Transcriptome analysis of differential gene expression in *Dichomitus squalens* during interspecific mycelial interactions and the potential link with laccase induction

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ABSTRACT

Interspecific mycelial interactions between white rot fungi are always accompanied by increased production of laccase. In this study, the potential of white rot fungi *Dichomitus squalens* for enhancing laccase production during interaction with two other white rot fungi *Trametes versicolor* or *Pleurotus ostreatus* was identified. To probe the mechanism of laccase induction and the role of laccase played during the combative interaction, we analyzed the laccase induction response to stressful conditions during fungal interaction related to the differential gene expression profile. We further confirmed the expression patterns of 16 selected genes by qRT-PCR analysis. We noted that many differential expression genes (DEGs) encoding proteins were involved in xenobiotics detoxification and ROS generation or reduction, including aldo/keto reductase, glutathione S-transferases, cytochrome P450 enzymes, alcohol oxidases and dehydrogenase, manganese peroxidase and laccase. Furthermore, many DEG-encoding proteins were involved in antagonistic mechanisms of nutrient acquisition and antifungal properties, including glycoside hydrolase, glucanase, chitinase and terpenoid synthases. DEGs analysis effectively revealed that laccase induction was likely caused by protective responses to oxidative stress and nutrient competition during fungal interspecific interaction.

1 **Keywords** white rot fungi · laccase · mycelial interactions · Transcriptome analysis · *Dichomitus*
2 *squalens*

3

4 Interspecific interactions between wood-decaying fungi are always associated with competition
5 for territory and resources to support fungal community development (Boddy, 2000;
6 Heilmann-Clausen and Boddy, 2005; Wells and Boddy, 2002). When niches of different species
7 overlap, a broad array of antagonistic responses can be triggered in the interaction zone where
8 mycelia contact occurs. These responses, including changes in mycelial morphology, production
9 of extracellular enzymes and secretion of secondary metabolites (Boddy, 2000; Eyre et al., 2010),
10 play an important role in intensifying the attack or defense mechanism and nutrient uptake to gain
11 advantage over the competitor (Arfi et al., 2013; Eyre et al., 2010).

12 It has been reported that a range of chemicals, such as alcohols, aldehydes, ketones, terpenes,
13 aromatic compounds and reactive oxygen species (ROS) are produced under antagonism within
14 interaction mycelia (Arfi et al., 2013; Evans et al., 2008), leading to the up-regulation of many
15 oxidative enzymes (Chi et al., 2007; Ferreira Gregorio et al., 2006; Hiscox et al., 2010). Laccase,
16 an enzyme for oxidizing a variety of phenolic and aromatic compounds, plays a defensive role in
17 reducing the oxidative stress caused by oxygen radicals originating from the reaction of toxic
18 molecules (Li et al., 2011; Yang et al., 2012). Furthermore, laccase also synthesized melanin,
19 which is involved in the absorption of toxic compounds and oxygen radicals to protect hyphae
20 against interspecific oxidative stress (Eisenman and Casadevall, 2012; Eisenman et al., 2007;
21 Nosanchuk JD, 2003). Many studies have shown that laccase plays a defensive role against
22 stressful conditions (Cho et al., 2009; Mayer and Staples, 2002; Piscitelli et al., 2011; Zhu and
23 Williamson, 2004), and laccase activity increased during interactions among many white-rot fungi
24 (Baldrian, 2004; Chi et al., 2007; Ferreira Gregorio et al., 2006; Flores et al., 2009; Hiscox et al.,
25 2010; Kuhar et al., 2015). Thus, laccase might be generally involved in the combative response of
26 different white-rot fungi to interspecific interactions. Moreover, increased laccase activity during
27 mycelial interaction also implied intensive nutrient competition (Hiscox et al., 2010). Based on
28 direct combative interactions between mycelia to defend or occupy resources for their own growth
29 (Boddy, 2000), a series of antagonistic metabolites were up-regulated, especially toxic or

antifungal compounds, which were likely induced by oxidative stress (El Ariebe et al., 2016; Peiris et al., 2007).

Our preliminary study showed that the laccase activity could be highly increased during the co-culture of *Dichomitus squalens* with two other white rot fungi *Trametes versicolor* and *Pleurotus ostreatus*. Considering that laccase is the most important oxidative enzyme secreted from white rot fungi, many studies focused on the laccase induction particularly in the mycelial interaction of different fungi (Baldrian, 2004; Flores et al., 2009; Kuhar et al., 2015; Wei et al., 2010), but lacking further exploration on the molecular mechanism of interspecific interaction and laccase induction among different fungi. As oxidative stress can occur in the interaction region of different fungi (Silar, 2005), and fungal laccase can be involved in the defense response to oxidative stress, which is also commonly accompanied by the overexpression of a large amount of other antioxidative enzymes (Jaszek et al., 2006; Yang et al., 2012). Thus, in this study, we performed a **transcriptional analysis** of gene expression changes in *D. squalens* under the interaction with two wood-decaying fungi *T. versicolor* and *P. ostreatus* to investigate the mechanism of fungal competitive interaction, and focus on the potential role including resistance against oxidative stress that laccase played during the interaction of *D. squalens* with other two competitors based on analysis of transcriptomic level.

MATERIALS AND METHODS

Strains and culture

Strains of *T. versicolor* and *P. ostreatus* were from the Biological Resource Center, NITE (NBRC), the strain number is NBRC30388 for *T. versicolor* and NBTC30776 for *P. ostreatus*. *D. squalens* was from the Deutsche Sammlung von Microorganismen and Zellkulturen (DSMZ), and the strain number is DSMZ9615. Strains were maintained on potato dextrose agar (PDA) slants and stored at 4°C. Before use, the stored fungi were inoculated onto the newly prepared PDA plates at 28°C for pre-culturing.

Co-culture growth conditions

1 A paired culture of *D. squalens* with *T. versicolor* or *P. ostreatus* was grown on Sc medium
 2 (Flundas and Hibbett, 2012), consisting of 10g/liter glucose, 1.5g/liter L-asparagine, 0.12mg/liter
 3 thiamine dichloride, 0.46g /L KH_2PO_4 , 1g/L K_2HPO_4 , 0.5g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 5 mg/L $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$,
 4 0.06 mg/L HBO_3 , 0.04 mg/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.2 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$,
 5 0.1 mg/L $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.4 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.2 mg/L $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 20g/L Agar. Co-culture
 6 experiments were inoculated with two plugs ($\Phi 7$ mm) of the appropriate fungal strains from
 7 pre-culture on opposite sides of a 90 mm petri dish containing Sc agar, and the two plugs were
 8 kept at a distance of 5 cm. The pure culture of Ds was set as control. All treatments were made
 9 with three replicates. After incubation for 9 days at 28°C, three plugs ($3 \times \Phi 5$ mm) taken from the
 10 plate at isolates and interaction zones were immersed in 0.1 M acetic acid buffer (pH 4.8) for 6
 11 hours at 4°C. The crude enzyme samples were centrifuged at a speed of 12000 rpm/min for 2 min
 12 at 4°C for laccase activity determination.

13

14 **NBT staining assay**

15 To determine ROS in the interaction zone and single species zone of each plate, the nitroblue
 16 tetrazolium (NBT) reduction assay was performed using a 0.3 mM NBT aqueous solution with 0.3
 17 mM NADPH (Lara et al., 2003). Mycelia were flooded with stain solution and incubated at room
 18 temperature with gentle rotation. Photographs were taken with a Nikon Coolpix camera to show
 19 purple coloration resulting from the reduction of NBT by ROS after 30 min staining.

20

21 **RNA extraction, library construction and sequencing**

22 The mycelium from single culture (*D. squalens* growing alone only) and two co-cultures DsTv
 23 (interaction zone of *D. squalens* and *T. versicolor*) and DsPo (interaction zone of *D. squalens* and
 24 *P. ostreatus*) were used as samples for the RNA extractions, each sample set three biological
 25 replicates for RNA-seq analysis. All samples were ground into a powder in liquid nitrogen using a
 26 mortar and pestle and extracted using the UNIQ-10 Trizol Total RNA Kit according to the
 27 manufacturer's instructions. The concentrations and quality of purified nucleic acids were checked
 28 using the ND-2000 (NanoDrop Technologies) and Agilent 2100 Bioanalyzer, and multiplexed
 29 libraries were constructed and sequenced on an Illumina HiSeq4000. All samples displayed a

1 260/280 ratio greater than 2.0 and RNA integrity numbers (RIN) ≥ 8.0 .

2 To obtain an overview of the gene expression profiles of *D. squalens* during interaction with
3 *T. versicolor* or *P. ostreatus*, a RNA-seq transcriptome library was prepared following the
4 instructions for the TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA) using 5
5 μg of total RNA. The mRNA was isolated according to the polyA selection method using oligo(dT)
6 beads and fragmented in fragmentation buffer. The double-stranded cDNA is then synthesized
7 using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer
8 primers (Illumina). The synthesized cDNA is subjected to end-repair, phosphorylation and 'A'
9 base addition, according to Illumina's library construction protocol. Libraries were size-selected
10 for cDNA target fragments of 200–300 bp on 2% Low Range Ultra Agarose and PCR amplified
11 using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantification by TBS380, a
12 paired-end RNA-seq sequencing library was sequenced with Illumina HiSeq4000 (1 $\times$ 51 bp read
13 length). The reference genome of *D. squalens* was download in NCBI
14 (<https://www.ncbi.nlm.nih.gov/genome>) (Floudas et al., 2012).

15

16 **Differential expression analysis and functional enrichment**

17 To identify differential expression genes (DEGs) between co-culture and pure culture samples, the
18 expression level of each gene was calculated according to the fragments per kilobase of exon per
19 million mapped reads (FRKM) method. RSEM (<http://deweylab.biostat.wisc.edu/rsem/>) (Li and
20 Dewey, 2011) was used to quantify gene and isoform abundances. The R statistical package
21 software EdgeR (Empirical analysis of Digital Gene Expression in R,
22 <http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>) (Robinson et al., 2010) was
23 utilized for differential expression analysis. In addition, functional-enrichment analysis including
24 GO and KEGG were performed to identify which DEGs were significantly enriched in GO terms
25 and metabolic pathways at the Bonferroni-corrected p-value < 0.05 compared to the
26 whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were
27 performed with Goatools (<https://github.com/tanghaibao/Goatools>) and KOBAS
28 (<http://kobas.cbi.pku.edu.cn/home.do>) (Xie et al., 2011).

29

Gene expression analysis by qRT-PCR

Quantitative real-time PCR was conducted in 96-well plates with a 7500 Real Time PCR System (Applied Biosystems) using a SYBR® Premix Ex Taq™ II Kit (TaKaRa, Japan). Each reaction mixture contained 10-μL volumes: 5 μl SYBR® Premix Ex Taq™ II, 0.2 μl ROX reference dye, 0.4 μl forward primer, 0.4 μl reverse primer, 1 μl cDNA template and 3 μl sterile distilled water. The recommended protocol for PCR was used according to the manual: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Three biological replicates per sample and three technical replicates were conducted for each cDNA template. To normalize the qRT-PCR data, 40S ribosomal protein S24 (DICSQDRAFT_46225), chitinase (DICSQDRAFT_98815) and α-tubulin (DICSQDRAFT_135086) with stable expression in all samples were used as the endogenous reference genes, and data were normalized using the average expression level of the three reference genes. The specific primers of target genes used in this work were listed in Table 1. Quantitative Relative quantification was established using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Assays of Laccase

Enzyme assays of crude enzyme samples were conducted in a 3 ml reaction mixtures according to the method described in Eggert et al.,1996 with slight modification (Eggert et al., 1996). Laccase activity was measured spectrophotometrically by monitoring the oxidation of 500 mM ABTS [2,20-azinobis-(3-ethylbenzthiazoline-6-sulphonate)] (molar extinction coefficient $36,000 \text{ M}^{-1}\text{cm}^{-1}$) buffered with 0.1 M acetic acid buffer (pH 4.8) at 420 nm. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the production of 1 μmol oxidized product per min. All measurements were performed in triplicate.

RESULTS

Laccase activity of *D. Squalens* in response to oxidative stress in mycelial interaction

1 When *D. squalens* (Ds) was co-cultured with *T. versicolor* (Tv) and *P. ostreatus* (Po) in Sc
2 medium, the laccases activity was significantly increased in the interaction zones compared to
3 single culture of *Ds* (Figure 1A), reaching 307.56 U/L in DsTv and 274.50U/L in DsPo, and
4 enhanced changes in laccase activity were as high as 190.04-fold in DsTv and 212.93-fold in
5 DsPo compared to the control. This strong laccase response matched previous findings for *Ds*
6 during interactions with other species, but the extent of increase in laccase activity was more
7 significant than that in other studies based on the different identity of the competitor and culture
8 conditions (Dong et al., 2011; Kannaiyan et al., 2015). Besides, the result showed laccase activity
9 in the interaction zones of DsTv and DsPo were all higher compared to other two single fungi Tv
10 and Po (Figure 1A). We also identified laccase activity neighboring the interaction zone. Although
11 laccase activity declined as distance increased from the interaction zone, it was still higher than
12 that of the *Ds* single culture. The result showed that secondary metabolites produced by
13 interspecific interaction could induce laccase activity. Since laccase production in the self-fungal
14 interaction zone did not increase compared to the control (Figure S6), it is highly possible that
15 interspecific antagonism contributed to the induction of laccase activity.

16 Research has suggested that overproduction of laccase during interspecific interaction is caused
17 by oxidative stress, which involves a series of competitive and defensive mechanisms (Jaszek et
18 al., 2006; Li et al., 2011; Yang et al., 2012). To determine oxidative stress during mycelial
19 interaction, we used Nitroblue tetrazolium (NBT) to stain the mycelium in both interaction zones
20 and single species (Figure 1D and E). Compared to the co-culture without NBT staining (Figure
21 1B and C), no color change was observed in the single species near the plate edge, but purple
22 formazan precipitate formation was obviously observed in both interaction zones of DsTv and
23 DsPo, which could indicate much higher accumulation of ROS during mycelial interactions.

24

25 **RNA-Seq analysis of gene expression**

26 To gain a better understanding of the interaction mechanisms, RNA-Seq analysis was used to
27 study the global gene expression of *Ds* when grown in pure culture and compared to the
28 interaction zone of DsTv and DsPo. Illumina sequencing data was deposited in the NCBI SRA
29 database with accession number SRR5328881. A minimum RPKM (Reads Per Kilo-base per

1 Million) value threshold was set at two-fold to limit the false-positive detection of low-abundance
 2 genes. To validate the technical reproducibility of the RNA-seq experiment, we compared the
 3 expression values of three biological replicates. After sequencing, over 128.88 million raw reads
 4 and 6.57 billion raw bases were obtained in a single sequencing run (Table S5). The raw data was
 5 firstly filtered to obtain high-quality clean data to ensure the success of subsequent analysis.
 6 Finally ,we obtained over 112.36 million clean reads and 5.48 clean bases (Table S5) . According
 7 to the related data (Table S6), reads from Ds transcripts data sets mapped to 86.86% (average
 8 value) of its genes, while reads from DsTv and DsPo transcripts data sets mapped to 14.49% and
 9 15.02% (average value) of Ds genes respectively. Although the mapping rate to Ds reads was low
 10 in two interactions, 98.23% of Ds genes could be detected in both interactions of DsTv and DsPo,
 11 and the total mapping rate was more than 60% when adding the mapping rate to Po and Tv reads.
 12 The raw data quality control (Figure S1-S3) indicated that the sequencing quality of three samples
 13 was high, and the sequencing data can meet the requirements of subsequent analysis.

14

Analysis of differential expression genes (DEGs)

16 Given the results of the RNA-seq data, gene expression with a competitor-to-control ratio of
 17 RPKM higher than 2 or below 0.5 and FDR<0.05 were considered differentially expressed (Figure
 18 S4). The sequences of genes were searched against the NCBI non-redundant sequences (NR)
 19 database using BlastX and setting a cut-off E-value of 10^{-5} . According to the result, a large number
 20 of Ds genes were found to be differentially expressed in the two interactions (DsPo and DsTv)
 21 compared to the control, and both two interactions comprised more up-regulated genes than
 22 down-regulated genes (Figure 2 and Table S1). Of these, 1085 genes were up-regulated and 873
 23 were down-regulated in DsTv, while 1182 genes were up-regulated and 783 were down-regulated
 24 in DsPo. In addition, over half of genes were up- or down-regulated in both interactions, with 621
 25 up-regulated genes and 441 down-regulated genes in both interactions.

26 Interestingly, 14 main oxidative stress-related and energy metabolism-related functional groups
 27 of DGEs annotated against NR database were found significantly up-regulated in both interactions
 28 DsTv and DsPo compared to the control (Figure 3). Of these functional groups, the largest one
 29 was cytochromes P450. This group composed of 52 DEGs in DsTv and 47 DEGs in DsPo, and

1 most of which were up-regulated in two interactions. The function group of glycoside hydrolase
2 also contained abundant DEGs, although the number of up-regulated DEGs in this group was less
3 than down-regulated DEGs, the change folds of up-regulated DEGs was more significant in both
4 interactions. For instance, one glycoside hydrolase-encoding gene (DICSQDRAFT_109102) could
5 reach to 556.80-fold in DsTv and 961.61-fold in DsPo (Table S1). Moreover, other oxidative
6 stress-related groups including alcohol oxidase, glutathione S-transferase, laccase, terpenoid
7 synthase, manganese peroxidase, multidrug resistance-associated ABC transporter, alcohol
8 dehydrogenase and FMN-linked oxidoreductase, and energy metabolism-related groups including
9 3-ketoacyl-CoA thiolase, S-adenosyl-L-methionine dependent methyltransferase, chitinase and
10 ATPase were all composed of more highly up-regulated DEGs than down-regulated DEGs in
11 DsTv and DsPo.

12 Importantly, glutathione S-transferase and laccase, which both function as detoxification and
13 ROS scavengers (Yang et al., 2012), were remarkably induced in two interactions. For the
14 glutathione S-transferase group, eight up-regulated and one down-regulated DEGs in DsTv, and
15 11 up-regulated and three down-regulated DEGs in DsPo, For the laccase group, eight and three
16 up-regulated DEGs in DsTv and DsPo respectively, only one gene was down-regulated in two
17 interactions. Among these, three laccase genes (Dslcc5, Dslcc10 and Dslcc11) were up-regulated
18 in both interactions, and the up-regulated folds of Dslcc11 even could reach up to 115-fold in
19 DsTv and 148-fold in DsPo (Table S7).

20 The 25 up-regulated genes with highest fold change under both conditions are presented in
21 Table 2 (not including genes with unknown function). This analysis showed that most highly
22 up-regulated genes encoded proteins which played a similar role in the interaction of DsTv or
23 DsPo. Of these, many significantly up-regulated genes encoding proteins were involved in
24 xenobiotic detoxification and generating or reducing ROS, including aldo/keto reductase,
25 glutathione S-transferases, NADH:flavin oxidoreductase/NADH oxidase, short-chain
26 dehydrogenases/ reductase, cytochrome P450 enzymes, the succinate dehydrogenase cytochrome
27 b560 subunit, manganese peroxidase, aldehyde dehydrogenase and laccase. In addition, some gene
28 encoding proteins were involved in an antagonistic mechanism of nutrient competition and
29 antifungal properties, including glycoside hydrolase, glucanase, alpha/beta-hydrolase, GroEs-like

protein and terpenoid synthases.

GO and KEGG analysis

Gene Ontology (GO) terms enrichment analysis globally provides significantly enriched GO terms in DEGs between co-cultures and single culture. Using GO analysis, a total of 45 and 27 GO terms were enriched in DEGs with a p-value <0.05 in two interactions of DsTv and DsPo, respectively. Interestingly, we found the same number of GO terms (20 GO terms) with FDR <0.05 (FDR adjusted p-value<0.05) showed significant enrichment in DsTv and DsPo. Although few enriched GO terms were distinct in DsTv and DsPo, most were the same and had similar enrichment ratios (Figure 4). According to the result, only two main categories, Biological Process (BP) and Molecular Function (MF), contained significantly enriched GO terms in both interactions. Genes categorized into “Cellular Component” showed no significant enrichment (FDR>0.05, Table S3). Under each GO categories, a large number of DEGs were most significantly (FDR<0.001) enriched in categories of “oxidation-reduction process,” “oxidoreductase activity,” “heme binding,” “oxidoreductase activity with incorporation or reduction of molecular oxygen,” “coenzyme binding,” “cofactor binding,” “iron ion binding” and “monooxygenase activity” in both interactions, which suggested that the response to stressful conditions was induced during interspecific interaction. We noticed that the highest percentage of DEGs were exclusively enriched in DsPo categories of “glutathione conjugation reaction” and “glutathione transferase activity,” while several DEGs were exclusively enriched in DsTv categories of “carbohydrate metabolic process,” “ion binding” and “flavin adenine dinucleotide binding.” As more DEGs of significantly enriched GO terms were up-regulated, these GO annotations also indicated that DEGs encoding proteins with antioxidant activity and detoxification properties were more active during the mycelial interaction.

DEGs usually cooperate to execute their biological functions, and KEGG pathway analysis is widely used to further understand the biological functions of genes. KEGG pathway enrichment analysis was used to identify significantly enriched metabolic pathways or signal transduction pathways in DEGs. Contrary to GO analysis, the DEGs of two interactions, DsTv and DsPo, showed distinct enrichment in KEGG pathways (Table S4). A total of 25 and 22 pathways

1 enriched in DEGs with a p-value <0.05 were selected from the interaction of DsTv and DsPo,
 2 respectively (Figure 5). We noticed that the pathways of “Drug metabolism-cytochrome P450”,
 3 “Glutathione metabolism,” “Metabolism of xenobiotics by cytochrome P450,” “Phenylpropanoid
 4 biosynthesis,” “chloroalkane and chloroalkene degradation,” “terpenoid backbone biosynthesis”
 5 and “Synthesis and degradation of ketone bodies” were significantly enriched in DEGs of both
 6 interactions. Moreover, pathways of “Aminobenzoate degradation,” “Dioxin degradation,”
 7 “Polycyclic aromatic hydrocarbon degradation” and “Riboflavin metabolism” were only
 8 significantly enriched in DEGs of DsPo, while “Bisphenol degradation,” and “Chloroalkane and
 9 chloroalkene degradation” were only significantly enriched in DEGs of DsTv. The KEGG analysis
 10 also showed that most KEGG pathways are mainly involved in xenobiotics metabolism and
 11 biodegradation, which may also function as defense responses to toxic substances and biological
 12 attacks.

13 Apart from a defensive function, nutrient competition also existed for mycelium growth and
 14 development during this interspecific interaction (Boddy, 2000; Heilmann-Clausen and Boddy,
 15 2005; Wells and Boddy, 2002). KEGG analysis indicated that some significantly enriched
 16 pathways were involved in energy production and metabolism of both interactions. The
 17 up-regulated “Nitrogen metabolism” and amino acid metabolism such as “Arginine and proline
 18 metabolism” and “Glycine, serine and threonine metabolism” were indispensable for fungal
 19 growth when interacting with its competitors. Carbohydrate metabolic pathways such as “Fructose
 20 and mannose metabolism” and “Starch and sucrose metabolism” and amino acid metabolic
 21 pathways such as “Alanine, aspartate and glutamate metabolism” were only significantly enriched
 22 in the DEGs of DsTv, suggesting that the antagonism mechanism of Ds in nutrition competition
 23 depended on the type of interaction partner. As oxidative phosphorylation pathway was intensified
 24 in both PoDs and PoTv (Figure S5), which also implied the ATP production and metabolism was
 25 enhanced for intensive nutrient competition.

26

27 **Validation of the DEGs results by qRT-PCR analysis**

28 Based on the RNA-Seq analysis, target genes were selected for quantitative real-time PCR to
 29 validate the gene expression profile. According to the RNA-seq result, we selected 10 genes that

1 were all up-regulated in both interactions and had functional annotation involved in ROS
2 production and regulation, including two laccases (Dslcc11 and Dslcc5), NAD(P)-binding protein
3 (DICSQDRAFT_70438), aldo/keto reductase (DICSQDRAFT_172438), glutathione S-transferase
4 (DICSQDRAFT_161976), GroES-like protein (DICSQDRAFT_182440), NADH:flavin
5 oxidoreductase (DICSQDRAFT_157369), cytochrome P450 monooxygenase
6 (DICSQDRAFT_55106) and acetyl-CoA synthetase-like protein (DICSQDRAFT_89216) and
7 Dyp-type peroxidase (DyP1). We also selected six down-regulated genes that all represented large
8 functional groups in both interactions, including cytochrome P450 (DICSQDRAFT_130358),
9 FAD/NAD(P)-binding domain-containing protein (DICSQDRAFT_172084), NAD(P)-binding
10 protein (DICSQDRAFT_58923), Aldo/keto reductase (DICSQDRAFT_167996), Clavaminate
11 synthase-like protein (DICSQDRAFT_108315) and glycoside hydrolase
12 (DICSQDRAFT_149582).

13 The relative expression values of a total of 16 genes in two interactions of DsTv and DsPo
14 provided by qRT-PCR were set compared to the control sample (Figure 6). The qRT-PCR results
15 of 10 up-regulated and 6 down-regulated genes were all in accordance with the RNA-seq data. Of
16 these five genes, DICSQDRAFT_70438, DICSQDRAFT_172438, DICSQDRAFT_157369, DyP1
17 and Dslcc11 were more highly up-regulated and one gene DICSQDRAFT_130358 was more
18 down-regulated than other genes. We also compared the relative expression of each gene
19 determined by qRT-PCR to values from the RNA-Seq analysis. The up- or down-regulated folds
20 of most genes in qRT-PCR and RNA-Seq were of the similar orders of magnitude, but there were
21 distinct differences between higher-value RNA-Seq and qRT-PCR data (Figure S7). The results
22 suggested that the RNA-Seq data and DEGs analysis during the interaction of DsTv and DsPo
23 were reliable.

24

25 **DISCUSSION**

26

27 In this study, the potential of Ds to enhance laccase production by co-culturing with Tv or Po was
28 identified. Notably, laccase activity were remarkably increased in DsTv and DsPo compared to the
29 control. As the expression of two major laccase genes Dslcc11 and Dslcc5 were also significantly

1 up-regulated based on RNA-seq analysis (Table S7), suggesting that Dslcc11 and Dslcc5 probably
2 play key roles in laccase activity induction. Although many studies have revealed that interspecific
3 fungal interactions contribute to the increase in laccase activity (Baldrian, 2004; Chi et al., 2007;
4 Ferreira Gregorio et al., 2006; Flores et al., 2009; Hiscox et al., 2010; Kuhar et al., 2015; Wei et al.,
5 2010), the mechanism of laccase production caused by mycelial interactions remains elusive. As
6 no research has included analysis of the differential expression of genes of Ds when Ds interacted
7 with other fungi, these two interactions of DsTv and DsPo were chosen for further study of
8 laccase in response to interspecific antagonism.

9 The RNA-seq data showed remarkable changes in Ds genetic expression, with 13.64% of the
10 genes in DsTv and 15.90% of the genes in DsPo differentially expressed, similar to the gene
11 expression change of other fungal interactions (Arfi et al., 2013). Moreover, the number of
12 up-regulated DEGs during the interactions was also similar to the number of down-regulated
13 DEGs, but the up-regulated folds of most DEGs were generally higher than the down-regulated
14 folds compared to the control (Table S1). The DEGs analysis by RNA-Seq was confirmed to be
15 reliable by performing qRT-PCR analysis of the expression of a 16-gene set.

16 It has been reported that interspecific interaction could induce an oxidative stress response by
17 accumulation of ROS (Iakovlev et al., 2004; Silar, 2005). Toxic ROS can lead to cellular death by
18 damaging DNA and proteins and triggering lipid peroxidation (Garcia-Sanchez et al., 2012; Ujor
19 et al., 2012), likely inducing the cellular functions of detoxification, defense and repair (Ujor et al.,
20 2012). In our study, oxidative stress/protective response may be active in the interaction zone
21 owing to the up-regulation of many DEGs encoding functional groups related to ROS generation,
22 including alcohol oxidases, which can produce H₂O₂ to support Fenton reactions (Daniel et al.,
23 2007), and Cytochrome P450 enzymes, which are involved in xenobiotic metabolism at the
24 expense of increasing ROS during the catalytic cycle process (Gonzalez, 2005).

25 To resist damage due to ROS, detoxification and defense response could be stimulated to
26 mediate oxidative stress, which is associated with a series of second metabolites secreted from
27 fungi (Evans et al., 2008; Jonkers et al., 2012). Interestingly, in our data, the putative functional
28 groups encoded by a large number of DEGs appear to play a similar role in removing ROS and
29 detoxifying xenobiotics (Table S2). For instance, aldo/keto reductase are responsible for the

1 reductive metabolism of biogenic and xenobiotic materials to primary and secondary alcohols,
2 leading to their detoxification (Mazhawidza et al., 2014). Aldehyde dehydrogenase belongs to
3 detoxification enzymes due to its role in the metabolism of intermediates and exogenous
4 aldehydes and consequently protect cell against oxidative stress (Chen et al., 2014). Glutathione
5 S-transferases are involved in xenobiotic metabolism and cellular detoxification during oxidative
6 stress response (Mariani et al., 2008). Lignin-modifying enzymes including laccase and
7 manganese peroxidase are also important for detoxification and resistance against oxidative stress
8 (Aust, 2004; Jaszek et al., 2006). Based on our RNA-seq analysis, the laccase group was highly
9 up-regulated in both interactions, which is consistent with strongly increased activity of laccase
10 during interactions. Although the previous study of the *Pycnoporus coccineus* also showed that
11 most highly up-regulated genes in *P. coccineus* under interaction with *Botrytis cinerea* and
12 *Coniophora puteana* were annotated as aldo/keto reductases, glutathione S-transferases and
13 cytochrome P450 enzymes (Arfi et al., 2013), there are still some differences compared with the
14 result in our study. The most important one is that no differential expressed laccase gene was
15 identified in this paper, which cannot be related to the overproduction of laccase in white-rot fungi
16 under interaction.

17 As laccases are considered to play a key role in the antagonistic mechanisms, their
18 overexpression also implies a strong competition of nutrients in the mycelial interaction zone.
19 When two different fungi competed for resource and territory, a series of antifungal metabolites
20 were released to protect against competitors and assist in the acquisition of nutrients (Wang M,
21 2013), such as toxic phenolic compounds and terpenoids, both of which can induce laccase
22 production (Ferreira Gregorio et al., 2006). The up-regulation of DEGs encoding terpenoid
23 synthases in RNA-seq data indicated the secretion of terpenoids, organic metabolites with
24 antifungal properties that are involved in the defense responses of fungi (Evans et al., 2008; Yap et
25 al., 2015). Phenolic compounds can directly generate the accumulation of ROS and consequently
26 produce oxidative stress (Cruz-Ortega R, 2007; Garcia-Sanchez et al., 2012). The role of laccase
27 may be to catalyze the monoelectronic oxidation of phenols released by its competitors to the
28 corresponding radical to detoxify or repress ROS (Ferreira Gregorio et al., 2006). The phenolic
29 substrates are also used as laccase mediators, whose oxidized radicals lead to the formation of new

1 dimers, oligomers and polymers via binding to non-laccase substrates (Jeon et al., 2012; Riva,
2 2006). Moreover, we found abundant genes encoding carbohydrate-active enzymes (CAZy) were
3 upregulated in both interaction zones, including glycoside hydrolase and glucanase, which also
4 reflected intensive competition in territory and nutrient uptake when different fungi species
5 confronted with each other.

6 The GO and KEGG analysis of the DEGs also suggested that oxidative stress exists in the
7 interaction zone. According to the significant enriched GO categories of DEGs, most of DEGs
8 with a role in the response to stressful conditions were up-regulated during the interaction of DsTv
9 or DsPo. The remarkably up-regulated genes mainly encode NAD(P)-binding protein
10 (DICSQDRAFT_70438), terpenoid synthase (DICSQDRAFT_159719), GroES-like protein
11 (DICSQDRAFT_182440), aldo/keto reductase (DICSQDRAFT_172438), NADH:flavin
12 oxidoreductase (DICSQDRAFT_157369), laccase B (Dslcc11), glutathione S-transferase
13 C-terminal-like protein (DICSQDRAFT_170658), and FAD-binding domain-containing protein
14 (DICSQDRAFT_139420), these proteins exactly belong to the significantly enriched GO
15 categories of “oxidation-reduction process,” “metabolic process,” “single-organism metabolic
16 process,” “oxidoreductase activity,” “catalytic activity,” “molecular function,” “transition metal
17 ion binding,” “coenzyme binding,” “cofactor binding,” “metal ion binding,” and “monooxygenase
18 activity,” which likely indicates that intracellular molecule binding, xenobiotics metabolism and
19 oxidative stress resistance were more active in Ds when it interacted with other fungi. This was in
20 agreement with the previous report that laccase was involved in the response to oxidative stress
21 (Jaszek et al., 2006; Piscitelli et al., 2011). Based on the analysis of KEGG pathways, enhanced
22 carbohydrate metabolism in cells was associated with the activation of defense and antioxidative
23 stress responses during the interspecific interaction (Shetty et al., 2007), and DEGs involved in
24 carbohydrate and nitrogen metabolism could be related to nutrition competition, in accordance
25 with the fungal ability to take up carbon from the competitor’s mycelium in the interaction zone
26 (Eyre et al., 2010; Wells and Boddy, 2002). In addition, the up-regulation of fatty acid metabolism
27 and amino acid metabolism with energy production was also correlated with the combative
28 mechanism and laccase induction.

29 In conclusion, our results provide insights into antagonistic mechanisms of interspecific

interaction between white rot fungi by analyzing differential gene expression and revealed that laccase induction was probably the result of oxidative stress/protective responses and nutrient competition with opposing competitors since many oxidative stress-resistant genes, antifungal genes and carbohydrate-active enzymes-encoding genes were significantly up-regulated. Importantly, we presented three highly up-regulated laccase genes (Dslcc5, Dslcc10 and Dslcc11) that can be examined for their transcription factor in future experiments.

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Author contributions

Z.Z. and N.L. performed the experiments and wrote the manuscript, and Y.I., B.H. and F.L. designed the experiments and revised the manuscript. All authors discussed and commented on the manuscript.

Additional Informantion

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2 **Figure 1 Interaction on Sc agar plate between *D. squalens* and other two competitors.** (A) Laccase production
3 of paired fungal interaction. Ds, Po, Tv: single culture of *D.squalens*, *P.ostreatus*, *T. versicolor*. DsTv: interaction
4 zone of *D.squalens* and *T. versicolor*, DsPo: interaction zone of *D.squalens* and *P.ostreatus*. DsTv: *D.squalens*
5 region adjacent to the interaction zone of *D.squalens* and *T. versicolor*. Tv/Ds: *T. versicolor* region adjacent to the
6 interaction zone of *D.squalens* and *T. versicolor*. Ds/Po: *D.squalens* region adjacent to the interaction zone of
7 *D.squalens* and *P.ostreatus*. Po/Ds: *P.ostreatus* region adjacent to the interaction zone of *D.squalens* and
8 *P.ostreatus*. Interaction zone of *D. squalens* and *P. ostreatus* (B) or *T. versicolor* (C) without nitroblue tetrazolium
9 (NBT) staining. Purple coloration was observed in the interaction zone of *D. squalens* and *P. ostreatus* (D) or *T.*
10 *versicolor* (E) with nitroblue tetrazolium (NBT) staining.

11 **Figure 2 Venn diagrams of number of DEGs compared to control sample in the interaction with *T. versicolor***
12 **(DsTv) and *P. ostreatus* (DsPo).** (A) Genes were up-regulated by at least 2-fold; (B) Genes were down-regulated
13 by at least 2-fold.

14 **Figure 3 Expression patterns for 14 oxidative stress-related and energy metabolism-related functional**
15 **groups of DEGs.** For each functional group, the number of up- or down-regulated DEGs in the interaction of
16 DsTv and DsPo compared to control are shown. Values between parentheses above each bar correspond to the
17 number of up- and down-regulated DEGs in each functional group.

18 **Figure 4 GO terms enrichment analysis on both interactions compared to control.**

19 (***) FDR<0.001, **FDR<0.01, * FDR<0.05)

20

21 **Figure 5 KEGG pathway enrichment analysis in *D. Squalens* under the interaction with (A) *T. versicolor* and**
22 **(B) *P. ostreatus* compared to the control.** (p-value<0.05)

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24 **Figure 6 Validation of up-and down-regulated genes expression during both interactions compared to**
25 **control by quantitative RT-PCR.** Expression levels of 16 genes in *D. squalens* were set as control and
26 normalized to 1. Black horizontal line corresponds to level equal to control. Values are averaged from three
27 biological replicates.

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2 **Table 1 PCR primers used for the q-RT-PCR experiments**

Primer Name	Forward sequence(5' to 3')	Reverse sequence (5' to 3')	NR annotation
DICSQDRAFT_70438	GCCTTCTTACGCTGTCTTCG	GCCTGTGACGTTTCCAATCT	NAD(P)-binding protein
DICSQDRAFT_172438	AGGAAGAGGCCATCAACCAT	TTTGGGATTGGAGCGAATAG	Aldo/keto reductase
DICSQDRAFT_182440	TCCTGAGAAGAATGGCGAGT	CACGGATATGCGGAGATCAT	GroES-like protein
DICSQDRAFT_161976	GAGGCTCTGCTCTCCAAGAC	ACGGTACGTTACCTTCACC	glutathion S-transferase
DICSQDRAFT_157369	CGAATCTTGCCTACCTCCAC	CGATCTTGATAGCGGTCTCC	NADH:flavin oxidoreductase/NADH oxidase
DICSQDRAFT_55106	AGCGCAAGATTGTTTCCAAC	TGATGTGCAACGCGAGATAG	cytochrome P450 monooxygenase pc-bph
DICSQDRAFT_89216	GATTCCATCGTGGCGTCTAT	GGAGGAATGACAAACGCAGT	acetyl-CoA synthetase-like protein
DyP1	TGTGGACGTGAGAACGATGT	TCTGCGTCAAGAAGGTGTTG	Dyp-type peroxidase
Dslcc11	CGAGCTCTCCTTTACCATCG	CTGCAGATGCCAGTCAATGT	laccase B
Dslcc5	CCCAGCGTTATTCATTCGTT	TCGTGCCATTGAAACTGAAG	laccase
DICSQDRAFT_130358	TGCGATTCTTCCATCTTTC	ATGGCATTCTTCGACCACTC	cytochrome P450
DICSQDRAFT_172084	TCATTGAGTTCTGCGACGAC	CCGTCAAGCATAGCGAGATT	FAD/NAD(P)-binding domain-containing protein
DICSQDRAFT_58923	CACCGTCATCAACATCTCGT	CATTCCCGACTTGTTTCGTCT	NAD(P)-binding protein
DICSQDRAFT_167996	ACAACCTGACCATCCTGGAG	TGGCTACTGGCTTGACCTTC	Aldo/keto reductase
DICSQDRAFT_108315	GACACCGGGACTGTAACGAT	TAGAAACCGCCAGAGAGCAT	Clavamate synthase-like protein
DICSQDRAFT_149582	GCCACGAAGACGTTTGACTT	ACGGTGATCGAGAATTCGTT	glycoside hydrolase
DICSQDRAFT_46225	CTCAGCACCGGTAACAATCC	CTGCCACCACCAAAGACAGT	40S ribosomal protein S24
DICSQDRAFT_98815	CGTCTTACGGCCACTCTTTC	ACCGCTCTCGTTACCACACT	chitinase
DICSQDRAFT_135086	CATCACTGCTTCCCTTCGTT	AATGACCGAGTTCTGCTCGT	α -tubulin

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2 Table 2 The 25 up-regulated genes with highest fold change under both co-culture conditions by RNA-seq analysis

Up-regulated genes in the interaction with <i>P. ostreatus</i>			Up-regulated genes in the interaction with <i>T. versicolor</i>		
Gene_ID	Fold change	Putative function	Gene_ID	Fold change	Putative function
18845230	2160.84	oxidoreductase	18845401	2057.23	Non-Catalytic module family EXPN protein
18845401	1525.30	Non-Catalytic module family EXPN protein	1884439	1157.90	UbiA prenyltransferase
18843716	976.27	NAD(P)-binding protein	18833825	556.79	glycoside hydrolase
18833825	961.61	glycoside hydrolase	18837948	328.53	terpenoid synthase
18837948	674.14	terpenoid synthase	18843669	316.56	Nitroreductase
18841174	622.22	GroES-like protein	18843554	288.29	cytochrome P450
18842297	570.29	Non-catalytic module family EXPN protein, partial	18843877	203.59	HAD-like protein, partial
18844395	547.52	UbiA prenyltransferase	18842297	190.84	Non-catalytic module family EXPN protein, partial
18839563	343.20	Aldo/keto reductase	18844746	126.84	amine oxidase
18837949	316.56	alpha/beta-hydrolase	18833943	114.88	laccase B
18837763	255.86	NADH:flavin oxidoreductase/NADH oxidase	18839529	92.21	succinate dehydrogenase cytochrome b560 subunit
18842217	196.82	Osmotin thaumatin-like protein	18837704	87.02	DUF323 domain-containing protein
18843669	148.74	Nitroreductase	18842217	84.90	Osmotin thaumatin-like protein
18833943	148.38	laccase B	18837323	84.90	arylalcohol dehydrogenase
18834029	133.96	carotenoid ester lipase	18839046	84.55	laccase
18839183	127.52	glutathione S-transferase C-terminal-like protein	18845343	60.52	secreted protein
18840270	115.66	Aldo/keto reductase	18842241	58.78	Manganese-dependent peroxidase

18835851	112.08	FAD-binding domain-containing protein	18835851	52.59	FAD-binding domain-containing protein
18836474	104.35	short-chain dehydrogenases/reductase	18844326	52.57	terpenoid synthase
18836780	94.17	barwin-like endoglucanase	18844314	48.29	GroES-like protein
18834127	82.64	NAD(P)-binding protein	18833814	42.25	cytochrome P450
18836407	78.55	NAD(P)-binding protein	18838354	42.20	RTA1-domain-containing protein
18842871	76.96	NAD(P)-binding protein	18842447	40.32	GroES-like protein
18844326	68.56	terpenoid synthase	18844012	40.10	carbonic anhydrase, partial
18837789	67.90	Aldo/keto reductase	18843657	39.62	O-methyltransferase

A **B**