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Transcriptome analysis reveals the role of nitric oxide in *Pleurotus eryngii* responses to Cd^{2+} stress

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HIGHLIGHTS

- A new insight into the transcriptional response of *P. eryngii* to heavy metals.
- NO plays an important role in alleviating fungal heavy metal stresses.
- Oxidoreductase and HSP contribute to heavy metal tolerance in NO-regulated *P. eryngii*.
- This study lays a foundation for potential application of NO in mushroom cultivation.

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ABSTRACT

Pleurotus eryngii is widely cultivated in China. However, our understanding of its transcriptional response to heavy metal stress and the underlying mechanism of nitric oxide (NO) in enhancing its tolerance to heavy metals is limited. In the present study, RNA-seq was used to generate large transcript sequences from *P. eryngii* exposed to cadmium chloride ($CdCl_2$) and exogenous NO. A total of 45,833 unigenes were assembled from the *P. eryngii* transcriptome, of which 32,333 (70.54%) unigenes matched known proteins in the nr database. Transcriptional analysis revealed that putative genes encoding heat shock proteins (HSPs) and genes participating in glycerolipid metabolism and steroid biosynthesis were significantly up-regulated in *P. eryngii* exposed to 50 μM Cd ($P < 0.05$). *P. eryngii* mycelia exposed to extremely high levels of heavy metals showed an increase in biomass when exogenous NO was added to the culture. The collaboration of putative oxidoreductase, dehydrogenase, reductase, transferase genes and transcription factors such as "GTPase activator activity", "transcription factor complex", "ATP binding", "GTP binding", and "enzyme activator activity", which were significantly up-regulated in samples induced by exogenous NO, contributed to the enhancement of *P. eryngii* tolerance to extremely high levels of heavy metals. The study provides a new insight into the transcriptional response of *P. eryngii* to extremely high levels of heavy metals and the mechanism of NO in enhancing heavy metal tolerance.

1. Introduction

Nitric oxide (NO) is a simple gaseous small molecule that can easily diffuse through the cell membrane and plays an important role in various organisms (Napoli et al., 2006, 2013). Extensive studies on NO have been conducted since its biosynthetic mechanism and physiological characteristics *in vivo* were confirmed in 1987 (Ignarro et al., 1987; Palmer et al., 1987). Thereafter, the majority of studies have focused on the biochemistry and related biological processes of NO, thereby creating a new era in biomedical research (Singh et al., 2017). NO can be synthesized by mammals and it plays major roles in the cardiovascular, immune, nervous, urinary, and reproductive systems (Palmer et al., 1987; Hofseth, 2008). NO is also involved in various plant physiological processes such as seed germination, growth, stomatal movement, photosynthesis, respiration, cell transportation, cell death, and stress response (Gadelha et al., 2017; He et al., 2017; Li et al., 2017; Mwaba and Rey, 2017). Studies have established plant cells can synthesize endogenous NO. However, despite decades of extensive research investigations on NO synthesis in plants, various

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processes, such as the mechanism of NO signaling, NO targets in plants, the cascade amplification mechanism, and NO removal, have remained unclear in plants.

Macrofungi, which are multicellular organisms, have several unique characteristics that are not observed in plants and animals. Recent studies have indicated that fungi are capable of synthesizing NO (Vieira et al., 2009). NO regulates the synthesis of cyclic guanosine monophosphate (cGMP), an important intracellular secondary messenger molecule that is involved in the control of a variety of signal transduction pathways in fungal cells (Vieira et al., 2009). NO also regulates cellular development, morphogenesis, sporulation, spore germination, reproduction, and apoptosis in fungi (Toritsuka et al., 1997; Chen et al., 2013; Canovas et al., 2016). Although the mechanism of NO in plants and animals has been widely studied, its activity in fungi remains elusive. Thus, in-depth investigations elucidating NO synthesis, degradation pathways, and the mechanism of signal transduction in the fungal system are warranted.

China is the biggest producer and exporter of *Pleurotus* in the world, accounting for 25% of the world's mushroom production. *P. eryngii* is a delicious food and traditional Chinese medicine; it contains a variety of biologically active agents such as terpenoids, sterols, and polysaccharides (Chen et al., 2016; Deng et al., 2016; Kang et al., 2016; Kikuchi et al., 2016; Sun et al., 2017). Macrofungi are exposed to various stresses in the environment, which include high pH, high temperature, drought, and high salinity or high levels of heavy metals in growth substrates. Because macrofungi are sessile and thus cannot move away from adverse stimuli, these have adopted strategies to enhance their tolerance of environmental stress by producing regulatory agents (Ntougias et al., 2012; Liu et al., 2015a, 2015b; Fu et al., 2016; Xiao et al., 2016). The cellular redox homeostasis of fungi would be broken when subjected to extreme abiotic stress, which caused oxidative damage to plasma membranes and led to lipid fluidity (Kong et al., 2012). Many previous studies have confirmed that exogenous NO could enhance fungal tolerance to abiotic stress (Chen et al., 2013; Nasuno et al., 2014; Canovas et al., 2016).

In the present study, the transcriptional responses of *P. eryngii* to heavy metal (cadmium chloride, $Cdcl_2$) stress and the mechanism of NO in enhancing fungal resistance to abiotic stress were investigated by RNA-Seq analysis. To the best of our knowledge, this is the first study of the gene expression of *P. eryngii* simultaneously exposed to heavy metal stress and exogenous NO. Our results can lay a foundation for similar studies on other fungi.

2. Materials and methods

2.1. Sample collection and preparation

P. eryngii mycelia were isolated from a cultivated strain in Sichuan, China. The obtained mycelia were identified by molecular and morphological analysis and were stored in the Sichuan Academy of Agricultural Sciences. The mycelia were initially incubated in potato dextrose agar medium (PDA) at 28 °C for 5 days and then 10 pieces (punch diameter: 5 mm) of mycelia from a solid medium were transferred to 100 mL of liquid potato dextrose medium (250-mL flask). The cultures were incubated with shaking at 28 °C and 110 rpm for 5 days. In our preliminary experiments on Cd dose, mycelia were subjected to stress using $CdCl_2$ at different concentrations (0 μM, 25 μM, 50 μM, 100 μM, and 200 μM) and incubated in a shaker at 28 °C and 110 rpm and harvested after 5 days for fresh weight (FW) determination. At the second part of our study, different concentrations (0 μM, 50 μM, 100 μM, 150 μM, 200 μM, 250 μM, and 300 μM) of SNP were added into 50 μM Cd-stressed mycelia. Then, potassium ferrocyanide ($K_4[Fe(CN)_6]$), an analog of SNP that does not release NO, was used as negative control of SNP (Guo et al., 2016). SNP and $K_4[Fe(CN)_6]$ were directly added into the flasks after filter sterilization. Each treatment was performed in triplicate under the same experimental conditions. The cultures were incubated with shaking at 28 °C and 110 rpm. The mycelia were harvested after 5 days for RNA extraction and transcriptome sequencing.

2.2. RNA extraction

All samples were ground into a fine powder in a frozen mortar while immersed in liquid nitrogen. Total RNA was then extracted from 100 mg of mycelium powder using a fungal RNA kit (Biomiga, San Diego, CA, USA) following the manufacturer's instructions. The quality and quantity of the RNA were determined according as follows: RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit® RNA assay kit in a Qubit® 2.0 fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using an RNA Nano 6000 assay kit on an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), taking 6.3 as a threshold for RNA integrity number (RIN).

2.3. Library preparation for transcriptome sequencing

A total amount of 1.5 μg RNA per sample was used as input for RNA sample preparation. Sequencing libraries were generated using an NEBNext® Ultra™ RNA library prep kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using polyT oligo-attached magnetic beads. Fragmentation was conducted using divalent cations under elevated temperature in NEBNext first strand synthesis reaction buffer (5 ×). First-strand cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA polymerase I. The remaining overhangs were converted into blunt ends using exonuclease/polymerase activities. After adenylation of the 3′ ends of the DNA fragments, NEBNext adaptors with hairpin loop structures were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with an AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3 μL of USER enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then, PCR was performed with Phusion High-Fidelity DNA polymerase, universal PCR primers, and an Index (X) primer. Finally, PCR products were purified (AMPure XP system), and library quality was assessed on the Agilent Bioanalyzer 2100 system. Raw reads were submitted to the Sequence Read Archive (SRA) database (Accession Numbers SRR3990812–SRR3990820).

2.4. Clustering, sequencing, quality control, and transcriptome assembly

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) following the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated.

Raw data (raw reads) in fastq format were initially processed using in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads from the raw data. At the