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Research Article
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Heme oxygenase-1/carbon monoxide signaling participates in the accumulation of triterpenoids of *Ganoderma lucidum*

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Abstract: Ganoderic triterpenoids (GTs) are the primary bioactive constituents of the Basidiomycotina fungus, *Ganoderma lucidum*. These compounds exhibit antitumor, anti-hyperlipidemic, and immune-modulatory pharmacological activities. This study focused on GT accumulation in mycelia of *G. lucidum* mediated by the heme oxygenase-1 (HO-1)/carbon monoxide (CO) signaling. Compared with the control, hemin (10 μmol/L) induced an increase of 60.1% in GT content and 57.1% in HO-1 activity. Moreover, carbon monoxide-releasing molecule-2 (CORM-2), CO donor, increased GT content by 56.0% and HO-1 activity by 18.1%. Zn protoporphyrin IX (ZnPPIX), a specific HO-1 inhibitor, significantly reduced GT content by 26.0% and HO-1 activity by 15.8%, while hemin supplementation reversed these effects. Transcriptome sequencing showed that HO-1/CO could function directly as a regulator involved in promoting GT accumulation by regulating gene expression in the mevalonate pathway, and modulating the reactive oxygen species (ROS) and Ca^{2+} pathways. The results of this study may help enhance large-scale GT production and support further exploration of GT metabolic networks and relevant signaling cross-talk.

Key words: *Ganoderma lucidum*; Triterpenoid; Heme oxygenase-1 (HO-1)/carbon monoxide (CO) signaling; Transcriptome sequencing

1 Introduction

Ganoderma lucidum [(Leyss.ex.Fr.) Karst], known as the treasure of Chinese medicinal fungi, has important pharmacological activities, such as antitumor (Cui et al., 2015), anti-chemotherapy (Liu et al., 2021), antimicrobial (Ahmad, 2018), anti-hyperlipidemic (Nie et al., 2013), and immune-modulatory (Hu et al., 2020) activities. Ganoderic triterpenoids (GTs) are considered the primary bioactive constituents of this fungus (Zhao et al., 2016; Su et al., 2020). The structural identity and pharmacological activity of GTs have attracted broad attention and research interest (Fatmawati et al., 2013; Cui et al., 2017; Peng et al., 2019; Sharma et al., 2019). In addition, augmenting GT content is a critical prerequisite for large-scale production of medicinal chemicals. An appreciation of GT synthesis mechanisms is necessary for inducing accumulation of GT.

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GT synthesis is regulated by a series of complex biochemical reactions of *G. lucidum* during fermentation. Modulation of these reactions through external stimuli may allow regulation of GT production and increase accumulation of these compounds (Gu et al., 2018; Ren et al., 2019). Hence, research on signal transduction mechanisms involved in GT synthesis is important for understanding the secondary metabolism of *G. lucidum*. Previous studies indicated that several signaling pathways and their cross-talk, such as reactive oxygen species (ROS) (Lian et al., 2021), Ca^{2+} (Gu et al., 2018), mitogen-activated protein kinase (MAPK) (Tan et al., 2018), and nitric oxide (NO) (Gu et al., 2017), are involved in regulating GT synthesis. Metabolic networks in *G. lucidum* are extremely complicated and still other signaling pathways may affect GT synthesis. Heme oxygenases (HOs; EC1.14.99.3) are sensitive and efficient microsomal enzymes, widely encountered in organisms. HOs catalyze the oxidative conversion of heme to biliverdin, accompanied by the release of carbon monoxide (CO) and free iron (Fe^{2+}). Biliverdin is then converted to bilirubin (BR) by biliverdin reductase (Carota et al., 2019). The involvement of HOs in the regulation of redox homeostasis and plant

growth has attracted increasing attention (Bai et al., 2012; Singh and Bhatla, 2016). Substantial differences in the function, subcellular localization, and cofactors of HOs exist, yet the mechanism of heme degradation is conserved among taxa. The heme oxygenase (HO) family in plants consists of HO-1, HO-2, HO-3, and HO-4. HO-1 has the highest expression, which can be induced by oxidative stressors, such as ultraviolet-B irradiation, salinity, and osmotic pressure. Moreover, plant growth and development processes are influenced by HO-1 expression (Liu et al., 2010; Zilli et al., 2014; Duan et al., 2016; Santa-Cruz et al., 2017).

CO is produced mainly by heme degradation and plays a vital role in plant metabolism. Several studies show that HO-1 is critical for plant resistance to oxidative damage, and this function is closely related to CO generation. HO-1 is likely the only enzyme that produces CO in plants, and HO-1/CO signaling participates in various metabolic processes, such as resistance to environmental stress, regulation of lateral and adventitious roots, induction of stomatal closure, and possibly seed germination and leaf senescence (Han et al., 2012; He and He, 2014; Amooaghaie et al., 2015). Further, HO-1/CO signaling always has cross-talk with NO, hydrogen sulfide (H_2S), and cyclic guanosine monophosphate (cGMP) signaling (Bai et al., 2012; Wu et al., 2013; Amooaghaie et al., 2015).

Research on HO-1/CO signaling has focused mainly on specific model plants. The involvement of HO-1/CO signaling in GT accumulation in *G. lucidum* was uncertain. In this study, we aimed to clarify the role of HO-1/CO signaling in GT biosynthesis in *G. lucidum*, and to analyze the differential expression of response genes regulated by HO-1/CO signaling.

2 Materials and methods

2.1 Liquid cultivation of *G. lucidum*

G. lucidum was cultivated in seed culture medium at 28 ℃ for 7 d with shaking at 180 r/min, which was then inoculated at 10% (volume fraction) into a fermentation culture medium at 28 ℃ for 7 d with shaking at 180 r/min.

The seed culture medium contained 10 g/L potato extract, 20 g/L glucose, 18 g/L peptone, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4$, and 0.05 g/L vitamin B_1 (VB_1), at pH 5.5. The fermentation culture medium contained 30 g/L glucose, 18 g/L peptone, 3 g/L KH_2PO_4, 1.5 g/L $MgSO_4$, and 0.05 g/L VB_1, at pH 5.5. All these reagents were obtained from Solarbio (Beijing, China) and Aladdin (Shanghai, China).

2.2 Chemicals and treatments

All chemicals were obtained from Sigma (St. Louis, MO, USA). Hemin was used at concentrations of 0–80 μmol/L as an HO-1 inducer. Zn protoporphyrin IX (ZnPPIX, a specific HO-1 inhibitor), carbon monoxide-releasing molecule-2 (CORM-2, CO donor), BR, and Fe^{2+} were all used at a concentration of 10 μmol/L.

Hemin, ZnPPIX, CORM-2, BR, Fe^{2+}, and ZnPPIX+hemin were respectively added to fermentation cultures on the 4th day of incubation and incubated for an additional 72 h. The treatment "ZnPPIX+hemin" was as follows: ZnPPIX was added on the 4th day and incubated for 48 h, and then hemin was added and incubated for 24 h. Finally, the mycelia were collected and analyzed on the 7th day.

2.3 Determination of mycelial biomass and GT content

Mycelia were collected by centrifuging at 3000*g* for 30 min, then washed and dried at 60 ℃ to a constant weight. The mycelia were mixed with 95% (volume fraction) ethanol (1∶50, mass (g)∶volume (mL) ratio) and extracted twice by ultrasound at 400 W and 50 ℃ for 20 min, and then the extracted supernatant was merged. The vanillin-glacial acetic acid-perchloric acid method was used for the determination of GT content and carried out as follows: 0.1 mL of sample diluent was added and heated to evaporate solvent at 60 ℃, and then 0.2 mL of 0.05 g/mL vanillin-glacial acetic acid solution and 0.5 mL of perchloric acid were added. The mixture was heated at 60 ℃ for 20 min and cooled for 15 min. The absorbance of the sample was measured at 550 nm after 5 mL of glacial acetic acid was added (Chen et al., 2017; Gu et al., 2017).

2.4 RT-PCR analysis

Total RNA isolation and real-time polymerase chain reaction (RT-PCR) were carried out as previously described (Tian et al., 2019). Complementary DNA (cDNA) was amplified by PCR using the following primers: for 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase (*hmgr*; accession number EU263989), forward 5'-GTCATCCTCCTATGCCAAAC-3' and

reverse 5'-GGGCGTAGTCGTAGTCCTTC-3'; for squalene synthase (*sqs*; accession number DQ494674), forward 5'-ACAGTTGTCAGCGAAGAGC-3' and reverse 5'-CGTAGTGGCAGTAGAGGTTG-3'; for lanosterol synthase (*ls*; accession number FJ195872), forward 5'-CTTCCGCAAGCACTACCCG-3' and reverse 5'-AGCAGATGCCCCACGAGCC-3'. Relative abundance was determined, using glyceraldehyde 3-phosphate dehydrogenase (*gpd*) as an internal standard. The primers used for validation of differentially expressed genes (DEGs) from transcriptome sequencing analysis are listed in Table S1.

2.5 HO-1 activity determination and gene expression analysis

HO-1 activity was determined using a microbial HO-1 enzyme-linked immunosorbent assay (ELISA) kit (Jijin Chemical Technology Co., Ltd., Shanghai, China).

Gene expression of *HO-1* was analyzed by RT-PCR using the following primers: forward 5'-GGACTCTTCTGGGACTTGGC-3' and reverse 5'-AAGGATACAGGTGTGCAGCC-3'.

2.6 Transcriptome sequencing analysis

G. lucidum mycelia treated with hemin and ZnPPIX were analyzed by transcriptome sequencing, including library construction, sequencing, data preprocessing, and gene annotation. Paired-end (PE) sequencing of libraries used the Illumina HiSeq platform (HiSeq X Ten; https://www.illumina.com) and next-generation sequencing (NGS) technology. FastQC (V0.11.8) was used to assess the quality of sequenced data.

Data analysis of transcriptome sequencing was used to match unigenes obtained by Illumina sequencing with unique known proteins against various protein databases, namely: National Center of Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov), non-redundant protein sequences (Nr), Swiss-Prot, Pfam 13, Gene Ontology (GO, 2.40.0), Kyoto Encyclopedia of Genes and Genome (KEGG, 3.16.1), and evolutionary genealogy of genes: non-supervised orthologous groups (eggNOG 4.5).

DESeq 1.39.0 was used to analyze genes expressed differentially between samples. The *P*-value was adjusted using the *q*-value; $P<0.05$ and $|\log_2(\text{fold change})|>1$ were set as thresholds for significant differential expression. GO annotation was extracted and used in GO functional enrichment analysis. This analysis was primarily used to identify which DEGs were significantly enriched in GO functional categories.

2.7 Statistical analysis

All experiments were carried out at least three times with identical or similar results. The results are expressed as mean±standard deviation (SD). Statistical analysis was performed using IBM SPSS statistics 20 and Duncan's multiple-range test, and $P<0.05$ was considered significantly different.

3 Results

3.1 Effects of hemin on GT accumulation and HO-1 activity

Different concentrations of hemin (0−80 μmol/L) were added to the fermentation cultures as described, and mycelial biomass, GT content, and HO-1 activity were determined (Fig. 1). Mycelial biomass significantly increased by 15.7% and 17.9% after induction with 20 and 40 μmol/L of hemin, respectively. Differences in biomass were not significant among hemin concentrations of 10, 20, and 40 μmol/L. GT content in the control was (21.06±2.36) mg/g, and increased by 34.6%, 60.1%, and 51.2% for hemin concentrations of 5, 10, and 20 μmol/L, respectively. HO-1 activity was (17.03±0.49) mU/g under the control condition, and increased by 57.1% and 52.5% for hemin concentrations of 10 and 20 μmol/L, respectively.

Exogenous hemin, an HO-1 inducer, significantly promoted GT accumulation over a certain concentration range. HO-1 activity was also affected and showed a similar trend. A functional relationship between HO-1 activity and GT content was hypothesized, and 10 μmol/L of hemin was chosen for follow-up studies.

3.2 Effects of HO-1/CO signaling on GT accumulation and HO-1 activity

Hemin, ZnPPIX, CORM-2, BR, Fe^{2+}, and ZnPPIX+hemin were added respectively to fermentation cultures as described above, at a concentration of 10 μmol/L. Compared with the control, mycelial biomass increased significantly (by 37.1%) only after CORM-2 treatment (Fig. 2). For GT content, hemin, CORM-2, and BR treatments induced significant increases of 60.1%, 56.0%, and 32.3%, respectively, while there was no significant

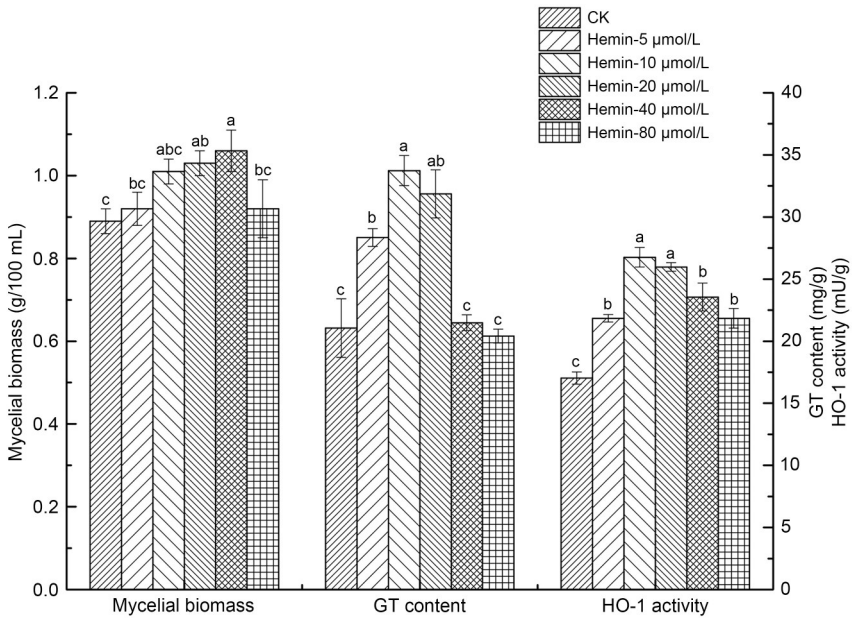

Fig. 1 Effects of different hemin concentrations (0−80 μmol/L) on the biomass, ganoderic triterpenoid (GT) accumulation, and heme oxygenase-1 (HO-1) activity of *Ganoderma lucidum*. Compared with the control (CK), mycelial biomass increased by 15.7% (20 μmol/L hemin) and 17.9% (40 μmol/L hemin); the GT content increased by 34.6% (5 μmol/L hemin), 60.1% (10 μmol/L hemin), and 51.2% (20 μmol/L hemin); HO-1 activity increased by 57.1% (10 μmol/L hemin) and 52.5% (20 μmol/L hemin). Data are expressed as mean±standard deviation (SD), *n*=3. Different letters indicate significant differences among groups at *P*<0.05.

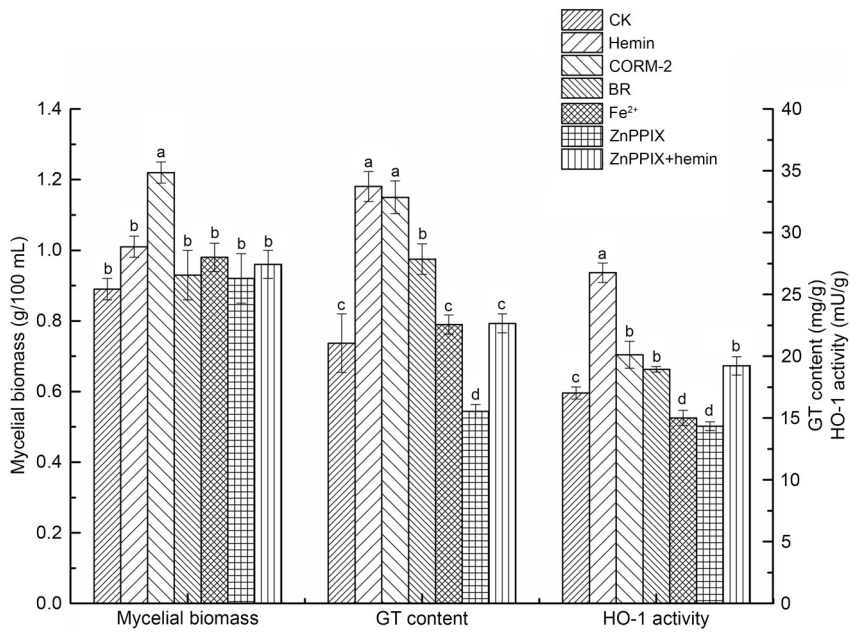

Fig. 2 Effects of different treatments on the biomass, ganoderic triterpenoid (GT) accumulation, and heme oxygenase-1 (HO-1) activity of *Ganoderma lucidum*. Hemin, Zn protoporphyrin IX (ZnPPIX), carbon monoxide-releasing molecule-2 (CORM-2), bilirubin (BR), Fe^{2+}, and ZnPPIX+hemin were added separately to fermentation cultures and induced for 72 h at a concentration of 10 μmol/L. Compared with the control (CK), the mycelial biomass increased by 37.1% (CORM-2); GT content increased by 60.1% (hemin), 56.0% (CORM-2), and 32.3% (BR), but decreased by 26.2% (ZnPPIX) and then recovered with hemin supplementation; HO-1 activity increased by 57.1% (hemin), 18.1% (CORM-2), and 11.2% (BR), but decreased by 15.8% (ZnPPIX), which was reversed by hemin supplementation. Data are expressed as mean± standard deviation (SD), *n*=3. Different letters indicate significant differences among groups at *P*<0.05.

difference under Fe^{2+} treatment. However, as a specific HO-1 inhibitor, ZnPPIX significantly reduced GT content by 26.2%. Hemin supplementation during the last day of ZnPPIX treatment largely reversed this effect, and GT content was then close to control levels. HO-1 activity was also analyzed. Compared with the control, hemin, CORM-2, and BR treatments increased HO-1 activity by 57.1%, 18.1%, and 11.2%, respectively. ZnPPIX inhibited HO-1 activity by 15.8%, but hemin supplementation largely eliminated this effect. We also found that the HO-1 activity was reduced by 11.9% under Fe^{2+} treatment, which indicated that Fe^{2+} negatively regulated the HO-1 activity.

From the above results, we found that the treatments of hemin, ZnPPIX, CORM-2, BR, and ZnPPIX+hemin had greater effects on GT content and HO-1 activity, and these treatments were chosen for the determination of *HO-1* gene expression levels. RT-PCR determination (Figs. 3 and S1, Table S2) showed that hemin and CORM-2 treatments significantly up-regulated *HO-1* gene expression levels: hemin to (1.64±0.10) times and CORM-2 to (1.48±0.15) times the control level. In contrast, the *HO-1* expression level was significantly down-regulated by ZnPPIX to (0.71±0.14) times the control but recovered after hemin supplementation.

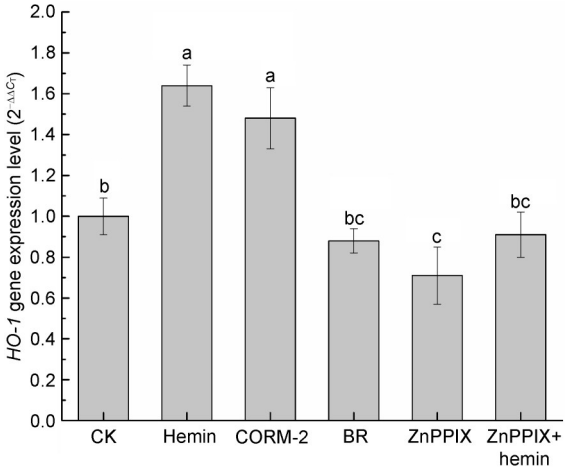

Fig. 3 Effects of different treatments on the heme oxygenase-1 (*HO-1*) gene expression level of *Ganoderma lucidum*. Compared with the control (CK), *HO-1* gene expression was significantly up-regulated to (1.64±0.10) times by hemin and (1.48±0.15) times by carbon monoxide-releasing molecule-2 (CORM-2), and were down-regulated to (0.71±0.14) times by Zn protoporphyrin IX (ZnPPIX), but hemin supplementation reversed this effect. Data are expressed as mean±standard deviation (SD), *n*=3. Different letters indicate significant differences among groups at *P*<0.05. BR: bilirubin.

BR treatment had no significant effect on *HO-1* gene expression. On the whole, the results of HO-1 activity and its gene expression level coincided, except for BR. As the gene expression determination by RT-PCR was more accurate, the RT-PCR results were taken as the main consideration.

In summary, GT content and HO-1 activity changed in parallel in *G. lucidum* mycelia under different treatment conditions. Importantly, GT content increased significantly when CORM-2, a CO donor, was added to the culture medium. These results were consistent with the hypothesis that HO-1, CO, and BR are involved in mediating GT synthesis, and indicated that CO plays the most important role in the process of GT synthesis. So the following study focused mainly on the role of HO-1/CO.

3.3 Effects of HO-1/CO signaling on gene expression of key enzymes in GT synthesis

Hemin, ZnPPIX, CORM-2, and ZnPPIX+hemin were added respectively to fermentation cultures as described above. Gene expression levels of key enzymes (*hmgr*, *sqs*, and *ls*) in the GT synthesis pathway were determined (Tables 1 and S3, Fig. S1).

Table 1 Effects of different treatments on gene expression of key enzymes in the GT synthesis pathway

Treatment	Expression level ($2^{-\Delta\Delta C_T}$)		
	hmgr	*sqs*	*ls*
CK	1.01±0.15^{a}	1.01±0.15^{c}	1.00±0.14^{c}
Hemin	0.40±0.05^{c}	1.25±0.02^{ab}	3.17±0.21^{a}
CORM-2	0.62±0.06^{b}	1.37±0.14^{a}	2.82±0.22^{a}
ZnPPIX	0.31±0.10^{c}	0.37±0.01^{d}	0.74±0.07^{d}
ZnPPIX+hemin	0.40±0.03^{c}	1.11±0.07^{bc}	1.43±0.24^{b}

Data are expressed as mean±standard deviation (SD), *n*=3. Different superscript letters after values indicate significant differences among groups at *P*<0.05. GT: ganoderic triterpenoid; CK: control; CORM-2: carbon monoxide-releasing molecule-2; ZnPPIX: Zn protoporphyrin IX; *hmgr*: 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase; *sqs*: squalene synthase; *ls*: lanosterol synthase.

Compared with the control, the gene expression of *hgmr* was significantly down-regulated in all treatment groups. Hemin and CORM-2 treatments significantly up-regulated the gene expression levels of *sqs* and *ls*, of which the *ls* expression was (3.17±0.21) times and (2.82±0.22) times the control, respectively. ZnPPIX treatment significantly down-regulated the expression of these genes, while hemin addition reversed ZnPPIX inhibition of *sqs* and *ls* expression, which then reached

respectively (1.11±0.07) times and (1.43±0.24) times the control level.

3.4 Analysis of transcript expression mediated by HO-1/CO signaling

To systematically study the effects of HO-1/CO signaling on the physiological processes and signal pathways of *G. lucidum*, the potential mechanisms were explored by transcriptome sequencing. Hemin and ZnPPIX were added separately to the fermentation culture as described above.

Compared with the control, a volcano plot showed that the transcription of 1559 genes was up-regulated (P<0.05, $|\log_2(\text{fold change})|$>2), and 2077 genes were down-regulated by hemin treatment (Fig. 4a). In contrast, ZnPPIX treatment induced up-regulation of 2491 genes and down-regulation of 2408 genes (Fig. 4b). Not surprisingly, alterations in HO-1/CO signaling induced transcriptional changes in a large number of genes.

3.4.1 GO annotation of DEGs

GO annotation was used to classify up- and down-regulated transcripts into putative functional groups to further explore the impacts of HO-1/CO signaling. Compared with the control, 4582 significantly modulated transcripts were identified from hemin-treated mycelia (Table S4): 1134 transcripts (605 up- and 529 down-regulated) were classified into 8 terms of the cellular component category, 843 (388 up- and 455 down-regulated) were classified into 80 terms of the biology process category, and 2605 (1347 up- and 1258 down-regulated) were classified into 67 terms of the molecular function category. Terms identified were those with a P-value of <0.05. As shown in Fig. 5a, the top three enriched terms for the cellular component category were "membrane" (GO:0016020, 388 DEGs), "integral component of membrane" (GO:0016021, 241 DEGs), and "intrinsic component of membrane" (GO:0031224, 241 DEGs). The top three enriched biology process terms were "carbohydrate metabolic process" (GO:005975, 102 DEGs), "oxidation-reduction process" (GO:0055114, 182 DEGs), and "transmembrane transport" (GO:0055085, 96 DEGs). The top three enriched molecular function terms were "hydrolase activity, hydrolyzing *O*-glycosyl compound" (GO:0004553, 72 DEGs), "hydrolase activity, acting on glycosyl bonds" (GO:0016798, 81 DEGs), and "metal ion binding" (GO:0046872, 217 DEGs). The term heme binding (GO:0020037, 50 DEGs) was significant in this category.

In contrast, compared with the control, 6587 transcripts were significantly annotated into 191 GO terms in ZnPPIX-treated mycelia (Table S5): 1484 (603 up- and 881 down-regulated) transcripts were classified into 9 terms of the cellular component category, 1229 (541 up- and 688 down-regulated) were classified into 117 terms of the biology process category, and 3874 (1945 up- and 1929 down-regulated) into 65 terms of the molecular function category. As shown in Fig. 5b, the top three enriched cellular component terms were "membrane" (GO:0016020, 508 DEGs), "intrinsic component of membrane" (GO:0031224, 316 DEGs), and "integral component of membrane" (GO:0016021, 315 DEGs). The top three enriched biology process terms were "carbohydrate metabolic process" (GO:005975, 129 DEGs), "oxidation-reduction

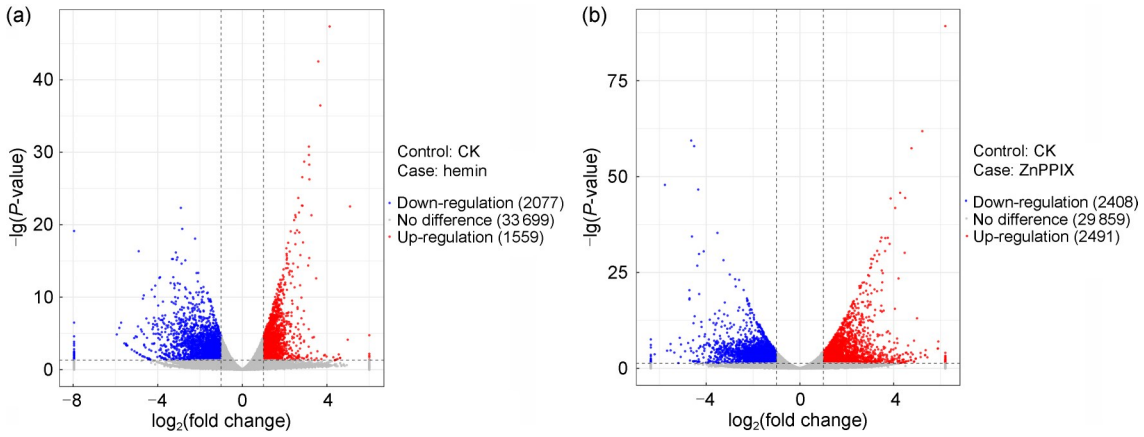

Fig. 4 Overview and volcano plot of differential gene expression of *Ganoderma lucidum* after hemin (a) and Zn protoporphyrin IX (ZnPPIX) (b) exposure relative to the control (CK). Each dot represents a differentially expressed gene.

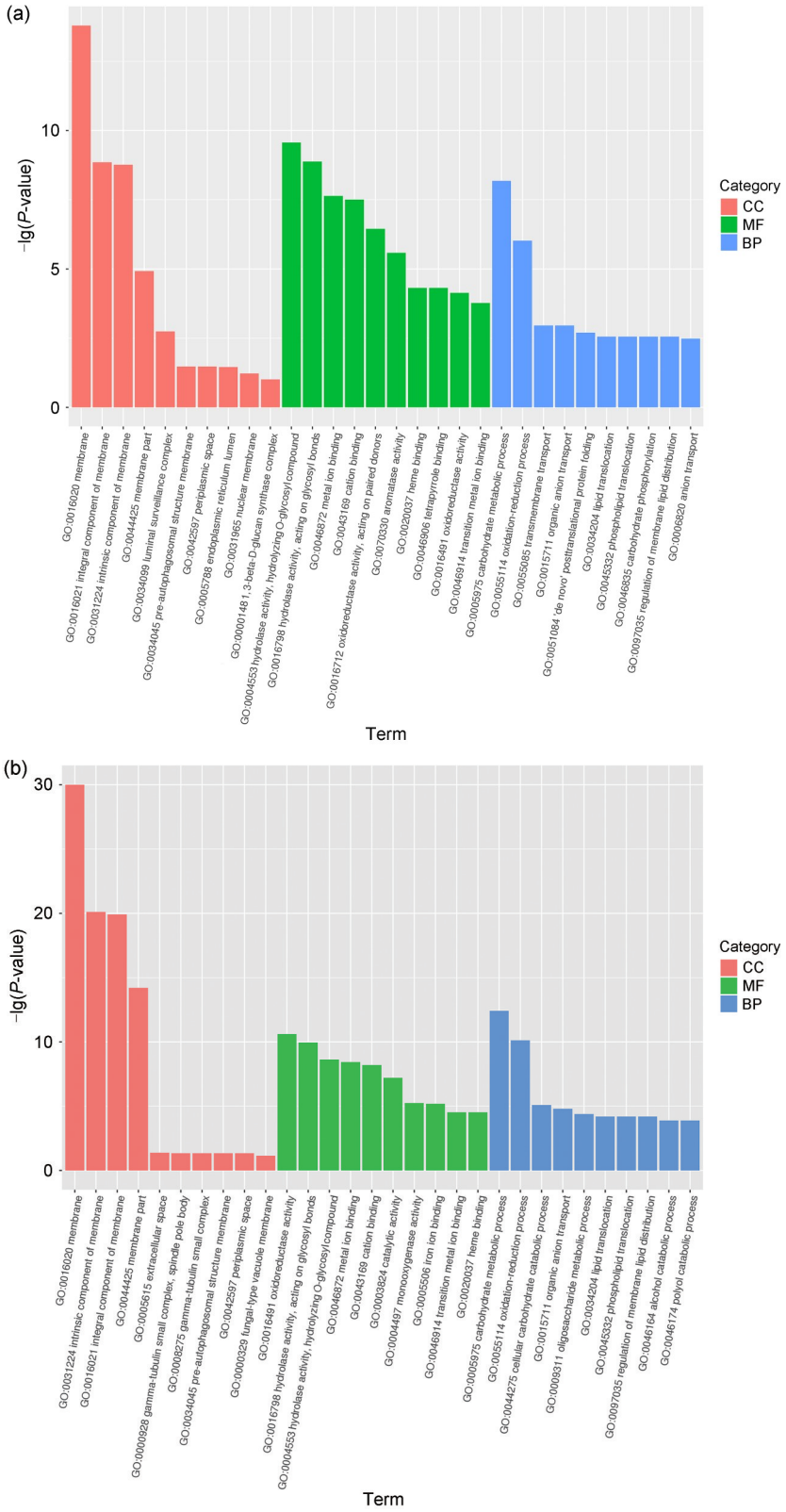

Fig. 5 Gene Ontology (GO) categories of differentially expressed unigenes identified after hemin (a) and Zn protoporphyrin IX (ZnPPIX) (b) treatments relative to control. CC: cellular component; MF: molecular function; BP: biology process.

process" (GO: 0055114, 230 DEGs), and "cellular carbohydrate catabolic process" (GO:0044275, 13 DEGs). The top three enriched molecular function terms were "oxidoreductase activity" (GO: 0016491, 296 DEGs), "hydrolase activity, acting on glycosyl bonds" (GO: 0016798, 95 DEGs), and "hydrolase activity, hydrolyzing *O*-glycosyl compound" (GO:0004553, 79 DEGs). The term heme binding (GO:0020037, 58 DEGs) was again annotated. Annotated data were combined to analyze specific differences in transcript expression following HO-1/CO induction.

3.4.2 Putative genes involved in GT biosynthesis

Compared with the control, treatments with hemin or ZnPPIX altered transcript levels of GT biosynthesis-related genes (Tables S6 and S7). Hemin treatments remarkably increased transcript levels of farnesyl-diphosphate synthase (*fps*), *sqs*, and *ls*, with $\log_2$(fold change) values of 1.18, 1.56, and 2.54, respectively. The transcript level of *hmgr* was significantly reduced with a $\log_2$(fold change) value of −1.53.

The ZnPPIX treatment decreased transcript levels of *hmgr*, phosphomevalonate kinase (*pmvk*), *sqs*, and *ls*, with $\log_2$(fold change) values of −1.73, −1.32, −1.66, and −1.82, respectively. These results were consistent with previous RT-PCR analysis, and provided further evidence that HO-1/CO signaling is involved in mediating GT biosynthesis.

3.4.3 Putative genes involved in antioxidant properties

HO-1/CO signaling may affect several signal transduction pathways. Compared with the control, the results of hemin treatment suggested that ten unigenes might be related to enzymes encoded in the ROS signaling pathway (Table S8). Seven up-regulated unigenes encode the enzymes/proteins peroxidase (POD), peroxiredoxin (PRDX), glutathione peroxidase (GPX), pyridoxamine 5'-phosphate oxidase (PNPO), thioredoxin (TXN), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform 1, and catalase (CAT). Three down-regulated unigenes encode the enzymes/proteins manganese superoxide dismutase (MnSOD), redoxin (REDX), and glutathione *S*-transferase (GST).

ZnPPIX treatment modulated seven unigenes that encode enzymes with antioxidant properties (Table S9). Three up-regulated unigenes were obtained, encoding the related enzymes/proteins TXN, GST, and NADPH oxidase isoform 1. Four down-regulated unigenes encode the related enzymes/proteins POD, MnSOD, CAT, and chloroperoxidase.

3.4.4 Putative genes involved in Ca^{2+} signaling transduction

Compared with the control, changes in transcript expression for genes associated with the Ca^{2+} signaling pathway were also induced by hemin and ZnPPIX treatments. Hemin treatment altered the expression of 11 unigenes predicted to encode enzymes involved in the Ca^{2+} signaling pathway (Table S10). Six up-regulated unigenes encode the enzymes/proteins calcium-activated cation channel, calcium-binding mitochondrial carrier protein (CAMP), calcium-dependent phosphotriesterase, calcium-channel protein cch1 (cch1), phospholipase/carboxylesterase, and phospholipase D. Five down-regulated unigenes encode enzymes related to calmodulin (CALM), calcium/proton exchanger, calcium/calmodulin-dependent protein kinase cmkB, calcium transporting adenosine-triphosphate enzyme (ATPase), and calcium/calmodulin-dependent protein kinase type 1B (CamK 1B).

After ZnPPIX treatment, ten unigenes related to Ca^{2+} signal transduction were identified (Table S11): three up-regulated unigenes correlated with genes encoding the enzymes/proteins CamK 1B, calcium-dependent phosphotriesterase, and phospholipase/carboxylesterase; seven down-regulated unigenes related to the enzymes/proteins CALM, calcium/proton exchanger, calcium ATPase transmembrane, vacuolar calcium ion transporter, calcium transporting ATPase, cch1, and phospholipase D1.

In summary, we found that genes associated with ROS and Ca^{2+} signaling pathways significantly changed after exposure of *G. lucidum* mycelia to hemin or ZnPPIX. Results indicated that HO-1/CO signaling may be involved in the regulation of GT synthesis and affect transduction of ROS and Ca^{2+} signaling.

3.4.5 Validation of mRNA expression profiles by RT-PCR

RT-PCR was used to validate RNA expression of 11 selected DEGs involved mainly in antioxidant processes and Ca^{2+} signaling. As shown in Fig. 6 and Table S12, the expression of five genes (*POD*, *TXN*, *CAT*, *CAMP*, and *cch1*) was up-regulated and the expression of six genes (*GST*, *MnSOD*, *REDX*, *CALM*, *CamK 1B*, and *calcium transporting ATPase*) was down-regulated after the hemin treatment. However,

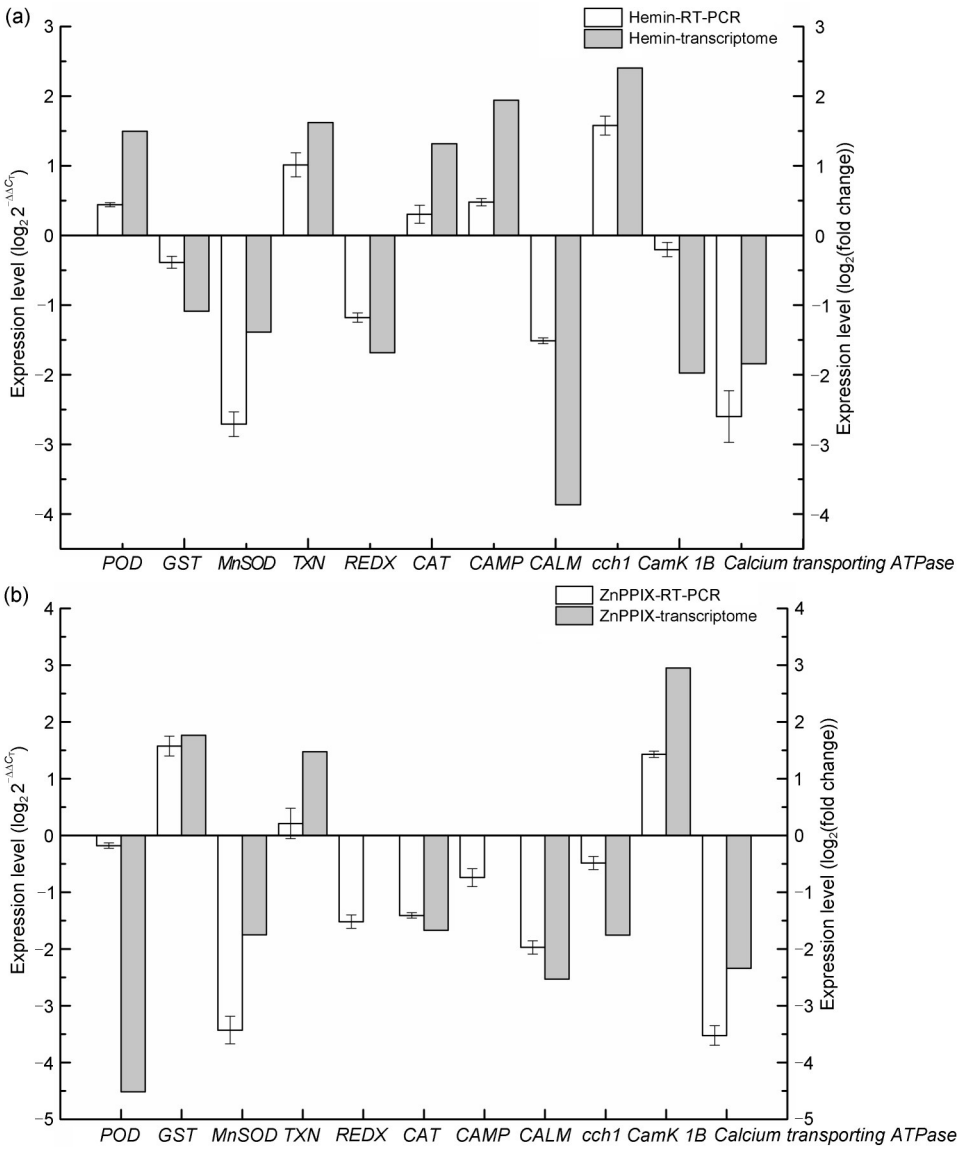

Fig. 6 Real-time polymerase chain reaction (RT-PCR) validation of transcriptome analysis. Eleven selected gene fragments involved in antioxidant properties and Ca^{2+} signaling after hemin (a) and Zn protoporphyrin IX (ZnPPIX) (b) treatments. After the ZnPPIX treatment, the expression levels of *REDX* and *CAMP* were not significantly different in the transcriptome analysis, which is not labeled in the figure. Other RT-PCR results bolstered the credibility of transcriptome experiments. Data are expressed as mean±standard deviation (SD), *n*=3. *POD*: peroxidase; *GST*: glutathione *S*-transferase; *MnSOD*: manganese superoxide dismutase; *TXN*: thioredoxin; *REDX*: redoxin; *CAT*: *catalase*; *CAMP*: calcium-binding mitochondrial carrier protein; *CALM*: calmodulin; *cch1*: calcium-channel protein cch1; *CamK 1B*: calcium/calmodulin-dependent protein kinase type 1B; *ATPase*: adenosine-triphosphate enzyme.

the ZnPPIX treatment induced up-regulation of *GST*, *TXN*, and *CamK 1B*, and down-regulation of eight genes (*POD*, *MnSOD*, *REDX*, *CAT*, *CAMP*, *CALM*, *cch1*, and *calcium transporting ATPase*). These results bolstered the credibility of the DEG results, except for *REDX* and *CAMP* which showed no significant expression from that of controls in the transcriptome analysis.

4 Discussion

The biosynthesis of secondary metabolites during *G. lucidum* fermentation is mediated by multiple signaling pathways and modulated by environmental conditions. GTs are a crucial source of bioactive ingredients and have attracted wide attention with

respect to biosynthesis mechanisms and signal transduction. Understanding of these processes provides in-depth knowledge of the relationship between the genetics and physiology of *G. lucidum*. Previous research provides evidence that two or more signaling pathways are involved, and that these pathways interact in mediating GT biosynthesis. Methyl jasmonate (MeJA) promotes GT accumulation by inducing production of intracellular ROS, and NADPH oxidase (NOX) plays a vital role in this process (Shi et al., 2015). In addition, the APSES (Asm1, Phd1, Sok1, Efg1, and StuA) transcription factor, Switching deficient-6 (*Swi6*), might mediate GT biosynthesis and fungal growth by regulating intracellular ROS levels. Compared with wild-type (WT) strains, the H_2O_2 and ganoderic acid levels in *GlSwi6* (*Swi6* in *G. lucidum*)-silenced strains were significantly reduced, but recovered with H_2O_2 supplementation (Zhang et al., 2018). Other studies reported that Ca^{2+} signaling also mediates GT synthesis. Changes in intracellular Ca^{2+} concentrations may activate downstream Ca^{2+}-related receptors and regulate related gene expression to affect GT content. For example, the addition of exogenous Ca^{2+} (10 mmol/L) enhanced GT production (Xu and Zhong, 2012). When subjected to heat stress, GT accumulation in *G. lucidum* was induced and cytosolic Ca^{2+} concentration increased. Transcription levels of Ca^{2+}-related genes showed that the Ca^{2+}-permeable channel gene (*cch*) and phospholipase C gene (*plc*) were prominently up-regulated, demonstrating that cytosolic Ca^{2+} participated in the regulation of GT biosynthesis under heat stress (Zhang et al., 2016). Moreover, NO, as a regulator of gene expression in the mevalonate pathway, might function directly to induce GT biosynthesis via activation of MAPK signaling. NO may also mediate cross-talk between Ca^{2+} and ROS signaling pathways (Gu et al., 2017). H_2S reduced GT biosynthesis by decreasing the intracellular Ca^{2+} concentration during heat stress. Further, transcriptome results indicated that related genes in multiple signaling pathways, including ROS, NO, and MAPK, showed significant changes after H_2S exposure and might eventually affect various physiological process in *G. lucidum* (Tian et al., 2019).

Since multiple signaling pathways participate in regulating GT biosynthesis, exploration of other signaling pathways involved is necessary to provide a comprehensive analysis of the regulation mechanism of GT biosynthesis.

HO-1 is an important regulatory signal with multiple biological functions in plants, and these functions are closely linked with CO. HO-1/CO signaling can participate in a variety of metabolic processes. For example, it was shown to protect plant cells against environmental stress by regulating glutathione and antioxidant metabolism, and by maintaining ion homeostasis (Han Y et al., 2008; Han B et al., 2014). CO is a by-product of heme degradation with HO-1, which was required for hair root development by increasing gene expression of *LeEXT-1*, accompanied by cross-talk with auxin, ethylene, and NO (Guo et al., 2009). Further, hemin and CO could up-regulate HO-1 transcription in auxin-depleted cucumber, NO production and adventitious root formation were also promoted, and these responses could be blocked by ZnPPIX or a specific NO scavenger. These results showed that NO might operate downstream of hemin to promote adventitious root formation, probably in a cGMP-dependent manner (Xuan et al., 2012). Moreover, abscisic acid (ABA) could induce stomatal closure by up-regulating *HO-1* gene expression and promoting CO release. NO and cGMP might function as downstream intermediaries in CO signaling (Cao et al., 2007). HO-1/CO signaling could also affect seed germination and retard leaf senescence (Liu et al., 2010; Huang et al., 2011; Amooaghaie et al., 2015).

Recently, research on HO-1/CO signaling has been focused primarily on plants. The involvement of HO-1/CO signaling in the growth and secondary metabolism of *G. lucidum*, especially GT biosynthesis, was unknown. In our study, we found that GT content was significantly enhanced by hemin induction (5, 10, and 20 μmol/L), and HO-1 activity showed parallel variation. Thus, HO-1 activity was likely related to GT biosynthesis. To further clarify this hypothesis, GT content was increased significantly by 60.1% after hemin, and by 56.0% after CORM-2 treatment. Conversely, ZnPPIX treatment reduced GT content, but heme treatment reversed this effect. More importantly, changes in HO-1 activity corresponded to changes in GT content, providing further evidence implying that HO-1 induced GT accumulation, primarily in association with its catalytic product CO. RT-PCR analysis showed that HO-1/CO signaling affected GT synthesis

by regulating gene expression of key enzymes in the GT synthesis pathway.

In general, interactions are found among HO-1/CO and other signaling pathways in the metabolic networks of plants and microorganisms. Previous studies showed that MAPK signaling, a crucial regulator in signal transduction for oxidative stress, was the primary functional target of CO (Otterbein et al., 2000). The physiological effects of CO were also mediated by NO and cGMP signaling (Wu et al., 2013). For example, the response of the epidermal stomata of *Vicia faba* to HO-1/CO signaling indicated that NO and cGMP signaling were involved downstream of CO signal transduction (Cao et al., 2007). In addition, CO donors regulated HO-1 transcription and produced NO in cucumber, promoting adventitious root formation via the cGMP pathway (Xuan et al., 2012). Low concentrations of CO significantly induced the accumulation of NO and glutathione, and ultimately activated the antioxidant system. CO might improve seed tolerance to low temperatures relying on NO-mediated glutathione homeostasis (Bai et al., 2012). Moreover, under drought stress, HO-1 could delay the programmed cell death of the rice aleurone layer via the cGMP pathway, accompanied by interactions with the ROS and NO signaling pathways (Wang et al., 2018). HO-1 also functions as a downstream component in H_2S-induced adventitious root formation by regulating related target genes including DNAJ-like gene (*CsDNAJ-1*) and CDPK gene (*CsCDPK1/5*) in *Cucumis sativus* (Lin et al., 2012). Other studies indicated that H_2S also delayed programmed cell death in gibberellic acid-treated wheat aleurone cells, in connection with modulation of glutathione homeostasis and HO-1 gene expression (Xie et al., 2014). In studies of microorganisms, it was found that cross-talk between CO and NO signaling improved adaptation to iron deficiency in *Chlamydomonas reinhardtii* (Zhang et al., 2013). Important interactions among HO-1/CO and other signaling pathways were prevalent and always jointly regulated secondary metabolism.

In this study, our transcriptome experiments revealed that HO-1/CO signaling affected the transduction of ROS and Ca^{2+} signaling in *G. lucidum*. Hemin treatment was associated with modulation of 10 unigenes (7 up- and 3 down-regulated) related to enzymes/proteins encoded in antioxidant processes and 11 unigenes (6 up- and 5 down-regulated) encoded in the Ca^{2+} signaling pathway. The ZnPPIX treatment modulated 7 unigenes (3 up- and 4 down-regulated) associated with enzymes/proteins encoded in antioxidant processes and 10 unigenes (3 up- and 7 down-regulated) encoded in the Ca^{2+} signaling pathway. Furthermore, RT-PCR was conducted to validate the RNA expression of 11 selected DEGs. The results added confidence to the DEG results, except for REDX and CAMP, which showed no significant expression from controls in the transcriptome analysis.

5 Conclusions

Our findings indicated that HO-1/CO signaling is involved in promoting GT accumulation by regulating related gene expression in *G. lucidum* mycelia. HO-1/CO signaling may also affect transduction of ROS and Ca^{2+} signaling (Fig. 7). The results of this study will support further exploration of the GT metabolic network, and may prove useful for large-scale GT production. In the future, more studies are needed to examine the in-depth mechanisms of GT biosynthesis and relevant signaling cross-talk.

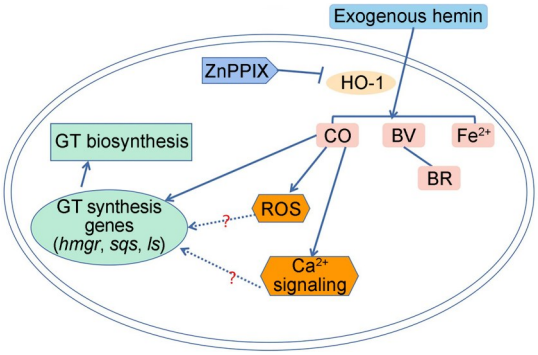

Fig. 7 Schematic representation showing that heme oxygenase-1 (HO-1)/carbon monoxide (CO) signaling participates in the regulation of ganoderic triterpenoid (GT) biosynthesis by mediating the gene expression of *hmgr*, *sqs*, and *ls* in *Ganoderma lucidum*. Transcriptome sequencing showed that HO-1/CO signaling also affects transduction of reactive oxygen species (ROS) and Ca^{2+} signaling. Question marks denote as yet undescribed or unverified pathways. The T-bar indicates inhibition. BV: biliverdin; BR: bilirubin; ZnPPIX: Zn protoporphyrin IX; *hmgr*: 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase; *sqs*: squalene synthase; *ls*: lanosterol synthase.

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Author contributions

Meilin CUI: project administration, conceptualization, data curation, writing, and editing. Yuchang MA: conceptualization. Youwei YU: conceptualization and 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, Yuchang MA, and Youwei YU 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

Fig. S1; Tables S1–S12