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Title: Evolution of substrate-specific gene expression and RNA editing in brown rot wood-decaying fungi

Running title: Evolution of substrate-specificity in fungi

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Abstract

Fungi that decay wood have characteristic associations with certain tree species, but the mechanistic bases for these associations are poorly understood. We studied substrate-specific gene expression and RNA editing in six species of wood-decaying fungi from the 'Antrodia clade' (Polyporales, Agaricomycetes) on three different wood substrates (pine, spruce, and aspen) in submerged cultures. We identified dozens to hundreds of substrate-biased genes (i.e., genes that are significantly upregulated in one substrate relative to the other two substrates) in each species, which are correlated with host ranges. Rapid evolution of substrate-biased genes is associated with gene family expansion, gain and loss of genes, and variation in cis- and trans- regulatory elements, rather than changes in protein coding sequences. We also demonstrated widespread RNA editing events in the Antrodia clade, which differ from those observed in the Ascomycota in their distribution, substitution types, and the genomic environment. Moreover, we found that substrates could affect editing positions and frequency, including editing events occurring in mRNA transcribed from wood-decay related genes. This work shows the extent to which gene expression and RNA editing differ among species and substrates, and provides clues into mechanisms by which wood-decaying fungi may adapt to different hosts.

Introduction

Wood-decaying fungi form an ecologically important guild, which is largely composed of species of Agaricomycetes (Basidiomycota) [1-4]. Two major modes of wood decay occur in Agaricomycetes: (1) white rot, in which all components of plant cell walls (PCW) are degraded, and (2) brown rot, in which a non-enzymatic

mechanism causes initial depolymerization of PCW carbohydrates, and sugars are selectively extracted without removal of large amounts of lignin [5-10]. There is considerable variation in host ranges of wood-decaying Agaricomycetes; some species occur only on particular hosts, while others have broad substrate ranges, sometimes including both conifers and hardwoods [11-13]. However, the mechanisms that determine host ranges in wood-decaying fungi are not well understood.

Regulation of gene expression and RNA editing (post-transcriptional modification of RNA sequences) both enable organisms to modulate genomic information. Various species have been shown to use transcriptional regulation to adjust to changes in their environments [14-17], but the role of RNA editing in such responses has not been widely studied [18, 19]. Transcriptomic analyses have been performed on different substrates for several wood-decaying Agaricomycetes, including both white rot (*Phanerochaete chrysosporium, Phanerochaete carnosa, Pycnoporus cinnabarinus, Dichomitus squalens*, *Heterobasidion annosum*) [20-25] and brown rot species (*Postia [Rhodonia] placenta,* and *Wolfiporia cocos*) [20, 23, 26, 27], and genome-wide RNA editing has been studied in the white rot fungus *Ganoderma lucidum* [28]. The latter study identified 8,906 putative RNA editing sites, without significant bias among substitution types, but did not investigate condition-specific RNA editing events. We recently studied transcriptional regulation and RNA editing in the brown rot fungus *Fomitopsis pinicola* [29], showing that it is able to modify both transcription and RNA editing levels on different wood types in diverse genes encoding enzymes with known or potential function in wood decay (including laccase, benzoquinone reductase, aryl alcohol oxidase, cytochrome P450s, and various glycoside hydrolases).

The prior studies, including our work on *F. pinicola*, demonstrate that wood-decaying Agaricomycetes can adjust gene expression on different substrates, but, due to sampling limitations and lack of standardization across studies, they do not permit comparative analyses of the diversity and evolution of substrate-specific responses. In the present work, we studied transcriptomes of six closely related species of brown rot fungi in the "Antrodia clade" of the Polyporales, which we grew on pine, aspen, and spruce sawdust in submerged cultures. Three of the species are most often found on angiosperms/hardwoods (*Daedalea quercina, W. cocos, Laetiporus sulphureus*) and two are almost always found on conifers/softwood (*Antrodia sinuosa*, *Postia [Rhodonia] placenta*), while *F. pinicola* is usually found on conifers, but also occurs on hardwoods [30]. Thus, this set of species presents an opportunity to explore the evolution of substrate-specific gene expression and RNA editing in wood-decaying fungi.

Materials and Methods

Culture conditions

Cultures of five species, with published genomes available on the Joint Genome Institute (JGI) MycoCosm portal (URLs below), were obtained from the USDA Forest Products Laboratory (Madsion, WI), including *A. sinuosa* (LD5-1) [https://genome.jgi.doe.gov/Antsi1/Antsi1.home.html], *P. placenta* (Mad-698-R) [https://genome.jgi.doe.gov/Pospl1/Pospl1.home.html], *W. cocos* (MD104 SS-10) [https://genome.jgi.doe.gov/Wolco1/Wolco1.home.html], *L. sulphureus* (93-53-SS-1) [https://genome.jgi.doe.gov/Laesu1/Laesu1.home.html], and *D. quercina* (L-15889

SS-12) [https://genome.jgi.doe.gov/Daequ1/Daequ1.home.html]. All strains are monokaryons, except *P. placenta,* which is a dikaryon. Culturing and harvesting of mycelium was conducted as in our prior study of *F. pinicola* (FP-58527) [https://genome.jgi.doe.gov/Fompi3/Fompi3.home.html]. Briefly, two-liter flasks containing 250 ml of basal salts media [26] were supplemented with 1.25 g of Wiley-milled wood of quaking aspen (*Populus tremuloides*), loblolly pine (*Pinus taeda*), or white spruce (*Picea glauca*) as the sole carbon source. Triplicate cultures for each substrate were inoculated with mycelium scraped from malt extract agar (2% w/w malt extract, 2% glucose w/w, 0.5% peptone, 1.5% agar) and placed on a rotary shaker (150 RPM) at 22-24 ℃. Five days after inoculation, the mycelium and adhering wood were collected by filtration through Miracloth (Calbiochem, San Diego, CA) and stored at -80 C°.

RNA extraction and library construction

Total RNA of samples from submerged culture was purified as described previously [29, 31]. Plate-based RNA sample prep was performed on a PerkinElmer Sciclone NGS robotic liquid handling system (PerkinElmer, Inc., Waltham, MA) using the Illumina TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA following the protocol outlined by Illumina in their user guide (Illumina, Inc., San Diego, CA). Total RNA starting material was 1 ug per sample and 8 cycles of PCR were used for library amplification. The prepared libraries were quantified using the KAPA Biosystems (Wilmington, MA) next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument (Roche Diagnostics Corp., Indianapolis, IN). The quantified libraries were then multiplexed and prepared

for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq Rapid paired-end cluster kit, v4. Sequencing of the flowcell was performed on the Illumina HiSeq2000 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 1x101 indexed run recipe.

Sequencing of one aspen sample from *D. quercina,* one pine sample from *A. sinuosa,* and one pine sample from P. *placenta* failed (Table S1). However, at least two biological replicates were obtained for each condition. RNAseq data are available via the JGI genome portal [https://genome.jgi.doe.gov/portal/] and have been deposited at DDBJ/EMBL/GenBank under the following accessions: SRP145276-SRP145283 (*D. quercina*: BOZCB, BOZGO, BOZCA, BOZGP, BOZHW, BOZHY, BOZGS, BOZHX), SRP145284-SRP145291 (*A. sinuosa*: BOZNU, BOZCZ, BOZHG, BOZCO, BOZNS, BOZNT, BOZHH, BOZCW), SRP145298-SRP145306 (*W. cocos*: BOZBY, BOZHU, BOZGG, BOZGH, BOZGN, BOZBX, BOZHT, BOZBW, BOZHS), SRP145308-SRP145315 (P. *placenta*: BOZHZ, BOZGT, BOZGU, BOZNB, BOZNA, BOZCG, BOZCH, BOZCC), and SRP164792, SRP164796, SRP164797, SRP164799-SRP164802 (*L. sulphureus*: BOZHB, BOZCU, BOZHA, BOZCT, BOZNG, BOZCS, BOZHC, BOZNC, BOZNH). RNAseq data for *F. pinicola* were taken from our prior study [29].

Identification and classification of substrate-biased genes

Raw reads were filtered and trimmed using the JGI QC pipeline. Using BBDuk (https://sourceforge.net/projects/bbmap/), raw reads were evaluated for sequence artifacts by kmer matching (kmer=25), allowing 1 mismatch, and detected artifacts were trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the

phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases or 1/3 of the original read length, whichever is longer). Filtered reads from each library were aligned to the corresponding reference genome using HISAT [32]. featureCounts [33] was used to generate the raw gene counts using gff3 annotations and mapped bam files. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p --primary options, because dUTPs strand RNAseq was used). FPKM (fragments per kilobase of transcript per million mapped reads) normalized gene counts were calculated by Cufflinks [34]. Based on recommendations from a previous study [35], edgeR [36] was subsequently used to determine which genes were differentially expressed between pairs of conditions using FDR (False Discovery Rate) < 0.05 and fold change ≥ 4 as cutoff for genes with FPKM >1 in at least one sample.

"Substrate-biased genes" were defined as ones that are significantly upregulated on one substrate relative to the other two substrates, by the criteria listed above (Fig. S1). For each pairwise comparison of substrates there are three possible outcomes (e.g., for pine vs. aspen, a gene could be upregulated on pine, upregulated on aspen, or not differentially expressed). Thus, with three substrates, there are 27 possible expression patterns, of which 15 correspond to substrate-biased genes (Fig. S1). Substrate-biased genes were further divided into "shared substrate-based genes" and "uniquely substrate-based genes". For example, a gene that is upregulated on pine vs. aspen and pine vs. spruce is a pine-biased gene; if that gene is also upregulated on spruce vs. aspen it would be considered a shared biased gene, but if it is not differentially expressed on spruce vs. aspen then it would be uniquely pine-biased (Fig. S1).

SignalP 4.0 [37] was used to search for secretory signal peptides in substrate-biased genes using the eukaryotic parameters. TMHMM 2.0 [38] was used to predict and characterize transmembrane domains in substrate-biased genes. Functional categories enriched with substrate-biased genes were identified using GOseq [39].

Analysis of RNA editing sites

Mapped strand-specific RNAseq reads were divided into sense- and antisense-strand groups and RNA editing sites were called separately for each group. Putative RNA editing sites from each sample were identified using JACUSA [40], with options to filter rare variants (ratio between reads with variant and total reads at specific position below 10%), variants with mapping quality less than 20, variants within 5bp of read start/end, indels or splice sites, and filtered variants with over 3 alleles per read pileup. In addition, reads were required to harbor at most 5 mismatches and variant sites to be covered by at least 2 reads. To further reduce false positives, a score threshold of 1.15 for variants was added. Sites that have the same position and type in all biological replications were determined, and only these reproducibly identified variants were analyzed. Thus, we minimized false positives due to potential sequencing and mapping errors. Annotation and functional consequences of RNA editing sites were assessed with SnpEff [41]. The nucleotides flanking editing sites were visualized using WebLogo3 [42]. Functional categories enriched in differentially edited genes were identified using GOseq [39].

Gain and loss of biased expression

The orthologs and paralogs among and within species were predicted by OrthoFinder v1.1.8 [43]. The substrate-biased genes and their non-biased orthologs were modeled as a two-state continuous-time Markov process, with states 1 (biased expression) and 0 (non-biased expression) on a maximum likelihood tree based on 500 orthologs, which was constructed using FastTree 2 (-gtr -gamma) [44]. If one copy of a gene family was a substrate-biased gene, the gene family was assigned as having biased expression. We then assessed the gain and loss of biased expression along each branch in the tree using the Dollo parsimony approach implemented in Count software [45].

Co-expression analysis, motif analysis, Ka/Ks and genetic distance

Co-expression network analysis was performed with the Comparative Co-Expression Network Construction and Visualization tool (CoExpNetViz) [46] using the Pearson correlation coefficient. The FPKM values were used as the matrix and twelve transcription factor and transcription factor-related genes in *W. cocos* were used as bait genes. The twelve transcription factor and transcription factor-related genes were retrieved from JGI annotations using GO terms GO:0006355, GO:0051090, and GO:0003700. The network was visualized using Cytoscape V3 [47]. We used 1 kb sequences upstream of co-expressed genes associated with TF 138100 to predict putative TF binding sites. We performed de novo motif discovery using frequencymaker and Weeder 2 [48]. We also compared the selection at coding regions and genetic distances 1 kb upstream of coding regions between *W. cocos* and *L. sulphureus*. Codon alignments, generated with PAL2NAL [49], were used for selection analyses. The Ka/Ks of ortholog pairs were calculated using the yn00

program from the PAML [50] package with default parameters (icode = 0, weighting = 0, commonf3x4 = 0). The pairwise genetic distance of upstream regions (1kb) of CDS was calculated using MEGA-CC [51] with the Jukes-Cantor model.

Results

Transcriptomes are clustered primarily by phylogenetic relatedness

Three substrates, aspen, pine and spruce, were used to explore how brown rot fungi adjust gene expression on different hosts. Transcriptome analyses show that most of the annotated genes from each species (e.g., 78%-88% of the annotated genes) were expressed. We used hierarchical clustering of expression levels in a single-copy (one-to-one) ortholog dataset to visualize global transcriptomic patterns among the six species. Each species displayed variation in gene expression across substrates, but the samples are clustered primarily by fungal species, rather than substrate type (Fig. 1A).

Magnitude and directionality of shifts in global gene expression on different substrates varies by species

Changes in global gene expression profiles on different substrates varied considerably across the six fungal species (Fig. 1B). For example, *W. cocos* has the highest fold change (up to log2FC=10) on aspen relative to spruce, whereas *F. pinicola* shows the lowest fold change for the same comparison, with most changes being smaller than log2FC=5 (Fig. 1B). Different fungal species also vary in terms of

the prevalence of up- vs. down-regulation in the same pairwise comparisons. For instance, on aspen vs. pine, *F. pinicola* and *L. sulphureus* show trends mainly toward up-regulation, while the other four species display both significant up- and down-regulation (Fig. 1B).

Numbers of substrate-biased genes vary widely across fungal species

The number of substrate-biased genes varied by an order of magnitude across the six species, ranging from 24 to 310 for aspen-biased genes, 16 to 359 for pine-biased genes, and 20 to 413 for spruce-biased genes. *F. pinicola* had the lowest number of aspen- and pine-biased genes, while *L. sulphureus* had the fewest spruce-biased genes. *W. cocos* had the greatest number of substrate-biased genes on all three wood types (Fig. 1C and Table S1). The numbers of substrate-biased genes are not biased by the numbers of annotated genes in each species. For instance, *F. pinicola* has a greater gene content and number of expressed genes than *W. cocos*, but the numbers of substrate-biased genes in *W. cocos* are seven to 22 times greater than those of *F. pinicola* for each substrate (Fig. 1C). The number of genes with biased expression indicates the degree of sensitivity of species to different substrates in terms of transcriptomic responses. Most of the substrate-biased genes in each fungal species are uniquely substrate-biased, not shared substrate-based, meaning that they are only up-regulated on one substrate type (see Methods for definition of terms; Fig. 1D and Fig. S1C).

Although the number of substrate-biased genes varies among species, their functions may be conserved to some extent. For example, although the number of aspen-biased genes from the six species are variable, eight GO terms were present

among the biased genes of all species, such as “monooxygenase activity” (including non-orthologous genes encoding cytochrome P450s) (Fig. 1E; see caption for all eight GO terms).

Among the substrate-biased genes, there are 17 to 210 “orphan” genes (i.e., genes that are unique to single species) per species (Fig. S2A). Because they are absent from five other genomes, it is unlikely that they reflect annotation errors. Around 10% of these biased orphan genes are predicted to have a signal peptide, and 15% have transmembrane domains (Fig. S2B). We examined GO enrichment among biased orphan genes belonging to *P. placenta* (Fig. S2C), which has the greatest number of biased orphan genes among the six species. Some enriched GO terms (molecular function), such as monooxygenase activity, are potentially associated with wood decay.

Gene expression bias turns over rapidly within orthogroups and is correlated with host ranges

To investigate the evolutionary pattern of biased expression, we first assessed the orthology status of all substrate-biased genes among the six studied species. Most (76-81%) of the substrate-biased genes from each species have orthologs in each of the other species (Fig. 2A). However, most orthogroups show substrate-biased expression in only one or a few species (Fig. 2A).

We mapped the substrate-biased genes and their orthologs on the organismal phylogeny. Generally, the presence and absence of biased expression are very dynamic for each orthogroup (Fig. 2B). We further used our orthogroup classification to quantify the turnover (gain and loss) of biased expression for each

orthogroup. To avoid the effect of gene gains and losses, we removed orthogroups in which there are missing orthologs in individual species. Biased expression displays rapid turnover across clades. For example, *W. cocos* has a net gain of substrate-biased expression on all substrate types, while *F. pinicola* and *L. sulphureus* have lost the most substrate-biased expression, but on different hosts (Fig. 2B).

To test whether biased gene expression is associated with substrates ranges (i.e., hardwood or softwood), we analyzed the correlations among expression of single-copy biased genes. Consistent with the global expression pattern (Fig. 1A), samples from the same species are clustered together independent of substrates. However, the species as a whole are clustered according to their host ranges (Fig. 2C). Thus, the three species most often found on hardwoods (*D. quercina, W. cocos*, and L*. sulphureus*) form one cluster, while the two conifer specialists (*A. sinuosa* and *P. placenta*) form another cluster, and *F. pinicola*, which is found often on hardwoods and softwoods, is separated from all other species. In four of the six species, expression patterns on conifers cluster together, although in *F. pinicola* the aspen and pine expression profiles are clustered, and in *A. sinuosa* the aspen and spruce profiles are clustered (Fig. 2C).

Gene duplications and mutations in cis-regulatory elements are correlated with turnover of substrate-biased expression

To assess the relationship between gene duplication and evolution of substrate-biased expression, we counted the number of paralogs of each substrate-biased gene across the six fungal species. For all species, gene families containing

substrate-biased genes are significantly larger than those lacking substrate-biased genes (Fig. 3A), suggesting that gene duplication facilitates neofunctionalization and emergence of biased expression.

To test whether origins of substrate-biased expression are related to the divergence in protein sequences, we analyzed Ka/Ks among ortholog pairs between *W. cocos* and *L. sulphureus* (Fig. 3B), which have very different numbers of biased genes (Fig. 1C). We divided the orthologs from the two species into two groups: the "biased" group was made up of substrate-biased genes from *W. cocos* and their non-biased orthologs in *L. sulphureus,* while the "non-biased" group was made up of orthologs that are non-biased in both species (as a control). Ka/Ks values of ortholog pairs in the biased group are no higher than those in the non-biased group (Fig. 3B). Thus, there is no evidence that the origin of biased expression in *W. cocos* is driven by divergence in coding sequences.

We also examined genetic distances in the 1-kb region upstream of each CDS (where the DNA sequences may impact transcription), using the same biased and non-biased groups. For each substrate, the genetic distances of the biased groups are higher than that in non-biased groups, with the results being significant for pine- and spruce-biased genes (Mann–Whitney U-tests) (Fig. 3C). These results suggest that divergence of cis-regulatory elements may be involved in the generation of biased expression.

Transcription factors orchestrate substrate-biased expression

Transcriptional changes have been suggested to follow the activity and expression of transcription factors (TFs) [52]. We found a significant positive correlation

(Spearman's rho = 0.93, p=0.008) between the number of TF-related biased genes (i.e, TF genes and their regulators that display substrate-biased expression) and total biased genes among the six species (Fig. 4A). We further explored the expression relationship between TF-related genes and total biased genes in individual species. A total of 12 TF-related uniquely substrate-biased genes (10 TFs and two regulators of TFs) were identified among the substrate-biased genes in *W. cocos*. 61% of the substrate-biased genes in *W. cocos* co-express with these 12 TF-related genes. Moreover, three out of the 12 TF-related biased genes, which co-express with 31% of the substrate-biased genes, were predicted to respond to environmental changes (Fig. 4 B). Specifically, ID 138100 and ID 17498 are predicted to respond to pH, while ID 104855, which contains a P450 domain, responds to iron. pH impacts the process of wood decay, by modifying the solubilization of ferric iron via oxalic acid chelation, which is central to the hydroquinone redox cycle that drives the Fenton reaction [53-57]. Furthermore, TFs could be co-expressed with their potential regulators in the network. For instance, there is one TF and one TF regulator (TFR) in each panel of Fig. 4B. To assess whether co-regulated genes possess a common regulatory signature, we searched for putative TF binding sites by de novo motif discovery in the 105 co-expressed genes associated with TFR 138100. We thus identified 25 highly conserved motifs ranging from 6nt to 10nt (Figure 4B and Table S1), further suggesting the these co-expressed genes might be regulated by the same TF/TFRs. Together, these results suggest that differential expression of trans-elements appears to be important in regulation of biased expression.

RNA editing is widespread in brown rot Polyporales

We analyzed RNA editing in five out of the six studied species (*P. placenta* was excluded as the sequenced strain is diploid). The number of normalized RNA editing sites is in the range of 10.8-98.9 sites/million reads (Fig. 5A). *A. sinuosa, L. sulphureus*, and *F. pinicola* have similar RNA editing levels, with 59.3-98.9 sites/million reads on the three substrates, but *D. quercina* and *W. cocos* have only 10.8-27.6 sites/million reads on each substrate (Fig. 5A). All 12 RNA editing types were found in each species, with more transitions than transversions observed (Fig. S3). Furthermore, the nucleotides surrounding the RNA editing sites (±1bp), either upstream or downstream, exhibit a relatively conserved preference for the same type of RNA editing across all five species (Figs. 5B and S4), which suggests the existence of common mechanisms of RNA editing in Polyporales of the Antrodia clade.

The RNA editing level varied from 10% to 90% at different editing sites (sites with frequency below 10% were filtered out), with the half of the total editing sites having frequency less than 40% (two examples in Fig. S5). Very few sites have an editing level in the range of 90-91%, with the maximum proportion (0.02%) found in *A. sinuosa* on aspen.

Genomic locations of RNA edited sites have fluctuating proportions among the five species we analyzed (Fig. S6). For instance, on aspen, the proportion of RNA editing sites in coding regions from *A. sinuosa* is significantly higher than that from *W. cocos* (Fisher test, p= 0.0059) (Fig. S6). Overall, 35-65% of RNA editing sites occurred in coding regions among the five species. Liu et al. identified 323 genes in *F. graminearum* that had stop (codon)-loss events [58], and Zhu et al. identified 66 such genes in *Ganoderma lucidum* [28]. In contrast, we found fewer than five

events of stop (codon)-loss events in each species (Table S2). We also analyzed the frequency of RNA editing at synonymous and non-synonymous sites in each species. The editing level of missense edits was significantly higher than that of synonymous editing sites in *F. pinicola* (Fig. 5C), but not in the other four species, which suggests that RNA editing in some species could be adaptive. Of the missense edits, 54%-65% resulted in changes of physicochemical properties of amino acid residues (Fig. 5D).

We detected 100 RNA editing sites in *W. cocos* that are shared by samples from all three different substrates. RNA editing at these sites is probably not dependent on substrate, and should be evident in *W. cocos* transcriptomes from diverse conditions. We searched for these 100 sites in EST sequences reported in the original publication of the *W. cocos* genome [8], which were produced on varous culture media (not milled wood), using the same strain as in the present study. In total, 69 out of 100 sites, with the same transitions, are found in the EST data. Given that only frequencies above around 50% can be called in EST analyses, these results support the identification of RNA editing sites in our RNAseq data.

RNA editing exhibits substrate specificity

There is considerable overlap among RNA-editing sites on the different substrates (Fig. 6A). In each of the five species we studied, the largest category of edited sites were those that occur on all substrates (100 to 907 sites, avg. 634 sites). Nevertheless, each species also had numerous sites that were edited only on a single substrate (29-433 sites, avg. 142 sites).

To further explore response of RNA editing to different substrates, we analyzed dynamic trajectories of shared sites from *L. sulphureus,* which has a relatively high number of shared sites on different substrates (Fig. 6A). Editing levels varied greatly across three different substrates in this species (e.g., “example 1” in Fig. 6B).

We identified the differentially RNA edited genes (DREGs) in all five species, which were defined as genes having unique nonsynonymous editing sites on one substrate relative to the other substrates (Fig. 6C). None of the DREGs were found among the substrate-biased genes, indicating that these two modes of gene regulation at the RNA level are independent during wood decay. Some DREGs have annotations that suggest potential roles in wood-decay. For example, there are several DREGs that encode glycosyl transferases (GT2, GT15), glycoside hydrolases (GH3, GH13, GH5, GH30, GH79) and decay-related oxidoreductases (AA3: GMC oxidoreductase) (Table S3). GO enrichment analysis of DREGs revealed four terms: iron ion binding, monooxygenase activity, oxalate oxidase activity, and glucosylceramidase activity (Fig. 6C). There is much evidence that the first three activities play key roles during wood decay by brown rot fungi [20, 23, 26], while glucosylceramidase (GH30) activity is involved in decomposition of hemicellulose [59, 60].

Discussion

The Antrodia clade is an ecologically important group of brown rot wood-decay fungi, with diverse and well-characterized substrate preferences [1]. Thus, the Antrodia clade presents an excellent system in which to explore mechanisms of

substrate-specificity and host-switching in wood decay fungi. Changes in gene expression on different substrates have been studied in individual species from Polyporales and Russulales [20-24, 26, 29, 62, 63], but the evolution of substrate-biased gene expression has not been addressed in a simultaneous, comparative study. Moreover, it is not clear if other forms of regulation at the transcriptional level could be involved in wood decay, such as RNA editing and methylation.

We first measured genome-wide gene expression employing one to one orthologs across six fungi species belonging to the Antrodia clade on three different substrates. If variation in gene expression is primarily adaptive, the clustering of expression patterns would be mainly based on substrates. In fact, clustering of global expression patterns in response to the three different substrates reflected the fungal phylogeny, with transcriptomes from each species forming a distinct group (Figs. 1A). Thus, variation in expression patterns of six-species orthologs is mainly associated with the random accumulation of neutral mutations rather than environmental adaptations. However, the clustering patterns do not exclude the possibility of stabilizing selection [64].

Previous studies have found similar patterns in which divergence in gene expression on the transcriptome scale is positively correlated with phylogenetic distance [65-67]. For example, in yeast species, Yang et al. [68] found that the transcriptome-based clustering of nine strains approximates the phylogeny, irrespective of their environmental origins. The great genetic distance between yeasts and Polyporales, suggests that a mode of neutral evolution of transcriptome profiles is a general attribute of fungi. While our result suggests the expression variations of six-species orthologs among the species are neutral, it does not exclude the possibility of adaptive evolution in one-to-one orthologs.

Within each species, dozens to hundreds of genes showed substrate-biased expression. By analyzing the pattern of biased expression among the six species, we showed that the rate of gain of biased expression is much higher in the lineage leading to *W. cocos* relative to the lineage leading to *P. placenta* (fold range of 4-45 depending on substrates), although the genetic distance (branch length) to their most recent common ancestor is almost equal (0.40 vs 0.35) (Fig. 2). This observation suggests that gain of substrate-biased expression may be under non-neutral (adaptive) evolution. Analyses of biased expression data revealed the correlation between species and their host ranges (Fig. 2C). We found that gene duplication, gain and loss and diversification of cis and trans-regulatory elements appear to contribute to the evolution of substrate-biased expression, rather than divergent changes in protein coding sequences (Figs. 3, 4, S2). Similar observations have been reported in comparisons of orthologs with different phenotypes in human and mouse, in which phenotypic differences were correlated with changes in non-coding regulatory elements and tissue-biased expression, rather than changes in protein sequences [69].

Other than our prior study in *F. pinicola [29]*, there has been only one genome-wide analysis of RNA editing in basidiomycetes, in fruiting body samples of the polypore *G. lucidum* [28]. *G. lucidum* is a member of the Polyporales, like the species analyzed here, but it is a white rot species of Polyporaceae, whereas the present study includes members of the Antrodia clade [70]. As in *G. lucidum*, all 12 types of RNA editing were found to be present in all five species (Fig. S3), and the nucleotides flanking the RNA editing sites are relatively conserved between the five species analyzed here and *G. lucidum* (Figs. 5B and S4). Compared with RNA editing of vegetative hyphae in Ascomycetes [58, 71], the RNA editing in basidiomycetes

has a greater diversity. In ascomycetes, A-to-G editing appeared to be the dominant form, with >95% of the identified editing sites belonging to this category. In the basidiomycetes [28, 72], including *G. lucidum, Pleurotus ostreatus* and the species in our study, A-to-G is not the only dominant transition and four of twelve possible editing types (A-to-G, C-to-T, G-to-A, and T-to-C) can account for up to 50% or more of total editing events. Given that A-to-G editing is dominant in animals and Ascomycetes, the expansion of editing types in basidiomycetes may suggest the occurrence of novel mechanisms of RNA editing.

Another difference between ascomycetes and basidiomycetes is that A-to-G editing sites do not share the same flanking nucleotides. Specifically, in Ascomycetes the enriched nucleotide upstream of edited sites is a T [58], whereas in basidiomycetes the enriched upstream nucleotide is a C. In cephalopods (animals), the enriched nucleotide upstream of the A-to-G editing sites is an A [73]. Orthologs of ADARs, the enzymes that are responsible for A-to-G RNA editing in animals, have not been found in fungal genomes [58]. Collectively, these observations suggest that there is much diversity in the enzymes and mechanisms for recognizing the editing motifs within fungi and between fungi and animals. RNA-edited genes could be functional in condition-specific processes among kingdoms. In ascomycetes, edited genes have been suggested to be involved in developmental regulation [58, 74], while behavioral complexity has been correlated with extensive editing in cephalopods [75].

To conclude, our study found that dynamic shifts in gene expression are associated with different substrates in wood decay fungi. The occurrence of substrate-biased expression is correlated with gene family expansion, divergence in cis-regulatory elements, and differential expression of transcription factors and their

regulators. In addition, we observed substrate-specific regulation of RNA editing, including editing events that cause amino acid replacements in genes implicated in decay. While our results do not address the functional significance of shifts in expression or RNA editing in specific genes, in aggregate they suggest that differential gene expression and RNA editing may enable wood decay fungi to adapt to different wood substrates.

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Figure legends

Figure 1. Patterns of gene expression in response to three different substrates from the six brown rot fungi species. (A) Neighbor-joining tree with branch length inferred using expression distance (1- Spearman's rho) for all pairs of species. (B) The fold change of all genes in response to one substrate relative to the other one. (C) Numbers of substrate-biased genes plotted on the branches of a simplified phylogenetic tree (branch lengths are labeled along the branches). (D) The proportion of uniquely substrate-biased and shared substrate-biased genes from each species. The two categories are illustrated in Figure S1. (E) Venn diagram showing overlap among GO terms for aspen-biased genes from six species. The eight GO terms shared among all six species are Molecular Function (MF): oxidoreductase activity, catalytic activity, monooxygenase activity, iron ion binding, heme binding; Biological Process (BP): metabolic process, regulation of nitrogen utilization; and Cellular Component (CC): mitochondrial intermembrane space. For panels A, B, D: A = *A. sinuosa*, P = *P. placenta*, W = *W. cocos*, L = *L. sulphureus*, D = *D. quercina*, and F = *F. pinicola*.

Figure 2. Turnover of substrate-biased expression among six species. (A) The proportion of substrate-biased genes having orthologs in six fungal species (for example, over 80% of aspen-biased genes have orthologs in six species). The large panel showing the number of species having biased genes within each orthogroup (horizontal axis). The vertical axis refers to how many orthogroups having the percentage shown on the horizontal axis. The number of orthogroups was shown as log2 scale. (B) Distribution and evolution of substrate-biased expression. The heatmap shows the distribution of substrate-biased expression (yellow) vs. absence of biased expression (blue) among orthologs/orthogroups (arranged vertically) among the six species, which are organized according to phylogenetic relationships. Ratios of gains and losses of substrate-biased expression at each tip were modelled by Dollo parsimony implemented in Count. The red dashed lines indicate a 1/1 ratio of gains to losses. Bars: A = aspen. P = pine S = spruce. The scale for *W. cocos* differs from that of the other species, due to its higher proportion of gains of substrate-biased expression. (C) Heatmap showing hierarchical clustering of 18 samples using expression data (FPKM) of single-copy biased genes. Blue branches group the species that occur primarily on conifers, red branches group hardwood specialists.

Figure 3. Factors contributing to turnover of biased expression. (A) The extent of gene expansion was compared between biased group and non-biased group. The y-axis represents the number of genes from each gene family. A = *A. sinuosa*, P = *P. placenta*, W = *W. cocos*, L = *L. sulphureus*, D = *D. quercina*, and F = *F. pinicola*. (B) Ratio of nonsynonymous substitutions (Ka) to synonymous substitutions (Ks) for ortholog pairs from non-biased and biased group between *W.*

cocos and *L. sulphureus*. (C) Genetic distance for upstream region (1 kb) of CDSs from the non-biased and biased groups between *W. cocos* and *L. sulphureus*.

Figure 4. Transcription factors orchestrating substrate-biased expression. (A) Correlation between numbers of total biased genes (y-axis) and TF/TF-related biased genes (x-axis) among six species. (B) Co-expression of TF-related biased genes with total biased genes in *W. cocos*. White squares represent four TF-related biased genes (TFR = TF regulator). The sequence logo shows a motif shared by all co-expressed genes associated with ID 138100. The other 24 shared motifs from the same clade were listed in table S1.

Figure 5. RNA editing in the Antrodia clade. (A) The number of normalized RNA editing sites among five species spanning the Antrodia clade. (B) The nucleotides neighboring the detected editing site (A to G) showing relative conserved preference. The RNA editing site is referred to as 0. Upstream to the editing site is referred to -1, while downstream is referred to +1. (C) Box plots showing the editing levels of RNA editing sites with different types of functional consequences in *F. pinicola*. (D) Physicochemical change of RNA edited sites. The change between any properties of amino acids (non-polar, polar uncharged, acidic and basic) was regarded as change of physicochemical properties. Absolute numbers of editing sites are indicated on the bars.

Figure 6. Condition-specific RNA editing events. (A) Venn diagrams showing the distribution of RNA editing sites on different substrates. A = aspen, P = pine, S = spruce. (B) Hierarchical clustering of the editing level of shared 892 editing sites

822 from *L. sulphureus*. (C) GO enrichment analysis of differentially edited genes
823 between any two substrates. Circled numbers correspond to the four enriched GO
824 categories.