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# Heat stress-induced reactive oxygen species participate in the regulation of HSP expression, hyphal branching and ganoderic acid biosynthesis in *Ganoderma lucidum*

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ABSTRACT

Heat stress (HS) is an important environmental factor that affects the growth and metabolism of edible fungi, but the molecular mechanism of the heat stress response (HSR) remains unclear. We previously reported that HS treatment increased the length between two hyphal branches and induced the accumulation of ganoderic acid biosynthesis and the gene expression of heat shock proteins (HSPs) in *Ganoderma lucidum*. In this study, we found that HS induced a significant increase in the cytosolic ROS concentration, and exogenously added ROS scavengers NAC, VC and NADPH oxidase (Nox) inhibitor DPI reduce the cytosolic ROS accumulation in *G. lucidum*. In addition, the phenomena of the increased gene expression and increased length between the two hyphal branches and the accumulation of GA biosynthesis induced by HS were mitigated. Furthermore, we investigated the effects of HS on Nox-silenced strains (NoxABi-10, NoxABi-11 and NoxRi-4, NoxRi-7) and found that the level of ROS concentration was lower than that in wild-type (WT) strains treated with HS. Additionally, Nox silenced strains reduced the HS-induced increase in HSP expression, the length between two hyphal branches and GA biosynthesis compared with the WT strain. These data indicate that HS-induced ROS participate in the regulation of HSP expression, hyphal branching and ganoderic acid biosynthesis in *G. lucidum*. In addition, these findings identified potential pathways linking ROS networks to HSR, physiological and metabolic processes in fungi and provide a valuable reference for studying the role of ROS in HSR, mycelium growth and secondary metabolites.

## 1. Introduction

The environment is a central parameter of growth, development and secondary metabolism. High temperature is one of the major environmental factors influencing almost all biological processes in microorganisms. Microbial cells lack the capacity to avoid heat and cannot flee extreme temperature conditions; thus, it is necessary to evaluate the growth of microorganisms in high temperature. Many studies have reported that heat stress (HS) affects fungal growth and development, primarily focusing on ascomycetes. It has been demonstrated that HS causes multifarious alterations in growth and pattern formation in the fission yeast *Schizosaccharomyces* (Vjestica et al., 2013), and HS resulted in cell death in *Saccharomyces cerevisiae* (Guyot et al., 2015). Relative to ascomycetes, less work has been conducted on basidiomycetes under HS conditions. *Agaricus bisporus* was severely reduced in both biomass and pileus diameter under high temperature (Lu et al., 2014). Furthermore, HS not only affects growth but also the secondary metabolism in the fungus. It was previously reported that HS led to trehalose accumulation in *Pleurotus* (Kong et al., 2012).

The mechanism of HS affects various physiological and metabolic processes in which heat shock proteins (HSPs) play a role (Leach et al., 2012; Tamayo et al., 2013; Vjestica et al., 2013). HSPs are divided into several classes based on molecular mass: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small HSPs (Morano et al., 2012). With the exception of HSPs, some small molecules also participating in the regulation of the physiological response of HS have been reported, with ROS being one of the most important signaling molecules. *Pleurotus* has been used to detect ROS accumulation and induction of apoptosis-like cell death upon exposure to HS (Song et al., 2014). In *Saccharomyces cerevisiae*, HS induces an increase in ROS production with concomitant the effects of thermotolerance (Cao et al., 2013). All of these studies have indicated that ROS signaling is closely related to the heat stress response (HSR). However, the precise roles of ROS in HS signaling transduction remain unclear.

Ganoderic acid (GA) is one of the major secondary metabolites in *Ganoderma lucidum*, a basidiomycete with high commercial value

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(Wang and Ng, 2006; Sanodiya et al., 2009). The GA content is often used as a standard to measure the overall quality of *G. lucidum*, and the lack of a relevant study has hindered its further application. Currently, reliable genetic tools of *G. lucidum* have been developed (Mu et al., 2012; Shi et al., 2012), making it a potential model species for the study of morphological development and regulation secondary metabolism in higher basidiomycetes. Current studies on *G. lucidum* have primarily focused on the study of GA based on the optimization of fermentation conditions. Acetic acid as an inducer was shown to increase GA accumulation in *G. lucidum* (Ren et al., 2014), and aspirin added to culture media also enhanced GA production (You et al., 2013). Further analysis of the mechanism revealed that among the regulatory factors associated with the biosynthesis of GA through various signaling molecules, ROS may prove to be an important one. Mu reported that ROS reduction in Nox-silenced strains affected fruiting body formation, GA biosynthesis and mycelium growth in *G. lucidum* (Mu et al., 2014). It is reported that methyl jasmonate (MeJA) induces the biosynthesis of GA and increases the distance between hyphal branches via ROS in *G. lucidum* (Ren et al., 2013; Shi et al., 2015). Furthermore, a previous study also found that HS resulted in the accumulation of GA and decreased the length between two hyphal branches in *G. lucidum* (Zhang et al., 2016). Thus, studies regarding whether HS causes the ROS burst in *G. lucidum* and the precise roles of ROS regulation in *G. lucidum* hyphal branching, GA biosynthesis and transcriptional levels of HSPs remain unknown.

As signaling molecules, ROS play important roles in the response to environmental stresses in plants, but the detailed mechanisms are still not very clear in fungi, especially in basidiomycetes. To investigate the physiological roles of ROS in *G. lucidum* under HS conditions, we studied the effect of HS on the ROS levels and measured HSP expression, hyphal branching and GA biosynthesis in the presence of ROS scavengers NAC and VC and Nox inhibitor DPI in the WT strain. The result demonstrated that HS-induced ROS accumulation participates in the regulation of HSR in *G. lucidum*. Next, we used the Nox-silenced strains to further investigate the connection between ROS and HSR. The investigation of ROS in *G. lucidum* is essential for the response to environmental factors and provides the basis for heat shock signal transduction studies of other fungi.

## 2. Materials and methods

### 2.1. Strains and culture conditions

*G. lucidum,* strain HG, was obtained from the culture collection of the Edible Fungi Institute, Shanghai Academy of Agricultural Science and was used as the wild-type (WT) control. The empty vector controls (SiControl-1, SiControl-2) and Nox-silenced strains were also constructed previously (Mu et al., 2014). The WT, SiControl and Nox-silenced *G. lucidum* strains were grown at 28 °C in PDB medium (Potato Dextrose Broth).

### 2.2. Treatments

For this the PDA plate, overlaid with a layer of cellophane, was inoculated with strains and cultured for 5 days at 28 °C. The fungal mycelium was then transferred into a PDA with NAC, VC and DPI for 30 min before HS treatment, respectively (Mu et al., 2014; Shi et al., 2015; Zhang et al., 2016). In previous research, the 42 °C was used as the HS treatment temperature in *G. lucidum* (Zhang et al., 2016). So, we chose 42 °C as HS treatment temperature in the present study. The *G. lucidum* strains were exposed to 42 °C for 0–60 min (Richthammer et al., 2012; Zhang et al., 2016), and then the ROS levels and related antioxidative enzymes genes were evaluated. The transcriptional expression levels of HSPs were detected after exposure to 42 °C for 20 min. Three-day-old *G. lucidum* strains were exposed to 42 °C for 90 min and then were allowed to recover at 28 °C for 2 d to evaluate hyphal branching.

*G. lucidum* strains were cultured in PDB liquid cultures with shaking for 5 days, and then were exposed to 42 °C for 12 h until the 7th day at 28 °C in stationary PDB liquid cultures, to detect the activities of antioxidant enzymes and the content of ganoderic acids (GA) and its mesostates. *G. lucidum* strains were pretreated for 30 min with ROS scavengers and Nox inhibitor before HS. Exogenously added 2 mM $H_2O_2$ functioned as a positive control under normal temperature. The scavengers of ROS used in detecting GA were 0.5 mM NAC and 1 mM VC. The inhibitors of Nox used in detecting GA were 10 μM DPI.

### 2.3. ROS detection assay

The ROS content was assayed according to a previously described method (Mu et al., 2014). *G. lucidum* strains were incubated in a 0.5-mg/mL NBT (10 mM potassium phosphate buffer, pH 7.5) solution for 2 h to detect superoxide. For the ROS fluorescence assay, the sterile glass coverslips were placed on the bottom of Petri dishes; when the mycelium grew on the cover slips, then were stained with DCFH-DA (20, 70-dichlorodihydrofluorescein diacetate) for 20 min to visualize ROS (Mu et al., 2014). The fluorescence was detected using a Zeiss Axio Imager A1 fluorescence microscope. ROS quantification was select all hyphae in the photos to detect the fluorescence intensity, and the average fluorescence intensities of DCFH-DA in the mycelia were analyzed using ZEN lite (Zeiss software). The $H_2O_2$ content in liquid hyphae was assessed using a commercial Hydrogen Peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). $H_2O_2$ bound to molybdenic acid forms a complex, measured at 405 nm, from which the content of $H_2O_2$ was calculated. Nox-silenced strains were also constructed as described (Mu et al., 2014). Real-time PCR analyses were used to determine the transcription levels of the NoxA, NoxB and NoxR gene in the silenced strains.

### 2.4. Real-time qPCR analysis of gene expression

For the determination of gene expression, the mycelia were cultured on PDA plates which was then overlaid with a layer of sterile cellophane as previously described (You et al., 2013), then were collected and frozen in liquid nitrogen. Total RNA was extracted, then reverse-transcribed to cDNA. Subsequently, real-time qRT-PCR-amplified fragments were detected using SYBR Green qPCR SuperMix. The relative quantification of gene expression was performed using the housekeeping gene 18S rRNA (Mu et al., 2012), the sample of the untreatment and WT served as the reference sample against which all other treatments and transformants were compared; expression of the reference sample was defined as 1.0 and the expression of the reference genes in all other treatments and transformants is reported as the fold increase over the reference sample. Post-qRT-PCR calculations of relative gene expression levels were performed according to the $2^{-\triangle\triangle CT}$ method described by Livak and Schmittgen (2001). The gene fragments were amplified by real-time qPCR using primers based on the *G. lucidum* genome sequence, as shown in Table S1 (Ren et al., 2017).

### 2.5. Enzyme activity assays

For the determination of enzymatic activity, the strains were cultured on PDA plates which was then overlaid with a layer of sterile cellophane as previously described (You et al., 2012). Then were frozen in liquid $N_2$ and stored at −70 °C until the mycelia were ground, and the enzymes were extracted. Frozen mycelia (0.3 g) were homogenized in 5 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000*g* for 20 min at 4 °C, and the supernatant was immediately used for subsequent enzymatic assays. The protein content was determined according to the Bradford method, using BSA as a standard.

Superoxide dismutase activity was determined according to