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E-mail: jzus_b@zju.edu.cn

Correspondence

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Abscisic acid-mediated cytosolic Ca^{2+} modulates triterpenoid accumulation of *Ganoderma lucidum*

Meilin CUI✉, Yitao ZHAO, Xiuhong ZHANG, Wei ZHAO

College of Food Science, Shanxi Normal University, Taiyuan 030031, China

Ganoderma lucidum is a mushroom widely used for its edible and medicinal properties. Primary bioactive constituents of *G. lucidum* are ganoderic triterpenoids (GTs), which exhibit important pharmacological activity. Abscisic acid (ABA), a plant hormone, is associated with plant growth, development, and stress responses. ABA can also affect the growth, metabolism, and physiological activities of different fungi and participates in the regulation of the tetracyclic triterpenes of some plants. Our findings indicated that ABA treatment promoted GT accumulation by regulating the gene expression levels (squalene synthase (*sqs*), 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgr*), and lanosterol synthase (*ls*)), and also activated cytosolic Ca^{2+} channels. Furthermore, under ABA mediation, exogenous Ca^{2+} donors and inhibitors directly affected the cytosolic Ca^{2+} concentration and related gene expression in Ca^{2+} signaling. Our study also revealed that ABA-mediated cytosolic Ca^{2+} played a crucial regulatory role in GT biosynthesis, accompanied by antioxidant defense modulation with increasing superoxide dismutase (SOD) activity and ascorbate peroxidase (APX) activity, and the resistance ability of $O_2^{\bullet-}$ and glutathione (GSH) contents.

G. lucidum is a traditional precious mushroom with extremely important pharmacological activities, including antitumor, antihypertensive, immunomodulatory, antiviral, and liver protection effects (Cui et al., 2017). To date, approximately 400 compounds have been isolated and characterized from *G. lucidum*; among them, GT are considered to be the major bioactive secondary metabolites responsible for its remarkable therapeutic functions, and the GT content is generally used to assess the overall quality of *G. lucidum*.

Several strategies are currently employed to manipulate the metabolic behavior of GT, such as the optimization of fermentation conditions and the overexpression of key genes for GT biosynthesis in the mevalonate (MVA) pathway, including *hmgr*, *sqs*, and *ls* (or 2,3-oxidosqualene cyclase (*osc*)) (Zhang et al., 2017). Numerous studies have shown a positive correlation between environmental elicitors (e.g., nitric oxide, methyl jasmonate, hydrogen sulfide, and calcium) and GT accumulation (Ren et al., 2019). Therefore, it is important to understand the related molecular mechanisms that underlie elicitor-induced GT accumulation in order to facilitate the successful commercial production of GT.

As an important plant hormone, ABA performs an essential role in multiple physiological processes through a series of signal transduction pathways (Siebeneichler et al., 2020) and is also involved in the induction of resistance against stressful conditions, especially excessive temperature, drought, and salinity (Marusig and Tombesi, 2020; Parveen et al., 2021). More importantly, increasing evidence shows that ABA is present and is synthesized in some fungi species (Arnesen et al., 2022), suggesting that it is likely important in fungal development. ABA addition could also affect the physiological functions and metabolite characteristics of different fungi, such as fungal trap formation and the capture of nematodes (Xu et al., 2011), fungal colonization in plants (Peskan-Berghöfer et al., 2015), and transcription and metabolite processes (Xu et al., 2018).

Furthermore, ABA also participates in the regulation of tetracyclic triterpenes (triterpenoid, ginsenoside,

✉ Meilin CUI, cuimeilin1988@163.com
Meilin CUI, https://orcid.org/0000-0003-2800-5770

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cucurbitacins, saponin, etc.), which is likely due to its regulation of the expression of transcription factors, key enzyme genes (such as *hmgr*), or specific gene promoters (Yin et al., 2020; Kong et al., 2023).

The occurrence of the Ca^{2+} signal in many distinct biological processes provides the potential ability to respond to various environmental stimuli and maintain normal development. Ca^{2+} signaling functions via Ca^{2+}-permeable channel-mediated influx and membrane transporter-mediated efflux. The activated Ca^{2+} influx systems induce an increase in cytosolic Ca^{2+} concentration and elicit Ca^{2+} signaling (Li et al., 2021); moreover, cytosolic Ca^{2+} can further activate downstream calcium-related receptors and modulate downstream genes (Ren et al., 2019). Additionally, several ABA-induced changes have been identified in plasma membrane ion channels and their potential signaling intermediates, especially cytosolic Ca^{2+} (MacRobbie, 2000). In ABA-mediated regulation of physiological metabolism, cytosolic Ca^{2+} concentration is activated and changed, and is even accompanied by interaction with other signals (reactive oxygen species (ROS), nitric oxide (NO), etc.) (Zehra et al., 2020; Li et al., 2021).

Comprehensively, based on the important breakthrough point of increasing GT content, we aimed to clarify the function of ABA-mediated cytosolic Ca^{2+} induction in GT biosynthesis of *G. lucidum*, with a view to providing further evidence for the mechanism of environmental regulation in fungal secondary metabolism.

Firstly, we investigated the effects of different ABA concentrations (0, 100, 200, 300, 400, and 500 μmol/L), ABA addition timings (Days 2, 3, 4, and 5 of cultivation), and ABA treatment durations (24, 48, 72, and 96 h) on GT synthesis in *G. lucidum* (Fig. S1). We found that exogenous ABA could promote GT accumulation by regulating the related gene expression of key enzymes (*hmgr*, *sqs*, and *ls*) of *G. lucidum*, and the appropriate treatment conditions were chosen to be ABA (300 μmol/L) added on Day 4 with a treatment duration of 48 h (Fig. S1).

Then, the Ca^{2+} concentration was determined using ion chromatography to exclude the possibility that ABA treatment affects the cytosolic Ca^{2+} concentration of mycelium. Compared to the respective control groups, the cytosolic Ca^{2+} concentrations showed increases of 37.53% and 14.83% after treatment for 48 and 72 h, respectively, while it decreased by 29.39% after 96 h. Whether or not it was ABA-induced, cytosolic Ca^{2+} concentration showed a downward trend with increasing incubation time, likely due to cell autolysis and Ca^{2+} leakage. All these results led to the preliminary hypothesis of a functional relationship in Ca^{2+} concentration mediated by ABA treatment (Figs. S2 and S3).

We also elucidated the role of ABA-mediated cytosolic Ca^{2+} induction in the regulation of GT biosynthesis. After ABA treatment for 24 h, an exogenous Ca^{2+} donor ($CaCl_2$, 10 mmol/L), a Ca^{2+} chelator (ethylene glycol tetraacetic acid (EGTA), 10 mmol/L), and a putative intracellular Ca^{2+} channel blocker ($LaCl_3$, 10 mmol/L) were added to respective cultures and the cultures were incubated for 24 h. The cytosolic Ca^{2+} concentration and related gene expression levels (calcium-channel protein *cch1*, calcium transporting ATPase, calcium-dependent mitochondrial carrier protein, vacuolar calcium ion transporter, and calcium/calmodulin-dependent protein kinase *cmkB*, which were selected via transcriptome sequencing) were all determined (Fig. S4, Table S1).

The results showed that $CaCl_2$-only addition could remarkably increase Ca^{2+} concentration by 2.59-fold compared to the contrast check (CK), and under ABA+$CaCl_2$ treatment, the Ca^{2+} concentration was 2.99-, 2.18-, and 1.15-fold compared to the CK, ABA, and $CaCl_2$-only treatments, respectively, indicating that exogenous Ca^{2+} supplementation and ABA+$CaCl_2$ could both increase the cytosolic Ca^{2+} concentration. However, the addition of EGTA or $LaCl_3$ showed negative feedback (Figs. 1a and S5). The related analysis of gene expression revealed that ABA and ABA+$CaCl_2$ could significantly upregulate the expression levels of *cch1* and calcium-dependent mitochondrial carrier protein. For the expression of calcium transporting ATPase, $CaCl_2$-only and ABA treatments could increase its expression level, respectively, but ABA+$CaCl_2$ treatment affected its expression due to Ca^{2+}. For the expression of *cmkB*, ABA treatment downregulated its expression, and ABA+$CaCl_2$ treatment did not significantly affect its expression, while on the contrary, ABA+$CaCl_2$ treatment inhibited the expression of vacuolar calcium ion transporter compared to the $CaCl_2$-only treatment. All these results suggested that ABA and ABA-mediated cytosolic Ca^{2+} could ultimately affect Ca^{2+} concentration by affecting the expression of the related genes, which present different changing characteristics (Fig. 1b).

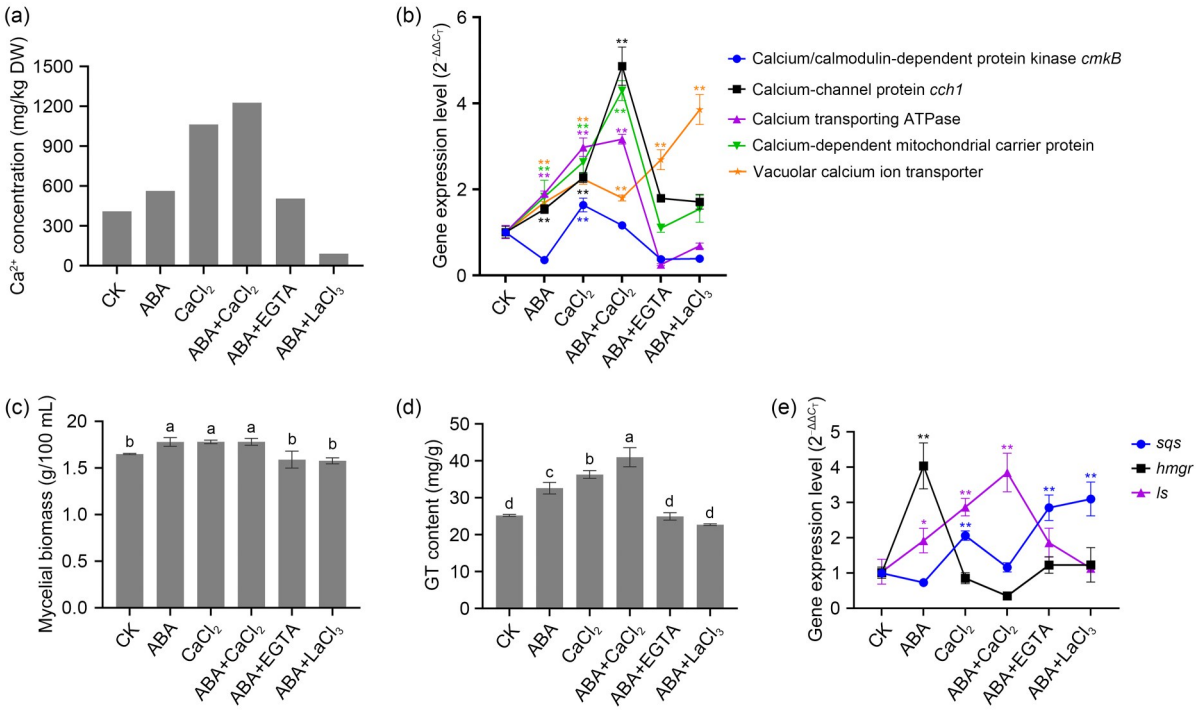

Fig. 1 Effects of ABA-mediated Ca^{2+} on cytosolic Ca^{2+} concentration (a), the related gene expression levels in Ca^{2+} signaling (b), the mycelial biomass (c), GT accumulation (d), and expression levels of the key enzyme genes of GT biosynthesis (e) of *Ganoderma lucidum*. Data were expressed as the mean±standard deviation (SD), *n*=3. Different lowercase letters indicate significant differences among various treatments (*P*<0.05). $^{*}P$<0.05, $^{**}P$<0.01 vs. CK. ABA: abscisic acid; CK: contrast check; DW: dry weight; EGTA: ethylene glycol tetraacetic acid; GT: ganoderic triterpenoids; *hmgr*: 3-hydroxy-3-methylglutaryl-CoA reductase; *ls*: lanosterol synthase; *sqs*: squalene synthase.

Furthermore, a comprehensive analysis of the effect of ABA-mediated cytosolic Ca^{2+} on GT biosynthesis was also performed (Figs. 1c–1e). For GT content, we noticed that compared to the CK, the $CaCl_2$-only and ABA+$CaCl_2$ treatments could increase the GT content by 43.95% and 62.57%, respectively, while the addition of EGTA and $LaCl_3$ decreased its content. The transcriptional levels of the key enzyme genes of GT biosynthesis (*sqs*, *hmgr*, and *ls*) were also detected. The treatments of ABA, $CaCl_2$-only, and ABA+$CaCl_2$ could promote the *ls* expression level. Contrarily, the *hmgr* expression levels under the ABA, $CaCl_2$-only, and ABA+$CaCl_2$ treatments were 404%, 85%, and 36%, respectively, of that of CK, indicating that cytosolic Ca^{2+} downregulated the *hmgr* expression level. Meanwhile, ABA+$CaCl_2$ decreased the *sqs* expression level. Collectively, these findings revealed that ABA-mediated cytosolic Ca^{2+} could affect GT accumulation by regulating the gene expression levels of key enzymes.

To further test the effect of ABA-mediated cytosolic Ca^{2+} on strain antioxidant capacity, nine antioxidant indexes were comprehensively analyzed. Notably, these antioxidant indexes showed different changing characteristics (Fig. 2).

The accumulation of $O_2^{\cdot-}$ causes lipid peroxidation and fission in the cell membrane, resulting in cell damage and even death. Superoxide dismutase (SOD) can catalyze the reaction of $O_2^{\cdot-}$ to reduce or eliminate the excess $O_2^{\cdot-}$ generated in the body's metabolic process, ultimately maintaining the body's metabolic balance. Compared to the CK group, the SOD activity level increased by 25.93% after ABA addition (Figs. 2a and 2b). Under the ABA+$CaCl_2$ treatment, the SOD activity level increased by 27.74% and 19.28% compared to the ABA and $CaCl_2$-only treatments, respectively. Similarly, we found the same trend of analysis results appeared in the resistance ability of $O_2^{\cdot-}$. Under the ABA+$CaCl_2$ treatment, the resistance ability of $O_2^{\cdot-}$ increased by 62.65%, 31.67%, and 14.81% compared to the CK, ABA, and $CaCl_2$-only treatments, respectively, showing that ABA and ABA-mediated cytosolic Ca^{2+} could improve SOD activity and the resistance ability of $O_2^{\cdot-}$. Ascorbate peroxidase (APX) is also a key antioxidant enzyme in scavenging H_2O_2 and ascorbic acid metabolism. We noticed that, under the ABA+$CaCl_2$ treatment, APX

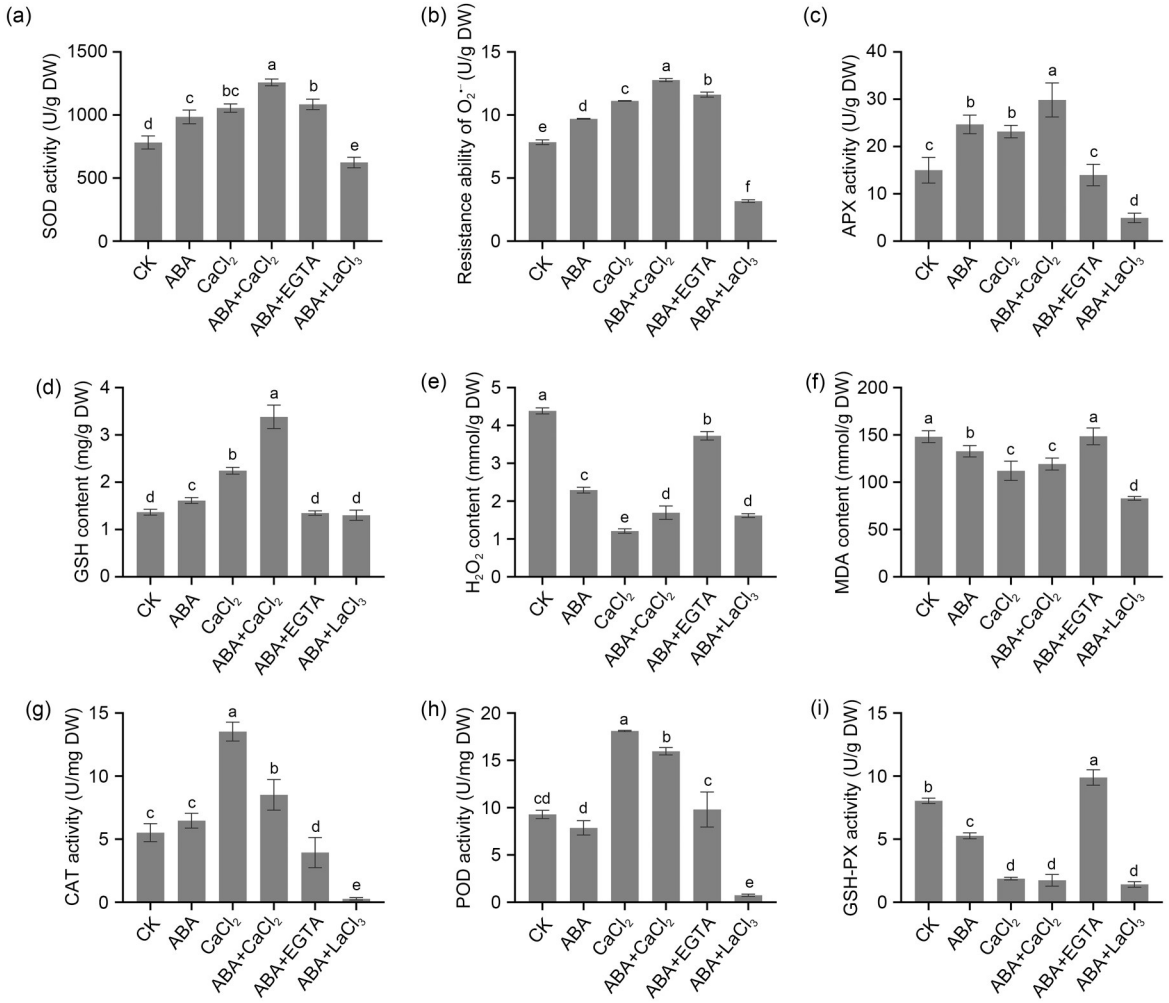

Fig. 2 Effects of ABA-mediated Ca^{2+} on the antioxidant capacity of *Ganoderma lucidum*, including SOD activity (a), resistance ability of $O_2^{\cdot-}$ (b), APX activity (c), the contents of GSH, H_2O_2, and MDA (d–f), CAT activity (g), POD activity (h), and GSH-PX activity (i). Data were expressed as the mean±standard deviation (SD), n=3. Different lowercase letters indicate significant differences among various treatments (P<0.05). ABA: abscisic acid; APX: ascorbate peroxidase; CAT: catalase; CK: contrast check; DW: dry weight; EGTA: ethylene glycol tetraacetic acid; GSH: glutathione; GSH-PX: GSH peroxidase; MDA: malondialdehyde; POD: peroxidase; SOD: superoxide dismutase.

activity increased by 98.88%, 20.85%, and 28.76% compared to the CK, ABA, and $CaCl_2$-only treatments, respectively (Fig. 2c), indicating that ABA, especially ABA-mediated cytosolic Ca^{2+}, could also significantly increase APX activity. Notably, as shown in Fig. 2d, ABA+$CaCl_2$ treatment could improve the glutathione (GSH) content by 2.48-, 2.09-, and 1.51-fold compared to the CK, ABA, and $CaCl_2$-only treatments, respectively, but GSH content was decreased after EGTA and $LaCl_3$ were added, suggesting that ABA and ABA-mediated cytosolic Ca^{2+} promoted GSH content.

Furthermore, the findings also showed that treatments with ABA and $CaCl_2$-only could reduce H_2O_2 content and malondialdehyde (MDA) damage to the strain and improve its antioxidant capacity, whereas the role of ABA+$CaCl_2$ was not more effective compared to the $CaCl_2$ treatment (Figs. 2e and 2f). Conversely, $CaCl_2$ addition could promote catalase (CAT) activity and peroxidase (POD) activity, while ABA treatment did not work in these cases, revealing that the ABA+$CaCl_2$ treatment could increase CAT activity and POD activity mainly due to the addition of Ca^{2+} rather than ABA (Figs. 2g and 2h). In addition, the results revealed that ABA and ABA-mediated cytosolic Ca^{2+} negatively regulated GSH peroxidase (GSH-PX) activity (Fig. 2i). All the above implied that ABA-mediated cytosolic Ca^{2+} could affect changes in the antioxidant

indicators, thereby affecting the antioxidant capacity and physiological metabolism of the body.

To sum up, our findings indicated that the ABA treatment was involved in promoting GT accumulation by regulating the gene expression levels, and in activating cytosolic Ca^{2+} channels. Furthermore, under ABA mediation, exogenous Ca^{2+} stimulation directly affected the cytosolic Ca^{2+} concentration and related gene expression in Ca^{2+} signaling. Our findings also revealed that ABA-mediated cytosolic Ca^{2+} significantly modulated GT biosynthesis, as well as presenting different characteristics for various antioxidant indicators. These results are expected to be useful for large-scale GT production and further exploration of the primary mechanism of GT accumulation by exogenous induction.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

Data availability statement

The data supporting the findings of this study are available within the paper and its supplementary information files. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

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Author contributions

Meilin CUI: project administration, conceptualization, data curation, and writing – review & editing. Yitao ZHAO: conceptualization and data analysis. Xiuhong ZHANG: conceptualization and supervision. Wei ZHAO: supervision. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Meilin CUI, Yitao ZHAO, Xiuhong ZHANG, and Wei ZHAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Figs. S1–S5; Table S1; Materials and methods