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The cloning, characterization and functional analysis of a gene encoding an acetyl-CoA acetyltransferase involved in triterpene biosynthesis in *Ganoderma lucidum*

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ABSTRACT

To better understand the functions of key genes involved in triterpene biosynthesis in *Ganoderma lucidum*, we cloned and characterized an acetyl coenzyme A acetyltransferase gene (AACT), designated Ganoderma lucidum AACT (*Gl-aact*). The open reading frame (ORF) of *Gl-aact* encoded a 417-amino acid polypeptide with a theoretical pI of 8.89. The Gl-AACT sequence was highly homologous to those of other fungal AACTs. Meanwhile, analysis of the *Gl-aact* expression profile revealed a positive correlation between *Gl-aact* transcript abundance and triterpene content changes during the development of *G. lucidum*. The treatment of mycelia with exogenous methyl jasmonate (MeJA) caused the accumulation of *Gl-aact* messenger RNA. *Gl-aact* over-expressing transformants were obtained by *Agrobacterium tumefaciens*-mediated transformation (ATMT), and triterpene production was observed to be increased when *Gl-aact* was over-expressed. Our results suggest that this enzyme may play an important role in triterpene biosynthesis.

1. Introduction

Ganoderma lucidum (Curtis) P. Karst. (Polyporaceae) has been used for centuries in East Asia to treat various human diseases. Triterpenoids are the most important pharmacologically active constituents of *G. lucidum* (Lin 1979). More than 200 triterpenes have been isolated from this mushroom, some of which inhibit cholesterol synthesis or exhibit antihistaminic, antioxidant, or anti-tumor effects, and several triterpenes have been reported to be active against the human immunodeficiency virus (Shi et al. 2010). However, the low yields of triterpene extraction strategies have inhibited its development as a clinical treatment. One of the potential solutions to this problem is the overproduction of triterpenes in *G. lucidum* using genetic engineering techniques. Therefore, a further understanding of the mechanism of triterpene biosynthesis in *G. lucidum* is necessary.

Although the pharmacological activities of triterpenes have been studied extensively, few researchers have investigated the mechanism of triterpene biosynthesis in *G. lucidum*. Several reports have confirmed that triterpenes are synthesized by the mevalonate pathway (MVP) in *G. lucidum* (Hirotani et al. 1990; Shiao 2003). To elucidate the molecular basis of triterpene accumulation in *G. lucidum*, we isolated and characterized several genes that encode HMG-CoA reductase (HMGR) (Shang et al. 2008), squalene synthase (SQS) (Zhao et al. 2007), lanosterol synthase (OSC) (Shang et al. 2010) and farnesyl-diphosphate synthase (FPS) (Ding et al. 2008).

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Researchers have found that the triterpene content of *G. lucidum* can be increased by up-regulating the expression of these genes. Ren et al. (2010) observed that exogenous MeJA exerted a positive effect on triterpene biosynthesis in *G. lucidum* and that the expression of the triterpene biosynthesis genes can be significantly induced by methyl jasmonate (MeJA). Liang et al. (2010) found that a maximal amount of total triterpene production of 41.4 ± 0.6 mg/g cell dry weight and a concomitant increase in the expression of *Gl-hmgr*, *Gl-sqs* and *Gl-osc* was achieved under an optimal level of phenobarbital. Fang et al. (2011) found that the *Gl-cyp51* transcript was over-expressed and triterpene production was increased in *Gl-cyp51* over-expressing transformants of *G. lucidum*, accompanied by an up-regulation of the expression of *Gl-aact*, *Gl-hmgr* and *Gl-osc*. These studies revealed a positive correlation between the expression of these genes and triterpene accumulation, which may imply that these genes play important roles in the control of triterpene biosynthesis in *G. lucidum*.

Acetyl-CoA acetyltransferase (AACT, EC 2.3.1.9) is specific for the thiolysis of acetoacetyl-CoA, and it is involved in poly-β-hydroxybutyrate synthesis in certain bacteria and isoprenoid biogenesis in eukaryotes (Vollack and Bach 1996). AACT catalyzes the formation of acetoacetyl-CoA by transferring an acetyl group from one acetyl-CoA molecule to another (Clinkenbeard et al. 1973). Because this is the first step of many biosynthetic pathways, AACT plays a fundamental role in carbon skeleton assembly patterns in many biological systems, including the synthesis of steroid hormones, cholesterol and ketone bodies (Modis and Wierenga 2000; Kursula et al. 2002). Sando et al. (2008) cloned and characterized several mevalonate pathway genes in *Hevea brasiliensis*. One recent study showed that *aact* can be up-regulated by yeast extract and Ag^+ elicitors along with the accumulation of diterpenoid tanshinones in *Salvia miltiorrhiza*. Acetyl-CoA acetyltransferase is a key enzyme in the MVP (Berges et al. 1997), however, relatively little is known about its genetic functions in the triterpene biosynthesis pathway in *G. lucidum*.

As the first step to better characterize the triterpene biosynthetic pathway and understand the role of AACT in *G. lucidum*, we cloned and characterized the *Gl-aact* gene. We found that triterpene production and the transcript levels of triterpene biosynthesis genes were increased by *Gl-aact* overexpression. Our results suggested that *Gl-aact* is an important gene in the triterpene biosynthesis pathway.

2. Materials and methods

2.1. Strains and growth conditions

Ganoderma lucidum, strain HG, was obtained from the culture collection of the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences and maintained on potato dextrose broth (PDB). *Escherichia coli* DH5α (Stratagene, Shanghai, China) was used as the recipient strain for recombinant plasmids and was grown in Luria–Bertani medium at 37 °C.

2.1.1. Genomic DNA and RNA extraction

Ganoderma lucidum mycelia were harvested after appropriate incubation times, frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The primordia culture medium was composed of 78% cottonseed shell, 20% wheat bran, 1% sucrose and 1% gesso in 60%–65% water. After inoculation, the cultivation bags containing 500 g of solid culture medium were then placed in an incubator at 28 °C for 30 d. Genomic DNA was extracted using the CTAB method (Saghai-Maroof et al. 1984). Total RNA was extracted using an RNA Isolation Kit (TaKaRa, Dalian, China), treated with RNase-free DNase I and then reverse-transcribed to cDNA using an oligo $(dT)_{17}$ primer using a 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China).

2.2. Amplification of the full-length aact sequence

Two degenerate primers, AACT-1 and AACT-2 (Table 1), were designed based on the highly conserved amino acid sequences of known *aact* genes from other fungi and used to amplify a fragment of the *Gl-aact* gene (266 bp). A BLASTX search confirmed that the isolated *Gl-aact* gene fragment was highly homologous to other fungal *aact* genes. Subsequently, the isolated sequence was used to design and synthesize gene-specific primers to clone the full-length sequence. Rapid amplification of cDNA ends (RACE)-PCR was performed to obtain the 3′-end cDNA using the specific primers AACT-RACE1 and AACT-RACE2 (Table 1). Self-Formed Adaptor PCR (SFEA-PCR) (Wang et al. 2007) was performed to amplify the

Table 1 – Oligonucleotide primers used in the study.

Name	Sequence
AACT-1	5′-TTCGARMTBAAYGARGCBTTC-3′
AACT-2	5′-GCNCCNCCNCCNCCRTTRCA-3′
ACAT-5Sp1	5′-TCAGTGAGTGGACAAGGGTGACGATGATGCG-3′
ACAT-5Sp2	5′-CCGAGAGCAACGGCACCGCTGTTCAAGATAC-3′
ACAT-5Sp3	5′-TCTTCGCAGGGTCAATNNNNNNNNNCTTCTC-3′
ACAT-RACE1	5′-GTTTGCTCTCCCCACCGCCC-3′
ACAT-RACE2	5′-GGCGTTCTCCGTTGTCGTCC-3′
AACT-BamHI	5′-GATCggatccATGTTTCTGGCCCGCATA-3′
AACT-XbaI	5′-GATCtctagaTTACAACTTCTGAATGAC-3′
AACT-sq1	5′-ATGTTTCTGGCCCGCATA-3′
AACT-sq2	5′-TTACAACTTCTGAATGAC-3′
Gl-GPD-F2	5′-TTGGTCTGGGTATGCGAGGAAG-3′
HPH-DET-F	5′-GTCGTGGCGATCCTGCAAGC-3′
HPH-DET-R	5′-CCTGCGGGTAAATAGCTGCGC-3′
AACT-real1	5′-CGAGCGTGCGTGGAAGGCGG-3′
AACT-real2	5′-TCCCTACCCTCAACCCCAGC-3′
Gpd-1	5′-GATGAAGGACTGGCGTGGT-3′
Gpd-2	5′-CCGTTGAGGCTGGGAATGAC-3′
Gpd-sq1	5′-CTCCTTCACGGAGACATT-3′
Gpd-sq2	5′-TAACACCCGCAGACGAACA-3′
Ls-sq1	5′-AGGGAGAACCCGAAGCATT-3′
Ls-sq2	5′-AAGCAGATGCCCCACGAGCC-3′
Hmgs-sq1	5′-CCCATCAACGCTTCCACCA-3′
Hmgs-sq2	5′-GTTGACGGCGTTGAAGAGGG-3′
Hmgr-sq1	5′-GTCATCCTCCTATGCCAAAC-3′
Hmgr-sq2	5′-GCACAATGGACGGGAAGTCG-3′
FPS-sq1	5′-GCCCTCCTCGGTTGGTGTGT-3′
FPS-sq2	5′-AGGGCGACGGGAAGGTAGAA-3′
SQS-sq1	5′-CTCTGGCACGAACCAAAACG-3′
SQS-sq2	5′-CGTAGTGGCAGTAGAGGTTG-3′

The sequences with lowercase letters, ggatcc and tctaga indicate *Bam*HI and *Xba* I restriction sites respectively.

5′-end DNA and promoter region using three gene-specific primers (AACT-5Sp1, AACT-5Sp2, AACT-5Sp3) (Table 1). The 3′RACE, 5′SFEA and originally isolated sequence were aligned and assembled to deduce the full-length sequence of *Gl-aact*. The full-length sequence was subsequently amplified by PCR using the primers AACT-sq1 and AACT-sq2 (Table 1).

2.3. *Sequence analysis*

The BLAST program and the ClustalW program at the US National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) were used for nucleotide sequence analysis, database searches, and amino acid sequence deduction. The molecular weight, theoretical pI and amino acid composition of the predicted protein were analyzed using ProtParam (http://us.expasy.org/tools/protparam.html).

2.4. *Expression profile analyses of* Gl-aact

Real-time (RT) PCR to determine the level of *Gl-aact* transcription was performed with SYBR *Premix Ex Taq* (TaKaRa, Dalian, China) using primers AACT-real1 and AACT-real2 (Table 1) by Eppendorf Mastercycler ep Realplex2. The relative level of amplified mRNA was normalized to the mRNA expression of the housekeeping gene *G. lucidum* glyceraldehyde-3-phosphate dehydrogenase (*Gl-gpd*) (Xu et al. 2006), which was amplified as an internal control using primers Gpd-1 and Gpd-2 (Table 1). Semi-quantitative reverse transcription PCR was carried out to confirm the results, using the newly designed primers AACT-sq1 and AACT-sq2 (Table 1) to amplify *aact* and the previously published primers Gpd-sq1 and Gpd-sq2 as the internal control (Table 1) (Ding et al. 2008).

2.5. *Over-expression analyses of* Gl-aact

The plasmid pGL-GPD was digested with *Bam*HI and *Xba*I and subsequently ligated with the *Gl-aact* complete cDNA to generate pGL-aact (Fig. 1). The plasmid was introduced into *Ganoderma lucidum* by *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Shi et al. 2012).

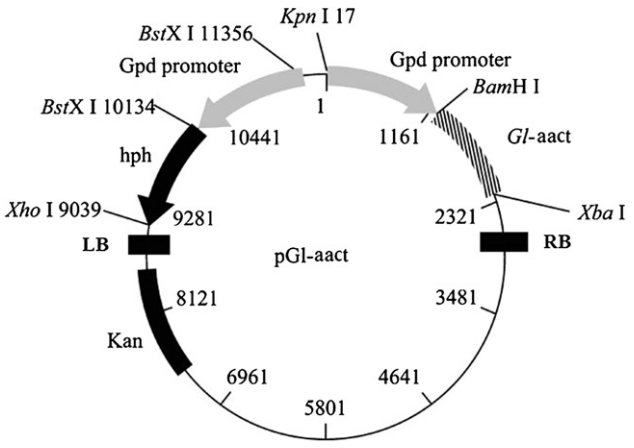

Fig. 1 – The plasmid (pGL-aact) used for *Gl-aact* over-expression.

Gl-aact over-expressing transformants (GLAO) were screened by PCR. The *hph* gene was detected using the primers HPH-DET-F and HPH-DET-R (Table 1). The fusion fragment containing the *Gl-gpd* promoter and *Gl-aact* gene was detected using the primers Gl-GPD-F2 and AACT-XbaI (Table 1).

RT-PCR was used to investigate the expression profiles of the GLAOs. The transcription of genes involved in the triterpene biosynthesis pathway was evaluated using RT-PCR with the primer sets shown in Table 1; transcripts of *Gl-gpd* were amplified as an internal control.

The GLAOs were cultured for 7 d before use in further experiments. The original *G. lucidum* strain HG was used as control. Triterpene content was determined as previously reported (Tang and Zhong 2002; Li et al. 2006).

3. Results

3.1. *Isolation of* aact *genomic DNA and cDNA in* G. lucidum

A 266-bp PCR product was amplified using the degenerate primers AACT-1 and AACT-2, which were designed based on the conserved regions of yeast and mold AACT sequences. Then, 5′SFEA and 3′RACE were carried out to obtain the full-length sequence. The full-length *Gl-aact* genomic sequence was 1484 bp and included four introns and a 1254 bp encoding region. The full-length *Gl-aact* cDNA contained a 1254 bp ORF, 82 bp of noncoding region, and a poly $(A)^+$ tail. The ORF encoded a 417-amino acid polypeptide with a theoretical pI of 8.89 and a theoretical molecular mass of 43.93 kDa. The nucleotide sequences of the *Gl-aact* gene and cDNA have been deposited in the GenBank database under accession numbers HQ596496 and HQ596497.

3.1.1. *Analysis of the deduced Gl-AACT amino acid sequence*

The deduced Gl-AACT amino acid sequence exhibited significant homology to other fungal AACTs, including those from *Laccaria bicolor* (72% identity, 83% positive) and *Coprinopsis cinerea* (75% identity, 86% positive). The highest similarities with fungi are consistent with the currently accepted phylogenies. As shown by InterProScan, the deduced Gl-AACT contains an acetyl-CoA acetyltransferase family distinctive domain, which is an acyl-enzyme intermediate active site. Hence, it was assumed that the catalytic mechanism of Gl-AACT is similar to that of acetyl-CoA acetyltransferases from other organisms.

3.1.2. *Expression of* Gl-aact *at different developmental stages and under MeJA treatment*

To determine the expression pattern of the *Gl-aact*, we monitored the level of corresponding mRNA at different developmental stages by RT-PCR. As shown in Fig. 2A, the *Gl-aact* transcript level was relatively low in the mycelia. However, the level of *Gl-aact* mRNA in the primordia was approximately 18.32-fold compared with 10-day-old mycelia. Similar results were also obtained by semi-quantitative reverse transcription PCR, as shown in Fig. 2B.

Prompted by previous work and the presence of a predicted MeJA-responsive element in *pGl-aact*, we investigated whether

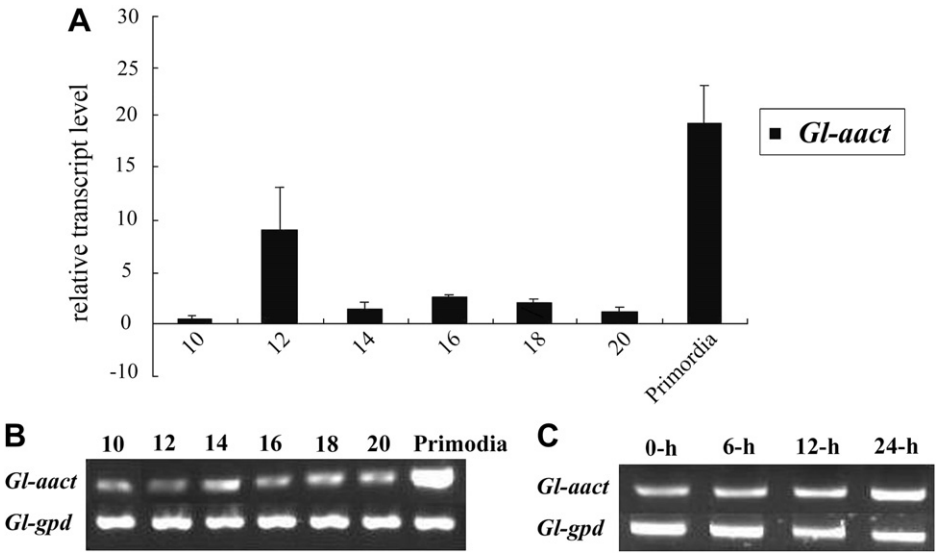

Fig. 2 – *Gl-aact* expression patterns. A. RT-PCR analysis of *Gl-aact* gene expression at different developmental stages. B. Semi-quantitative reverse transcription PCR analysis of *Gl-aact* gene expression at different developmental stages. C. RT-PCR analysis of *Gl-aact* gene expression in *G. lucidum* treated with 254 μM MeJA on day 6 for different times.

Gl-aact transcript levels were modulated by MeJA treatment (Ren et al. 2010). Fig. 2C shows the effect of MeJA on the *Gl-aact* mRNA level in the mycelia. It was observed that *Gl-aact* was up-regulated by approximately 2-fold after a 12 h MeJA induction compared with the control. The highest mRNA level, approximately 3-fold higher than the control, was observed at 24 h.

3.2. Gl-aact *over-expression analyses*

Due to the putative role of *Gl-aact* in MVP, we investigated its function using an over-expression strategy *in vivo*. Using the ATMT method, the wild type *G. lucidum* strain HG was transformed with pGL-aact. We selected 19 hygromycin-resistant transformation isolates randomly. The 19 obtained isolates were tested by PCR to determine the presence of the *hph* fragment and the fusion fragment. The *hph* fragment was amplified from all 19 transformants, and 11 of the transformants also contained the fusion fragment in their genomic DNA (data not shown). We randomly selected GlAO-2 for further analyses.

We checked the levels of several transcripts in GlAO-2. The results showed that *Gl-aact* was actually over-expressed in GlAO-2 (Fig. 3A), and its transcript levels were approximately 3-fold higher than those of the control. At the same time, we measured the triterpene content in GlAO-2. As shown in Fig. 3B, the triterpene content of GlAO-2 was approximately 75.73% higher than that of HG. We also checked the triterpene production of the other GlAOs and found that the triterpene levels were approximately 16.9%–73.7% higher than those of the control group (data not shown). This result further confirmed that *Gl-aact* plays a key role in triterpene biosynthesis.

We also investigated the transcription of other genes involved in the triterpene biosynthesis pathway in GlAO-2. Fig. 3C shows that some of these genes, including *Gl-fps*, *Gl-osc*, *Gl-idi* and *Gl-sqs*, were clearly up-regulated, while others were not changed significantly.

4. Discussion

To obtain a better understanding of the physiological functions of AACT in triterpene biosynthesis in *G. lucidum*, we isolated and characterized *Gl-aact*, which encodes an active AACT. A previous study demonstrated that the triterpene content of *G. lucidum* mycelia was much lower than that of the fruiting bodies (Hirotani et al. 1990). Analysis of the *Gl-aact* transcript profiles revealed a positive correlation between *Gl-aact* expression and the accumulation of triterpenes in *G. lucidum* during development. This result is consistent with observations of other genes (*Gl-fps*, *Gl-sqs*, *Gl-hmgr* and *Gl-osc*) involved in MVP (Zhao et al. 2007; Ding et al. 2008; Shang et al. 2008; Shang et al. 2010). This suggests that *Gl-aact* may play an important role in the control of *G. lucidum* triterpene biosynthesis.

In many previous studies, researchers have found that the yield of triterpenes can be influenced by different environmental factors, including the culture medium, fermentation process parameters, and use of chemical agents (Tang and Zhong 2002; Li et al. 2006; Ren et al. 2010). Moreover, studies of the regulation of triterpene biosynthesis at the molecular level showed that several genes involved in *G. lucidum* triterpene biosynthesis could be up-regulated by different factors. For example, Xu et al. (2010) found that the triterpene content was considerably enhanced by using a two-stage process combining shake-flask fermentation and static culture. Ren et al. (2010) found that exogenous MeJA exerted a positive effect on triterpene biosynthesis from *G. lucidum*, and several key genes (*Gl-hmgs*, *Gl-hmgr*, *Gl-fps*, *Gl-sqs*, and *Gl-osc*) were up-regulated by MeJA. Zhang et al. (2010) and Liang et al. (2010) found that the triterpene content and levels

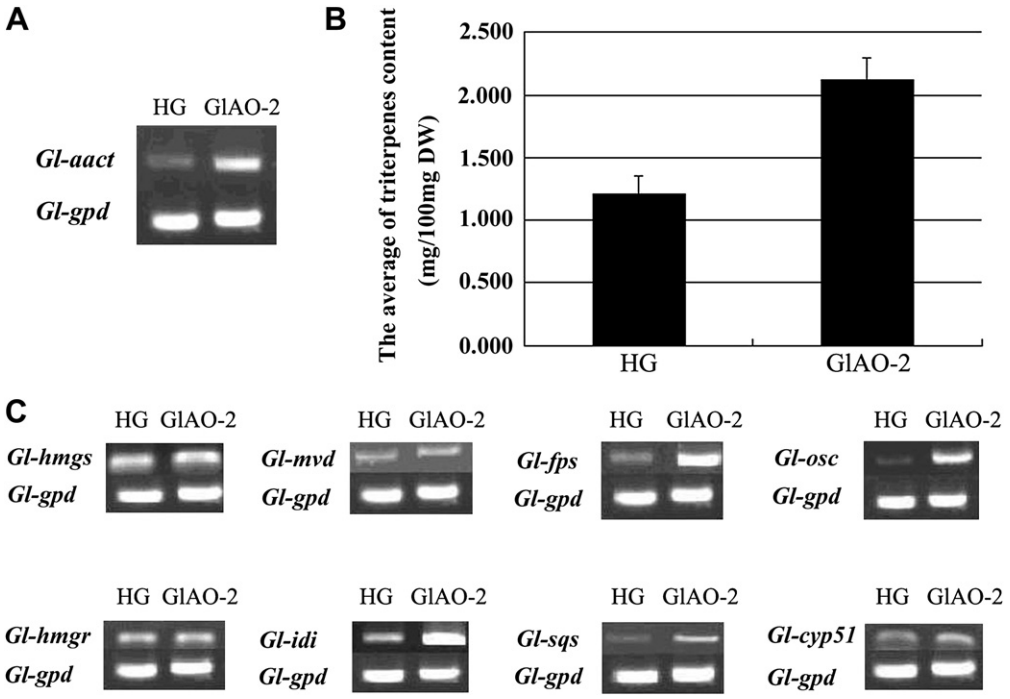

Fig. 3 – Characterization of *Gl-aact* over-expressing (GlAO) transformants of *G. lucidum*. A. Transcription level of *Gl-aact* in GlAO. B. The average triterpene content in GlAO. C. RT-PCR analysis of the genes involved in triterpene biosynthesis in GlAO.

of *Gl-hmgr*, *Gl-sqs* and *Gl-osc* mRNAs were increased at an optimal level of 80% gaseous O_2 and induced by phenobarbital, respectively. These data suggest that triterpene biosynthesis is controlled via the induction of enzymes in *G. lucidum* through a complex regulatory system. Each gene has a distinct regulatory effect on triterpene biosynthesis, and the interactions of these genes are complex. In our study, over-expression analyses of *Gl-aact in vivo* showed that the levels of *Gl-fps*, *Gl-sqs*, *Gl-osc* and *Gl-idi* mRNA were increased. These results confirmed the importance of *Gl-sqs* and *Gl-osc* in triterpene biosynthesis, consistent with Zhang and Liang's findings (Liang et al. 2010; Zhang et al. 2010). However, the mRNA levels of other genes (*Gl-hmgs*, *Gl-hmgr*, *Gl-mvd* and *Gl-cyp51*) were not changed appreciably in GlAO-2. These results were basically consistent with the above descriptions, and indicated the importance of *Gl-aact*. This study indicated that the regulation of triterpene biosynthesis in *G. lucidum* was very complex and that *Gl-fps*, *Gl-sqs*, *Gl-osc* and *Gl-idi* played important roles in this process.

MeJA and related derivatives have been implicated as signal transduction molecules with multiple effects on plant growth, development, and response to stress (Zhang et al. 2010). Our previous study showed that MeJA could induce triterpene biosynthesis through transcriptional induction in *G. lucidum* (Ren et al. 2010), including the up-regulation of the *Gl-aact* transcription. Analysis of the *pGl-aact* sequence identified two potential MeJA responsive elements (data not shown). These results suggest that, like other genes (*Gl-fps*, *Gl-osc*, *Gl-hmgr*), *Gl-aact* is most likely involved in the triterpene biosynthesis pathway that is regulated by MeJA signaling via its putative MeJA-response elements. However, further studies are needed to precisely define the *Gl-aact* promoter elements that are involved in the regulation of triterpene biosynthesis.

Disclosure

We confirm that this paper has no conflict of interest among the authors and compliance of laws and regulations about the experimentation.

Acknowledgments

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