Transcriptomic Profile of Lignocellulose Degradation from *Trametes versicolor* on Poplar Wood

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The *Trametes versicolor* genome is predicted to encode many enzymes that effectively degrade lignin, making it a potentially useful tool for biopulping. However, the wood degradation mechanism of *T. versicolor* is not clear. To identify the enzymes that contribute to lignocellulose degradation, changes in the *T. versicolor* transcriptome during growth on poplar wood, relative to growth on glucose medium, were evaluated. Eight hundred and fifty-three genes were differentially expressed, with 360 genes up-regulated and 493 genes were down-regulated on poplar wood. Notably, most genes involved in lignin degradation were up-regulated, including eight lignin peroxidase (LiP) genes. Genes encoding cellulose and hemicellulose degrading-enzymes were mostly down-regulated, including six endo-β-1,4-glucanase genes and three cellobiohydrolase I genes. These results characterized transcriptomic changes related to lignocellulose degradation. This information could be used to develop *T. versicolor* as a tool to improve the efficiency of lignin degradation or to provide a theoretical foundation for a new paper pulp manufacturing process.

Keywords: Trametes versicolor; Transcriptome; Lignin degradation; Cellulose; Hemicellulose degradation; Poplar

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INTRODUCTION

Many microorganisms use cellulose and hemicellulose as carbon and energy sources. Only a small group of filamentous fungi in the phylum Basidiomycota, however, have the unique ability to efficiently depolymerize and mineralize lignin, the most recalcitrant component of plant cell walls (Chang *et al.*2012). These fungi are commonly called "white rot" fungi, and are usually found on fallen trees or in forest litter. The majority of these species belong to the genera *Poria*, *Polyporus*, *Pleurotus*, *Phanerochaete*, *Fomes*, *Bjerkandera*, and *Trametes* (Lundell *et al.* 2014).

White rot fungi may degrade lignin by secreting ligninolytic enzymes, including peroxidases (PODs), multicopper oxidase (MCO), laccases, and H_2O_2-generating enzymes (Yadav and Yadav 2015; Manavalan *et al.* 2015). Complete enzymatic hydrolysis and breakdown of cellulose chains require the combined activities of the β-1,4-glycosidic bond-cleaving enzymes endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL). β-1,4-endoglucanases hydrolyze the non-crystalline regions of cellulose microfibrils, while CBH enzymes digest the ends of cellulose chains (Medie *et al.* 2012).

In addition, both the hydrolysis and degradation of hemicellulose are even more complicated due to its chemical composition and the presence of branched sugar units, acetyl groups, and covalent cross-linkages in hemicellulose (Sindhu *et al.*2016). The

expression of genes encoding diverse lignocellulose-degrading enzymes is affected by fungi, substrates, culture conditions, *etc.* (Cragg *et al.* 2015).

Biological delignification with lignin-degrading fungi saves energy and chemicals in the manufacture of cellulose pulp from wood. It could also be useful for the production of second-generation bioethanol or for the decoloration, decontamination, and degradation of other macromolecular compounds (Patt *et al.*2006; Alexieva *et al.*2010; Bischof *et al.* 2013; Nguyen *et al.* 2014). *T. versicolor* is a common fungi species native to temperate climate zones that can secrete PODs, laccases, and carbohydrate-active enzymes. It has strong similarities to *Phanerochaete chrysosporium* and is a useful model organism for the study of white rot fungus (Barrasa *et al.*1995; MacDonald *et al.*2011; Macdonald *et al.* 2012; Varma *et al.* 2015) and for testing wood resistance to white rot decay (Plaschkies *et al.* 2014).

T. versicolor and *P. chrysosporium* are often found in dead deciduous wood and coniferous wood. Both fungi can simultaneously degrade cellulose, hemicelluloses, and lignin. *P. chrysosporium* has been thoroughly researched as a white rot fungi model organism. The *T. versicolor* genome, however, contains more oxidoreductase-encoding genes, which are involved in lignin degradation and plant biomass-decomposition, than *P. chrysosporium*. For instance, *T. versicolor* has 26 PODs class II peroxidases, 10 MCOs, 13 MnPs, and 2 dye-decolorizing peroxidases (DyPs) encoded in its genome, while *P. chrysosporium* has only 12 PODs, 5 MCOs, 5 MnPs, and no DyP genes (Eastwood *et al.* 2011; Floudas *et al.* 2012). Furthermore, previous studies have found no laccase-encoding genes in most *P. chrysosporium* strains (Kersten and Cullen 2007; Wymelenberg *et al.* 2010). Therefore, *T. versicolor* may be a superior model for studying the degradation of deciduous wood. Unfortunately, few studies have investigated transcriptomic differences regarding genes involved in wood degradation in *T. versicolor* or described expression of its genes at the RNA level (Barrasa *et al.*1995; Furukawa *et al.* 2014; Lundell *et al.* 2014).

To better understand the potential industrial applications of *T. versicolor* and its differences from other fungi at the RNA level, transcriptomic analyses are needed to complement the knowledge of the *T. versicolor* proteome and genome (Mahajan and Master 2010; Rytioja *et al.* 2014).

Previous transcriptomic studies have mainly focused on the white rot fungi *P. chrysosporium* and *Ceriporiopsis subvermispora* and the brown rot fungus *Postia placenta* (Martinez *et al.* 2009; Wymelenberg *et al.* 2009; Fernandez-Fueyo *et al.* 2012). Recent studies related to transcriptomic analysis of other white rot fungi were performed to evaluate the degrading mechanism of white rot fungi (Couger *et al.*2015; Munir *et al.* 2016). However, changes in the transcriptome of *T. versicolor* during growth on a wood substrate have not been studied.

Poplars are fast-growing trees that have been extensively cultivated and widely used in pulping in China (Georgieva *et al.* 2008; Singh *et al.* 2013). To elucidate the mechanism of *T. versicolor* degradation of lignocellulose in poplar wood, and to discover the key genes and enzymes that regulate this process, this study investigated how the transcriptome of *T. versicolor* changed when it was grown on a poplar medium *versus* on a glucose medium. This analysis revealed substantial differences in the expression of the genes involved in lignocellulose degradation and illustrated the diversity of the degradation mechanisms of *T. versicolor*.

EXPERIMENTAL

Fungal Strain and Culture Conditions

T. versicolor was obtained from the China Center of Industrial Culture Collection (CICC) 14001 and determined by ITS sequencing. Wood samples were prepared using a blender to grind the poplar clone 107 (*Populus* × *euramericana* 'Neva'). Air-dried samples were sifted through sieves with 0.425 mm and 0.250 mm openings. Fibers that passed through the 0.425 mm sieve but were retained by the 0.250 mm sieve were recovered. Wood samples of 2.0 g were transferred to 500 mL jars, which were steam-sterilized for 60 min. This culture medium was inoculated with one of the 20 mm circular mycelium (without agar) taken from the growing edge of the *T. versicolor* grown on potato dextrose agar (PDA; 20% potato, 2% dextrose and 2% agar, w/v), by even mixing of the wood sample and mycelium.

A microporous film (0.5 mm) was placed on the mixed cultures, and they were incubated under the film at stationary conditions at 25 °C. The cultures remained at this condition until the hyphae of *T. versicolor* penetrated though the microporous film and the diameter of the mycelial mat covered the jars (7 days), at which point the central 30 mm of growth was harvested. Because the culture was initially grown on PDA agar plugs, the transcriptome of *T. versicolor* grown on poplar wood was compared with its transcriptome when grown on PDA medium (7 days). Solid PDA medium (200 g potato extract, 20 g glucose, 20 g agar, 3 g KH_2PO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 1 L in H_2O) was prepared in 500 mL jars and autoclaved at 121 °C for 20 min. Three biological replica samples of *T. versicolor* were provided growing on the poplar wood and PDA medium, respectively. Each biological replica sample of *T. versicolor* (growing on the poplar wood and PDA medium) was prepared for quantitative real-time PCR. Another three replica samples of *T. versicolor* were pooled together growing on the poplar wood and PDA medium. Excess liquid was removed using Miracloth, and mycelia (without agar) were flash frozen in liquid nitrogen and stored at -80 °C for RNA sequencing.

RNA Extraction and Sequencing

Total RNA was isolated from the *T. versicolor* growing on a glucose carbon medium and on poplar medium. RNA samples were first treated with RNAse-free DNAse I to remove contaminating DNA. mRNA was enriched using oligo(dT) magnetic beads. After adding fragmentation buffer (40 mMTris-OAc, 100 mMKOAc, 30 mM $Mg(OAc)_2$, pH 8.3), the mRNA was fragmented into short fragments (about 200 bp). Next, the first strand of cDNA was synthesized using random hexamer-primers. Buffer, dNTPs, RNase H, and DNA polymerase I were added to synthesize the second cDNA strand. The double-stranded cDNA was purified using magnetic beads. End reparation and 3'-end single nucleotide A (adenine) addition was then performed. Finally, sequencing adaptors were ligated to the fragments.

The fragments were enriched by PCR amplification. During the QC step, an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and ABI Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) were used to perform quality and to quantify the sample library. The library was then ready for sequencing using an Illumina HiSeqTM 2000 (GATC Biotech, Konstanz, Germany) or another sequencer if necessary (Firon *et al.* 2013).

Reads Mapping and Annotations

Reads were mapped in relation to the *T. versicolor* genome (EBI, http://www.ebi.ac.uk/ena/data/view/PRJNA56097).Differential gene expression levels were calculated using Cufflinks software (v.2.0.1; UC Berkeley, Berkeley, CA, USA). Differentially expressed genes (DEGs), including Pfam, were annotated using the *T. versicolor* genome database (ncbi.nlm.nih.gov). Gene expression levels were normalized and reported as fragments per kilobase of exon per million mapped reads (FPKM). Genes were considered as induced or repressed only if their $\log_2$ fold change was >2 or <2, respectively, and their *P*-value was <0.001. For each differentially expressed gene, up to date gene ontology (GO) annotations were obtained using Blast2GOv.2.3.5 (https://www.blast2go.com/) with the default parameters. The "GO slim" option was employed to reduce the number of functional classes. Gene ontology slim annotation results were then used as queries against the AgBase database to classify genes into to the three main categories (molecular function, biological process, and cellular component) and to be further assigned to secondary categories (Ries *et al*. 2013).

Gene Ontology and KEGG Pathway Enrichment Analysis of Differentially Expressed Genes

Gene ontology is an international standardized gene function classification system that offers a dynamic, updated, and controlled vocabulary and strictly defined concepts to comprehensively describe the properties of genes and their products in any organism. Gene ontology (GO) covers three domains: cellular components, molecular functions, and biological processes. The basic unit of GO is GO-term. Every GO-term belongs to a type of ontology. GO enrichment analysis provides all the GO terms that are significantly enriched in DEGs compared with the genome background and filters the DEGs that correspond to biological functions. This method maps all DEGs to GO terms in the database (www.geneontology.org), calculates gene numbers for every term, and uses a hyper-geometric test to find significantly enriched GO terms in DEGs compared with the genome background. Genes with similar biological functions usually interact with each other.

Pathway-based analysis further elucidates the biological functions of genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is the major public database used for pathway analysis. Pathway enrichment analysis identifies significantly enriched metabolic pathways, or signal transduction pathways, in DEGs compared with the whole genome background. The formula used for KEGG pathway analysis is the same formula used in GO analysis (Hadibarata *et al*.2012; Khudhair and Salim 2012).

Quantitative Real-Time PCR (qRT-PCR)

To confirm the quality of the differential gene expression (DGEs) data, real time qRT-PCR was carried out. Twelve genes implicated in lignocellulose degradation were randomly selected for verification. The total RNA was extracted from *T. versicolor* grown on poplar or glucose medium using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Three biological replica samples of *T. versicolor* were provided. The first strand of cDNA was synthesized from 1 µg of total RNA with a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. Specific primers for the twelve genes used to validate the RNA-seq results and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed for real-time PCR amplification. cDNA templates derived from

poplar and glucose media were used for real-time PCR amplification using a SYBR Green kit (Takara, Dalian, China) and a 7500 Real-Time PCR System (Applied Biosystems). All reactions were run in triplicate. The threshold cycle (CT) was determined using the default threshold settings. The ΔΔCt method was employed to calculate the relative expression levels of Dim-2 and DmtA, using GAPDHas the internal control for each sample. All data are presented as the mean (±S.E.) of three replicates (Livak and Schmittgen 2001). All of the primers used are listed in Supplemental Table S1.

RESULTS AND DISCUSSION

Analysis of RNA-seq Datasets

To identify genes that are differentially expressed between the two carbon media, the expression levels of target genes were calculated by normalizing the number of unambiguous tags in each library to reads per kb per million reads (RPKM). To determine if the different carbon media could cause significant changes in gene expression, differential DGE analysis of the two groups was performed. A false discovery rate (FDR) ≤0.001 and an absolute value of $\log_2$Ratio≥1 were adopted as the thresholds to determine significant differences in gene expression.

Sequencing of cDNA samples of *T. versicolor* on glucose and poplar medium yielded 57.3 and 57.1 million reads, respectively, and exceeded 2.81and 2.80 billion nucleotides of cDNA per sample, respectively. Good quality scores of the reads were obtained, and the Q20 percentages (sequencing error rate lower than 1.05%) were 97.56% and 97.04%, respectively. N percentages were all approximately 0.00% (Supplemental Figs.1 and 2), and 65.84% and 66.63% of reads that were generated for *T. versicolor* growing on glucose medium (the control sample) and poplar medium (the treatment sample) could be mapped to the genome, respectively (Table1). There were 853 genes differentially expressed (FDR≤0.001 and |$\log_2$Ratio|≥1) between the poplar and glucose medium. A total of 360 genes were up-regulated on poplar wood, and 493 genes were down-regulated (Supplemental Table S2).

Table 1. Summary of RNA-Seq Data and Mapping to Reference Genome

Map to Gene	Control (glucose carbon source)		Treatment (poplar carbon source)	
	No. of Reads	Percentage (%)	No. of Reads	Percentage (%)
Total Reads	5,739,925	100	5,714,357	100
Total Base Pairs	281,256,325	100	280,003,493	100
Total Mapped Reads	3,779,343	65.84	3,807,352	66.63
Perfect Match	2,067,175	36.01	2,040,938	35.72
<=2bp Mismatch	1,712,168	62.97	1,766,414	30.91
Unique Match	3,614,612	62.97	3,679,844	64.40
Multi-position Match	164,731	2.87	127,508	2.23
Total Unmapped Reads	1,960,582	34.16	1,907,005	33.37

To assess how well the DEG data could be compared between growth conditions, the distributions of sequencing coverage were analyzed. The distribution of sequencing coverage was similar, ensuring that comparisons of sequencing data between the control (glucose medium) and the treatment (poplar medium) were valid (Fig. 1).

Gene Ontology Enriched Analysis

To better understand the variety of genes involved in *T. versicolor* growth on poplar medium *versus* on glucose, functional classes of differentially expressed genes were determined using GO analysis.

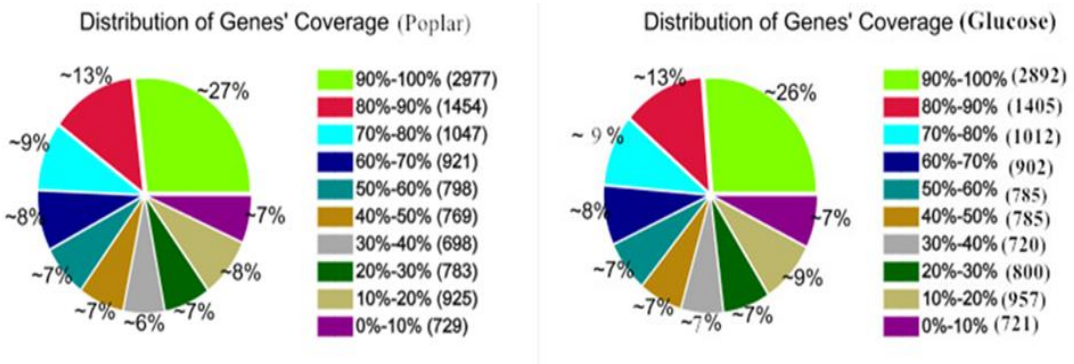

Fig. 1. Distribution of gene coverage in each RNA-Seq library

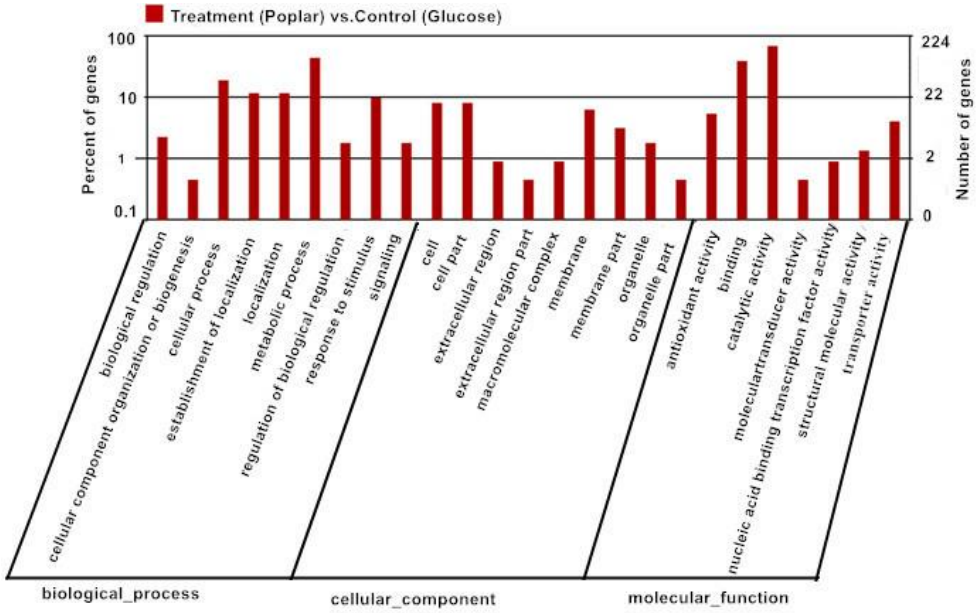

Fig. 2. GO functional classification of DEGs. The DEGs were placed into the three main GO categories: biological process, cellular component, and molecular function

The DEGs were placed into one of the three main GO categories: biological process, cellular component, and molecular function. Blast2 GO software was used to determine the functions of the differentially expressed genes and novel transcripts. Generally, more genes were enriched into the biological process and molecular function categories than into the cellular component category. Furthermore, GO functional classification of the 853 DEGs revealed the potential functions of the genes whose expression changed when the fungus was cultured on poplar wood. These results showed that the 853 DEGs were categorized into 25 functional groups within all three of the main GO domains: biological processes (9), cellular components (9), and molecular functions (7). Metabolic process, cell, and catalytic activity were the most common annotation terms in each of the three GO term categories (Fig. 2). Among these groups, a higher

percentage of genes was found in the categories of metabolic processes, cellular processes, catalytic activity, and binding (Fig.3). The GO functional analysis indicated that multiple biological processes were involved in the response to different carbon sources.

Lignin Degradation

Fungal degradation of lignin is mediated by the ligninolytic system of enzymes, which includes PODs, multicopper oxidase (MCO), H_2O_2 generating-enzymes, and other related enzymes. The results clearly confirmed that most of the ligninolytic enzymes were up-regulated when *T. versicolor* was grown on poplar medium compared with glucose medium (FDR≤0.001 AND $|\log_2 Ratio| \geq 1$) (Table 2).

There was significant expression of genes encoding lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) in the *T. versicolor* transcriptome (Supplemental Table S2). One peroxidase gene (EIW63170) was significantly up-regulated (with a 5.72-fold induction) on the poplar medium. This gene is possibly important for the degradation of aromatic compounds (Table 2). LiP-encoding genes can catalyze the C_α-C_β cleavage of propyl side chains in lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to aldehydes or ketones, oxidation of phenols, and aromatic ring cleavage in nonphenolic lignin model compounds (Hammel and Cullen 2008). Two MnP-encoding genes were up-regulated on the poplar wood compared to glucose medium. Three genes (mp, EIW62513; mp EIW55327; mp_2, and EIW55249) encoding manganese peroxidases were down-regulated on poplar medium relative to glucose medium. Only one VP gene was expressed in *T. versicolor*, and it was significantly down-regulated by growth on poplar wood. In addition, *T. versicolor* also expressed one dye-decolorizing peroxidase (DyP) gene, and two heme peroxides genes, but they were not significantly different across growth conditions (Supplemental Table S2). *T. versicolor* multicopper (MCO)-encoding genes are implicated in lignin degradation. Only one laccase-encoding gene (EIW64151) was significantly down-regulated on the poplar medium (Table 2).

In addition, several enzymes have been hypothesized to provide the H_2O_2 that is necessary for LiP and MnP activity, including GMC oxidoreductases, alcohol oxidase, aryl-alcohol oxidase, pyranose 2-oxidase, copper radical oxidase, and glyoxal oxidase. Many genes that encode enzymes, including aromatic compound dioxygenase, pyranose 2-oxidase, GMC oxidoreductase, and alcohol dehydrogenase, significantly increased growth on the poplar medium (Table 2). In addition, two alcohol oxidase genes were up-regulated, which potentially supported Fenton chemistry through the generation of extracellular H_2O_2. Peroxide and free-radical generation are considered key components of ligninolysis. In this study, the expression of other genes that may be related to lignin degradation were detected, including a family of lipases, glucosides, proteases, quinone reductases, and nitrate reductases (Supplemental Table S2). Lipases, oxidases, and esterases along with LiP that may enter the cell wall and break the lignin barrier (Zeng *et al.* 2014). Glucosides were employed as effective precursors in lignin biosynthesis (Matsui *et al.* 2000).

Proteases in the presence of glucose or wood that are known to promote or suppress LiP activity and lignin degradation (Dass *et al.* 1995). Quinone reductases are capable of reducing substrate during oxidation of lignin model compounds (Deller *et al.* 2008). Nitrate reductases often located in the periplasmic compartment conducting lignin metabolism (Baek *et al.* 2003).

Table 2. Genes Encoding Lignin- or Aromatic Compound-Degrading in *T. versicolor*

Function	Predicted activity	ID	RPKMLog$_2$Poplar	RPKMLog$_2$Glucose	P/G
PODs	Peroxidase	EIW63170	7.63	1.90	5.72
	AA2, Lignin peroxidase	EIW53446	8.22	3.50	4.72
		EIW63274	6.36	1.90	4.47
		P20013	10.94	9.11	1.83
		EIW53199	193.10	12.34	3.97
		EIW52610	228.65	31.71	2.85
		CAA53333	218.69	41.26	2.41
		EIW53370	386.51	113.55	1.77
		EIW53190	190.48	84.91	1.17
	AA2, Manganase peroxidase	EIW63279	9.17	6.55	2.62
		EIW55302	7.95	6.05	1.90
		EIW62513	38.16	367.25	-3.27
		EIW55327	1.30	12.28	-3.23
		EIW55249	27.32	122.42	-2.16
VP	AA2,Versatile peroxidase	EIW53183	1.97	10.97	-2.48
MCO	AA2 ,Laccase	EIW64151	2.92	5.21	-2.30
	Fet3	EIW55589	3.22	9.50	-1.56
H_2O_2 generation	AA2, Aromatic compound dioxygenase	EIW61517	8.53	7.40	1.14
	AA3, Pyranose 2-oxidase	EIW52665	11.18	9.95	1.22
	Alcohol oxidase	EIW59672	8.56	7.37	1.19
		EIW62184	12.05	10.97	1.08
		EIW59941	3.17	7.07	-1.00
	GMC oxidoreductase	EIW61651	5.71	2.89	2.82
	Alcohol dehydrogenase	EIW53227	6.07	5.48	1.96
	Aryl-alcohol oxidase	EIW51595	10.82	35.80	-1.72
Lipase	Lipase	EIW57858	8.92	7.40	1.52
	Carotenoid ester lipase	EIW58887	6.66	4.21	2.45
		EIW58852	7.28	5.21	2.08
		EIW58851	18.45	7.35	1.33
		EIW58877	7.79	23.76	-1.61
Protease	β-glucosidase	EIW64808	8.46	6.45	2.01
	Acid protease	EIW59393	5.48	2.46	3.01
		EIW59132	9.06	6.97	2.10

		EIW59394	5.83	3.96	1.87
		EIW62808	4.22	2.57	1.66
OXO	Oxalate decarboxylase	EIW54513	7.05	51.59	-2.87
		EIW56396	17.55	54.30	-1.63
QRD	Quinone reductase	EIW64611	6.97	0.99	2.81
		EIW57190	7.30	3.10	1.63
	epoxide hydrolase	EIW59656	0.58	6.41	-3.46
		EIW59618	26.84	10.37	1.37
P450	CyP450 monooxygenase	EIW58876	1.08	4.45	-3.37
	CytochromeP450 (Max-expression)	EIW61050	93.62	4.58	4.35

Among these genes, four lipases, one β-glucosidase, four acid proteases, and one quinone reductase were significantly up-regulated by growth on the poplar medium (Supplemental Table S2). In addition to lignin degradation, two genes (EIW59656 and EIW59618) encoding epoxide hydrolases in *T. versicolor* degraded polycyclic aromatics (Table 2). The *T. versicolor* genome encoded a large repertoire of P450 genes. These genes have various roles in secondary metabolism, and are thought to be involved in the degradation of lignin and other xenobiotic compounds. This study detected significant expression of 30 cytochrome P450-encoding genes (FDR ≤ 0.001 and $|\log_2 \text{Ratio}| \geq 1$). Of these, 19 were up-regulated on the poplar medium. The largest increase in P450-encoding gene expression was a 4.35-fold increase (RPKM Log_2 Poplar/Glucose) in gene EIW61050 (Table 2).

Cellulose and Hemicellulose Degradation

In contrast to oxidative systems, cellulose degradation is mediated by at least three concerted cellulolytic activities: endoglucanases, cellobiohydrolases, and β-glucosidases. As predicted, the mRNAs of these genes were detected in *T. versicolor* growing on the poplar medium, and most cellulase-encoding genes were down-regulated compared to glucose medium (Table 3). *T. versicolor* is a kind of white-rot fungi that degrades much more lignin than cellulose and hemicelluloses. Therefore, these genes of upregulation on lignin degradation and downregulation cellulose degradation were observed.

Seven gene-encoding endoglucanases (endo-1,4-β-glucanase), which break cellulose internal glycosidic bonds, were detected in the *T. versicolor* transcriptome. Six of these were down-regulated on the poplar medium. There was a significant expression of two genes (EIW63632 and EIW60068) that belong to a third family of β-glucosidases, which cleave cellobiose by hydrolyzing it to glucose. These genes were also down-regulated on poplar medium.

In addition to the three categories of hydrolytic enzymes mentioned above, *T. versicolor* also expressed an extracellular oxidative enzyme called cellobiose dehydrogenase (CDH). It might be vital for limiting the inhibitory effects of cellobiose and other oligosaccharide hydrolysis products. One cellobiose dehydrogenase gene (EIW55840) was detected in the transcriptome, and was found to be down-regulated (-1.35 folds) on the poplar medium. Besides typical cellulases, other enzymes that promote cellulose degradation by helping cellulase degrade cellulose were also detected. Transcripts of three genes predicted to encode GH61 proteins, which were -3.19, -1.37, and -1.17 folds, decreased on poplar relative to glucose.

Similar to the genes with cellulolytic activity, transcripts of genes that encode main chain hemicellulases were also detected. xylanases can degrade the linear polysaccharide β-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. Three xylanase genes were down-regulated (-2.59, -1.75, and -1.49 folds) on poplar wood. Another enzyme, mannanase, which hydrolyzes terminal, non-reducing β-D-mannose residues in β-D-mannosides, was also down-regulated on poplar wood. The expression of eleven complex and diverse hemicellulases (Table 3) were detected; these coordinate their activities with other hemicellulase groups to hydrolyze glucoside residues. From this, it was inferred that there was no single enzyme that could individually degrade hemicellulose.

In addition to hemicellulose-specific glycoside hydrolases, transcripts were detected that were predicted to encode carbohydrate esterase family members (CE). Two

genes (EIW55232 and EIW55233) encoding feruloyl esterases (CE1), which act on carboxylic ester bonds, were up-regulated on poplar wood (Table 3). Transcripts encoding carboxyl esterase (CE1), acetyl xylanesterases (CE1), and chitin deacetylase (CE4) changed 1.20, -2.69, and -1.11 folds on the poplar medium, respectively (Table 3). Carboxyl esterase and feruloyl esterase (CE1) are involved in several degradation processes, including aminobenzoate degradation and toluene degradation.

Lyase and GH28 catalyze the breakdown of various chemical bonds by means of hydrolysis and oxidation, and often form new double bonds or a new ring structure, especially during breakdown of pectin in wood. Specifically, the carboxy-lyases can cleave C–C bonds. Evidence was found of the expression of two such genes in *T. versicolor*, and it was observed that they were down-regulated on poplar medium.

Chitin is a component of the cell walls of fungi. Like cellulose, chitin is an abundant biopolymer that is relatively resistant to degradation. The expression of four chitinase-encoding genes, which may be involved in cell wall morphogenesis, was detected. Four genes (EIW52530, EIW59804, EIW60464, and EIW54971) encoding glycosyl transferases, which catalyze the transfer of activated carbohydrate moieties from donor molecules to an acceptor carbohydrate, nucleic acid, lipid, or protein molecules, were also detected in the *T. versicolor* transcriptome (Table 3; Supplemental Table S2).

KEGG Pathway Enrichment Analysis of DEGs

To further assess the functions of genes that were differentially expressed between the two different carbon sources, the KEGG database was used to analyze gene interaction pathways. The results of KEGG pathway enrichment analysis are reported in Supplemental Table S3. One-hundred and ninety pathways were found in the *T. versicolor* that was cultivated on the poplar medium, and 22 of these pathways were connected with degradation, including 13 pathways that were significantly different between growth conditions (P-value<0.0001) (Table 4). Aminobenzoate degradation was the most significantly enriched pathway, including 63 regulating genes (Table 4, Fig. 3). Among these genes, 36 genes were up-regulated on poplar wood, including 19 cyp450 genes, three carotenoid ester lipases (EIW58887, EIW58851, and EIW58851), one carboxylesterase (EIW54801), and four esterases (EIW63971, EIW59789, EIW51601, and EIW59808) (Supplemental Fig 3). In this data set, four pathways important for aromatic compound degradation (Table 4) were found to be enriched. These pathways contained genes that were also implicated in the regulation of aminobenzoate degradation.

Validation of the Gene Expression Profiles by qRT-PCR

To verify the quantitative results of the RNA-Seq experiments, 12 genes were selected for analysis with qRT-PCR, based on their expression levels in RNA-Seq data and importance in regulation of lignocellulose degradation. Seven genes that were significantly down-regulated (EIW57149, EIW62722, EIW59656, EIW62376, EIW57875, EIW57445, and EIW60188), and five genes that were significantly up-regulated (EIW63279, EIW61651, EIW55930, EIW63274, and EIW63170) (Supplemental Table S2) were chosen. The results (Fig. 4) confirmed that the expression profiles of genes from the two carbon sources (poplar *vs.* glucose) were similar as determined by RNA-Seq transcriptomic analysis and as by qRT-PCR. This supports the validity of the transcriptomics results.

Table 3. Genes Encoding Enzymes that Degrade or Oxidize Cellulose, Hemicellulose, and Other Carbohydrate Compounds in *T. versicolor*

Function	Predicted activity	ID	RPKMLog$_2$Poplar	RPKMLog$_2$Glucose	P/G
Cellulase	GH5, endoglucanase	EIW62496	5.83	2.75	3.08*
	(endo-β-1,4-glucanase)	EIW54636	3.81	5.76	-1.95*
		EIW62722	4.75	8.25	-3.50*
		EIW62895	7.35	10.21	-2.86*
		EIW53033	8.24	6.38	-1.53*
		EIW57949	7.63	9.56	-1.32*
		EIW55688	4.85	8.92	-1.29*
	GH6,cellobiohydrolase I	EIW57445	7.42	8.92	-1.50*
		EIW64214	11.18	12.62	-1.44*
		EIW64126	9.25	10.60	-1.35*
		EIW58677	9.80	8.29	1.51*
	GH7,cellobiohydrolase II	EIW60188	10.69	11.92	-1.23*
	CDH,cellobiose dehydrogenase	EIW55840	6.06	7.41	-1.35*
	GH3, glucan,exo-β-1,3- glucosidase	EIW63632	6.02	8.10	-2.09*
		EIW60068	6.85	7.91	-1.06*
	GH61,glycoside hydrolase	EIW65011	2.90	6.09	-3.19*
		EIW58784	4.05	5.42	-1.37*
		EIW61483	10.37	11.54	-1.17*
	GH79,glycoside hydrolase	EIW61463	2.87	5.74	-2.88*
		EIW56405	8.86	6.99	1.87*
		EIW62362	8.20	7.16	1.05*
Hemi-	GH10, endo-1,4-β-xylanase	EIW62376	7.44	10.02	-2.59*
cellulase		EIW58084	23.34	78.71	-1.75*
		EIW54084	240.80	674.35	-1.49*
	GH10,Mannanase	EIW63496	7.64	10.03	-2.38*
	GH3,N-acetylhexosaminidase	EIW52408	4.94	3.50	1.44*
	GH3,β-D-xylosidase	EIW63936	5.81	4.49	1.32*
	GH4, α-galactosidase	EIW63555	6.81	9.60	-2.78*
	GH16,glycoside hydrolase	EIW64599	7.94	6.69	1.25*
	GH15,glucoamylase	EIW58421	5.13	8.25	-3.12*
	GH20, hexosaminidase	EIW63852	3.78	2.58	1.20*
	GH25,glycoside hydrolase	EIW55257	6.52	8.56	-2.04*
	GH28,endo-polygalacturonase	EIW55930	4.38	1.25	3.13*

	GH43,endo-1,5-a-L-arabinosidase	EIW59347	4.91	5.94	-1.03*
	GH98,endo-1,4-β-galactosidase	EIW61652	7.42	9.04	-1.63*
Chitinases	GH18,Chitinase	EIW54504	6.89	4.36	2.53*
		EIW58781	7.50	6.23	1.27*
		EIW57149	0.27	4.33	-4.06*
		EIW58782	36.79	0.47	-1.10*
Cleavec-c bonds	CE1,feruloyl esterase	EIW55232	2.88	0.90	1.98*
		EIW55233	6.18	4.87	1.32*
	CE1,carboxyl esterase	EIW54801	5.03	3.84	1.20*
	CE1,acetyl xylan esterase	EIW63589	6.14	8.84	-2.69*
	CE4,chitin deacetylase	EIW61936	6.47	7.58	-1.11*
	GT15，α-1,2-mannosyltransferase	EIW52530	4.63	6.37	-1.73*
		EIW59804	4.28	5.50	-1.21*
Pectin	GT15，glycosyl transferase	EIW60464	8.90	7.66	1.24*
breakdown	O-methyltransferase	EIW54971	7.93	6.87	1.06*
	dihydrolipoamidesuccinyltransferase	EIW56277	6.39	7.64	-1.25*
	GH28,rhamnogalacturonase	EIW62809	6.90	8.04	-1.14*
H_2O_2		EIW59045	7.49	5.70	1.79*
Generation	isocitratelyase	EIW56396	2.82	5.69	-2.87*
	oxalate decarboxylase	EIW54513	5.23	6.92	-1.70*
	oxalate decarboxylase/oxidase	EIW55840	66.73	169.78	-1.35*
Cofactor	cellobiose dehydrogenase	EIW56720	4.13	4.17	-1.06*
	amine oxidase	EIW54618	2.68	5.76	-2.32*
	dehydrogenase	EIW55702	1.23	5.22	-3.98*
polysaccharide	oxidoreductases	EIW65088	2.25	5.29	-3.04*
degradation	2OG-Fe-IIoxygenase oxidoreductase	EIW62837	1.34	4.74	-3.40*
	Flavin oxidoreductase/NADH	EIW53457	4.86	2.93	-2.21*
	aldo/keto reductase	EIW51597	71.52	270.87	-1.92*
		EIW62562	4.50	5.34	1.58*
		EIW56834	6.44	8.13	1.10*
		EIW60601	9.87	5.39	1.74*
	sorbose reductase	EIW63330	3.14	5.00	-2.25*
	D-xylose reductase	EIW54430	4.13	4.12	-1.63*
	glyoxylate dehydrogenase				

* Significant difference (P-value < 0.001)

Table 4. Percent of DEGs with Pathway Annotation for Mapped Genes and its Pathway ID

Pathway	DEGs withpathway annotation	*P*-value	Pathway ID
Aminobenzoate degradation	15.00	2.84E-13*	ko00627
Polycyclic aromatic hydrocarbon degradation	12.14	1.22E-10*	ko00624
Microbial metabolism in diverse environments degradation	23.81	3.41E-09*	ko01120
Bisphenol degradation	13.10	6.49E-09*	ko00363
Limonene and pinene degradation	13.81	3.95E-08*	ko00903
Toluene degradation	4.76	4.25E-08*	ko00623
Diterpenoid biosynthesis	1.90	8.54E-08*	ko00904
Chlorocyclohexane and chlorobenzene degradation	5.00	5.69E-07*	ko00361
Stilbenoid, diarylheptanoid and gingerol biosynthesis	7.38	8.19E-07*	ko00945
Starch and sucrose metabolism	8.10	1.30E-06*	ko00500
Metabolic pathways	43.10	2.15E-06*	ko01100
Biosynthesis of secondary metabolites	23.81	6.36E-06*	ko01110
Tropane, piperidine and pyridine alkaloid biosynthesis	2.86	6.16725E-05*	ko00960

* Significant difference (P-value < 0.0001)

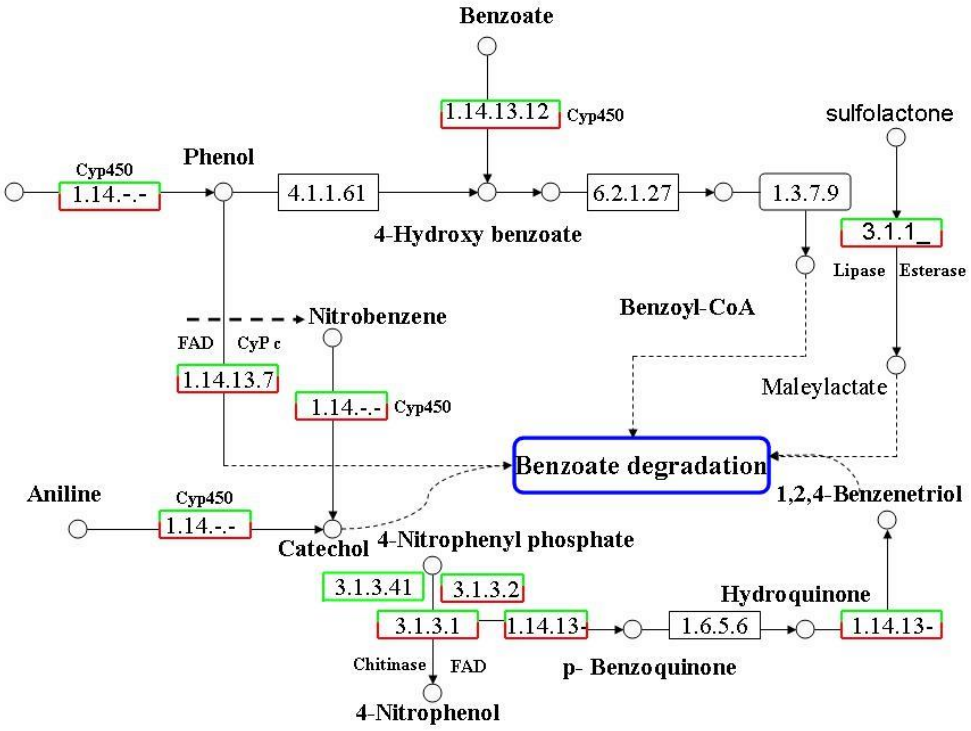

Fig. 3. Simplified aminobenzoate degradation pathways generated by KEGG enrichment analysis of DEGs (up-regulated and down-regulated genes are shown in red and green boxes, respectively. Genes shown in boxes lined with both red and green are annotated by different transcripts, which may be either up-regulated or down-regulated)

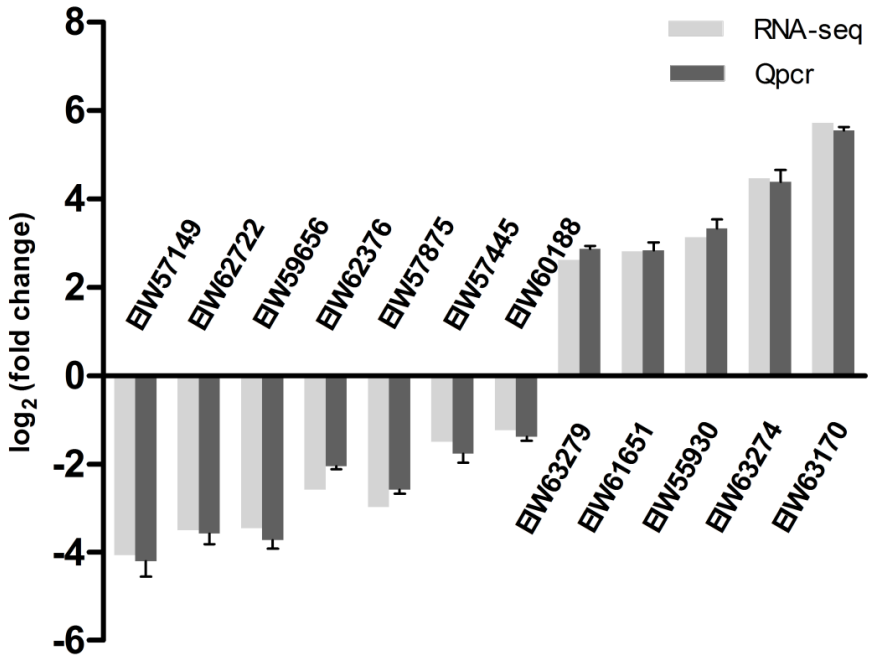

Fig. 4. qRT-PCR validation of DEGs identified in RNA-Seqanalysis. The qRT-PCR data represent the mean ± SE (standard error) of three biological replicates. All primers and gene abbreviations are listed in Supplemental TableS1).

T. versicolor is a member of the order Polyporales with wide industrial applications because it can efficiently degrade plant cell walls, including the recalcitrant cell wall component lignin. *T. versicolor* differed sharply from the model white rot fungi *P. chrysosporium* in terms of the genes that it employed for lignin degradation. Eight genes encoding LiPs were detected in *T. versicolor* and found to be up-regulated on poplar wood; transcripts of only two LiPs, however, were observed in *P. chrysosporium* growing on aspen medium (Martinez *et al.* 2009; Wymelenberg *et al.* 2009; Fernandez-Fueyo *et al.* 2012). This study clearly showed that poplar wood played a vital role in promoting the expression and secretion of LiP during lignin degradation in *T. versicolor*. Maybe *T. versicolor* expressed and secreted extracellular enzymes, such as LiP, more efficiently than in *P. chrysosporium*. Substantial evidence implicated MnP in lignin degradation, but this enzyme cannot directly cleave the dominant nonphenolic structures within lignin. One manganese-repressed peroxidase-encoding gene (EIW62513) was found expressed in *T. versicolor*, though it was significantly decreased (with a -3.27-fold change) on poplar wood (Supplemental Table S2). This further suggested that poplar wood had a positive effect on lignin degradation. Furthermore, only one significant laccase-encoding gene was down-regulated on poplar wood, perhaps because laccase was not essential for fungal wood decay in this experiment (Table 3).

There was a clear increase in the expression of 19 P450s-encoding genes, which are important for aromatic compound degradation, in the *T. versicolor* grown on poplar wood (Supplemental Table S2). Based on this result, it was deduced that the induction of P450 was important for lignin degradation. Overall, the expression patterns observed in *T. versicolor* grown on a complex lignocellulose substrate suggested that there were diverse strategies for lignin degradation, and thus provided clues about different mechanisms of delignification (Subramanian and Yadav 2009; Syed *et al.* 2010; Ichinose 2013).

Overall numbers and family distributions of GH-encoding genes in *T. versicolor* and *P. chrysosporium* were found to be similar, but sharp differences in expression were observed. Six β-1-4-endoglucanases (GH5) encoding-genes were significantly down-regulated in *T. versicolor* grown on poplar medium, while two similar genes were up-regulated in *P. chrysosporium* grown on an aspen-containing medium (Martinez *et al.* 2009; Wymelenberg *et al.* 2009; Fernandez-Fueyo *et al.* 2012). Four GH7-encoding genes in *P. chrysosporium* were significantly up-regulated by growth on wood. In contrast, only one GH7 gene was down-regulated in *T. versicolor* growing on poplar wood. These results indicated that the expression patterns of cellulase vary greatly between species, and may depend on the microorganisms themselves, or their substrates.

The KEGG analysis identified the aminobenzoate degradation pathway as the most enriched pathway for genes that were differentially expressed between glucose and poplar media. This suggested that growth on poplar wood significantly altered *T. versicolor'*s expression of enzymes that hydrolyze methyl and benzoic acid groups. Catechol and benzoate were important products in lignin degradation (Aranda *et al.* 2010; Sari *et al.*2012; Cruz-Morató *et al.* 2013; Anasonye *et al.* 2014), but were also produced during aminobenzoate degradation. From these results it was inferred that lignin degradation may have been related to aminobenzoate degradation or have similar intermediate compounds. In addition to aminobenzoate degradation, there were six significantly enriched pathways that were closely connected to macromolecular compound degradation (Table 4). These seven pathways contained relatively similar genes encoding P450 and esterase enzymes. Maybe the degradation pathways of lignin

and other compounds shared common enzymes, key genes, or recycling mechanisms. Therefore, *T. versicolor* did not only have the ability to degrade lignin, but may have also degraded other pollutants.

In conclusion, this study provided a comprehensive analysis of the *T. versicolor* gene expression profile when cultured on a poplar wood medium. A range of glycoside hydrolases and enzymes with oxidative activity that were involved in the process of lignocellulose degradation were examined. The DEGs and pathways identified in this study could facilitate more detailed investigations into the molecular mechanisms of lignin degradation and provide a foundation for future studies of *T. versicolor*. Furthermore, manipulation of these genes may be a useful tool for strain improvement, and the regulation of enzymatic activity. This study also identified novel putative genes that could not be annotated on the basis of predicted protein sequences. These genes could provide a starting point for the discovery of novel enzymes that could accelerate the performance of lignocellulose-degrading enzymes.

Availability of supporting data: The Illumina sequencing data used in this study are deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE81742 at NCBI.

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