on wood treated with MCQ. The total numbers of genes with GO terms for the early and late groups were 271 and 273, respectively.

nals for the peroxisome, while that of the succinate/fumarate antiporter had a signal peptide motif but lacked a secretion signal. Presumably, the antiporter was trading isocitrate produced in the mitochondria for succinate produced in the peroxisome. The genes with annotations for ODC1 and ODC2 showed increased expression at different time points; the former was more highly expressed at day 31 (7.0 $\times$) and the latter at day 154 (4.0 $\times$).

Other genes (see Table S4 in the supplemental material) that were expressed more at day 31 were gene 1430 (2.5 $\times$; annotation for COP), 26 genes with annotations for cytochrome P450 (cytochromes P450 belong to a large and diverse superfamily of proteins involved in the degradation of xenobiotics, the metabolism of fatty acids, and the biosynthesis of steroids), 6 genes with annotations related to oxidative phosphorylation (gene 5697, mitochondrial phosphate carrier protein, 9.8 $\times$; gene 8032, hemerythrin domain protein, 5.3 $\times$; gene 5659, alternative oxidase, 4.4 $\times$; gene 9070, cytochrome *c*, 3.3 $\times$; gene 2563, NADH-ubiquinone oxidoreductase, 3.3 $\times$; and gene 718, cytochrome *c* peroxidase), 4 genes related to signal transduction (gene 7796, mitogen-activated protein kinase phosphatase, 4.8 $\times$; gene 1553, casein kinase α 1-like, -4.5 $\times$; gene 2071, mitogen-activated protein kinase, 2.7 $\times$; and gene 2947, cmgc mitogen-activated protein kinase, 2.5 $\times$), and 3 genes related to transcriptional regulation (gene 1147, transcription factor, 4.5 $\times$; gene 2241, transcription factor *sfp1*, 3.5 $\times$; and gene 3983, transcriptional regulator *prz1*, 3.5 $\times$). Genes with annotations related to hydroxyl free radical production were also expressed more at day 31. They were gene 4739

(LCC, 11.2 $\times$), gene 1487 (tyrosinase, 4.7 $\times$), and gene 1213 (phenylalanine ammonia lyase, 4.0 $\times$). Of the last three, only the predicted protein for LCC had a motif for extracellular localization.

Genes that were more highly expressed at day 154 included 16 genes with annotations for cytochrome P450, as well as 3 genes whose annotations and detected signal motifs suggested that they could be regulating extracellular levels of H_2O_2 (see Table S4 in the supplemental material). The three genes were gene 8299, AAOX, 5.9 $\times$; gene 7478, copper radical oxidase, 5.5 $\times$; and gene 6682, CAT, 2.9 $\times$. AAOX and copper radical oxidase are oxidoreductases that couple oxidative dehydrogenation of their substrates with the reduction of O_2 to form H_2O_2 . CAT eliminates H_2O_2 by converting it to H_2O and O_2 .

Our analysis detected 18 differentially regulated genes whose predicted proteins had annotations related to extracellular polysaccharide degradation (see Table S4 in the supplemental material). Of the three genes that had annotations for pectinase, genes 1513 and 2569 (GH28 polygalacturonase and GH43 endo-1,5- α -L-arabinanase, respectively) were expressed more at day 31 (4.0 $\times$ and 2.7 $\times$, respectively), while gene 3577 (GH28 exorhamnogalacturonase b) was expressed more at day 154 (4.0 $\times$). The remaining genes were expressed more at day 154 and included 5 genes with functions related to cellulose degradation (genes 509 and 508, GH5 endo-1,4- β -glucanases, 19.7 $\times$ and 9.5 $\times$, respectively; gene 1962, GH61, 7.0 $\times$; gene 3737, GH3 β -glucosidase, 5.0 $\times$; and gene 4759, GH5, 4.7 $\times$) and 10 genes related to hemicellulose degradation (genes 2726, 2724, and 2727, GH10 endo- β -xylanases,

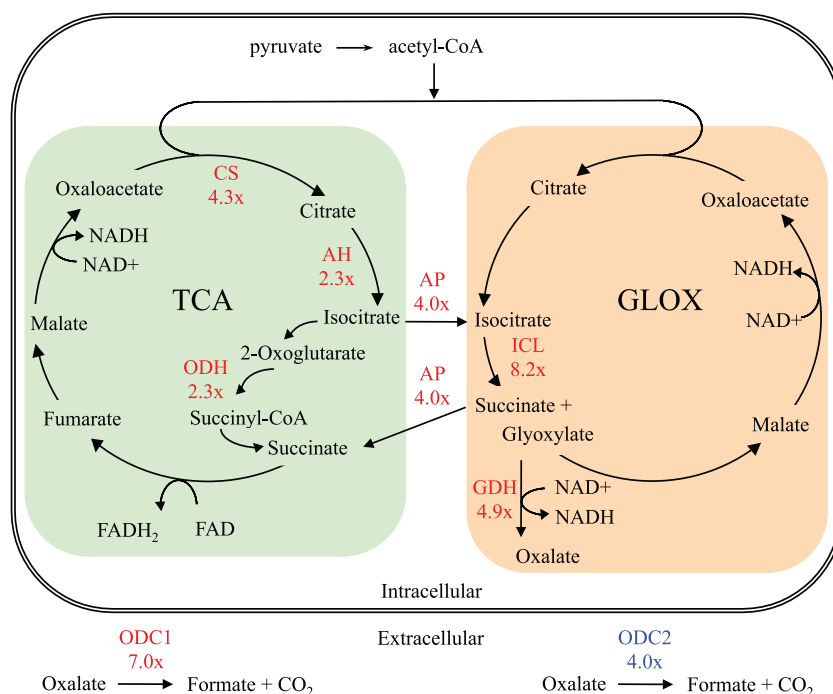


FIG 4 Proposed gene regulation pathway of oxalate metabolism in response to MCQ. The green- and apricot-shaded areas denote the mitochondria and the peroxisome, respectively. The double lines represent the cell membrane. Red and blue text colors indicate that a gene was more highly expressed before or after the MCQ-treated wood showed high strength loss, respectively ($FDR < 1E-4$). TCA, tricarboxylic acid cycle; GLOX, glyoxylate cycle; CS, citrate synthase, gene 6899; AH, aconitate hydratase, gene 7902; AP, succinate/fumarate antiporter, gene 5088; ICL, gene 415; ODH, 2-oxoglutarate dehydrogenase e1 component, gene 4245; GDH, gene 1257; ODC1, gene 7157; ODC2, gene 6399. Based on reference 59 (copyright 2001 National Academy of Sciences USA).

16.6 $\times$, 6.1 $\times$, and 5.8 $\times$, respectively; genes 7952 and 7362, GH5 endo- β -mannanases, 4.4 $\times$ and 4.3 $\times$, respectively; gene 583, GH53 endo-1,4- β -galactanase, 4.3 $\times$; gene 2755, GH115 α -glucuronidase, 4.1 $\times$; gene 1713, GH3 β -xylosidase, 3.3 $\times$; gene 1433, GH43 galactan-1,3- β -galactosidase, 2.7 $\times$; and gene 187, GH2 β -mannosidase, 2.7 $\times$). GH61 is a family of copper-dependent polysaccharide monooxygenases that enhance the breakdown of lignocellulose when mixed with cellulases. Gene 3723 (GH12 endo-1,4- β -glucanase) was also expressed more at day 154 (20.8 $\times$), but its predicted protein had a mitochondrial target, which did not make sense given the annotation.

Several genes with annotations for sugar transport were also differentially regulated (see Table S4 in the supplemental material). Genes 7926 and 8655 (annotations for generic sugar transporters) were expressed more at day 31 (4.2 $\times$ and 2.9 $\times$, respectively). Genes 9158, 6103, 7444, and 7081 had annotations for hexose transporters/carriers and showed increased expression at day 154 (5.4 $\times$, 3.6 $\times$, 3.6 $\times$, and 2.9 $\times$, respectively). Motifs for a signal anchor and signal peptide were detected in genes 6103 and 7444, respectively.

Several other families of glycoside hydrolase genes were differentially regulated, but their annotations involved fungal cell wall chemistry (see Table S4 in the supplemental material). The genes (annotation and FC value) that were expressed more at day 31 were gene 1342 (GH55 glucan-1,3- β -glucosidase, 11.7 $\times$), gene 6334 (GH47 α -mannosidase, 4.0 $\times$), gene 782 (GH18 class III chitinase, 3.2 $\times$), and gene 7135 (GH30 glucan endo-1,6- β -glucosidase, 2.4 $\times$). The genes that were expressed more at day 154 were genes 7277 and 6272 [GH16 endo-1,3(4)- β -glucanases, 4.6 $\times$ and 4.1 $\times$, respectively], gene 6020 (GH79, 3.8 $\times$), gene 4867 (GH89

α -N-acetylglucosaminidase, 3.3 $\times$), gene 4006 (GH55 glucan-1,3- β -glucosidase, 3.1 $\times$), and gene 424 (GH18 type II chitinase, 2.5 $\times$). GH79 includes endo- β -N-glucuronidases and heparanases. The predicted proteins of these 10 genes all had motifs for extracellular localization.

The last set of 14 genes that were differentially regulated had annotations related to membrane lipid metabolism, and all showed increased expression at day 154 (see Table S4 in the supplemental material). The genes (annotation and FC values) involved in lipid synthesis were gene 5564 (squalene synthase, 23.5 $\times$), gene 5558 (squalene synthase, 11.1 $\times$), gene 2210 (cyclopropane-fatty-acyl-phospholipid synthase, 4.5 $\times$), and gene 7624 (Δ -12 fatty acid desaturase, 5.3 $\times$). Squalene synthase regulates the first committed step of sterol biosynthesis, cyclopropane-fatty-acyl-phospholipid synthase introduces a cyclopropane ring into acyl chains of phospholipids, and Δ -12 fatty acid desaturases are oxidoreductases that remove two hydrogen atoms to insert a double bond at the C-12 position of a fatty acid. The genes for lipid catabolism were genes 7689 and 7325 (lysophospholipase plb1, 7.8 $\times$ and 3.6 $\times$, respectively); gene 2492 (lipase class 3, 4.9 $\times$); genes 6060 and 8157 (GDSL lipase acylhydrolases, 4.8 $\times$ and 5.3 $\times$, respectively); genes 5409, 8303, 7565, and 4208 (carbohydrate esterase family 10, 7.5 $\times$, 4.1 $\times$, and 3.3 $\times$, respectively); and gene 8701 (carbohydrate esterase family 16 choline esterase, 5.6 $\times$). Several of these predicted proteins had extracellular motifs and are listed in Table S4 in the supplemental material.

qRT-PCR. The relative expression levels of the genes selected for a more in-depth study are graphed in Fig. 5. The results of the two-sample Wilcoxon test indicated significant differences in relative gene expression ($P < 0.002$) between the MCQ-treated and

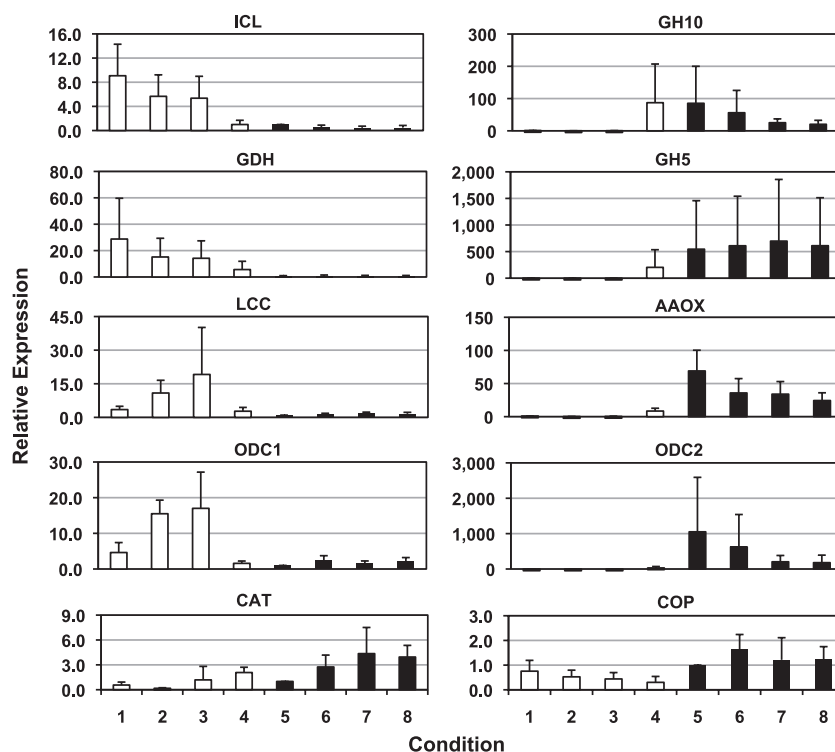


FIG 5 Relative changes in gene expression of 10 genes over time on MCQ-treated and untreated wood as determined by qRT-PCR. For conditions 1, 2, 3, and 4 (open bars), fungus was grown on MCQ-treated wafers for 25, 46, 70, and 154 days, respectively. For conditions 5, 6, 7, and 8 (filled bars), fungus was grown on untreated wafers for 6, 8, 10, and 12 days, respectively. Expression (+SD; $n = 4$) was calculated relative to condition 5 (untreated day 6) for genes with annotations for ICL, GDH, LCC, ODC1, CAT, and COP, or condition 1 (MCQ day 25) for GH10, GH5, AAOX, and ODC2. Expression values were normalized against 18S rRNA. All genes showed a significant difference ($P < 0.002$) in a two-sample Wilcoxon test comparing MCQ-treated and untreated wood.

untreated samples for all 10 genes analyzed. The genes that showed significantly higher expression on MCQ-treated wood were genes 415, 1257, 4739, and 7157 (annotations for ICL, GDH, LCC, and ODC1, respectively). The genes that showed significantly higher expression on untreated wood were genes 2726, 509, 8299, 6399, 6682, and 1430 (annotations for GH10, GH5, AAOX, ODC2, CAT, and COP, respectively). For the GH5 and ODC2 genes, expression could be as much as 500 and 1,000 times higher, respectively, on untreated wood (day 6) than on MCQ-treated wood (day 25). Genes 2726, 509, 8299, and 6399 (annotations for GH10, GH5, AAOX, and ODC2, respectively) from MCQ-treated wood at days 46 and 70 exhibited expression levels below the limits of detection.

In some cases, gene expression appeared to be correlated with wood strength loss (Fig. 1A and 5). Genes 415, 1257, 4739, and 7157 (annotations for ICL, GDH, LCC, and ODC1, respectively), showed the highest levels of expression when the MCQ was protecting the wood (zero strength loss) and the lowest levels of expression when the MCQ lost its effectiveness or when grown on untreated wood (nonzero strength losses in both cases). Genes 2726, 509, 8299, and 6399 (annotations for GH10, GH5, AAOX, and ODC2, respectively), exhibited very low expression when MCQ was protecting the wood, with higher levels of expression after MCQ lost its effectiveness or when grown on untreated wood. The other two genes (genes 6682 and 1430, annotations for CAT and COP, respectively) did not show any general correlation with strength loss.

Pairs of genes that exhibited overall similarity in their relative

expression patterns had annotations for ICL and GDH, LCC and ODC1, and ODC2 and AAOX. Upon exposure to MCQ, genes with annotations for ICL and GDH were induced almost immediately to peak expression levels but then gradually dropped to normal levels after MCQ lost its effectiveness. Induction of peak expression was somewhat slower for genes with annotations for LCC and ODC1, occurring at day 70 and then dropping back to normal levels once MCQ was no longer protecting the wood. Expression of genes with annotations for ODC2 and AAOX were repressed by MCQ. On untreated wood, these two genes showed their highest levels of expression initially and then gradually decreased with time. Expression of genes for GH10 and GH5 were repressed as long as MCQ was protecting the wood, but once MCQ lost its effectiveness, gene expression was as high or almost as high as for the controls. On untreated wood, peak expression of the putative GH10 gene appeared to occur earlier than for the GH5 gene.

Microscopy. Scanning electron micrographs showed that the predominant crystals formed on the MCQ-treated and untreated wood were copper oxalate and calcium oxalate, respectively (Fig. 6). In each case, the predominant crystal increased in abundance with time. The respective crystal types differed in their sizes and morphologies. The copper oxalate formed large moolooite crystals (up to 15 μm in diameter at day 154) that had 4 bulbous, wedge-shaped sections built up from many flaky layers. The calcium oxalate, on the other hand, formed solid crystals (up to 8 μm in length by day 12) that were tetragonal in shape, with or without pyramidal ends, and were often found coalesced together in large

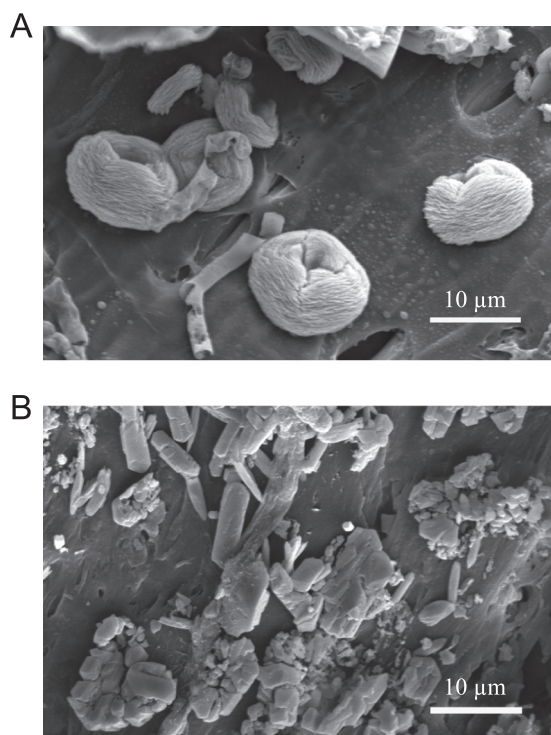


FIG 6 Scanning electron micrographs of copper oxalate crystals on MCQ-treated wood after 154 days of exposure (A) and calcium oxalate crystals on untreated wood after 12 days of exposure (B) to fungus. The metal in the crystal was verified by EDX spectroscopy.

table-like aggregates. The only abundant copper-containing particles found in the unexposed MCQ-treated wafers bore no resemblance to the moolooite copper oxalate crystals. They were $<2\ \mu\text{m}$ in diameter and had a low, domed profile with a smooth surface (not shown). On the unexposed, untreated wood, calcium-containing crystals were similar in shape to the calcium oxalate crystals but did not exceed $1\ \mu\text{m}$ in width and length and did not occur in large aggregates.

DISCUSSION

By exploiting the natural copper tolerance of brown rot fungi, we used RNA-Seq as a discovery tool to identify 917 genes whose expression levels were being regulated during decay of wood treated with MCQ. Our results clearly showed that *F. radiculosa* was responding to the MCQ by altering its growth, which affected the normal progression of wood decay. Genes involved in oxalate production and degradation, laccase activity, quinone biosynthesis, pectin degradation, ATP production, cytochrome P450 activity, signal transduction, and transcriptional regulation were upregulated while the preservative was still protecting the wood, while genes related to oxalate degradation; cytochrome P450; H_2O_2 production and degradation; degradation of cellulose, hemicellulose, and pectin; hexose transport; fungal cell wall chemistry; and membrane lipid metabolism were upregulated once MCQ lost its effectiveness and wood showed high loss of compression strength. Although the design of this analysis lacked samples from untreated controls, it was a critical component of our investigation because it gave us our first glimpse of how brown rot cellular networks can change in response to copper-based wood

preservatives. Furthermore, it provided comprehensive baseline information regarding which genes were being differentially regulated, allowing us to select 10 for a more in-depth study.

For example, in the RNA-Seq analysis, genes with annotations for ICL and GDH showed increased expression when MCQ was protecting the wood from strength loss, suggesting that rates of oxalate production were being upregulated to remove copper in the form of copper oxalate crystals. By quantifying the relative expression of these two genes in the qRT-PCR study, we demonstrated that each was highly induced by the MCQ treatment, gradually decreasing back to normal levels after the MCQ was no longer protecting the wood. In addition, the close similarity in the gene expression patterns of these two genes over time on treated and untreated wood suggests that their expression was coregulated by the same factor(s). Previous gene expression studies in *P. placenta* demonstrated functional TCA and GLOX cycles, but significant differences in gene regulation of oxalate biosynthesis were not observed in liquid cultures or on ground wood from different tree species (24, 25, 28).

A putative gene for ODC (ODC1) was also induced by MCQ. Its induction lagged behind that of transcripts for ICL and GDH, but like transcripts for ICL and GDH, it showed higher levels of expression on MCQ-treated wood than on untreated wood, and its expression fell back to control levels when MCQ lost its effectiveness. This suggests that the fungus was upregulating oxalate biosynthesis and degradation together in response to the MCQ treatment.

A different ODC gene (ODC2) was repressed by MCQ. ODC2 was only expressed on untreated wood when expression of ICL and GDH genes was constitutive (i.e., not upregulated) and calcium oxalate crystals were being produced. The expression of ODC2 was also at least 1 to 2 orders of magnitude higher than that of ODC1. Thus, these two genes exhibited very different mechanisms of regulation involving different factors. Studies that compare the gene promoter regions, regulation in response to other metals, and properties of their respective enzyme activities should provide further evidence to substantiate the factors that contribute to the different expression patterns exhibited by these two ODC genes.

Of the three putative laccase genes in *F. radiculosa*, only one was induced by the MCQ treatment, and its pattern of expression was very similar to that of the ODC1 gene. Since laccase may be involved in hydroxyl free radical production in the presence of high oxalate levels (29), it is possible that the four genes induced by MCQ (annotations for ICL, GDH, LCC, and ODC1) share some aspect of their regulation to ensure that laccase gene expression increases when oxalate metabolism increases. Factors that regulate their expression, though, are still poorly understood, since these genes have only recently been described in brown rot fungi (28, 48, 61). The high concentration of copper found in the MCQ is a possible candidate. Copper has been found to be a strong inducer of laccase gene expression in several species of white rot fungi, like *Pleurotus ostreatus* (62), *Trametes versicolor* (63), and *Ceriporiopsis subvermispora* (64). It is important to note, though, that not all laccase genes respond similarly to copper, nor is gene expression necessarily correlated with enzyme activity (62). Other factors that have been found to induce laccase gene expression are silver in *C. subvermispora* (64), nitrogen in *T. versicolor* (63), and certain aromatic compounds that act as laccase substrates (63).

One reason why oxalate figures so prominently in the biochemistry of brown rot fungi stems from its metabolic role as an energy by-product of glucose. In *F. palustris*, Munir et al. (59) found that glucose was not completely oxidized to CO₂ by the TCA cycle but was oxidized to oxalate in the GLOX cycle, with the two cycles linked through isocitrate lyase. This may explain why we observed increased expression of genes involved in oxidative phosphorylation with genes involved in oxalate biosynthesis in our RNA-Seq study on MCQ-treated wood. However, more studies that examine how these energy-related genes behave at different times compared to controls will need to be done to confirm their coregulated expression.

Metabolic shifts in response to an exogenous xenobiotic have been detailed for the white rot fungus *Phanerochaete chrysosporium* (65). Proteomic analysis showed that the response to exogenous vanillin resulted in increased abundance of proteins with roles in vanillin metabolism, glycolysis, the TCA cycle, and energy production (65). Shimizu et al. (65) also noted that stress caused a metabolic shift from the GLOX cycle (isocitrate lyase activity, 3.7×) to the TCA cycle (isocitrate dehydrogenase activity, 920×) (65). Although these were enzyme activity studies, the results contrasted with our gene expression data. None of the three candidate genes for isocitrate dehydrogenase in *F. radiculosa* (48) showed changes in expression during growth on MCQ-treated wood. It is possible that differences in how brown rot and white rot fungi regulate genes of the TCA and GLOX cycles may have been one of the fundamental shifts in energy metabolism that eventually led to the evolution of high-oxalate-producing brown rot fungi, capable of generating an efficient laccase-based Fenton system for decay initiation.

Our gene regulation data suggest that oxalate production occurs in the peroxisome, requiring an antiporter that trades isocitrate produced in the mitochondria for succinate produced in the peroxisome. This interpretation is consistent with the subcellular-localization studies done in *F. palustris* by Sakai et al. (60). The terminal steps of oxalate production in the two species, though, are quite distinct. In *F. palustris*, glyoxylate is converted to malate within the peroxisome; malate is transported to the cytoplasm, where it is oxidized to oxaloacetate; and then oxaloacetate is cleaved to oxalate and acetate (59). These conclusions were based on greater activity levels of oxaloacetate hydrolase than glyoxylate dehydrogenase (59). Our gene expression results suggest that a much shorter pathway is being regulated in *F. radiculosa*, that is, oxalate is synthesized directly by glyoxylate dehydrogenase acting on glyoxylate within the peroxisome. This was further supported by our genomic analysis of *F. radiculosa*, which detected peroxisomal targeting signals for both ICL and GDH but no annotation for oxaloacetate hydrolase (48).

When the MCQ was no longer protecting the wood and it showed high strength loss, RNA-Seq analysis identified numerous genes that were upregulated and had annotations related to saccharification and hexose transport. Quantification of two of these genes, a putative GH10 endo-β-xylanase and a GH5 endo-1,4-β-glucanase that have roles in hemicellulose and cellulose degradation, respectively, showed that both genes were highly upregulated on untreated wood at all time points examined (17 to 66% wood strength loss) but were generally inhibited when the MCQ was still protecting the wood (0% strength loss). Once the MCQ was effectively inactivated, the expression of both glycoside hydrolases returned to normal or near-normal levels, suggesting involvement

of a repressor and/or inducer. On untreated wood, the relative expression of the GH10 gene also peaked earlier than that of the GH5 gene, which is consistent with data showing that early loss of wood bending strength was correlated with removal of the hemicelluloses with subsequent removal of cellulose (37).

The brown rot fungi *F. radiculosa*, *P. placenta* (24), and *S. lacrymans* (22) showed increased expression of genes with putative annotations for GH3, GH5, GH10, GH28, and GH43 when grown on wood/shavings/particles (versus glucose-based cultures for *P. placenta* and *S. lacrymans*) or on MCQ-treated wood that showed high strength loss (versus no strength loss for *F. radiculosa*). Increased expression of genes for GH115 and GH53 occurred only in *F. radiculosa*, while increased expression of genes for GH35, GH51, and GH79 was unique to *P. placenta* and increased expression of genes for GH1 and GH74 was unique to *S. lacrymans*. Genes for GH12 and GH61 were both upregulated in *F. radiculosa* and *S. lacrymans*, but not in *P. placenta*, and a gene for GH2 was upregulated in *F. radiculosa* and *P. placenta*, but not in *S. lacrymans*. Thus, even though the substrate for *F. radiculosa* and *S. lacrymans* was softwood and that for *P. placenta* was hardwood, the three brown rot fungi shared more similarities than differences in terms of the gene families of glycoside hydrolases that showed differential regulation. Similarly, when comparing *P. placenta* grown on softwood versus hardwood, no differences were found in the expression levels of genes encoding the GH families that had functions related to wood degradation (25). All three species also exhibited concurrent upregulation of sugar transporters when the majority of the glycoside hydrolases were highly expressed. Genes for four hexose transporters were upregulated in *F. radiculosa*, while genes for three sugar transporters were upregulated in *P. placenta* (24) and genes for two sugar transporters were upregulated in *S. lacrymans* (22).

Brown rot fungi have several FAD-binding oxidases and copper radical oxidases that have been implicated in Fenton chemistry as sources of H₂O₂ because their genes were found to be upregulated during growth on wood. In *P. placenta*, transcripts for LCC and copper radical oxidase showed increases of 3.2× and 1.8×, respectively, on balled milled aspen versus glucose (24) and 3.9× and 4.3×, respectively, on balled milled aspen versus balled milled pine (25). In *F. radiculosa*, although we did not conduct an in-depth expression analysis of gene 7478, which encodes the putative copper radical oxidase that was differentially expressed during decay of MCQ-treated wood, our results for the putative AAOX gene showed that it was not coexpressed with the gene for LCC. This suggests that the AAOX gene was probably not contributing H₂O₂ to the Fenton system.

In conclusion, our differential gene expression study led to many new insights into the complex network of metabolic processes that regulate and link wood decay and copper tolerance. These biological processes were tightly coordinated to make nutrients quickly available while ensuring the survival of the fungus under the harsh and changing conditions of extracellular digestion. Despite the tremendous advantage that system biology approaches have for accelerating our knowledge, we still face two major challenges. The first is that the functions for all the genes were inferred from electronic annotation and have not been confirmed by experimental methods. The second is that we still lack gene ontology annotations for 40% of the *F. radiculosa* genes and have no homologs for 11% of the genes. Our hope, though, is that as we understand more about the functional networks that regu-

late wood decay and copper tolerance, we will be able to identify molecular targets for rational wood preservative development, as well as engineer efficient microbes that can convert biomass to bioenergy and biochemicals.

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