Redox Biology 16 (2018) 388–400

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research paper

SA inhibits complex III activity to generate reactive oxygen species and thereby induces GA overproduction in *Ganoderma lucidum*

Rui Liu[1], Pengfei Cao[1], Ang Ren, Shengli Wang, Tao Yang, Ting Zhu, Liang Shi, Jing Zhu, Ai-Liang Jiang, Ming-Wen Zhao*

Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture; Microbiology Department, College of Life Sciences, Nanjing Agricultural University, No 1 Weigang, Nanjing 210095, Jiangsu, People's Republic of China

ARTICLE INFO

Keywords:
Salicylic acid
Reactive oxygen species
Mitochondrial complex III
Ganoderic acids

ABSTRACT

Ganoderma lucidum has high commercial value because it produces many active compounds, such as ganoderic acids (GAs). Salicylic acid (SA) was previously reported to induce the biosynthesis of GA in *G. lucidum*. In this study, we found that SA induces GA biosynthesis by increasing ROS production, and further research found that NADPH oxidase-silenced strains exhibited a partial reduction in the response to SA, resulting in the induction of increased ROS production. Furthermore, the localization of ROS shows that mitochondria are sources of ROS production in response to SA treatment. An additional analysis focused on the relationship between SA-induced ROS production and mitochondrial functions, and the results showed that inhibitors of mitochondrial complexes I and II exert approximately 40–50% superimposed inhibitory effects on the respiration rate and H_2O_2 content when co-administered with SA. However, no obvious superimposed inhibition effects were observed in the sample co-treated with mitochondrial complex III inhibitor and SA, implying that the inhibitor of mitochondrial complex III and SA might act on the same site in mitochondria. Additional experiments revealed that complex III activity was decreased 51%, 62% and 75% after treatment with 100, 200, and 400 μM SA, respectively. Our results highlight the finding that SA inhibits mitochondrial complex III activity to increase ROS generation. In addition, inhibition of mitochondrial complex III caused ROS accumulation, which plays an essential role in SA-mediated GA biosynthesis in *G. lucidum*. This conclusion was also demonstrated in complex III-silenced strains. To the best of our knowledge, this study provides the first demonstration that SA inhibits complex III activity to increase the ROS levels and thereby regulate secondary metabolite biosynthesis.

1. Introduction

Ganoderma lucidum (*G. lucidum*) is a well-known medicinal basidiomycete widely used in Southeast Asia, because it containing a wide range of immuno-modulatory and bioactive compounds [14,71]. The triterpenes and polysaccharides isolated from *G. lucidum* have numerous biological activities, including anticancer, antioxidant, and hypocholesterolemic effects as well as inhibitory effects on adipocyte differentiation [59,65]. Ganoderic acids (GAs) are some of the major secondary metabolites found in *G. lucidum*, and these compounds belong to the class of triterpenoids [50]. Because of their significant biological activity, the potential of GAs as medicinal agents has been proposed [35,36]. In addition, researches on the GAs also provide a valuable foundation for studying the secondary metabolic processes in fungi. Therefore, many studies have investigated the different regulatory effects of various fermentation conditions on the biosynthesis of GAs. For example, limiting the nitrogen sources in liquid culture leads to an increase in the GA content [77], aspirin treatment is a powerful approach for triggering GA production [75], and acetic acid acts as an inducer to significantly increase GA biosynthesis [54]. Based on these findings, it can be concluded that many factors can influence GA biosynthesis, but defects in basic biology studies have hindered further increases in the commercial value of *G. lucidum*. In recent years, the genomic sequence and genetic transformation system of *G. lucidum* have been established [45,63,7]. These discoveries provide opportunities for the development of basic biological tools for investigating *G. lucidum*, and the resulting developments have promoted the use of *G. lucidum* as a potential model system for studying the complicated mechanisms of secondary metabolic pathways in higher basidiomycetes. Therefore, research on the regulatory pathways of secondary metabolism in *G. lucidum* has become more important.

Salicylic acid (SA), which is considered a plant hormone, shows a

* Corresponding author.
E-mail address: mwzhao@njau.edu.cn (M.-W. Zhao).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.redox.2018.03.018
Received 8 March 2018; Received in revised form 27 March 2018; Accepted 29 March 2018
Available online 31 March 2018

broad distribution in plants [28], plays a key role in the regulation of plant growth and development, and is involved in disease resistance in plants in response to various pathogenic attacks [11,31,56]. In addition, SA has recently been the focus of intensive research efforts due to its function as a signaling molecule during the plant responses to abiotic stresses, such as heavy metal, salinity, drought and temperature stresses [26,32,69], and a few studies have investigated its role in enhancing the production of secondary metabolites in plants. In *Salvia miltiorrhiza* cells, SA treatment exerts an obvious effect on the accumulation of phenolic compounds [13], and in *Ulmus minor*, SA treatment induces the accumulation of sinapyl alcohol and enhances resistance to pathogens [40]. In addition, SA induces salvianolic acid B production in *Salvia miltiorrhiza* [21] and increases volatile oil biosynthesis in *Atractylodes lancea* plantlets [70]. However, the function of SA in microorganisms is still not well understood. In *G. lucidum*, it has been reported that SA treatment can enhance GA accumulation [5], and this interesting phenomenon indicates that plant hormones can induce the biosynthesis of secondary metabolites in fungi. However, the mechanism through which SA regulates the secondary metabolism of *G. lucidum* remains unclear.

To investigate the signaling events induced by SA that result in GA accumulation, the ROS level under SA treatment was analyzed, and our results showed that GA accumulation was observed due to an SA-induced burst of ROS. Additional experiments found that the source of ROS overproduction induced by SA was not only dependent NADPH oxidase (NOX) but also included the mitochondria. To determine the effect of SA treatment on the mitochondria, the ROS levels and respiratory rate after co-treatment with various inhibitors of the mitochondria complex and SA were measured, and the data showed that mitochondria complex III is involved in SA treatment-induced ROS generation.

2. Materials and methods

2.1. Materials and growth conditions

G. lucidum strain ACCC53264 (obtained from the Agricultural Culture Collection of China) was used as the wild-type (WT) strain and grown at 28 °C in potato dextrose agar medium for 7 days. Seed cultures were grown in potato dextrose broth (PDB) medium and placed on a rotary shaker incubator at 150 rpm and 28 °C for 7 days. The fermentation experiments were performed at 28 °C in CYM (1% w/v maltose, 2% w/v glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4{\cdot}7H_2O$, and 0.46% KH_2PO_4, with an initial pH of 5.5) for 7 days after inoculation with 4% (v/v) seed culture. NOX-silenced strains were also established as previously described [46].

2.2. Extraction and quantification of SA

SAs were extracted from the fungal mycelia using a previously described method [53,73]. The *G. lucidum* strain was grown as described above in CYM for 7 days with or without SA (Sigma, USA), and the mycelia were then frozen in liquid nitrogen for extraction of endogenous free salicylic acid. The levels of free SA were quantified by HPLC based on a previously described method [72]. All the data were corrected based on an internal salicylic acid standard, and the free SA was measured.

2.3. Detection and quantification of GA and intermediates

The total ganoderic acids (GA) and cellular squalene and lanosterol were extracted from fungal mycelia and measured according to a previously described method [62]. To detect GAs and its mesostates under SA treatment, the mycelia were treated with 100 μM SA dissolved in ethanol for 0.5 h according to a previously described method [5]. In the pretreatment experiments, 7-day-old *G. lucidum* strains were pretreated with ascorbic acid (VC, 2 mM), N-acetyl cysteine (NAC, 1 mM), diphenyleneiodonium chloride (DPI, 50 μM), rotenone (Rot, 5 μM), 4,4,4-trifluoro-1-(2-thienyl) − 1,3-butanedione (TTFA, 10 μM) or antimycin A (AA, 5 μM) for 2 h prior to treatment with 100 μM SA.

2.4. ROS detection assay

The production of ROS was assessed according to a previously described method [46] with slight modifications. For fluorescent detection of the ROS, the mycelia were stained with 2′, 7′-dichlorodihydrofluorescein diacetate (DCHF-DA) for 20 min, the fluorescence was detected using a Zeiss Axio Imager A1 fluorescence microscope, and the average fluorescence intensities of DCFH-DA in the mycelia were analyzed using ZEN lite software (Zeiss). The H_2O_2 content of the hyphae liquid was measured by monitoring the A415 of the titanium-peroxide complex according to the method described by [3].

2.5. Detection of mitochondrial ROS production

The mitochondrial ROS production was measured using samples that were double-stained with DCFH-DA and Mito-Tracker Red CMXRos, as described by [74]. The fluorescence was detected using a Zeiss Axio Imager A1 fluorescence microscope, and the average fluorescence intensities were analyzed using ZEN lite software (Zeiss).

2.6. Isolation of G. lucidum mitochondria and measurement of the respiratory rate

The mitochondria were isolated as previously described [15,20], with some modifications. All the steps were performed at 4 °C. The mycelia were frozen and powdered under liquid nitrogen with a mortar and pestle and suspended in a three-fold volume of ice-cold extraction buffer containing 250 mM sucrose, 1 mM EDTA, 0.5% (w/v) polyvinylpyrrolidone-40, 10 mM β-mercaptoethanol, and 50 mM Tris-HCl (pH 7.2). The mixture was homogenized extensively for 30 min on ice, and the homogenate was then centrifuged for 15 min at 1200 × *g*. The supernatants were decanted and centrifuged for 20 min at 17,000 × *g*. The pellets were resuspended and washed twice with wash buffer (250 mM sucrose and 50 mM Tris-HCl, pH 7.2). The mitochondria were finally resuspended in a small volume of wash medium.

The oxygen consumption was measured as described previously [48,8], with some modifications. The oxygen consumed by isolated mitochondria was measured at 25 °C with a Clark-type oxygen electrode (Hansatech Ltd., UK) in 1 mL of reaction medium (0.25 M sucrose, 10 mM KCl, 5 mM EDTA, 20 mM HEPES/Tris pH 7.2, and 0.15% (w/v) bovine serum albumin). The mitochondrial oxygen consumption was measured with 5 mM malate plus 10 mM glutamate (complex I substrate) or 10 mM succinate (complex II substrate). In the experiments requiring the addition of different concentrations of SA, 5 μM Rot, 10 μM TTFA or 5 μM AA was added to the pre-treated samples.

2.7. Assays of the activities of respiratory chain complexes I, II and III

The enzyme activity assays were performed using the Tissue Mitochondrial Complex I, II and III Assay Kit (Comin Biotechnology, China) according to the instructions provided in the kit's manual. After 5 min of incubation in assay buffer at 25 °C, the Complex I activity was measured based on the decrease in the oxidation of NADH to NAD^+, which was assessed by the absorbance at 340 nm. The Complex II activity was measured indirectly by monitoring the reduction of 2,6-dichloroindophenol based on the absorbance at 605 nm. Under catalysis by cytochrome reductase (complex III), the reduced ubiquinone (50 μM decylubiquinol) or coenzyme Q substrates were converted into ubiquinone or coenzyme Q, and ferricytochrome c (ferricyt c^{3+}) was reduced to ferrous cytochrome c (ferrocyt c^{2+}). After 5 min of incubation at 25 °C, the absorbance at 550 nm was obtained to assess the reduction