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# Impacts of calcium signal transduction on the fermentation production of antitumor ganoderic acids by medicinal mushroom *Ganoderma lucidum*

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ABSTRACT

Recently signal transduction engineering of secondary metabolism is receiving great interest as a powerful tool towards efficient production of valuable secondary metabolites. This work found that the calcineurin-signal transduction was significant to triterpene biosynthesis by higher fungus (mushroom). Addition of calcium ion (at 10 mM) to static liquid cultures of *Ganoderma lucidum*, a famous traditional medicinal mushroom, was proved as a useful strategy to enhance the production of antitumor ganoderic acids (GAs), which resulted in 3.7-, 2.6-, 4.5-, 3.2- and 3.8-fold improvement of total GAs, individual GA-Mk, -T, -S, and -Me, respectively. Experiments using $Ca^{2+}$ sensor inhibitors indicated the involvement of calcineurin signal in regulating GAs biosynthesis. Quantitative gene transcription analysis revealed that the expression levels of genes of GAs biosynthesis and $Ca^{2+}$ sensor were up-regulated with calcium addition while down-regulated under the inhibitors addition, suggesting that higher GAs production may be resulted from higher expressions of those genes. Based on the results obtained, a possible model on the effect of external calcium ion on the GAs biosynthesis via calcineurin signal transduction pathway was proposed.



## 1. Introduction

Higher fungi, especially mushrooms, produce numerous unique bioactive compounds, and their fermentation studies have received much interest in recent decades (e.g., Wagner et al., 2003; Yoshida et al., 1965; Zhong and Xiao, 2009). *Ganoderma lucidum*, a traditional Chinese medicinal mushroom, has been used for the prevention and treatment of various human diseases for several thousand years in Asia (Shiao et al., 1994). Ganoderic acids (GAs) are one type of main bioactive components in *G. lucidum*, and they exhibit various significant pharmacological activities such as anti-tumor, anti-metastasis and anti-HIV (Xu et al., 2010). However, low GAs yield from both field cultivation and fermentation is the bottleneck for its wide application including large-scale preclinical tests. Until now, various fermentation conditions have been studied to enhance the GAs production such as culture media and various parameters of fermentation process, but the GAs content was yet not so high, around 12–49.6 mg/g DW (Hsieh and Yang, 2004; Wagner et al., 2003; Xu et al., 2010).

Increase of secondary metabolite production by signal transduction, among various strategies, has drawn great interest as reported in plant cell cultures (Zhao et al., 2005). Strategies based on signal transduction to enhance metabolite production in mushroom were summarized in Table 1. For GA production, fungal elicitors (Zhu et al., 2008), methyl jasmonate (Ren et al., 2010) and $H_2O_2$ (Zhang et al., 2010) were added to culture medium to enhance the production, but those work did not elucidate how the signals induced GA biosynthetic genes. The related mechanism study may be important to elevate GA production by signal transduction engineering.

Reported signal pathways in mushroom were listed in Table 2, and most of them were related to cell development. Although some signal transduction strategies were adopted to enhance metabolite production by mushrooms (Table 1), there is lack of information on the mechanism investigation. $Ca^{2+}$ signals were reported important in the elicitor-induced accumulation of secondary metabolites in plant cells and fungi (Chung, 2003; Yue and Zhong, 2005; Zhao et al., 2005). For triterpenes, both brassinosteroid and ginsenoside biosyntheses were demonstrated to be regulated by $Ca^{2+}$ signals in *Arabidopsis* plant and *Panax notoginseng* plant cells (Du and Poovaiah, 2005; Yue and Zhong, 2005), but gene transcription alteration remains unknown. In mushrooms, the mechanism of $Ca^{2+}$ signals in regulating triterpene biosynthesis is yet unknown, in spite of the potential significance of such information for biotechnology application.

Calcineurin signal pathway is one of important $Ca^{2+}$ signal pathways (Stie and Fox, 2008). Calcineurin, a serine/threonine-specific protein phosphatase, is activated by binding $Ca^{2+}$/calmodulin complex when intracellular $Ca^{2+}$ level increases, and it further activates the transcription factor CRZ1 (calcineurin-responsive zinc finger) to bind with the CDRE (calcineurin-dependent responsive element) motif in the promoter of downstream genes to activate them as

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**Table 1**
Reported signaling transduction strategies for metabolites production in mushroom fermentation.

| Treatment | Organism | Metabolites | Content (mg/g DW) | Production titer (mg/L) | Acting mechanism | Reference |
|---|---|---|---|---|---|---|
| Naphthalentacetic acid | *Phellinus linteus* | Extracellular polysaccharides | NA | 6240 | NA | Guo et al., 2009 |
| Tween 80 | *Pleurotus tuber* | Extracellular polysaccharides | NA | 930 | NA | Zhang and Cheung, 2011 |
| $NH_4^+$ | *Cordyceps militaris* | Cordycepin | NA | 420.5 | NA | Mao and Zhong, 2006 |
| $CaCl_2$ | *Paecilomyces sinclairii* | Red pigments | NA | 4400 | NA | Cho et al., 2002 |
| Ascorbic acids | *Hericium erinaceus* | Extracellular polysaccharides | NA | 1260 | NA | Lee et al., 2010 |
| Adenosine | *C. militaris* | Cordycepin | NA | 8570 | NA | Das et al., 2009 |
| Fungal elicitors | *G. lucidum* | Extracellular polysaccharides | NA | 920 | NA | Zhu et al., 2008 |
| | | Intracellular polysaccharides | 129.1 | 1940 | NA | Zhu et al., 2008 |
| | | Total crude GAs | 28.3 | 315.5 | NA | Zhu et al., 2008 |
| Ether extract from *C. molossus* | *G. lucidum* | Triterpenoids | NA | 313.7 | Induction by hexadecanoic acid | Liu et al., 2011 |
| Light | *G. lucidum* | Total crude GAs | 41 | 720.8 | NA | Zhu et al., 2010 |
| Methyl jasmonate | *G. lucidum* | Total crude GAs | 45.2 | NA | Up-regulation of biosynthetic genes | Ren et al., 2010 |
| $H_2O_2$ | *G. lucidum* | Total and individual GAs | 41.8 | 902.9 | Up-regulation of biosynthetic genes | Zhang et al., 2010 |
| $CaCl_2$ | *G. lucidum* | Total and individual GAs | 86.5 | 1902.8 | Up-regulation of biosynthetic genes | In this work |

NA: not available.

reported in *Aspergillus* and yeast (Soriani et al., 2010, Stathopoulos and Cyert, 1997). Research on calcineurin signals was focused on the morphology and virulence related work (Stie and Fox, 2008). To our best knowledge, calcineurin signal was only found in Ascomycota higher fungi involved in biosynthesis of chitin (Fortwendel et al., 2010), 1, 3-β-D-glucan (Fortwendel et al., 2009) and sesquiterpene (Pinedo et al., 2008), and there have been no reports in Basidiomycota (including mushrooms), another major division (phyla) of higher fungi. Moreover, there is still lack of information regarding whether and how calcineurin signals regulate triterpene biosynthesis, not to mention how $Ca^{2+}$ sensor and GA biosynthetic genes would respond to calcium ion during fermentation process.

The biosynthetic pathway of GAs is reported through the mevalonate/isoprenoid pathway but its details are yet unclear (Hirotani et al., 1990; Shiao, 1992; Yeh et al., 1989). 3-Hydroxy-3-methyglutaryl coenzyme A reductase (HMGR) is the rate-limiting enzyme of the mevalonate/isoprenoid pathway; the squalene synthase (SQS) catalyzes the first step specific to the triterpenoid or sterol biosynthesis, and the formation of lanostane-triterpene skeleton is catalyzed by lanosterol synthase (LS). These three enzymes are important in the GA biosynthetic pathway, and until now the genes encoding those enzymes of GA biosynthesis have been cloned (Shang et al., 2010; Xu et al., 2010).

In this work, the impact of medium $Ca^{2+}$ on the GA production was investigated in the static liquid culture of *G. lucidum* by addition of $CaCl_2$ and inhibitors of calmodulin (chlorpromazine) and calcineurin (cyclosporin A). The levels of total GA and four individual GAs (GA-Mk, -T, -S, and -Me) were analyzed quantitatively. Furthermore, transcription profiles of three genes encoding HMGR, SQS and LS in the GA biosynthetic pathway and three genes encoding calmodulin (CAM), calcineurin subunit A (CNA) and transcription factor CRZ1 in the calcium signal pathway were also investigated to elucidate the role of $Ca^{2+}$ sensors in the GA biosynthesis regulation.

## 2. Materials and methods

### 2.1. Maintenance of mycelial cultures of G. lucidum

*G. lucidum* CGMCC 5.616 from China General Microbiological Fermentation Center was maintained on potato-dextrose-agar slants. Details of the culture medium and procedures of the two-stage liquid static cultures of *G. lucidum* were reported earlier (Fang and Zhong, 2002).

### 2.2. Addition of $CaCl_2$, chlorpromazine, and cyclosporin A

$CaCl_2$ and chlorpromazine were dissolved in water, and cyclosporin A was dissolved in dimethyl sulphoxide (DMSO). All the solutions were sterilized by filtering through 0.22-μm polyvinylidenedifluoride syringe filters (Millipore, Germany) before their additions into *G. lucidum* cultures. Three final concentrations (1, 10, and 50 mM) of $CaCl_2$ were added when shaking flask cultures were converted to static liquid cultures in 10 cm diameter plate for incubation. Each plate contained 50 mL broth. To study the effects of calmodulin and calcineurin inhibitors on GA production, 100 μM chlorpromazine and 15 μM cyclosporine A were added with or without 10 mM $CaCl_2$. Each plate was harvested only once and then stored in a freezer for analysis.

**Table 2**
Reported signaling pathway in mushroom.

| Mushroom | Signal pathway | Function | Reference |
|---|---|---|---|
| *Lentinus edodes* | Two-component system | Development and osmotic response | Szeto, 2008 |
| *L. edode* | Mitogen-activated protein kinase | Fruit-body initiation and development | Szeto et al., 2007 |
| *L. edode* | Ras | Mycelial development | Hori et al., 1991 |
| *Coprinus cinereus* | G protein | Mating | Olesnicky et al., 1999 |
| *C. cinereus* | Calcium | Mycelial growth | Kameshita et al., 2007; Kaneko et al., 2009 |
| *Schizophyllum commune* | Ras and cAMP | Nutrient sensing and mushroom development | Yamagishi et al., 2005 |
| *S. commune* | G protein | Fruit-body formation | Yamagishi et al., 2002 |
| *G. lucidum* | Calcium | GA biosynthesis | In this work |

**Table 3**
Sequences of primer pairs for qRT-PCR assay.

| Target gene | Primer name | Primer sequence (5′–3′) |
|---|---|---|
| *Cam* | CAM-forward | GAGGTACATCTCCGCCGCC |
| | CAM-reverse | TCACGAACTCCTCGCAGTTGAT |
| *Cna* | CNA-forward | TCGGGGTCGTATAGGTCGG |
| | CNA-reverse | CTTTGCGCTTTTGCGTGAG |
| *Crz1* | CRZ1-forward | GGGTGGCTGATGCAGAAATAC |
| | CRZ1-reverse | CGAGGAGAGCGAGACGGG |
| *Hmgr* | HMGR-forward | TCGCAGTGGCACAGGAGC |
| | HMGR-reverse | CCCGGTGTTGGTGTTAGAAG |
| *Sqs* | SQS-forward | TGACGCTTCCTGACGAGA |
| | SQS-reverse | GTGGCAGTAGAGGTTGTA |
| *Ls* | LS-forward | CTTCCGCAAGCACTACCCG |
| | LS-reverse | AGCAGATGCCCCACGAGCC |
| *18S* | 18S-forward | TATCGAGTTCTGACTGGGTTGT |
| | 18S-reverse | ATCCGTTGCTGAAAGTTGTAT |

### 2.3. Sampling, analyses of dry cell weight, medium sugar, total crude GAs, and individual GA

For sampling, three plates were taken each time and the data were averaged from the triplicate samples. Dry cell weight (DW) and residual sugar concentration were measured by gravimetric method and phenol-sulfuric acid method, respectively (Fang and Zhong, 2002). Total crude GAs and individual GA were extracted and measured according to the method described elsewhere (Fang and Zhong, 2002; Liang et al., 2010).

### 2.4. Isolation of cDNA of genes of CAM and CNA from G. lucidum

The *G. lucidum* CAM, CNA and CRZ1 partial cDNAs were isolated by PCR amplification using degenerated forward/reverse primers designed on the basis of the conserved regions of fungi in the previous reported sequences: forward-CAM, 5′-AAGAGCARATYTCTGAGTTCAAGG-3′R (A, G),Y (C,T); reverse-CAM, 5′-MYCCTCGTAGTTGATYTGGCC-3′ M (A,C), Y (C,T); forward-CNA, 5′-WCGCGTACTSGGACTCGTCAT-3′W (T, A), S (G, C); reverse-CNA, 5′-TCYGATCCTGTWGAAGACTTTGG-3′ Y(C,T), W (T, A); forward-CRZ1, TGCAAGTSGARAARCAGCA, R (A, G), S (G,C); reverse-CRZ1, GHGCYTGGTGTCGYTTGC, H (A, T, C), Y (C,T). The amplified fragments were fused into the pMD19-T vector with a TA cloning system (Takara, Dalian, China) and sequenced (Invitrogen, Carlsbad, CA). The CAM amino acid sequence showed 98% and 96% identity with the available CAM amino acid sequences of *Schizophyllum commune* (Genbank accession number: XP_003025993.1) and *Postia placenta* (Genbank accession number: XP_002473838.1), respectively. The CNA amino acid sequence had 69% and 74% identity with the available CNA amino acid sequences of *Cryptococcus neoformans* (Genbank accession number: XP_567518.1) and *Neurospora crassa* (Genbank accession number: XP_961193.2), respectively. The CRZ1 amino acid sequence showed 15% and 18% identity with the available CRZ1 amino acid sequences of *Schizosaccharomyces japonicus* (Genbank accession number: XP_002175006.1) and *Penicillium marneffei* (Genbank accession number: XP_002144329.1), respectively. The CAM, CNA and CRZ1 nucleotide sequences were submitted to Genbank and their accession numbers were HQ456914, HQ456915 and JN052923 .

### 2.5. Measurement of gene expression by real-time quantitative RT-PCR (qRT-PCR)

The total RNA of 100 mg fresh weight cell was extracted by using 1 mL TriZol (Invitrogen, Carlsbad, CA), treated with RNase-free DNase I (MBI Fermentas, The Republic of Lithuania) to remove residual genomic DNA. RNA concentration was determined by Biophotometer Plus (Eppendorf, Germany). Reverse transcription was achieved by using RevertAid™ M-MuLV Reverse Transcriptase system (MBI Fermentas, The Republic of Lithuania) according to the manufacturer's protocol.

The transcriptional levels of *cam*, *cna*, *crz1*, *hmgr*, *sqs*, and *ls* genes were measured by qRT-PCR, whose procedure was the same as reported earlier (Liang et al., 2010). Transcriptional levels were calculated using the standard-curve method and were normalized against the *G. lucidum* 18S rDNA gene (an internal control) with untreated cells as a reference. For each gene, the reference sample was viewed as having an expression level of 1.0, and the results of other samples were expressed as the fold increase of mRNA level over the reference sample. Primers used for qRT-PCR were listed in Table 3.

### 2.6. Statistical analysis

The significance of samples was determined by analysis of variance, and sample means were separated by the Student paired *t* test ($P<0.05$).

## 3. Results and discussion

### 3.1. Effects of calcium ion addition time and concentration

Experiments on the addition time and concentration of external calcium ion were carried out at first in this work since both of them are generally main factors for an additive to affect cell growth and metabolite accumulation in a fermentation process. To determine the optimal addition time, 10 mM $CaCl_2$ was added to the cultivation medium every two days. The DW and GAs content (GAs amount based on DW) measured on day 16 were summarized in Table 4. There was no significant difference in cell growth for different time addition of $CaCl_2$. But, the GA yield showed different stimulatory responses to $Ca^{2+}$ induction at different growth stages. The content of total crude GAs was enhanced significantly when the cells were exposed to 10 mM $CaCl_2$ before day 6 (middle growth stage). As shown in Table 4, $Ca^{2+}$ induction on day 2 (the beginning of the static culture) led to the highest GA yield. The content of individual GAs

**Table 4**
Effects of $Ca^{2+}$ addition time (at 10 mM) on maximal biomass and GA content in *G. lucidum* fermentation (on day 16).

| Addition time | DW (g/L) | GA content (mg/g DW) | | | | |
|---|---|---|---|---|---|---|
| | | Total | Mk | T | S | Me |
| Control | 16.22 ± 0.41 | 23.48 ± 0.67 | 1.07 ± 0.10 | 2.50 ± 0.15 | 1.00 ± 0.41 | 1.04 ± 0.17 |
| Day 0 | 16.42 ± 0.58 | 67.90 ± 1.78* | 0.73 ± 0.17* | 13.02 ± 2.33* | 1.73 ± 0.36 | 2.46 ± 0.26* |
| Day 2 | 16.79 ± 0.89 | 71.12 ± 1.03* | 1.91 ± 0.32* | 11.94 ± 2.13* | 2.33 ± 0.23* | 3.03 ± 0.37* |
| Day 4 | 16.17 ± 0.22 | 62.55 ± 2.05* | 1.21 ± 0.20 | 6.09 ± 0.78* | 1.23 ± 0.25 | 1.50 ± 0.20* |
| Day 6 | 16.35 ± 0.22 | 30.15 ± 1.57* | 0.95 ± 0.08 | 3.65 ± 0.23* | 0.90 ± 0.08 | 1.31 ± 0.05 |
| Day 8 | 16.65 ± 0.66 | 26.10 ± 1.40 | 1.20 ± 0.09 | 2.42 ± 0.14 | 0.84 ± 0.05 | 1.17 ± 0.10 |
| Day 10 | 17.05 ± 1.04 | 25.77 ± 1.19 | 0.83 ± 0.03* | 3.01 ± 0.55 | 0.91 ± 0.04 | 1.27 ± 0.12 |
| Day 12 | 16.68 ± 0.65 | 25.17 ± 1.46 | 0.79 ± 0.15 | 2.44 ± 0.43 | 0.69 ± 0.14 | 1.83 ± 0.25* |
| Day 14 | 16.33 ± 0.60 | 23.75 ± 1.24 | 0.70 ± 0.14* | 2.12 ± 0.28 | 0.65 ± 0.09 | 0.58 ± 0.09* |

* $p<0.05$.

showed a similar response like the total GA. Thus, day 2 was the optimal addition time.

Regarding the effect of external calcium concentration on cell growth and GA production, a different amount of $CaCl_2$ (1, 10, and 50 mM final concentration) was added on day 2 during fermentation. The time profiles of DW, spore number and GA content were measured and their maximal values were shown in Table 5. There was no significant difference in DW when different $CaCl_2$ was added, and the highest values were all obtained on day 16. For the spore numbers under different $Ca^{2+}$ concentrations, the peak values appeared on day 8, except that on day 12 under 50 mM $Ca^{2+}$ addition. At the dosage of 10 mM $Ca^{2+}$, the spores harvested were most. While increasing the dosage to 50 mM, the spore number slightly declined; and under treatment of 1 mM $Ca^{2+}$, it was almost the same as that of control. Regarding GA content, the maximal content of total crude GAs reached 86.45 ± 5.24 mg/g DW on day 12 by 10 mM $Ca^{2+}$ induction. Compared to that case, the total GAs content slightly decreased at an increased $Ca^{2+}$ concentration of 50 mM; and under induction of 1 mM $Ca^{2+}$, it did not show significant difference from that of control. Accumulation of four individual GAs was also analyzed, and their maximum values were obtained on day 16, which showed a similar response to that of total crude GAs. Therefore, 10 mM $Ca^{2+}$ was the optimal dosage for the GA biosynthesis induction.

In this study, day 2 was the optimal $Ca^{2+}$ induction time, suggesting that the response of GA biosynthesis to induction time was closely related to the cell physiology. The similar observation was found in enhanced GA production by phenobarbital elicitation (Liang et al., 2010). The optimal dosage for $Ca^{2+}$ induction of GA production was 10 mM, and it might be owing to the up-regulation of gene expression of $Ca^{2+}$ sensors and GA biosynthesis, which will be discussed later. Similar concentration-dependent induction was also reported in calcium-induced alkaloid production in plant (Pinol et al., 1999). The most spore number was obtained at 10 mM $Ca^{2+}$ induction, which was in coincidence with the highest GA content under the same condition. This was in good agreement with the previous report that more spores led to higher GA content in static liquid culture of *G. lucidum* (Zhang and Zhong, 2010).

Our results indicated that induction with external calcium ion was an efficient strategy for enhancing GA production since contents of total GAs and four individual GAs were increased by around 2.6- to 4.5-fold, higher than the previous reports (e.g., Liang et al., 2010; Tang and Zhong, 2003). Furthermore, our results suggested that $Ca^{2+}$ not only enhanced GA biosynthesis but also slightly altered the distribution (heterogeneity) of individual GAs in the static liquid culture of *G. lucidum*. A similar result was reported in the ginsenoside biosynthesis by external $Ca^{2+}$ addition to *Panax notoginseng* plant cell cultures (Yue and Zhong, 2005). This work may help to produce specific GAs efficiently by regulating the biotransformation of individual GAs; and this is of potential industrial impact since different individual GAs have different bioactivities for application.

### 3.2. Effect of calcineurin signaling on GA production

In order to investigate whether calcineurin signaling system was involved in the $Ca^{2+}$ regulation of GA biosynthesis by *G. lucidum*, the inhibitors of calmodulin (chlorpromazine) and calcineurin (cyclosporine A) were used. Various concentrations of those inhibitors were tested in preliminary experiments (data not shown). The results indicated that the treatment of 100 μM chlorpromazine or 15 μM cyclosporine A was effective in the inhibition. Under those conditions, the dynamic profiles of cell growth and GA accumulation with or without $Ca^{2+}$ addition were analyzed. As shown in Fig. 1A, there were no significant differences in cell growth when chlorpromazine or cyclosporine A was added, compared to the situation with $Ca^{2+}$ addition. This suggested that $Ca^{2+}$ signals might not affect the cell growth in this case. The results of Fig. 1B indicated that the sugar consumption patterns in all cases were similar, which showed a continuous decline, in agreement with the cell growth profile. Spore number (Fig. 1C) and contents of total and four individual GAs were depressed by chlorpromazine or cyclosporine A treatment and partially relieved when added with $Ca^{2+}$ (Fig. 1D–H). Chlorpromazine addition decreased contents of total GA, individual GA-Mk, -T, -S, and -Me by 18%, 55%, 31%, 31% and 67% on day 16, respectively, and similar results were observed in case of cyclosporine A addition, which depressed the above GA contents by 14%, 72%, 81%, 69% and 72%, respectively on day 16.

GA contents were enhanced by $Ca^{2+}$ and decreased by antagonists of calmodulin and calcineurin, suggesting $Ca^{2+}$ signals might regulate GA biosynthesis through calmodulin-dependent calcineurin signaling pathway. By complementing with $Ca^{2+}$ addition, GA contents were partially recovered, which supported the hypothesis. In the biosynthesis of individual GAs, we suppose that acetyltransferase and P450 monooxygenase may participate in the biotransformation process by comparing their chemical structures. Those two enzymes were found to be regulated by calcineurin in Ascomycetes (Pinedo et al., 2008); in addition, our recent report showed that P450 inducer (phenobarbital) could also change the biosynthesis and distribution of individual GAs (Liang et al., 2010). Regarding the further mechanism on the regulation of GA biosynthesis by calcineurin signals, it was supposed that calcineurin signals might regulate GA biosynthesis in the transcriptional levels. In addition, we consider that CRZ1 transcription factor might also participate in the regulation of GA biosynthesis; and a similar phenomenon was observed in the glucan and chitin biosynthesis by *A. fumigates* (Fortwendel et al., 2009). Related points would be further discussed in the following experiments.

In another aspect, the time profile of spore number (Fig. 1C) was found similar to that of total GA content (Fig. 1D). This fact implies that calcineurin signals may regulate GA biosynthesis via affecting asexual sporulation of the mushroom. The effect of calcineurin signals on asexual sporulation was reported in *Aspergillus*, *Botrytis* and *Magnaporthe* (Choi et al., 2009; Cramer et al., 2008; Schumacher et al., 2008; Zhang et al., 2009).

### 3.3. Gene expressions responding to $Ca^{2+}$ signals

To probe the molecular events underlying the enhanced GA production by calcineurin signals, the transcriptional levels of three $Ca^{2+}$ sensor genes (*cam*, *cna* and *crz1*) and three GA biosynthetic genes (*hmgr*, *sqs* and *ls*) were detected by qRT-PCR in cultures with addition of $Ca^{2+}$, chlorpromazine and cyclosporine A (Fig. 2A–F). For *cam*, the

**Table 5**
Effects of $Ca^{2+}$ concentration (added on day 2) on maximal biomass, spore number and GA content.

| Addition amount | DW (g/L) | Spore number ($\times 10^8$/cm$^2$) | GA content (mg/g DW) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Total (crude) | Mk | T | S | Me |
| Control | 18.64 ± 0.40 | 1.10 ± 0.16 | 23.12 ± 1.37 | 0.98 ± 0.13 | 2.43 ± 0.78 | 0.89 ± 0.07 | 1.34 ± 0.31 |
| 1 mM | 18.01 ± 0.32 | 1.13 ± 0.19 | 25.46 ± 1.64 | 0.87 ± 0.17 | 1.93 ± 0.20 | 0.79 ± 0.09 | 1.20 ± 0.16 |
| 10 mM | 22.01 ± 1.38 | 1.82 ± 0.18* | 86.45 ± 5.24* | 2.55 ± 0.45* | 10.90 ± 2.07* | 2.79 ± 0.87* | 5.08 ± 0.73* |
| 50 mM | 21.06 ± 0.59 | 1.61 ± 0.23* | 72.69 ± 1.64* | 1.67 ± 0.12* | 8.20 ± 0.39* | 1.57 ± 0.37* | 2.50 ± 0.42* |

* $p<0.05$.

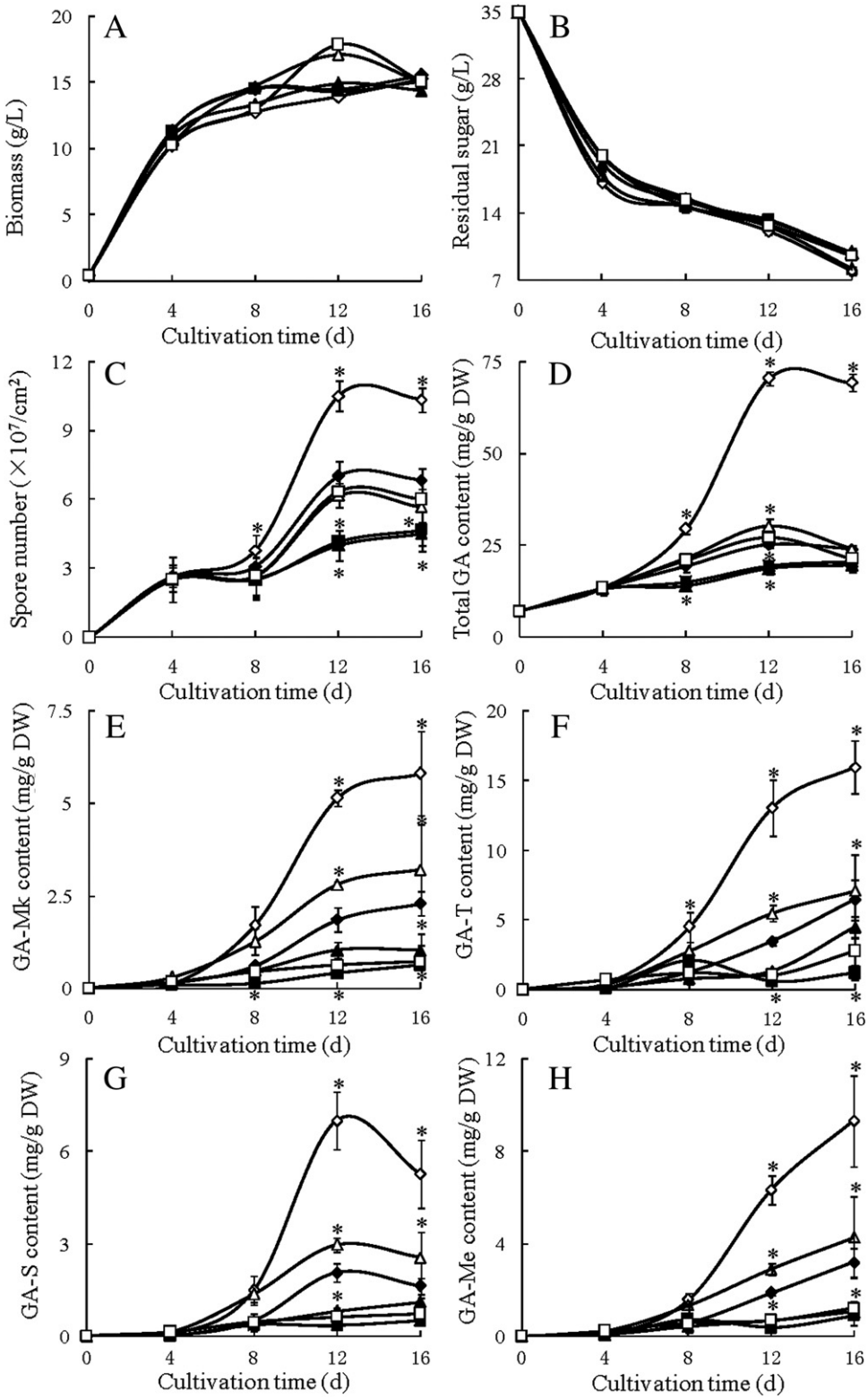


**Fig. 1.** Effects of addition of $Ca^{2+}$ and $Ca^{2+}$ sensor inhibitors on the biomass (by dry weight) (A), sugar consumption (B), spore number (C), and the content of total GA (D), GA-Mk (E), GA-T (F), GA-S (G) and GA-Me (H) in static liquid cultures of *G. lucidum* in shake flasks. Symbols for control (filled diamond), 10 mM $CaCl_2$ (blank diamond), 100 μM chlorpromazine (filled triangle), 100 μM chlorpromazine plus 10 mM $CaCl_2$ (blank triangle), 15 μM cyclosporine A (filled square), and 15 μM cyclosporine A plus 10 mM $CaCl_2$ (blank square). The error bars in the figure indicate the standard deviations from three independent samples, * $p<0.05$.

transcription level significantly enhanced on day 4 and day 12 under $Ca^{2+}$ addition; it remarkably decreased on day 12 under treatment of chlorpromazine; it slightly increased on day 4 and day 12 under supplementation of cyclosporine A. As to *cna*, this gene was up-regulated on day 4, day 8 and day 12 under $Ca^{2+}$ induction; it significantly down-regulated from day 8 when adding chlorpromazine; it declined on day 4 when cyclosporine A was added. Regarding *crz1*, the gene transcription markedly enhanced under $Ca^{2+}$ stimulation and repressed under

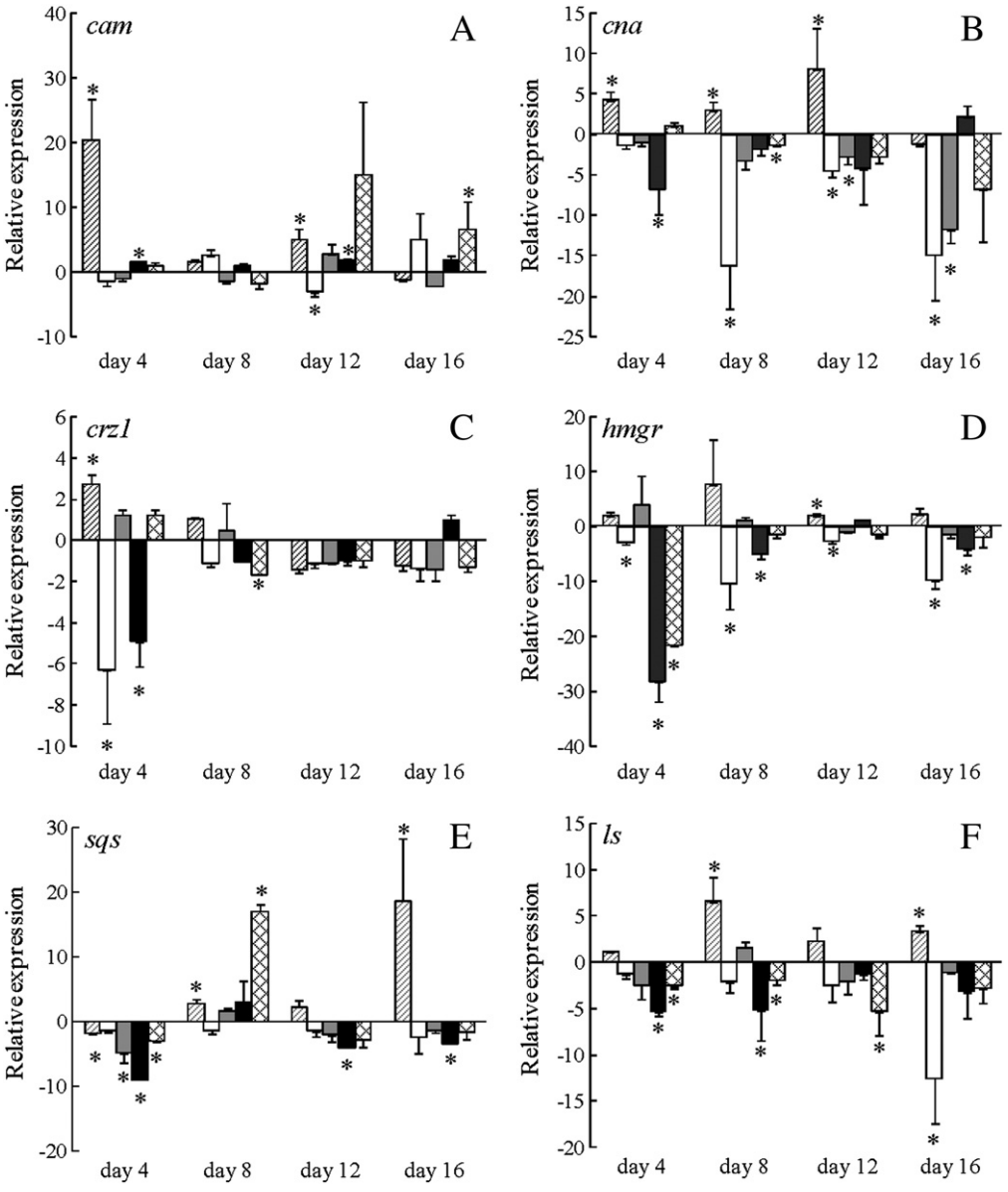


**Fig. 2.** Transcriptional levels of $Ca^{2+}$ sensor and GA biosynthetic genes (mean fold of gene transcriptional level over control) when $CaCl_2$, chlorpromazine and cyclosporine A were added. (A) *cam*; (B) *cna*; (C) *crz1*; (D) *hmgr*; (E) *sqs*; and (F) *ls*. Symbols for 10 mM $CaCl_2$ (hatched bar), 100 μM chlorpromazine (blank bar), 100 μM chlorpromazine plus 10 mM $CaCl_2$ (grey bar), 15 μM cyclosporine A (dark bar) and 15 μM cyclosporine A plus 10 mM $CaCl_2$ (double hatched bar). The error bars in the figure indicate the standard deviations from three independent samples,* $p<0.05$.

chlorpromazine and cyclosporine A addition on day 4. About *hmgr*, its transcriptional level was increased on day 12 under $Ca^{2+}$ addition and decreased under supplementation of chlorpromazine and cyclosporine A. With regard to *sqs*, the gene transcription was significantly up-regulated on day 8 and day 16 under $Ca^{2+}$ induction; it was not affected by chlorpromazine and down-regulated on day 4, day 8 and day 12 by cyclosporine A. To *ls*, the transcription level ascended on day 8 and day 16 by $Ca^{2+}$ stimulation; it descended on day 16 under chlorpromazine; it also declined on day 4 and day 8 under cyclosporine A. The inhibition of the transcriptional levels of all those genes was partially relieved when complemented with $Ca^{2+}$ in most cases. By summarizing all the above results, the model of the predicted involvement of calcineurin signaling system in GA biosynthesis was illustrated in Fig. 3.

In this work, the transcriptional levels of *hmgr*, *sqs* and *ls* were analyzed to investigate the relationship of those genes' expression with the GA production, and it was concluded that higher transcriptional levels of GA biosynthetic genes led to higher GA accumulation, which coincided with recent reports (Liang et al., 2010; Ren et al., 2010; Zhang et al., 2010). GA accumulation was elevated sharply during day 8 and day 12 by $Ca^{2+}$ stimulation, which was in agreement with the significant up-regulation of gene expressions from day 8. Previous researches also revealed a positive correlation between the gene expression of *hmgr*, *sqs* and *ls* and the content of triterpenes in *Arabidopsis* (Ohyama et al., 2007), *Eleutherococcus senticosus* (Seo et al., 2005) and *G. lucidum* (Shang et al., 2010).

GA production was significantly reduced by adding inhibitors of calmodulin and calcineurin. Earlier work indicated that calcineurin signals were important for responses to environmental signals by fungi (Stie and Fox, 2008). Therefore, the partial cDNA sequences of *cam*, *cna* and *crz1* were cloned and their expression levels were investigated quantitatively in this work to clarify whether genes of calcineurin signal in *G. lucidum* would respond to. As shown in Fig. 2A–F,

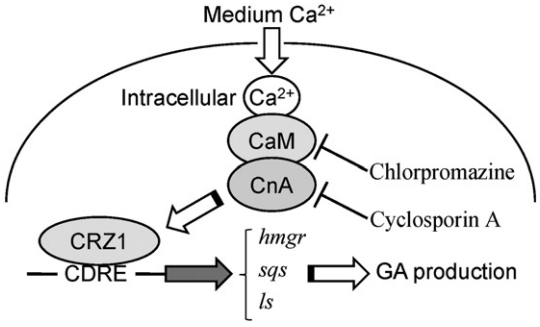


**Fig. 3.** Schematic of the predicted involvement of calcineurin signaling system in GA biosynthesis by *G. lucidum*. CaM, calmodulin; CnA, calcineurin subunit A; CRZ1, calcineurin-responsive zinc finger (transcription factor); CDRE, calcineurin-dependent response element; *hmgr*, 3-hydroxy-3-methyglutaryl coenzyme A reductase gene; *sqs*, squalene synthase gene; *ls*, lanosterol synthase gene; chlorpromazine, inhibitor of calmodulin; cyclosporin A, inhibitor of calcineurin. Symbols:⟶, promoter regions;⟹, increasing effect;⊣, inhibition effect.

$Ca^{2+}$ induction up-regulated the transcriptional levels of $Ca^{2+}$ sensor and GA biosynthetic genes while the addition of inhibitors of calmodulin and calcineurin had down-regulation effect. The results suggested that calmodulin and calcineurin were important for regulating GA biosynthetic genes at the transcriptional level. The role of calmodulin in affecting peroxidase isozyme genes at the transcriptional level was reported in *Phanerochaete chrysosporium* (Sakamoto et al., 2010). Our previous work also showed that transcriptional level of *cam* was higher in the liquid static culture with higher GA production, compared to that in the shaking flask culture, suggesting that higher level of $Ca^{2+}$ sensor gene expression might be favorable to the GA biosynthesis (Xu et al., 2011). Moreover, we found that a putative calcineurin-dependent response element (CDRE) motif existed in the promoters of *sqs* and *ls* by bioinformatic analysis, implying the potential role of the transcription factor CRZ1 in GA biosynthesis. As the results illustrated in Fig. 2C, the transcription of this gene was induced by $Ca^{2+}$ stimulation and repressed by inhibitors significantly. Similar observations were reported in chitin synthesis of *Aspergillus fumigates* (Fortwendel et al., 2010) and *Magnaporthe oryzae* (Choi et al., 2009). Further work such as knocking down the *cam*, *cna* and *crz1* genes in *G. lucidum* with RNAi or using a mass spectrometry-based approach to identify the signal-inducible proteins (Chen et al. 2005) would be helpful to clarify the more detailed role of each calcineurin signal in GA biosynthesis regulation.

## 4. Conclusions

The findings in this study provided an example of enhancing valuable secondary metabolite production by manipulating signal transduction in mushroom cultures. The production of antitumor GAs was significantly enhanced in *G. lucidum* fermentation by administering medium calcium ion, which proved to be a simple and efficient strategy. The results indicated that $Ca^{2+}$ signal could induce the GA biosynthesis through calmodulin-dependent calcineurin signal pathway to up-regulate its biosynthetic genes in the transcriptional level. The work would be helpful to the large-scale GA production (applied aspect) as well as to the further research on secondary metabolism regulation (basic research) in mushrooms.

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