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Label-free differentially proteomic analysis of interspecific interaction between white-rot fungi highlights oxidative stress response and high metabolic activity

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ABSTRACT

The laccase production by mycelial antagonistic interaction among white-rot fungi is a very important pathway for lignin degradation research. To gain a better understanding of competitive mechanisms under mycelial antagonistic interaction among three lignin-degrading white-rot basidiomycetes of Trametesversicolor (Tv), Pleurotusostreatus (Po) and Dichomitussqualens (Ds), mycelial morphology and proteins in three co-culture combinations TvPo (Tv cocultivated with Po), PoDs (Po cocultivated with Ds), TvDs (Tv cocultivated with Ds) were compared with corresponding each two mono-cultures. In this study, scanning electron microscopy detection of co-cultures indicated a highly close attachment of fungal hyphae with each other and conidiation could be inhibited under fungal interaction. In addition, a label-free proteomic analysis revealed changes on fungal proteomes existed in their counterpart competitors of co-culture. The maximum number of 1020 differentially expressed proteins (DEPs) were identified in PoDs relative to Po while the minimum number of 367 DEPs were identified in PoDs relative to Ds. Notably, we also found a large number of overexpressed proteins were oxidative stress-related proteins, followed by carbohydrate metabolism-related proteins and energy production-related proteins in all three co-culture combinations compared with control. These results were important for the future exploration of molecular mechanisms underlying lignin-degrading fungal interaction.

1. Introduction

Mycelial antagonistic interaction between different white-rot fungus species in overlap niches is inevitable to cause fungal competition for territory and resources (Boddy, 2000; Heilmann-Clausen and Boddy, 2005). This competitive interactions are always accompanied by the changes of mycelial morphology and secretion of second metabolites and production of extracellular enzymes (Bertrand et al., 2013; Boddy, 2000; Ferreira Gregorio et al., 2006; Freitag and Morrell, 1991; Hiscox et al., 2010; Hynes et al., 2007; Peiris et al., 2007; Rodriguez Estrada et al., 2011). Many studies reported that the production of oxidative enzymes, such as laccase and manganese peroxidase which played function to oxidize a wide range of phenolic and aromatic compounds, could be obviously increased during fungal interspecific interaction (Baldrian, 2004; Chi et al., 2007; Ferreira Gregorio et al., 2006; Hiscox et al., 2010; Pan et al., 2014; Wei et al., 2010). In addition, fungal antagonistic interaction could induce toxic reactive oxygen species (ROS) production (Silar, 2005) and further caused oxidative stress. A large amount of ROS accumulation also could act as singling molecule to stimulate cell defensive system against oxidative damage (Nath et al., 2016).

Previous studies have reported the change of mRNA level in the interaction between different fungi (Arfi et al., 2013; Iakovlev et al., 2004; Zhong et al., 2017). However, as change in protein expression levels cannot be accurately revealed only by measuring mRNA levels (Gygi et al., 1999), it's necessary to explore the global changes at proteomic level under fungal interactions by using proteomic strategy. This attractive strategy is currently used to provide systematic understanding of proteins act as virulence factors in the host–pathogen interaction among plants, bacteria and fungi (Bhadauria et al., 2009; Gonzalez-Fernandez et al., 2010; Marra et al., 2006; Mehta et al., 2008; Paper et al., 2007). However, much less has

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been done on identification of specific proteins that play a primary role in the fungal–fungal interactions, which leading to a poor understanding about regulatory proteins and biochemical pathways in response to stressful condition caused by fungal combative interaction. Nowadays, a new proteomic strategy label-free is widely used in analyzing change level of protein abundance (Gonzalez-Fernandez and Jorrin-Novo, 2012; Lochner et al., 2011). This approach has become available to explore the more complex mechanisms underlying variations in plant pathogenic fungi and identify potential virulence factors (Gonzalez-Fernandez et al., 2013).

Trametes versicolor (Tv), *Pleurotus ostreatus* (Po) and *Dichomitus squalens* (Ds) all belong to effective lignin-degrading white-rot basidiomycetes. These three fungi can secret a range of laccases and manganese peroxidases (MnP) as their lignin-modifying enzymes which give them ability of dye decolorization and pretreatment of lignocellulosic biomass (Arora et al., 2002; Casieria et al., 2008; Perie et al., 1998; Perie and Gold, 1991; Valmaseda et al., 1991). In our previous study, we found laccase could also be significantly induced under interspecific mycelial Tv, Po and Ds (Luo et al., 2017). Thus, we analyzed the intercellular proteins obtained from three mono-culture Tv, Po, Ds and three co-cultures (TvPo, PoDs and TvDs) by using label-free proteomic approach to shed light on proteomic level changes during fungal interspecific interaction. This study focused on the common or different pathways among three co-culture combinations compared with the three mono-cultures. The goal of this work was to get an in depth understanding of antagonistic mechanism under fungal–fungal interaction.

2. Materials and methods

2.1. Strains and culture

Strains of *T. versicolor*, *P. ostreatus* from the Biological Resource Center, NITE (NBRC) and *D. squalens* from the Deutsche Sammlung von Microorganismen and Zellkulturen (DSMZ) 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. Cultures were routinely sub-cultured every 7 d.

2.2. Mono- and co-culture

For mono-culture, a 7 mm agar plug of one fungal pre-culture was inoculated in the center of a 90 mm petri dish containing 30 mL of Sc agar media (Floudas et al., 2012), consisting of 10 g/L glucose, 1.5 g/L L-asparagine, 0.12 mg/L Thiaminiumdichloride, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 mg/L $FeCl_3 \cdot 6H_2O$, 0.06 mg/L HBO_3, 0.04 mg/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 2 mg/L $ZnSO_4 \cdot 7H_2O$, 0.1 mg/L $MnSO_4 \cdot 4H_2O$, 0.4 mg/L $CoCl_2 \cdot 6H_2O$, 1.2 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 20 g/L Agar. The petri dishes were incubated at 28 °C for 9 d and set as control sample. Similarly, co-culture experiments were inoculated with two 7 mm agar plugs from different fungal pre-culture on opposite sides of a petri dish containing Sc agar media. Three combinations in co-culture were TvDs, TvPo and PoDs, and the petri dishes were also incubated at 28 °C for 9 d. Each mono- and co-cultures were set three biological replicates.

2.3. Assays of laccase

Laccase activity was assayed in a 3-ml reaction system. It included 300 μL 0.5 mM ABTS (molar extinction coefficient 36 000 M^{-1} cm^{-1}), 2.4 mL 0.1 M acetic acid buffer (pH 4.8) and 300 μL samples 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.

2.4. Scanning electron microscopy

The effect of fungal mycelial morphology underlying interspecific interaction was observed by Scanning Electron Microscope (SEM). Placing coverslips on the interaction zone in co-culture media and any blank space in mono-culture media respectively before inoculating strain agar. After being incubated for 9 d at 28 °C, the co-cultured and mono-cultured mycelium were fixed with 2.5 % (v/v) glutaraldehyde in 0.1 M PBS (Phosphate Buffer Saline, pH 7.4) for 6 h at 4 °C, and subsequently washed three times by 0.1 M PBS (pH 7.4). After that the samples were dehydrated in graded series of 30–100 % ethanol. The specimens were then dried in vacuum dryer, coated with gold and finally examined using SEM (JEOL, JSM-6510LV, Japan).

2.5. Sample preparation and protein extraction

Mycelium from the interaction zones (~2 mm wide) of three co-cultures (TvDs, TvPo, PoDs) and isolates of three mono-cultures (Tv, Po, Ds) were excised with a razor blade and then freeze dried at −80 °C as protein extraction samples. Each co- and mono-cultured samples were set three biological repeats.

Samples were ground to a powder in liquid nitrogen by using a pestle and mortar and transferred to a pre-chilled centrifuge tube. For cell lysis, samples were suspended in 200 μL lysis buffer (4 % SDS, 100 mM DTT, 150 mM Tris–HCl pH 8.0) on ice. Cells were disrupted with agitation using a homogenizer (Fastprep-24®, MP Biomedical), and boiled for 5 min. The samples were further ultrasonicated and boiled again for another 5 min. Undissolved cellular debris were removed by centrifugation at 14 000 rpm for 15 min. The supernatant were collected and quantified with a BCA protein assay kit (Bio-Rad, USA).

2.6. Protein digestion

Digestion of protein (50 μL for each sample) was performed according to the FASP procedure (Wiśniewski et al., 2009). Briefly, the detergent, DTT and other low-molecular-weight components were removed using 200 μL UA buffer (8 M Urea, 150 mM Tris–HCl pH 8.0) by repeated ultrafiltration (Microcon units, 10 kD) facilitated by centrifugation. Then 100 μL 0.05 M iodoacetamide in UA buffer was added to block reduced cysteine residues and the samples were incubated for 20 min in darkness. The filter was washed with 100 μL UA buffer three times and then 100 μL 25 mM NH_4HCO_3 twice. Finally, the protein suspension was digested with 40 μL 4 μg trypsin buffer (4 μg trypsin from Promega in 25 mM NH_4HCO_3) overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm.

2.7. LC–MS/MS analysis

The peptide of each sample was desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma), then concentrated by vacuum centrifugation and reconstituted in 40 μL of 0.1 % (v/v) trifluoroacetic acid. MS experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). 5 μg peptide was loaded onto a C18-reversed phase column (Thermo Scientific Easy Column, 2 cm × 100 μm, 5 μm particle size) in buffer A (2 % acetonitrile and 0.1 % Formic acid) and separated with a linear gradient of buffer B (84 % acetonitrile and 0.1 % Formic acid) at a flow rate of 400 nL/min controlled

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