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ATP deficiency triggers ganoderic acids accumulation via fatty acid β-oxidation pathway in *Ganoderma lucidum*

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Abstract

Background Ganoderic acids (GAs), recognized as significant triterpenoid bioactive components in *Ganoderma lucidum*, exhibit a broad spectrum of pharmacological activities, including immunomodulation, anti-cancer, and anti-aging properties. Despite their significant pharmacological potential, the low yield of GAs from natural sources has emerged as a critical bottleneck hindering their broader application in the pharmaceutical and health care industries. Previous studies have suggested that environmental perturbations can influence energy metabolism, potentially impacting the biosynthesis of bioactive compounds. However, the specific influence of environmental changes on energy metabolism and subsequent effects on GAs synthesis in *G. lucidum* remains an understudied area.

Results We demonstrated that intracellular ATP deficiency significantly influences GAs accumulation induced by alterations in energy metabolism. Intracellular ATP deficiency was consistently observed under all four known conditions that induce GAs accumulation: heat stress (HS), nitrogen limitation, treatment with 50 μM methyl jasmonate (MeJA), and treatment with 200 μM salicylic acid (SA). Consistent with these findings, silencing the ATP synthase beta subunit (ATPsyn-beta) or treating with oligomycin (Oli), an ATP synthase inhibitor, increased GAs accumulation and induced intracellular ATP deficiency in *G. lucidum*. Our results revealed an increase in the GAs biosynthetic pathway and increased levels of the GAs precursor acetyl-CoA in mycelia with intracellular ATP deficiency. Enhanced fatty acid β-oxidation was identified as the primary source of additional acetyl-CoA, indicating that this process, induced by intracellular ATP deficiency, is crucial for GAs accumulation.

Conclusions This study demonstrated that changes in intracellular ATP content respond to environmental perturbations and impact the biosynthesis of GAs, holding substantial implications for production practices. Modulating ATP levels could increase GAs yields, cater to market demands, and reduce costs. The research also furnishes a scientific foundation for optimizing cultivation conditions, employing genetic engineering to refine biosynthetic pathways, and leveraging environmental control to boost production efficiency.

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Keywords Ganoderic acids, ATP, *Ganoderma lucidum*, Acetyl-CoA, Fatty acid β-oxidation, Energy metabolism

Background

Fluctuations in environmental parameters, such as moisture, temperature, and nutrient availability, typically exert adverse effects on the life activities of organisms. To survive, all organisms must respond and adapt to these altered environmental conditions by regulating their metabolism and undergoing physiological changes [1–3]. Energy metabolism, a cornerstone of metabolic processes, breaks down nutrients to generate the energy required for life activities. In response to environmental changes, organisms initiate compensatory regulatory mechanisms through energy metabolism to maintain physiological homeostasis [4–8]. Recent studies have indicated that energy metabolism plays a significant regulatory role in the response of microorganisms to environmental changes. For example, in *Saccharomyces cerevisiae*, a mechanism for regulating cytosolic viscosity during periods of heat and starvation has been identified, potentially triggered by ATP levels [9]. Similarly, in *Arabidopsis thaliana*, hypoxia-induced ATP depletion modulates oleoyl-CoA synthesis, initiating a response to low-oxygen stress [10]. These findings suggest that adjustments in energy metabolism may be crucial for organisms in response to environmental changes by influencing other biological processes.

Secondary metabolites, traditionally considered non-essential for growth and development, have recently been recognized for their critical roles in regulating the survival, reproduction, and environmental adaptation of fungi [11]. Notably, secondary metabolites such as lovastatin, penicillin, and artemisinin have had a profound impact on human civilization, highlighting the importance of studying secondary metabolism [12, 13]. As a result, research into the regulation of secondary metabolism has gained significant interest over the past decade. Various physical factors, including temperature variations, solid-state culture, and pH adjustments, have been documented to enhance the accumulation of secondary metabolites in fungi. Additionally, chemical, biological, and biochemical signals, such as SA and ethylene, have also been shown to enhance the biosynthesis of secondary metabolites in organisms. Studies have further revealed that enzymes associated with energy production may significantly influence the accumulation of secondary metabolites during these induction processes. For example, Liu (2021) reported that the inhibition of aconitase activity reduced heat stress-induced biosynthesis of GAs in *G. lucidum* [14]. However, there has been a notable scarcity of research examining the effects of cofactors, such as ATP and NADH, on secondary metabolism in fungi. The role of energy metabolism in modulating secondary metabolism in response to environmental changes remains largely unexplored.

G. lucidum is a renowned edible and medicinal fungus native to East Asia. GAs are among its primary medicinal constituents, with their concentration serving as a critical determinant of the mushroom quality. Thus, enhancing the content of GAs is of considerable importance in production, and understanding the regulatory mechanisms of their biosynthesis and accumulation has become a significant research focus. The biosynthesis of Ganoderic Acids (GAs) in *G. lucidum* represents a complex and multifaceted metabolic pathway that involves several key enzymes. A substantial body of research has conclusively demonstrated that HMG-CoA Reductase (HMGR), Squalene Synthase (SQS), and Oxidosqualene Cyclase (OSC) serve as rate-limiting enzymes in the synthesis of GAs [15, 16]. Moreover, the expression levels of these enzymes have been shown to correlate significantly with the accumulation of GAs [17]. While many eukaryotic organisms are known to possess multiple copies of the genes encoding HMGR, SQS, and OSC, current genomic studies have revealed the presence of only a single copy of each gene in the *G. lucidum* genome [18–21]. Researches also indicate that the biosynthesis and accumulation of GAs are closely associated with environmental conditions and are significantly influenced by stimuli such as heat shock, low pH, MeJA treatment, and SA treatment, demonstrating the environmental sensitivity of GAs biosynthesis. Subsequent analyses have revealed that reactive oxygen species signaling and mitochondrial activity are also implicated in the regulation of GAs biosynthesis during environmental shifts [14, 22, 23]. However, the precise roles of energy content and metabolism in modulating GAs biosynthesis in response to various environmental changes remain to be elucidated.

In this study, mycelium of *G. lucidum* was subjected to various environmental stimuli, revealing a correlation between intracellular ATP deficiency and the accumulation of GAs. By knocking down the ATP synthase beta subunit (ATPsyn-beta) or treating with Oli, strains with ATP deficiency were generated, confirming that ATP deficiency could induce GAs accumulation. Our analysis of key acetyl-CoA synthesis pathways revealed that intracellular ATP deficiency upregulates fatty acid β-oxidation. To assess the role of fatty acid β-oxidation in GAs accumulation induced by ATP deficiency, etomoxir(eto) treatment and co-silencing analysis were employed. This study explored the intricate relationship between ATP levels and the biosynthesis of GAs within *G. lucidum*, shedding light on a potential regulatory mechanism. These findings introduce a novel strategy

for enhancing the production of GAs, which are bioactive compounds with a wide range of pharmacological activities. This enhancement is crucial for increasing the economic value of GAs-derived products, optimizing the efficiency of industrial-scale fermentation processes, and amplifying the health-promoting properties of these medicinal compounds.

Materials and methods

Strain and material culture

The *G. lucidum* strain G20 (*G. lucidum* ACCC53264) was provided by the China Center for Agricultural Microorganisms Conservation. The strains were cultivated on a complete yeast medium (CYM:10 $g{\cdot}L^{-1}$ maltose (biochemical grade, Sangon, Shanghai, China), 20 $g{\cdot}L^{-1}$ glucose (biotech grade, Sangon, Shanghai, China), 2 $g{\cdot}L^{-1}$ yeast extract (microbiological grade, Sangon, Shanghai, China), 2 $g{\cdot}L^{-1}$ peptone (microbiological grade, Sangon, Shanghai, China), 0.5 $g{\cdot}L^{-1}$ $MgSO_4{\cdot}7\ H_2O$ (biochemical grade, Sangon, Shanghai, China), and 2 $g{\cdot}L^{-1}$ K_2HPO_4 (biochemical grade, Sangon, Shanghai, China)) solid plate (10 $g{\cdot}L^{-1}$ agar (Reagent Grade, Sangon, Shanghai, China)). Subsequently, the mycelia were transferred into 100 mL of CYM liquid medium. The medium was incubated in a shaking incubator at 28℃ and 150 $rpm{\cdot}min^{-1}$ for 5 days in the dark. Next, the mycelia were evenly broken under aseptic conditions using a cantilever electric stirrer at 2000 $rpm{\cdot}min^{-1}$ for 5 min (NY-20L3, Enpei, Changzhou, China). Finally, 2.5 mL of the mycelium homogenate was transferred into 100 mL of CYM liquid medium and incubated at 28℃ and 150 $rpm{\cdot}min^{-1}$ for 5 days in the dark.

Detection of the intracellular ATP content in *G. lucidum*

The detection was performed using the ATP Assay Kit manufactured by Beyotime Biotechnology (Cat: S0026).

Determination of GAs content in *G. lucidum* via HPLC

Ganoderic acid levels were extracted from dried mycelium and measured using previously described methods [24–26]. GAs were determined using high-performance liquid chromatography (HPLC) instrument (Shimadzu, Kyoto, Japan). The monitoring wavelength for ganoderic acid was set at 252 nm, and a Shim-pack GISTC18 column (5 μm, 4.6 mm I.D. × 250 mm) was used. Calibration curves were established using ganoderic acid A, specifically (7,15,25R)-7,15-dihydroxy-3,11,23-trioxolanost-8-en-26-oic acid, to evaluate the total ganoderic acid production. The standard concentration of ganoderic acid A used for calibration was greater than 98%, and it was sourced from Solarbio (SG8810, Beijing, China). The total ganoderic acid content was calculated based on the peak area obtained from the chromatograms and the established calibration curves.

Construction of transformants

The specific transformation procedure was as previously described [27]. The fungal RNAi vector pAN7-ura3-dual was used to construct transformant strains of *G. lucidum*. Fragments of the coding sequences of *ATPsyn-β*, *hcd*, *acd* and co-silenced (*ATPsyn-β* and *hcd* or *acd*) were amplified by polymerase chain reaction (PCR) using *G. lucidum* cDNA as the template and the primers listed in Supplemental Table S1. The amplified fragments were ligated into the pAN7-ura3-dual vector and Sanger sequenced before transformation into *G. lucidum* protoplasts. The transformants were screened on CYM medium containing 100 μg/mL hygromycin B [28].

To screen for valid silenced strains, mycelia were incubated on CYM plates at 28 °C for 5 days, then collected and frozen in liquid nitrogen. The silencing efficiency of candidate transformants was detected by quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis [29]. Total RNA was extracted from 100 mg of mycelium using RNAiso Plus (9109, TaKaRa). cDNA was synthesized using the RT MasterMix kit (G488, ABM). The transcript levels of related genes were detected using SYBR Green qPCR SuperMix (Q321-02, Vazyme) according to the manufacturer's instructions.

Quantitative analysis of gene expression levels through real-time fluorescence PCR

In this experiment, the qPCR SYBR Green Master Mix kit from Vazyme was utilized to conduct the qRT-PCR reaction. The qRT-PCR data were processed using the 18S rRNA gene of *G. lucidum* as the endogenous control. The relative changes in target gene expression were determined according to the $2^{-\Delta\Delta CT}$ method [30]. Here, ΔΔCT represents the difference between the ΔCt of the treatment group and the ΔCt of the control group. The relative expression level of the gene of interest was calculated using the following formula: $2^{-\text{(treatment group }\Delta Ct - \text{control group }\Delta Ct)}$. Specifically, the ΔCt for the treatment group was defined as the difference between the Ct value of the target gene and the Ct value of the 18S rRNA internal reference gene within the same sample. Similarly, the ΔCt for the control group is the difference between the Ct value of the target gene and the Ct value of the 18S rRNA internal reference gene within that sample. Primer sequence information is provided in the supplementary material.

Detection of acetyl-CoA content in *G. lucidum*

Detection of acetyl-CoA was performed using the immunofluorescence end-point method, as described in the Acetyl-CoA detection kit (Cat: MAK039) produced by Merck.

Detection of lipase (LPS) and pyruvate dehydrogenase (PDH) activities

The experiment utilized the copper soap lipase (LPS) activity detection kit (Cat: BC2340) and the pyruvate dehydrogenase (PDH) activity detection kit (Cat: BC038), both produced by Shanghai Solarbio Bioscience & Technology Co., Ltd.

Statistical analysis

All data were obtained from three independent repeated experiments, and the final data are presented as the mean ± standard deviations (SD). Asterisks indicate significant differences between the control and treated samples as determined by Student's t-test: * means $p < 0.05$, and ** means $p < 0.01$. Different asterisks represent different levels of significance. Letters (a, b, c, d, etc.) indicate significant differences ($p < 0.05$) between different treatments as determined by Duncan's multiple range test. Different letters represent different levels of significance.

Data availability

All data generated or analyzed during this study are included in this published article.

Results

Intracellular ATP decrease induces intracellular GAs accumulation in *G. lucidum*

Previous studies have demonstrated that the accumulation of GAs in *G. lucidum* can be augmented by exposure to HS, nitrogen starvation (NS), MeJA and SA. To explore the interplay between GAs biosynthesis and energy metabolism, this study measured both GAs and ATP levels in *G. lucidum* subjected to these stressors. The results revealed significant increases in GAs content—by 170.5%, 46.3%, 52.4%, and 50.7% following HS, NS, MeJA, and SA treatments, respectively—aligning with previous findings (Fig. 1A through D). Concurrently, the intracellular ATP content, an indicator of energy metabolism status, was markedly reduced by 60.0%, 67.0%, 74.5%, and 52.0% under the same treatments (Fig. 1E through H). These findings lead to the hypothesis that there is a potential inverse correlation between the content of GAs and the levels of intracellular ATP in *G. lucidum* when subjected to specific environmental stimuli.

To further investigate the proposed correlation between intracellular ATP deficiency and the accumulation of GAs in *G. lucidum*, the fungus was treated with Oli, an inhibitor of ATP synthase. Compared with those in the control group, the intracellular ATP levels were significantly decreased by 68.9%, 91.7%, and 91.1% in the 0.5 μM, 1.0 μM, and 2.0 μM Oli treatment groups, respectively (Fig. 1I). Concurrently, GAs contents were significantly elevated by 66.7%, 62.9% and 72.8% in the corresponding Oli-treated groups (Fig. 1J). These results indicated that defects in ATP synthesis can cause GAs accumulation in *G. lucidum*.

Given the pivotal role of the ATP synthase beta-subunit in modulating intracellular ATP levels, this gene was targeted for RNA interference (RNAi) to elucidate its role in the ATP deficiency and GAs accumulation relationship. Two independent RNAi strains, *ATPsyn-beta*-1i and *ATPsyn-beta*-12i, were successfully generated (Fig. 2A). Compared with that in the wild-type (WT) strain, the ATP content in the *ATPsyn-beta*-1i and *ATPsyn-beta*-12i strains substantially decreased by 69.3% and 75.2%, respectively (Fig. 2B). Similarly, the GAs content in these RNAi strains was elevated by 47.8% and 39.1% relative to that in the WT strain (Fig. 2C). These findings collectively suggest that a deficiency in intracellular ATP can indeed lead to an increase in GAs content in *G. lucidum*, thereby supporting the hypothesis that there is a regulatory link between energy metabolism and the biosynthesis of secondary metabolites such as GAs.

To further explore the molecular mechanisms underlying this phenomenon, the transcriptional response of key genes in the GAs biosynthetic pathway was examined, including HMGR, SQS, and OSC. Quantitative real-time PCR (qRT-PCR) analysis revealed that the transcription levels of these genes were notably upregulated under 2 μM Oli treatment (*hmgr*: 172.5%; *sqs*: 148.8%; *osc*: 147.4 %) (Fig. 2D-F) and following *ATPsyn-beta* knockdown (hmgr: 178.7%, 172.8%; sqs: 151.8%, 155.8%; osc: 139.9%, 145.9%) (Fig. 2G-I). These findings indicate that intracellular ATP deficiency enhances the transcription of enzymes critical to GAs biosynthesis.

Intracellular ATP deficiency changes the activities of the major source pathways for acetyl-CoA

GAs are biosynthesized through the mevalonate (MVA) pathway, which commences with acetyl-CoA. Combined with the increased transcription of *hmgr*, *sqs*, and *osc*, the acetyl-CoA content might be the main reason for GAs synthesis being regulated by ATP levels. Thus, the effect of intracellular ATP deficiency on acetyl-CoA content was investigated. Treatment with 2 μM Oli, an inhibitor of ATP synthase, could lead to a substantial increase in intracellular acetyl-CoA content by 104.1% compared to the control group (Fig. 2J). Similarly, in the *ATPsyn-beta* RNAi strains (*ATPsyn-beta*-1i and *ATPsyn-beta*-12i), which exhibit reduced ATP levels due to the knockdown of the ATP synthase beta-subunit, the intracellular acetyl-CoA content increased by 118.3% and 105.9%, respectively, when compared to the WT strain (Fig. 2K). We have revised "These findings demonstrate that a deficiency in intracellular ATP results in significant acetyl-CoA accumulation in *G. lucidum*. Additionally, the accumulation of acetyl-CoA, a key precursor in the MVA pathway, indicated a potential redirection of metabolic

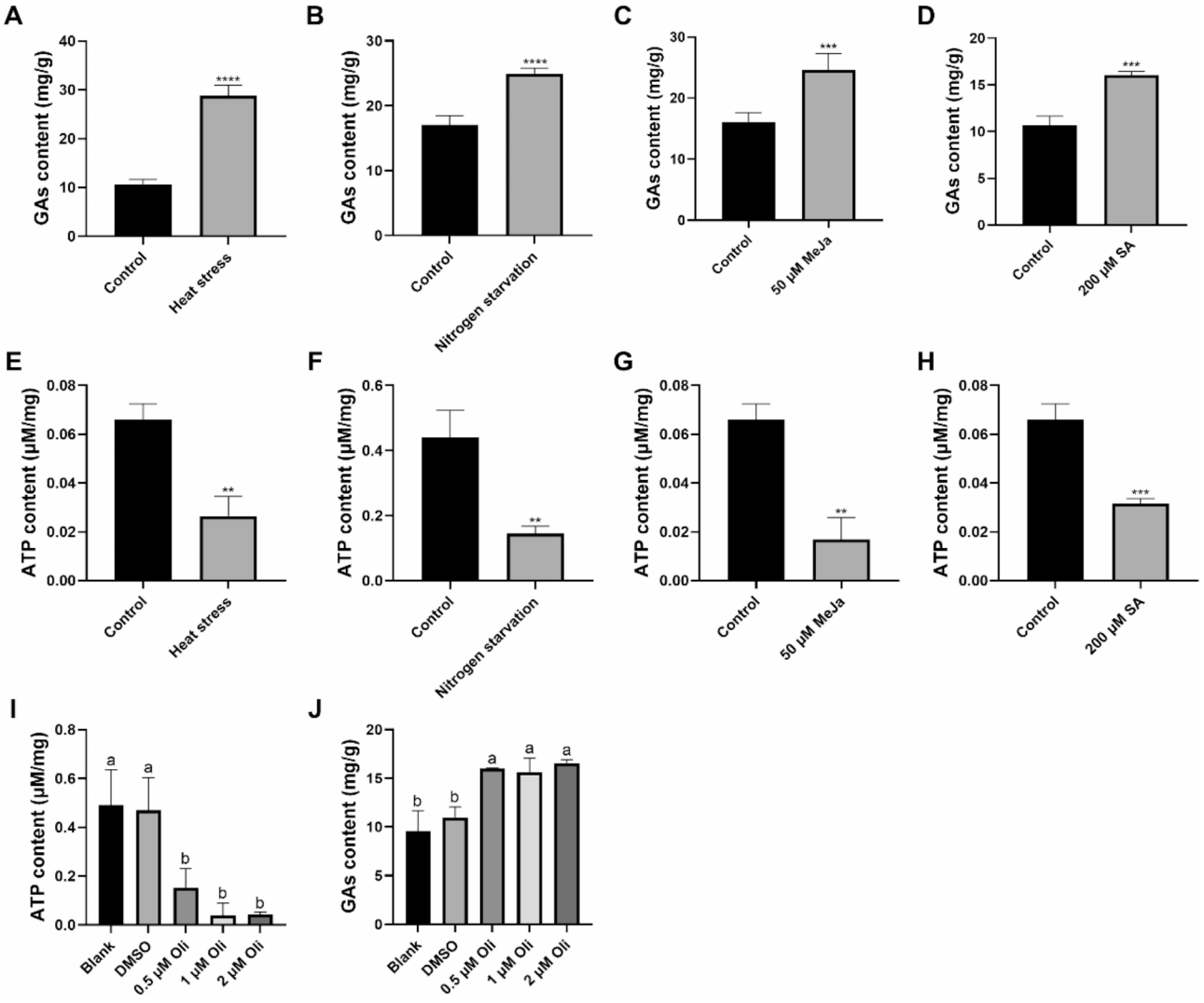

Fig. 1 Contents of intracellular ATP and GAs in *G. lucidum* under different treatments. (**A**, **E**) HS. The *G. lucidum* WT mycelium was cultured in CYM liquid culture medium at 28 °C for 5.5 days, subjected to HS at 42 °C for 12 h, and then incubated at 28 °C for 24 h to measure ATP and GAs. (**B**, **F**) Nitrogen limitation. The *G. lucidum* WT mycelium was cultured in CYM liquid culture medium for 4 days and then transferred to synthetic medium containing glutamine as the sole nitrogen source (control: 30 mM; nitrogen limitation: 3 mM), and the mycelium was cultured for 3 days to measure ATP and GAs. (**C**, **G** and **D**, **H**) MeJA/SA treatment. The *G. lucidum* WT mycelium was cultured in CYM liquid culture medium for 4 days, 50 μM MeJA or 200 μM SA was added, and the culture was continued for 3 days to measure ATP and GAs, respectively. Ethanol was added as a control. (**I**, **J**) Oli treatment. The *G. lucidum* WT mycelium was cultured in CYM liquid culture medium for 4 days, 0.5 μM, 1 μM or 2 μM Oli (dissolved in DMSO) was added, and the culture was continued for 3 days to measure ATP and GAs. Blank: blank control; DMSO: solvent control. (**A**-**H**) All experiments were performed in three independent replicates, and the results are presented as the means ± SDs, with asterisks indicating significant differences from untreated samples (Student's t test: ** $p < 0.01$). (**I**, **J**) All experiments were performed in three independent replicates, and the results are presented as the means ± SDs, with different letters indicating significant differences (Duncan's multiple range test, $p < 0.05$)

flux towards GAs biosynthesis under conditions of ATP scarcity.

The mechanism of acetyl-CoA accumulation caused by intracellular ATP deficiency was further elucidated. The main sources of acetyl-CoA are the oxidative decarboxylation of pyruvate (a product of glycolysis) and the β-oxidation of fatty acids. Thus, the transcription of key enzymes involved in the oxidative decarboxylation of pyruvate, glycolysis and β-oxidation was analyzed. As the key rate-limiting enzymes involved in the glycolysis pathway, the transcription of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) was detected. Compared to the control, the transcription of *pk* and *hk* was upregulated in both the *ATPsyn-beta* silenced strains and Oli treatments (Fig. 3A, C, D, F), whereas *pfk* was upregulated exclusively following *ATPsyn-beta* silenced (Fig. 3B, E). However, there was no significant change in transcription of genes encoding subunits of the pyruvate dehydrogenase complex (PDC) (Fig. 3G, H, I, L, M, N).

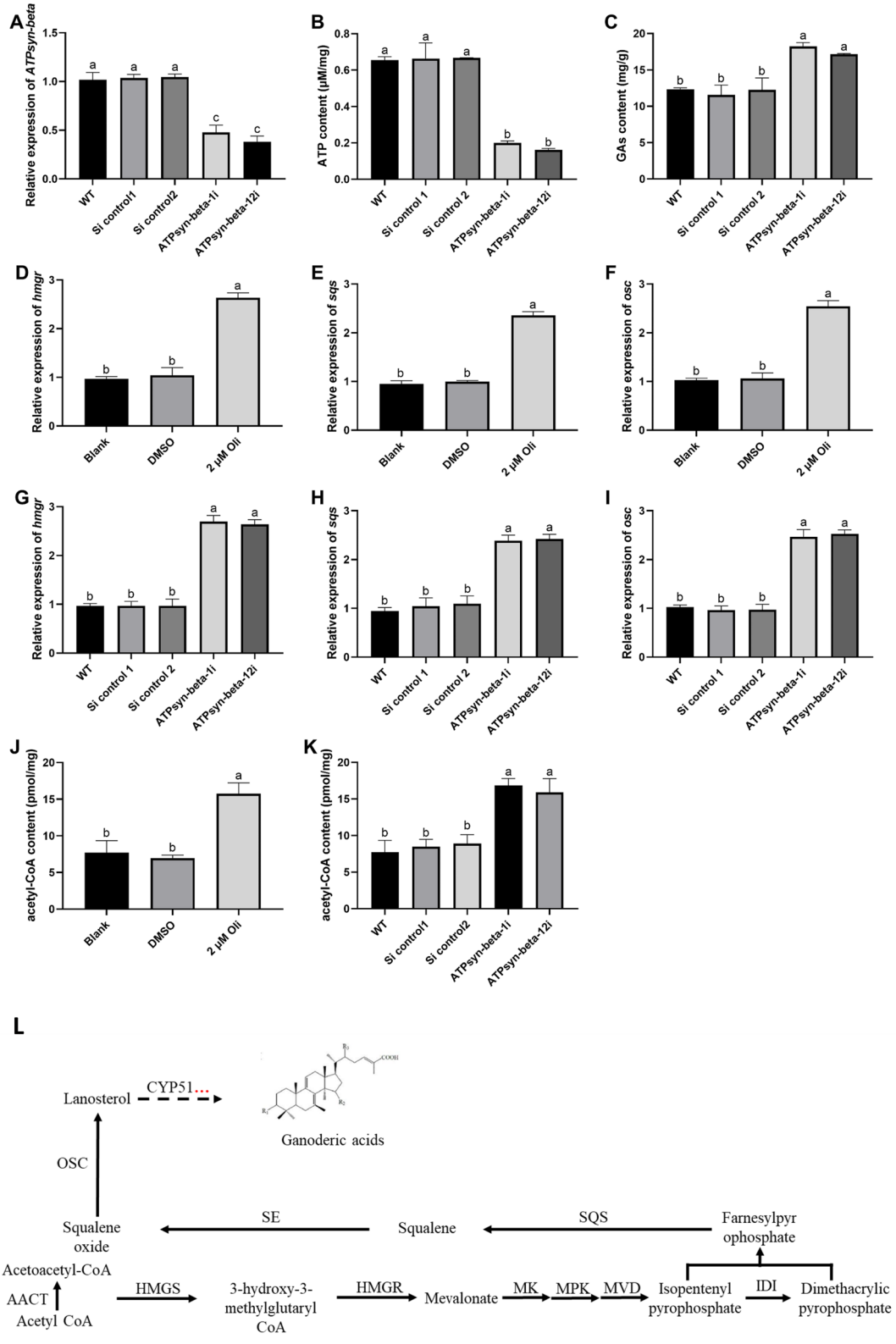

Fig. 2 (See legend on next page.)

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Fig. 2 Screening of *ATPsyn-beta*-silenced transformants and effects of *ATPsyn-beta* silenced or Oli treatment on the contents of intracellular ATP, GAs, and acetyl-CoA and the biosynthesis of GAs. (**A**) Relative expression levels of *ATPsyn-beta* in WT and *ATPsyn-beta*-silenced transformants. The relative expression levels of *ATPsyn-beta* were determined via RT–qPCR. The 18S rRNA gene was used as a qRT–PCR reference gene, and the relative gene expression level of the WT was defined as 1.0. Si control: Empty vector control transduction for the knockdown vector. (**B**) Intracellular ATP content in WT and *ATPsyn-beta*-silenced transformants. (**C**) GAs content in WT and *ATPsyn-beta*-silenced transformants. (**D**-**F**) Relative expression levels of key mevalonate (MVA) pathway enzymes in WT treated with 2 μM Oli and the control. The *G. lucidum* WT mycelium was cultured in CYM liquid culture medium for 4 days, after which 2 μM Oli (dissolved in DMSO) or DMSO was added, and the culture was continued for 3 days. Blank: Blank control; DMSO: Solvent control. Changes in the acetyl-CoA content in *G. lucidum*. (**G**-**I**) Relative expression levels of key MVA pathway enzymes in WT and *ATPsyn-beta*-silenced transformants. The 18S rRNA gene was used as a qRT–PCR reference gene, and the relative gene expression level of the WT was defined as 1.0. (**J**) Effects of the addition of 2 μM Oli in WT on the content of acetyl-CoA in *G. lucidum*. The Oli treatment method is the same as Fig. 2D. Blank: Blank control; DMSO: Solvent control. (**K**) Acetyl-CoA content in WT and *ATPsyn-beta*-silenced transformants. (**L**) Synthetic pathway of ganoderic acid [31]. All the experiments were performed in three independent replicates, and the results are presented as the means ± SDs, with different letters indicating significant differences (Duncan's multiple range test, $p < 0.05$)

The genes encoding medium-chain acyl-CoA dehydrogenase (ACD) and 3-hydroxyacyl-CoA dehydrogenase (HCD-R2), two of the five key rate-limiting enzymes involved in β-oxidation, were upregulated at the transcriptional level (Fig. 4E, F, K, L), while the transcription of the triacylglycerol lipase (TGL) gene also increased (Fig. 4A, B). Meanwhile, changes in the activity levels of key enzymes involved in the oxidative decarboxylation of pyruvate and lipolysis were also analyzed. Compared to the control group, the intracellular ATP deficiency induced a phenotype characterized by a decrease in the activity of pyruvate dehydrogenase and an increase in total lipase activity (Figs. 3O and J and 4C and D). The above results demonstrate that both the oxidative decarboxylation of pyruvate and fatty acid β-oxidation can respond to intracellular ATP deficiency, suggesting that fatty acid β-oxidation may be a primary contributor to acetyl-CoA accumulation under such conditions.

To confirm the role of fatty acid β-oxidation in acetyl-CoA accumulation under energetic stress, *acd* and *hcd-r2* were selected for further analysis. Co-silenced transformants of *ATPsyn-beta* and *acd*, as well as *ATPsyn-beta* and *hcd-r2*, were generated (Fig. 5C, D). Using qRT-PCR, we obtained the silencing efficiency of two transformants (ATPsyn-ACD-4i and ATPsyn-ACD-8i) for the ATPsyn-beta and acd co-silenced genotypes (Fig. 5C), and two transformants (ATPsyn-HCD-3i and ATPsyn-HCD-5i) for the ATPsyn-beta and hcd co-silenced genotypes (Fig. 5D). Upon measuring the acetyl-CoA content, knockdown of *acd* or *hcd-r2* mitigated the acetyl-CoA accumulation induced by intracellular ATP deficiency (Fig. 6E, F). These results indicate that fatty acid β-oxidation plays an important role in acetyl-CoA accumulation under conditions of intracellular ATP deficiency.

Fatty acid β-oxidation plays an important role in GAs accumulation induced by decreased intracellular ATP

To further clarify the role of fatty acid β-oxidation in GAs accumulation induced by decreased intracellular ATP, eto., a fatty acid β-oxidation pathway inhibitor, was used to treat the mycelia of *ATPsyn-beta* silenced transformants, WT and Si control. Following the addition of 10 μM eto. to the culture medium, the GAs content of the WT remained unchanged. However, the GAs content increase in the *ATPsyn-beta*-1i and *ATPsyn-beta*-12i strains was significantly reversed by 36.9% and 30.5%, respectively, and returned to WT levels in the absence of eto. treatment (Fig. 5A, B). In the WT strain group treated with 2 μM Oli, the addition of 10 μM eto. reduced the induced GAs content to the level of the control group by 31.2% (Fig. 5B). These results indicate that fatty acid β-oxidation in *G. lucidum* plays a crucial role in GAs accumulation induced by intracellular ATP deficiency.

Because key fatty acid β-oxidation enzymes ACD and HCD responded to intracellular ATP deficiency, co-silenced transformants of *ATPsyn-beta* and HCD were used to further confirm the role of fatty acid β-oxidation in GAs accumulation induced by intracellular ATP deficiency (Fig. 6). The transcription levels of genes involved in GAs biosynthesis and the contents of GAs were analyzed. The results showed that the transcription levels of the genes in the co-silenced strains were similar to those in the WT strain, indicating that silenced *acd* or *hcd* abolished the accumulation of acetyl-CoA and GAs caused by *ATPsyn-beta* silenced. Moreover, co-silencing reversed the transcriptional upregulation of genes encoding key enzymes involved in GAs biosynthesis induced by *ATPsyn-beta* silenced, as measured by qRT−PCR, resulting in levels comparable to those of the WT strains. These results further indicated that fatty acid β-oxidation is involved in GAs accumulation induced by intracellular ATP deficiency.

In summary, these results indicate that intracellular ATP deficiency leads to the accumulation of intracellular GAs in *G. lucidum* and that the fatty acid β-oxidation pathway significantly contributes to increasing acetyl-CoA levels and increasing the transcription of key enzyme-encoding genes involved in GAs biosynthesis. These findings underscore the pivotal role of the fatty acid β-oxidation pathway in the cellular response to energy deficiency and its impact on secondary metabolite production.

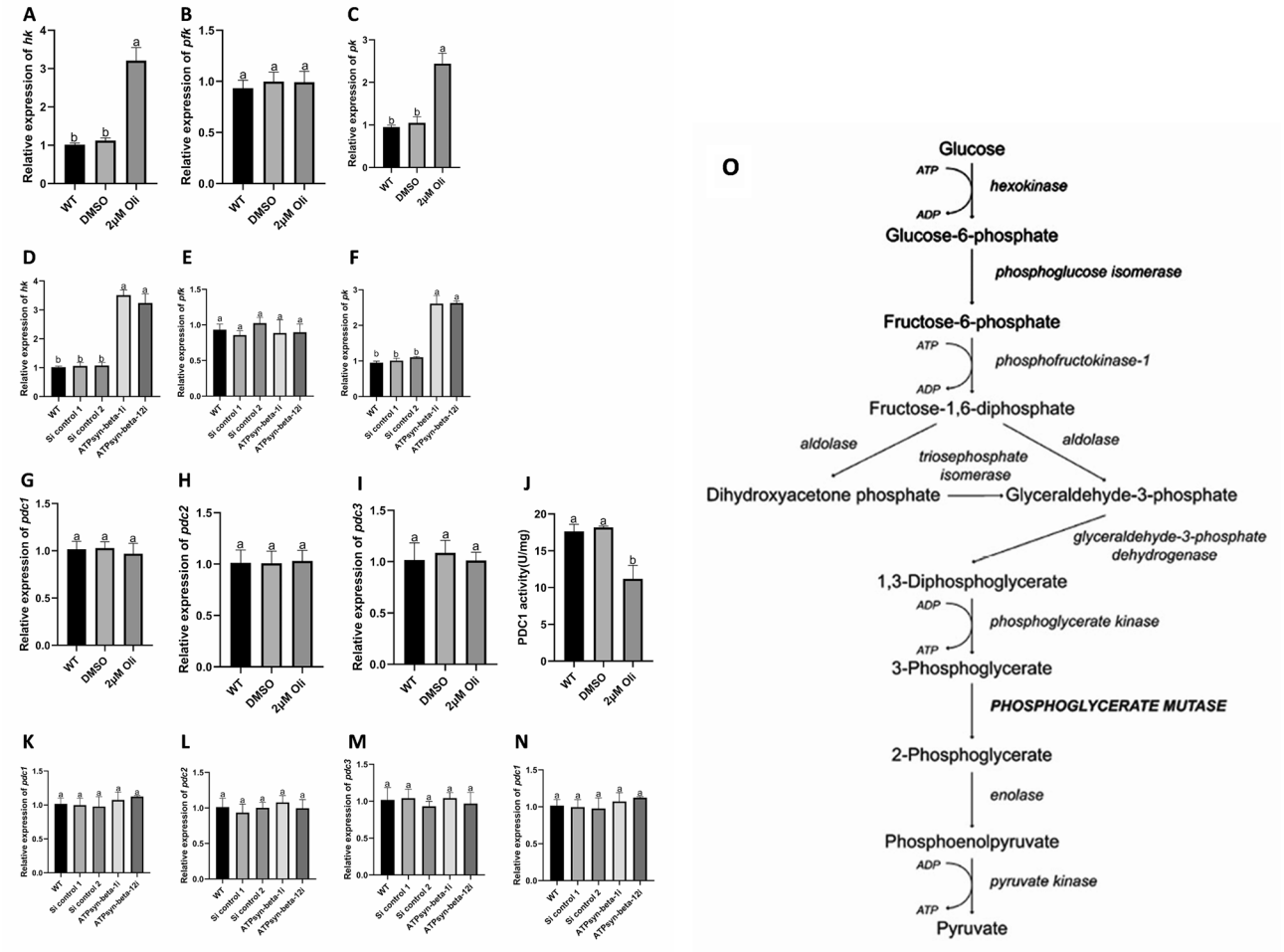

Fig. 3 Effects of *ATPsyn-beta* silenced or Oli treatment on pyruvate decarboxylation and glycolysis in *G. lucidum*. (**A**-**C**) Relative transcription levels of key glycolytic enzymes in WT treated with 2 μM Oli exogenously and in the control. The 18S rRNA gene was used as a qRT–PCR reference gene, and the relative gene expression level of the WT was defined as 1.0. The Oli treatment method is the same as that in Fig. 2D. Blank: Blank control; DMSO: Solvent control. (**D**-**F**) Relative transcription levels of key glycolytic enzymes in WT and *ATPsyn-beta*-silenced transformants. The 18S rRNA gene was used as a qRT–PCR reference gene, and the relative gene expression level of the WT was defined as 1.0. (**G**-**I**) Relative transcription levels of pyruvate dehydrogenase enzyme-related subunit genes in WT treated with 2 μM Oli exogenously and the control. Blank: Blank control; DMSO: Solvent control. The 18S rRNA gene was used as a qRT-PCR reference gene, and the relative gene expression level of the WT was defined as 1.0. (**J**) PDC1 enzyme activity in WT treated with 2 μM Oli and control. The Oli treatment method was the same as that described in Fig. 2D. Blank: Blank control; DMSO: Solvent control. (**K**-**M**) Relative transcription levels of pyruvate dehydrogenase enzyme-related subunit genes in WT and *ATPsyn-beta*-silenced transformants. The 18S rRNA gene was used as a qRT–PCR reference gene, and the relative gene expression level of the WT was defined as 1.0. (**N**) PDC1 enzyme activity in WT and *GLATPsyn-beta*-silenced transformants of *G. lucidum*. (**O**) Schematic diagram of yeast cytoplasmic glycolysis [32]. All the experiments were performed in three independent replicates, and the results are presented as the means ± SDs, with different letters indicating significant differences (Duncan's multiple range test, $p < 0.05$)

Discussion

Energy metabolism provides energy and helps adjust life activities to survive environmental challenges. In response to environmental changes, energy metabolism is restructured to maintain cellular energy homeostasis, affecting the efficiency of many biological processes due to altered energy status and metabolite levels, including the biosynthesis of some secondary metabolites. However, little is known about how energy metabolic restructuring regulates life activities during environmental challenges. GAs represent a class of secondary metabolites of significant interest in the context of *G. lucidum*. The biosynthesis of these compounds was reported to be regulated by many environmental effectors. However, the mechanisms of accumulation of this secondary metabolite in response to different environmental stimuli remain unclear. Jiang et al. used proteomics and metabolomics to show that MeJA treatment induces metabolic rearrangement, inhibiting normal glucose metabolism, energy supply, and protein synthesis, while promoting the synthesis of secondary metabolites, including GAs [34]. Previous studies have demonstrated that four environmental stresses—nitrogen deficiency, heat stress, MeJA treatment, and salicylic acid treatment—significantly enhance

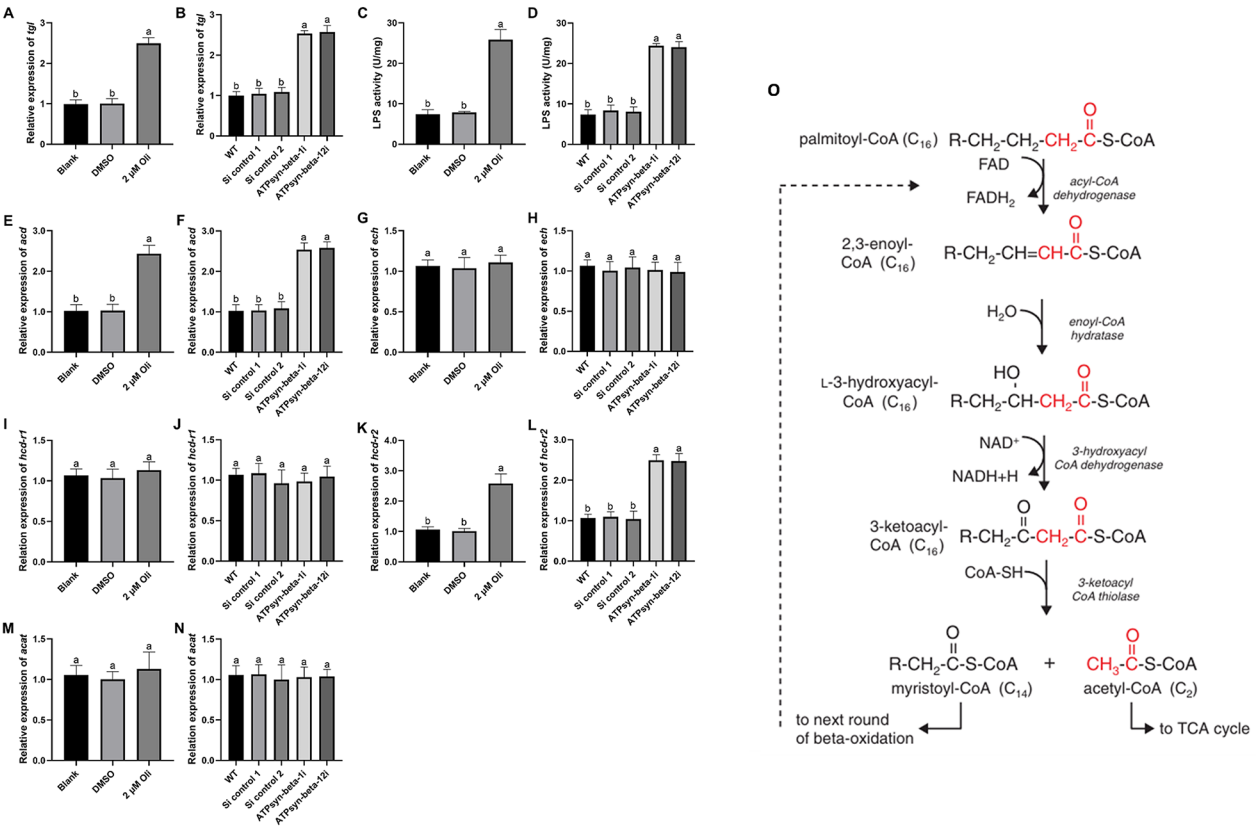

Fig. 4 Effects of *ATPsyn-beta* silenced or Oli treatment on lipolysis and fatty acid beta oxidation in *G. lucidum*. (**A**) Relative transcription levels of tgl in WT treated with 2 μM Oli and the control. The Oli treatment method is the same as that in Fig. 2D. Blank: Blank control; DMSO: Solvent control. (**B**) Relative transcription levels of tgl in WT and *ATPsyn-beta*-silenced transformants. (**C**) LPS enzyme activity in WT treated with 2 μM Oli and the control. The Oli treatment method is the same as that in Fig. 2D. Blank: Blank control; DMSO: Solvent control. (**D**) LPS enzyme activity in WT and *ATPsyn-beta*-silenced transformants. (**E**, **G**, **I**, **K**, **M**) Relative transcription levels of key fatty acid beta oxidation enzymes in WT treated with 2 μM Oli and the control. The Oli treatment method was the same as that described in Fig. 2D. Blank: Blank control; DMSO: Solvent control. (**F**, **H**, **J**, **L**, **N**) Relative transcription levels of key fatty acid beta oxidation enzymes in WT and *ATPsyn-beta*-silenced transformants. (**O**) Classical fatty acid β oxidation cycle in eukaryotes [33]. All the experiments were performed in three independent replicates, and the results are presented as the means ± SDs, with different letters indicating significant differences (Duncan's multiple range test, $p < 0.05$)

the synthesis of GAs in *G. lucidum* [35–38]. Our experimental results further show that these stresses lead to a marked increase in GAs in *G. lucidum*, accompanied by a decrease in ATP content. This suggests that when *G. lucidum* encounters environmental stress, it undergoes metabolic rearrangement that inhibits energy metabolism, which may represent a common adaptive strategy. We linked this process to GAs accumulation by showing that intracellular ATP deficiency enhances the activity of the MVA pathway and increases the acetyl-CoA content by activating the fatty acid β-oxidation pathway. Our findings highlight the role of energy metabolism in the regulation of secondary metabolism, providing insights into how the cellular energy status can influence the production of valuable secondary metabolites.

ATPsyn-beta is a component of the ATP synthase complex catalytic core and plays an important role in maintaining energy homeostasis in cells [39, 40]. Disruption of ATPsyn-beta impairs ATP synthase activity, which may not only decrease ATP levels as previously reported [41], but also play a key role in other biological processes, such as glucose metabolism and lipid metabolism [14, 42]. Given its critical role in biological metabolism, RNAi-mediated knockdown of *ATPsyn-beta* has been shown to increase the mortality rate of *Euscelidius variegatus* and delay larval development [43]. Guan et al. found that *ATPsyn-beta* may play a protective role in AGEs-related renal fibrosis through siRNA-mediated downregulation of *ATPsyn-beta* [40]. Furthermore, overexpressed *ATPsyn-beta* may improve immune responses in plants by controlling physiological metabolism and fundamental cellular processes [42]. In this study, it was found that intracellular ATP deficiency is inversely correlated with GAs accumulation in *G. lucidum*. As shown in Fig. 2, both *ATPsyn-beta* gene silenced and Oli treatment upregulated the transcription of key genes, subsequently increasing GAs accumulation. These findings indicate that inhibiting ATP synthase could reprogram

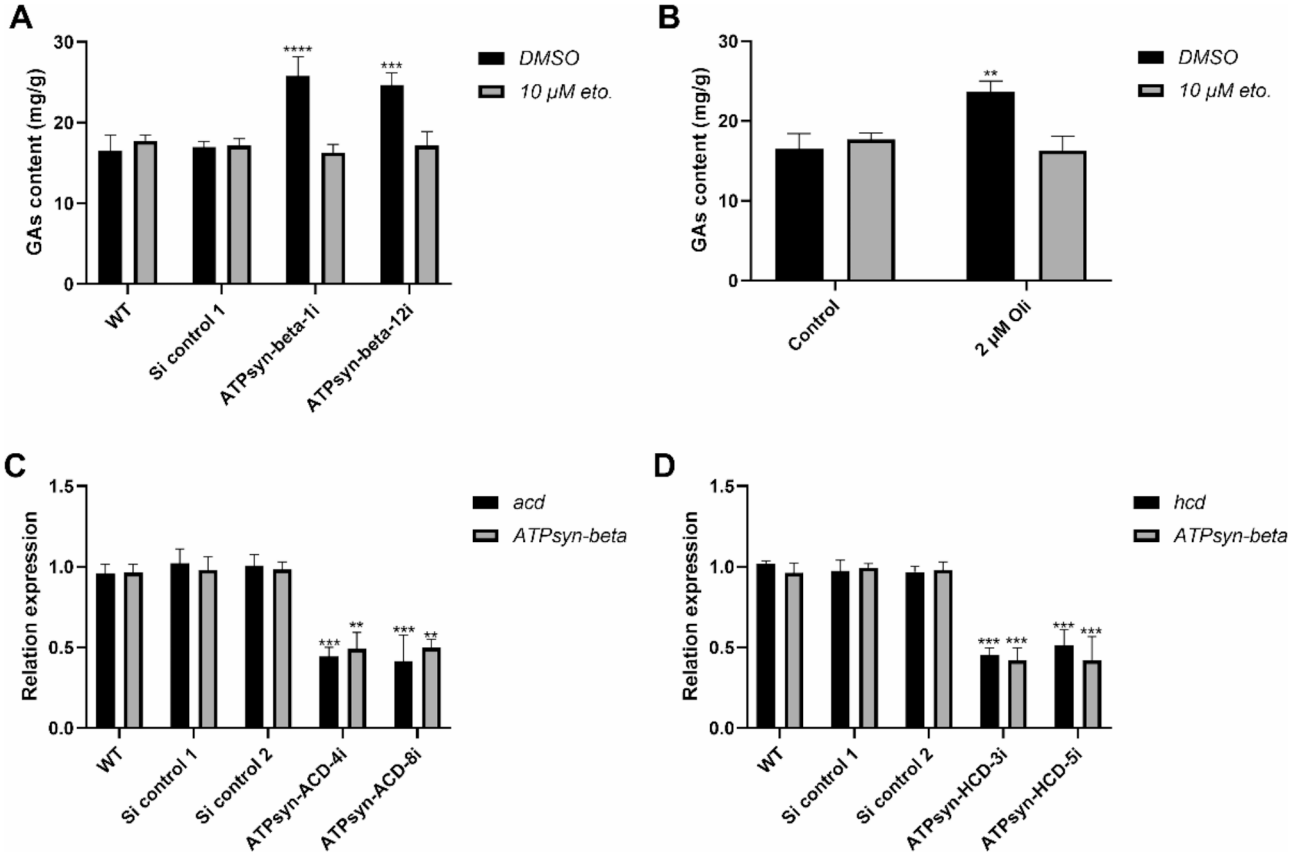

Fig. 5 Effects of eto. treatment GAs content in *G. lucidum*, and screening of ATPsyn-ACD and ATPsyn-HCD co-silented transformants. (**A**) GAs contents in WT and *ATPsyn-beta*-silenced transformants treated with 10 μM eto. and the control. *G. lucidum* WT mycelium was cultured in CYM liquid culture medium for 4 days, followed by the addition of 10 μM eto. The mixture was added, and the culture was continued for 3 days to measure the GAs content. DMSO was used as a control. (**B**) *G. lucidum* WT mycelium was cultured in CYM liquid culture medium for 4 days, followed by the addition of 10 μM eto, or 2 μM Oli was added, and the culture was continued for 3 days to measure the GAs content. (**C**, **D**) Screening of ATPsyn-ACD and ATPsyn-HCD co-silented transformants. *G. lucidums*' ATPsyn-ACD and ATPsyn-HCD double-silented transformants were screened via qRT-PCR. Using *G. lucidum*'s 18S rRNA as the qRT-PCR reference gene, the relative gene expression level of the WT was defined as 1.0. All experiments were performed in three independent replicates, and the results are presented as the means ± SDs, with different letters indicating significant differences (Duncan's multiple range test, $p < 0.05$) and asterisks indicating significant differences from untreated samples (Student's t test: * * $p < 0.01$)

energy metabolism and carbon metabolism. In addition, our study also observed a significant imbalance between the decreased levels of ATP and the increased levels of GAs (Fig. 1). The emergence of this phenomenon could possibly be attributed to the existence of an unknown feedback regulatory mechanism in GAs synthesis [44]. Another possibility is that the abrupt depletion of ATP forces cells to prioritize the remaining energy for maintaining basic cellular processes, thereby creating a discrepancy between the levels of ATP and GAs [45].

GAs are biosynthesized from the precursor acetyl-CoA, proceeding through the MVA pathway, followed by a series of enzymatic modifications. However, the MVA pathway is characterized by a relatively low flux toward acetyl-CoA in organisms. Scott et al. (2021) reported that increased acetyl-CoA supply in *Saccharomyces cerevisiae* could enhance MVA pathway activity [46]. Therefore, the level of acetyl-CoA is considered a critical factor affecting GAs biosynthesis and accumulation. High levels of acetyl-CoA accumulation were observed in both *ATPsyn-beta* gene-silenced strains and oligomycin-treated cultures, both of which exhibited high GAs production. Thus, identifying the primary source of acetyl-CoA is crucial for understanding how metabolic flux, triggered by ATP insufficiency, directs synthesis toward GAs. Reports indicate that glycolysis and the fatty acid oxidation pathway can be stimulated to counteract energy depletion [47]. Additionally, acetyl-CoA is derived primarily from the oxidative decarboxylation of pyruvate and β-oxidation of fatty acids during ATP biosynthesis [48]. However, our findings show that intracellular ATP deficiency enhances the activity of the glycolytic and fatty acid β-oxidation pathways, but does not significantly impact the oxidative decarboxylation of pyruvate.

In addition, our observations revealed that the changes in the transcription and enzymatic activity of PDC1 were

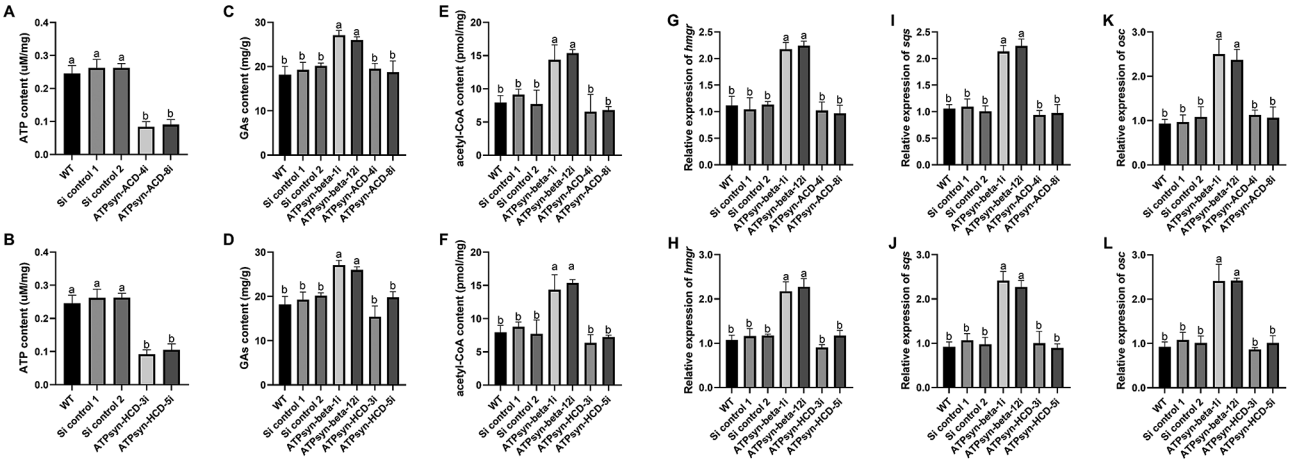

Fig. 6 Effects of *ATPsyn-acd* or *ATPsyn-hcd* co-silenced on the contents of intracellular ATP, GAs, acetyl-CoA and biosynthesis of GAs. (**A**, **B**) Intracellular ATP contents in WT, *ATPsyn-acd* and *ATPsyn-hcd* co-silenced transformants. (**C**, **D**) GAs contents in WT, *ATPsyn-acd* or *ATPsyn-hcd* co-silenced transformants. (**E**, **F**) Acetyl-CoA contents in WT, *ATPsyn-acd* or *ATPsyn-hcd* co-silenced transformants. (**G**-**L**) Relative expression levels of key MVA pathway enzymes in WT, *ATPsyn-acd* or *ATPsyn-hcd* co-silenced transformants. All experiments were performed in three independent replicates, and the results are presented as means ± SDs, with different letters indicating significant differences (Duncan's multiple range test, $p < 0.05$)

not consistent. This discrepancy suggests that the availability of mRNA for PDC is not the sole limiting factor in PDC1 activity. Enzyme activity is likely regulated by a multitude of factors beyond mRNA levels [49]. In conditions of energy deficiency, cells may employ post-translational modifications to regulate PDC1 activity. These modifications may include phosphorylation, acetylation, or ubiquitination, which can alter the activity, stability, or localization of the enzyme [50]. Oli, as an ATP synthesis inhibitor, disrupts the normal production of ATP [51]. PDC is a critical link between glycolysis and the tricarboxylic acid (TCA) cycle. The decrease in PDC1 activity could be a cellular strategy to conserve energy. By reducing the rate at which pyruvate enters the TCA cycle, the cell can lower the metabolic intensity of the entire respiratory chain, thereby adapting to the state of energy deficiency [52, 53]. Thus, the elevated levels of acetyl-CoA observed under conditions of intracellular ATP deficiency in *G. lucidum* may be predominantly attributed to the fatty acid β-oxidation pathway. Further results showed that the increase in acetyl-CoA content was prevented in co-silenced transformants of *ATPsyn-beta* and genes involved in fatty acid β-oxidation. This indicates that acetyl-CoA, a product of fatty acid β-oxidation, serves as a substrate for other secondary metabolic pathways, facilitating GAs biosynthesis in *G. lucidum*. These results were consistent with previous reports and indicated that intracellular ATP deficiency could upregulate the fatty acid β-oxidation pathway, increase acetyl-CoA accumulation, and subsequently increase the MVA pathway in *G. lucidum* [46, 54, 55]. These results further establish that energy homeostasis is a key regulatory mechanism for environmental stress in secondary metabolism, particularly for pathways with acetyl-CoA as the initial substrate.

Energy metabolism is a biological process that involves adjusting a series of metabolic pathways in cells to support complex life activities. This study revealed that *G. lucidum* activates the intracellular β-oxidation pathway under conditions of ATP deficiency, producing more acetyl-CoA and entering the tricarboxylic acid cycle to produce ATP. At the same time, part of acetyl-CoA enters the MVA pathway, promoting the synthesis of GAs. Thus, this study offers a new perspective for investigating energy homeostasis and metabolic rearrangement mechanisms in fungi.

Conclusions

This study investigated the role of energy metabolism in regulating secondary metabolism in *G. lucidum*, particularly under environmental stress. Researchers have discovered an evident correlation between energy metabolic restructuring induced by intracellular ATP deficiency and the biosynthesis and accumulation of secondary metabolites, including GAs.

Furthermore, inhibition of the ATP synthase beta subunit can reprogram cellular energy and carbon metabolism, leading to upregulation of the MVA pathway and an increase in acetyl-CoA content. This metabolic shift was driven primarily by the activation of the fatty acid β-oxidation pathway, which in turn increased GAs production. The findings also suggested that the level of acetyl-CoA is a critical factor affecting GAs biosynthesis. High acetyl-CoA accumulation, observed in *ATPsyn-beta* gene silenced strains and in cultures treated with Oli, was correlated with increased GAs production. This indicated that intracellular ATP deficiency could stimulate

metabolic pathways to counteract energy depletion, redirecting metabolic flux towards the synthesis of valuable secondary metabolites.

To conclude, this study established a link between energy homeostasis and the regulation of secondary metabolism, emphasizing the potential of targeting energy metabolism pathways to enhance the production of bioactive compounds in *G. lucidum*. These results provide new insights into the mechanisms by which the cellular energy status influences secondary metabolism and offer a promising strategy for improving the yield of biosynthesized products in fungi.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02668-2.

Supplementary Material 1

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Not applicable.

Author contributions
WDL conceived and designed the study. WDL, SNY, YK, QQC, YFL, and YS conducted the experiments and analyzed the data. WDL and YS drafted the manuscript. LS, and, MWZ supervised the research and revised the manuscript. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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