ARTICLE

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Induced Effect of Na^+ on Ganoderic Acid Biosynthesis in Static Liquid Culture of *Ganoderma lucidum* via Calcineurin Signal Transduction

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ABSTRACT: Na^+/Ca^{2+} exchange is important to cell physiology and metabolism, but its role in the secondary metabolite biosynthesis by fungi is yet unclear. In this work, in static liquid cultures of *Ganoderma lucidum*, which is an efficient process for hyper-production of anti-tumor ganoderic acids (GAs), it was interestingly found that Na^+ addition could enhance the GAs production, but K^+ did not. Further investigation by intracellular Ca^{2+} imaging and using a calcineurin inhibitor (i.e., cyclosporin A) revealed that addition of Na^+ led to the influx of Ca^{2+} from culture broth to the cells and calcineurin signals were also triggered. Addition of 100 mM Na^+ at the beginning of the static liquid cultivation, in which the addition dosage and timing were optimized, resulted in 2.8-fold improvement of total GAs production. Quantitative gene transcription analysis indicated that the expression levels of the genes of Ca^{2+} sensors and GA biosynthesis were upregulated with Na^+ induction while downregulated by using the calcineurin inhibitor, implying that higher GA production might result from higher expression of those genes. This work not only provided a simple and efficient induction strategy by Na^+ addition for the improved GAs production but also suggested the regulation mechanism of Na^+ on the GA biosynthesis through calcineurin signaling transduction.

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KEYWORDS: *Ganoderma lucidum*; ganoderic acid production; calcium sensor; calcineurin signal; secondary metabolism; medicinal mushroom fermentation

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Introduction

Mushrooms, as higher fungi, can produce numerous unique bioactive compounds and are receiving great attentions for healthcare industry (Zhong and Xiao, 2009). *Ganoderma lucidum* (Ling-zhi in Chinese, Reishi in Japanese), a famous traditional Chinese medicinal herb, has been used for the prevention and treatment of various human diseases for several thousand years in Asia (Shiao et al., 1994), and its current market scale in the mainland China alone has reached billions of Chinese Yuan (RMB). Ganoderic acids (GAs), one type of main biologically active components in *G. lucidum*, are synthesized through the mevalonate/isopenoid pathway like other triterpenoids, but the details of later steps from lanosterol to GAs in its biosynthetic pathway is yet unclear (Hirotani et al., 1990; Shiao et al., 1994; Yeh et al., 1989). The fact implies that heterologous biosynthesis of GAs using a microbial host is not feasible at this stage, although a few secondary metabolites like plant sesquiterpenes were successfully produced in microorganisms such as *Saccharomyces cerevisiae* (Asadollahi et al., 2008).

Several individual GAs have been demonstrated to exhibit significant pharmacological activities such as anti-tumor, anti-metastasis, and anti-invasion (Chen et al., 2008, 2010; Tang et al., 2006). However, low GAs yield from time-consuming and labor-intensive field cultivation is the bottleneck for its commercial application. Submerged fermentation was viewed as a promising alternative to satisfy the need for high-quality individual GAs for large-scale clinical trials. Some strategies such as optimizing medium, adding inducers and manipulating fermentation

process (Liang et al., 2010) as well as innovation of separation/purification technology (Wang et al., 2012a) were developed to enhance GA yield but further increase in the production of GAs, especially individual GAs, is greatly required (Xu et al., 2010b).

Channel ions were reported to have significant impacts on secondary metabolite biosynthesis in plants (Zhao et al., 2005). K^+ and Na^+, the two main channel ions, may have similar impacts on the metabolite production (Wang et al., 2012b), but they may also show different effects. For example, the stimulating effect by Na^+ but not by K^+ was observed in aflatoxin production by *Aspergillus flavus* (El-Kady et al., 1997). Yet, the underlying mechanism of the different impacts of Na^+ and K^+ on the metabolite biosynthesis was not elucidated in those reports.

High level of extracellular Na^+ was reported to increase intracellular Ca^{2+} to cope with salt stress (Na^+ tolerance) in yeast (Feske et al., 2007) and rice (Kader and Lindberg, 2008) by triggering Ca^{2+} signals (Ariño et al., 2010). Among the targets to deal with the high level of extracellular Na^+, ENA1 (sodium transport ATPase 1) and Ca^{2+}-ATPase were reported to be involved in by activating calcineurin signals to mediate ion homeostasis (Ariño et al., 2010). Although our recent work showed that calcineurin signals participated in the regulation of GA biosynthesis (Xu and Zhong, 2012), it is yet unknown whether ENA1 and Ca^{2+}-ATPase would participate in inducing calcineurin signals. Furthermore, there is no information regarding whether a relatively higher level of channel ions will trigger signaling transduction to induce secondary metabolite biosynthesis in mushrooms.

Calcineurin signaling pathway is one of the 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 in yeast and *Aspergillus* (Soriani et al., 2010; Stathopoulos and Cyert, 1997). To our best knowledge, there is no information on whether an elevated level of medium Na^+ would enhance the cytosolic Ca^{2+} level and activate calcineurin signals to enhance GA biosynthesis.

In this work, the impact of channel ions (K^+ and Na^+) on the GA production was investigated in the static liquid culture of *G. lucidum*. The levels of total GA and four individual GAs (GA-Mk, -T, -S, and -Me) were analyzed. The time courses of intracellular and extracellular Na^+ and Ca^{2+} were detected. Transcription profiles of *hmgr* (encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase), *sqs* (encoding squalene synthase), *ls* (encoding lanosterol synthase), *cam* (encoding calmodulin), *cna* (encoding calcineurin A), *crz1*, *ena1*, and *Ca^{2+}-ATPase* genes were further investigated to try to understand how calcineurin signals regulated the GA biosynthesis. Here, our hypothesis is that medium Na^+ might regulate GA biosynthesis through calcineurin signaling pathway. The information obtained may be helpful to the more efficient production of this valuable bioactive compound as well as to the better regulation of its biosynthesis in *G. lucidum* fermentation.

Materials and Methods

Microorganism and Culture Condition

G. lucidum CGMCC 5.616 from China General Microbiological Fermentation Center was maintained on potato-dextrose-agar slants and incubated at 30°C for 7 days. Details of the culture medium and procedures of the two-stage liquid static cultivation of *G. lucidum* were described elsewhere (Fang and Zhong, 2002). K^+ and Na^+ were added aseptically by filtering through 0.22-μm polyvinylidenedifluoride syringe filters (Millipore, Darmstadt, Germany) as chloride salts. Their dosage and addition time varied according to the experiment requirement.

To study the effect of calcineurin inhibitor on GA production, 15 μM cyclosporin A (Sigma, Saint Louis, MO) was dissolved in dimethyl sulfoxide (DMSO). Then, it was sterilized by filtering through 0.22-μm polyvinylidenedifluoride syringe filters (Millipore) before addition into *G. lucidum* cultures.

Sampling, Analyses of Dry Cell Weight, Medium Sugar, Total Crude GAs, and Individual GA

Mycelia mats on the liquid surface in each 10 cm diameter plate were harvested only once and washed for three times with distilled water. The mycelia mats were stored in a freezer for analysis. 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 GAs were extracted and then measured by spectrophotometer and HPLC, respectively, as described previously (Fang and Zhong, 2002; Xu et al., 2010a).

Determination of Extracellular and Intracellular K^+, Na^+, and Ca^{2+} Concentrations

Extracellular and intracellular K^+, Na^+, and Ca^{2+} concentration were measured with inductively coupled plasma emission spectrometer (iCAP6300, Thermo Fisher, Cambridge, UK) by standard procedures. Fluo-3/AM (Sigma) was used as a Ca^{2+}-specific probe to assess the cytoplasmic calcium in *G. lucidum* according to the manufacture's protocol. Fluo-3/AM was prepared from a 1 mM stock solution in DMSO and added to the small pieces (1 cm × 1 cm) of *G. lucidum* to a final concentration of 150 μM. Pluronic F-127 (Sigma) was added to a final concentration of 2% to facilitate dye penetration. The cultures were incubated at 37°C for 1 h for dye loading. Images of calcium green fluorescence were

Table I. Primers used in this study.

Primer	Sequence (5′–3′)
ena1-F	GACACGAAGACATCCTCACCC
ena1-R	CCATCCCATCCTCCCACTC
soce-F	TAAAGTGTAAAGCCTGGGGTGC
soce-R	GCGAGGAAGCGGAAGAGC
Ca^{2+} *channel*-F	GCGGTGTCGTATCAAAGTAGTC
Ca^{2+} *channel*-R	GGCTGGCGGAGGCATT
Ca^{2+}-*ATPase*-F	GGCACTTATCCCCGTCCG
Ca^{2+}-*ATPase*-R	GATTGAGGGTCCGCCAGAG

observed under a Nikon microscope by using a 450- to 490-nm excitation filter and a 520-nm barrier filter.

Measurement of Gene Expression by Real-Time Quantitative RT-PCR (qRT-PCR)

The total RNA of 100 mg fresh weight cells was extracted by using 1 mL TriZol (Invitrogen, Carlsbad, CA), treated with RNase-free DNase I (MBI Fermentas, Vilnius, Republic of Lithuania) to digest residual genomic DNA. RNA concentration was quantified by Biophotometer Plus (Eppendorf, Hamburg, Germany). One microgram of total RNA was reverse-transcribed by using RevertAid^{TM} M-MuLV Reverse Transcriptase system (MBI Fermentas) according to the manufacturer's protocol.

The transcriptional levels of *hmgr*, *sqs*, *ls*, *cam*, *cna*, *crz1*, *ena1*, *soce* (encoding store-operated calcium entry), Ca^{2+} *channel* and Ca^{2+}-*ATPase* were analyzed by qRT-PCR. The procedures and the primers of *hmgr*, *sqs*, *ls*, *cam*, *cna*, and *crz1* used for qRT-PCR were described by Xu and Zhong (2012). For *ena1*, *soce*, Ca^{2+} *channel*, and Ca^{2+}-*ATPase* genes, the primers used for qRT-PCR were listed in Table I. The gene expression of 18S rDNA was found stable under our experimental conditions, so transcriptional levels of different genes were analyzed by the standard-curve method and normalized by that of *G. lucidum* 18S rDNA gene (an internal control) of untreated cells. For each gene, the expression level of reference sample was taken as 1.0, and the results of samples under other conditions were presented as the fold increase of mRNA level over the reference sample.

Nucleotide Sequence Accession Number

The GenBank accession numbers of *ena1*, *soce*, Ca^{2+} *channel*, and Ca^{2+}-*ATPase* genes of *G. lucidum* were JX840403, GR367417.1, GR367374.1, and FJ381967, respectively.

Statistical Analysis

Data were averaged from three independent sample measurements. The error bars presented as the standard deviations from the means of triplicates. The significance of samples was determined by analysis of variance, and sample means were separated by the Student paired *t*-test ($P < 0.05$).

Results and Discussion

Effects of Different Channel Ions on GA Production

The homeostasis of intracellular ion concentrations is fundamental to cell physiology and metabolism, including secondary metabolite biosynthesis. Among them, the maintenance of K^+ and Na^+ homeostasis becomes even more crucial (Radhakrishnan et al., 2010; Serrano et al., 1986). The particular concentration range of K^+ (1–100 mM) or Na^+ (1–200 mM), which was added at the beginning of the static culture as NaCl or KCl, was chosen to study their effects on the GA production. As presented in Table II, Na^+ addition significantly enhanced the GA production but K^+ did not. One hundred millimolar Na^+ led to a significant increase in individual GA contents. The production of GA-Mk, GA-T, GA-S, and GA-Me was 263.73 ± 9.06, 237.62 ± 5.89, 83.13 ± 2.76, and 100.16 ± 6.12 mg/L, respectively, which was 3.65, 4.30, 2.74, and 3.18 times higher than that of control. Higher or lower concentration of Na^+ than 100 mM resulted in a

Table II. Effect of metal ions on cell growth and individual GA contents (added at the beginning of liquid static cultivation).

		GA content (mg/g DW)			
Metal ion	Biomass (g/L)	GA-Mk	GA-T	GA-S	GA-Me
Control	17.52 ± 0.43	4.42 ± 0.27	3.40 ± 0.22	1.90 ± 0.16	1.93 ± 0.12
1 mM K^+	17.52 ± 0.41	$2.68 \pm 0.04^*$	$2.13 \pm 0.07^*$	$0.89 \pm 0.05^*$	$0.98 \pm 0.07^*$
10 mM K^+	17.18 ± 0.10	4.31 ± 0.22	$4.81 \pm 0.34^*$	1.72 ± 0.10	2.07 ± 0.17
100 mM K^+	17.40 ± 0.37	$5.12 \pm 0.19^*$	$6.34 \pm 0.18^*$	2.15 ± 0.08	$2.80 \pm 0.02^*$
1 mM Na^+	17.37 ± 0.40	$5.30 \pm 0.19^*$	$10.99 \pm 0.18^*$	$2.75 \pm 0.09^*$	$3.09 \pm 0.06^*$
10 mM Na^+	17.61 ± 0.55	4.42 ± 0.87	$16.17 \pm 2.12^*$	$3.53 \pm 0.25^*$	$3.68 \pm 0.59^*$
100 mM Na^+	17.39 ± 0.47	$15.76 \pm 0.52^*$	$14.03 \pm 0.34^*$	$4.93 \pm 0.16^*$	$5.97 \pm 0.35^*$
200 mM Na^+	17.28 ± 0.20	$14.16 \pm 0.54^*$	$10.87 \pm 0.44^*$	$3.95 \pm 0.14^*$	$4.79 \pm 0.20^*$

$^*P < 0.05$.

decrease of GA yield. Compared with Na^+, GA production was only slightly improved by 10 mM and 100 mM K^+ but decreased by 1 mM K^+. The reduction of GA production by 1 mM K^+ might be due to the antagonist effect of K^+ on Ca^{2+} (Iranbakhsh et al., 2007), while the latter was important to GA biosynthesis (Xu and Zhong, 2012). The slight stimulatory effect of 10 mM and 100 mM K^+ on GA production might be owing to the salt stress caused by relatively higher concentration of K^+, which was reported on the alkaloid accumulation by *Catharanthus roseus* (Zhao et al., 2001). To find an optimal timing of Na^+ addition, 100 mM NaCl was supplemented every 2 days. The biomass and individual GA content on Day 16 were summarized in Table IIIA, showing that addition of NaCl at different time did not significantly affect the cell growth. However, individual GA contents displayed different inductive responses to Na^+ addition at different growth stages. Adding 100 mM NaCl to the medium before Day 6 (middle growth stage), the GA content was elevated significantly. Na^+ stimulation on Day 2 (the start of the static culture) brought about the highest GA yield. Hence, Day 2 was the optimal addition time. Regarding the addition time of K^+, as shown in Table IIIB, the GA production was not significantly enhanced by its various additions.

The above results revealed that Na^+ addition increased the GA production but K^+ addition did not. It might be due to the fact that cells maintain characteristically high concentrations of K^+ and low concentrations of Na^+ in the cytosol (Zhu, 2001), which might result in the different responses to the medium Na^+ and K^+ addition. Therefore, fluxes of Na^+ and K^+ were detected to elucidate the difference in the next part. The optimal dosage for NaCl to induce GA biosynthesis was 100 mM since contents of four individual GAs were enhanced at least 2.74-fold. Similar dose-dependent effect was observed in the polysaccharide biosynthesis by the medicinal mushroom *Phellinus linteus* to NaCl addition (Lytton, 2007). The optimal induction time for NaCl was day 2, indicating that the response of GA biosynthesis was closely related to cell growth phase (cell physiology). The similar phenomenon was also found in the enhanced GA production by $CaCl_2$ elicitation (Xu and Zhong, 2012). Our results also indicated that Na^+ stimulation was an efficient strategy to elevate GA production. The reason might be due to the alteration of biosynthetic genes expression, which will be discussed later.

Fluxes of Na^+/K^+ and Ca^{2+} in Response to Na^+ or K^+ Addition

In order to study the fluxes of channel ions during the fermentation process, extracellular and intracellular Na^+ concentrations were measured (Fig. 1B). The levels of extracellular and intracellular Na^+ kept nearly constant in control. In contrast, the intracellular Na^+ level at the beginning was significantly raised by medium Na^+ addition. The concentration of intracellular Na^+ reached maximum on Day 4 and declined gradually, while the concentration of extracellular Na^+ was dropped to minimum on Day 4 and increased gradually, implying that medium Na^+ flew into cells at first in response to the higher Na^+ concentration (stress) but from Day 4 intracellular Na^+ gradually effused to the medium.

Cytosolic Ca^{2+} was reported to burst upon high salt stress and further induce Ca^{2+} signals to regulate cellular responses (Ariño et al., 2010). The time courses of extracellular and intracellular Ca^{2+} levels were also assayed. As shown in Figure 1A, concentration of medium Ca^{2+} kept almost unchanging for control but it descended significantly under Na^+ induction from Day 4 and kept nearly constant. In contrast, intracellular Ca^{2+} level ascended significantly under Na^+ stimulation during the whole fermentation process. It reached maximum on Day 4 and declined gradually. Compared with that of Na^+ addition,

Table III. Effect of addition time of 100 mM NaCl (A) and 100 mM KCl (B) on cell growth and individual GA content.

		GA content (mg/g DW)			
Cultivation time	Biomass (g/L)	GA-Mk	GA-T	GA-S	GA-Me
(A)					
Control	17.35 ± 0.31	5.26 ± 0.54	3.66 ± 0.17	2.12 ± 0.05	2.37 ± 0.23
Day 0	17.41 ± 0.20	15.09 ± 0.47*	10.36 ± 0.27*	3.31 ± 0.08*	4.94 ± 0.06*
Day 2	17.41 ± 0.35	17.96 ± 0.36*	14.15 ± 0.25*	5.19 ± 0.15*	6.15 ± 0.07*
Day 4	17.61 ± 0.20	14.16 ± 0.54*	10.87 ± 0.44*	3.95 ± 0.14*	4.79 ± 0.20*
Day 6	17.41 ± 0.40	5.80 ± 0.04	8.38 ± 0.16*	2.72 ± 0.10*	4.58 ± 0.08*
Day 8	17.34 ± 0.30	6.33 ± 0.12	5.07 ± 0.25*	1.85 ± 0.02*	2.26 ± 0.06
Day 10	17.54 ± 0.30	4.64 ± 0.09	4.23 ± 0.08*	2.02 ± 0.04	2.60 ± 0.05
Day 12	17.48 ± 0.42	4.82 ± 0.22	3.61 ± 0.08	2.13 ± 0.04	2.16 ± 0.04
(B)					
Control	17.87 ± 0.23	4.74 ± 1.26	3.25 ± 1.46	1.58 ± 0.25	1.86 ± 0.33
Day 0	17.86 ± 0.58	4.59 ± 0.58	3.44 ± 0.78	1.87 ± 0.29	2.24 ± 0.39
Day 2	17.77 ± 0.48	4.57 ± 0.57	3.35 ± 1.13	1.84 ± 0.48	2.10 ± 0.56
Day 6	17.88 ± 0.59	4.38 ± 0.52	3.38 ± 0.99	1.83 ± 0.39	2.16 ± 0.49

*$P < 0.05$.

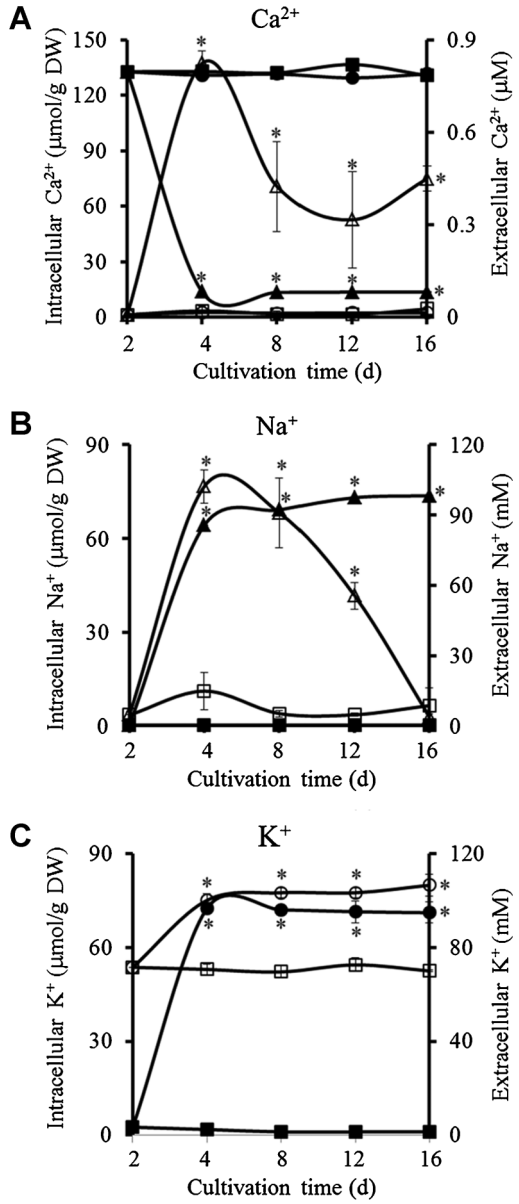

Figure 1. Levels of extracellular and intracellular Ca^{2+} (**A**), Na^+ (**B**), and K^+ (**C**) in static liquid culture of *G. lucidum*. Symbols for intracellular Ca^{2+} of control (blank square), intracellular Ca^{2+} under addition of 100 mM NaCl (blank triangle), intracellular Ca^{2+} under addition of 100 mM KCl (blank circle), extracellular Ca^{2+} of control (filled square), extracellular Ca^{2+} under addition of 100 mM NaCl (filled triangle) and extracellular Ca^{2+} under addition of 100 mM KCl (filled circle); intracellular Na^+ of control (blank square), intracellular Na^+ under addition of 100 mM NaCl (blank triangle), extracellular Na^+ of control (filled square) and extracellular Na^+ under addition of 100 mM NaCl (filled triangle); intracellular K^+ of control (blank square), intracellular K^+ under addition of 100 mM KCl (blank circle), extracellular K^+ of control (filled square) and extracellular K^+ under addition of 100 mM KCl (filled circle); The error bars in the figure indicate the standard deviations from three independent samples, $^*P < 0.05$.

concentration of cytosolic Ca^{2+} in the control was lower and kept nearly not varied. Results implied that high medium Na^+ would result in the influx of Na^+, causing the increment of intracellular Ca^{2+} by Ca^{2+} influx from medium. Similar phenomenon was observed in *S. cerevisiae* (Feske et al., 2007) and rice (Kader and Lindberg, 2008). In those cases, calcineurin signaling system was reported to be involved in, and the same signaling system was also demonstrated to participate in GA biosynthesis regulation in our previous work (Xu and Zhong, 2012). Compared to the effect of Na^+ on Ca^{2+} flux, Ca^{2+} flux was not altered by K^+ flux (Fig. 1A) although a relatively higher level of medium K^+ was observed to enter the cells (Fig. 1C). This may be due to the non-existence of specific K^+/Ca^{2+} exchanger. Until now there are no reports on the increase of cytosolic Ca^{2+} and triggering Ca^{2+} signals under a relatively higher level of extracellular K^+, except one example in *Drosophila* (Suzuki et al., 2002).

In order to study the different roles of Na^+ and K^+ flux played in Ca^{2+} flux, transcriptional levels of *Ca^{2+} channel*, *soce*, and *Ca^{2+}-ATPase* were detected. SOCE is a major mechanism for Ca^{2+} influx regulated by the Ca^{2+} content of the intracellular pool (Feske et al., 2007). *Ca^{2+}-ATPase*, a transport protein, is vital for regulating the amount of Ca^{2+} within cells, which is affected by the concentrations of Na^+ and K^+ in rice (Surach et al., 2006). These three genes were reported to upregulate in the static culture (higher GA production) in our previous suppression subtractive hybridization study (Xu et al., 2012), suggesting that they might involve in the GA biosynthesis regulation. As shown in Figure 2A and B, gene expressions of *Ca^{2+} channel* and *soce* did not respond to the relatively higher levels of K^+ and Na^+, implying that these two genes did not participate in the regulation of Ca^{2+} flux. On the other hand, transcriptional expression of *Ca^{2+}-ATPase* was upregulated with addition of Na^+ but it was not significantly changed by K^+ (Fig. 2C), suggesting that *Ca^{2+}-ATPase* played a critical role in Ca^{2+} flux. The impact of *Ca^{2+}-ATPase* on Ca^{2+} flux for Na^+ tolerance was also reported in *S. cerevisiae* (Cunningham and Fink, 1996).

Effect of Calcineurin Signals on GA Production

Since K^+ did not alter the concentration of intracellular Ca^{2+} (Fig. 1A) and did not stimulate GA biosynthesis (Tables II and IIIB), it was reasonably considered not to trigger calcineurin signals. Thus, only the effect of calcineurin signals induced by Na^+ on GA biosynthesis was studied in the following experiments. In order to investigate whether medium Na^+ regulated the GA biosynthesis through calcineurin signaling system, cyclosporine A (a calcineurin inhibitor) was applied. The dynamic profiles of biomass, sugar consumption, spore number, and GA accumulation were analyzed with or without Na^+ and cyclosporine A. As revealed in Figure 3A, there were no significant differences in biomass in all cases, suggesting that Na^+ or cyclosporine A did not influence cell growth. In agreement with the time course of biomass, residual sugar profiles under all conditions were similar and they fell continuously (Fig. 3B). Spore number

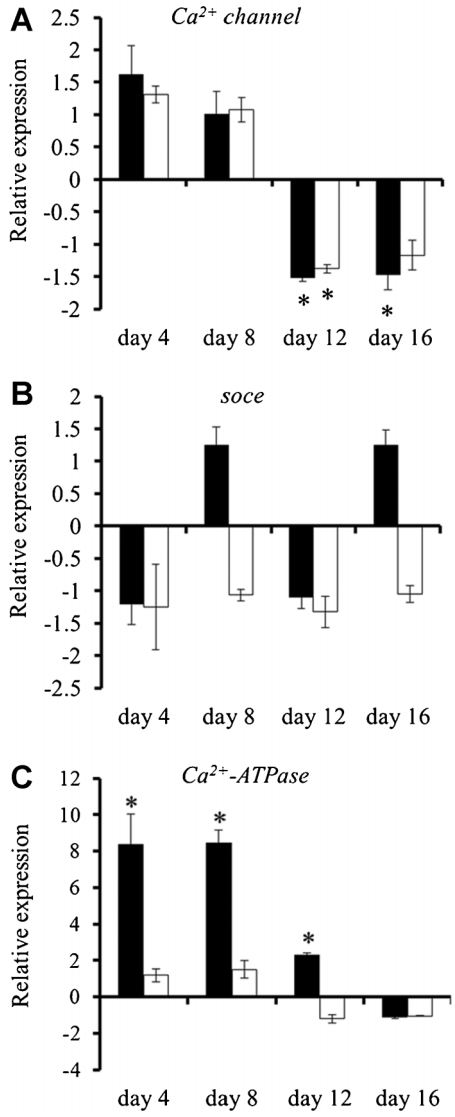

Figure 2. Transcriptional levels (mean fold of gene transcriptional level over control) of *Ca^{2+} channel* (**A**), *soce* (**B**), and *Ca^{2+}-ATPase* (**C**) when 100 mM NaCl or KCl was added. Symbols for 100 mM NaCl (dark bar) and 100 mM KCl (blank bar). Control: without addition of NaCl or KCl. The error bars in the figure indicate the standard deviations from three independent samples, $^{*}P < 0.05$.

(Fig. 3C) and contents of total crude GAs and four individual GAs were enhanced with Na^+ induction, declined by adding cyclosporine A and partially relieved by supplementing with Na^+ (Fig. 3D–H). Here, it is also noted that different from the production of other secondary metabolites like antibiotics, which are generally produced during stationary phase of growth under different nutritional and hydrodynamic stresses (Mehmood et al., 2011), the GAs were biosynthesized from the beginning of fermentation. This phenomenon coincides with our previous reports (Fang and Zhong, 2002; Xu and Zhong, 2012).

GA contents were increased by Na^+ induction and depressed by antagonist of calcineurin (cyclosporine A), indicating that Na^+ stimulation might regulate GA biosynthesis through calcineurin signaling pathway. By supplementing Na^+ to the cultures added with cyclosporine A, GA contents were partially recovered, which supported the hypothesis. The time course of spore number (Fig. 3C) was similar to that of GA contents (Fig. 3D–H), implying that calcineurin signals might regulate GA biosynthesis by influencing asexual sporulation of *G. lucidum*. The significance of asexual sporulation in GA biosynthesis was previously reported (Zhang and Zhong, 2010). How calcineurin signals affect asexual sporulation has been reported in *Aspergillus* (Cramer et al., 2008), *Botrytis* (Schumacher et al., 2008), and *Magnaporthe* (Choi et al., 2009; Zhang et al., 2009). The above results were in agreement with those we observed when applying Ca^{2+} to induce GA biosynthesis by calcineurin signals (Xu et al., 2012).

Cytosolic Ca^{2+} Image by Using Fluo-3/AM Fluorescence

In order to examine the changes of cytosolic Ca^{2+} of cells with or without Na^+ and cyclosporine A, we used Fluo-3/AM fluorescent dye for a qualitative fluorescence analysis. Fluo-3/AM is a non-fluorescent dye but emits green fluorescence after crossing over cell membrane and binding with Ca^{2+} (Takahashi et al., 1999). According to the time course of intracellular Ca^{2+} concentration measured under Na^+ induction, we chose to examine the cytosolic Ca^{2+} on Day 4 since the difference on that day was the largest. The intensities of fluorescence represented the relative amounts of free intracellular Ca^{2+}. As shown in Figure 4, strong green fluorescence was observed in the cells under Na^+ addition. The fluorescence decreased notably when the cells were treated with cyclosporine A, but it became comparatively stronger when supplementing Na^+. Results suggested that calcineurin signals might be involved in regulating the content of intracellular Ca^{2+} under Na^+ induction. Similar phenomenon was observed in *S. cerevisiae* (Feske et al., 2007). The molecular mechanism underlying the enhanced GA production through calcineurin signals by Na^+ induction will be discussed later.

Gene Expression Response to Na^+ Induction

Under environmental stress, the biosynthesis of secondary metabolites often involves various intracellular signals and their mediated effects on biosynthetic genes expression (Peebles et al., 2009; Zhou and Zhong, 2011). The transcription levels of *hmgr*, *sqs*, and *ls* were measured to reveal their relationship with GA biosynthesis. As shown in Figure 5A, the highest transcription level of *hmgr* was reached on Day 8 under Na^+ stimulation, which was about 20-fold that of control. For *sqs* and *ls* (Fig. 5B and C), their largest change of transcription levels was observed on day 4, nearly 109 and 4 times higher than that of control, respectively. The transcription levels of GA biosynthetic

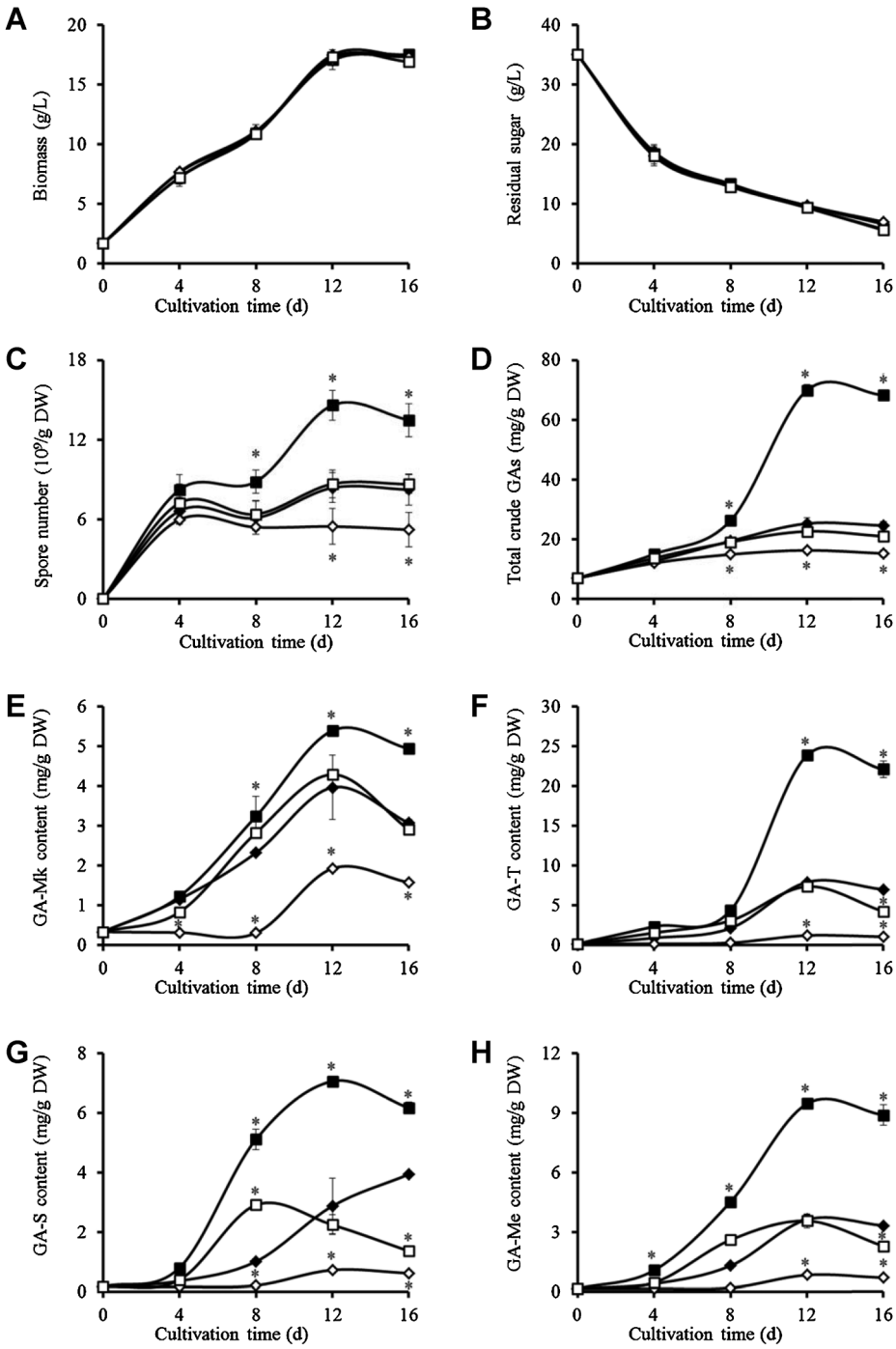

Figure 3. Effects of addition of Na^+ and cyclosporine A (calcineurin inhibitor) on the biomass (by dry weight) (**A**), residual sugar (**B**), spore number (**C**), and the content of total crude GAs (**D**), GA-Mk (**E**), GA-T (**F**), GA-S (**G**), and GA-Me (**H**) in static liquid cultures of *G. lucidum*. Symbols for control (filled diamond), 100 mM NaCl (filled square), 15 μM cyclosporine A (blank diamond), and 15 μM cyclosporine A plus 100 mM NaCl (blank square). The error bars in the figure indicate the standard deviations from three independent samples, $^*P < 0.05$.

genes (*hmgr*, *sqs*, and *ls*) were significantly depressed by adding cyclosporine A and the maximal alteration was nine-, four-, and four-fold lower than that of control, respectively. By supplementing Na^+ to the cyclosporine A added medium, the expression levels of GA biosynthetic genes were enhanced in comparison with those of control, suggesting that medium Na^+ partially relieved the inhibitory effect of cyclosporine A. GA production was enhanced

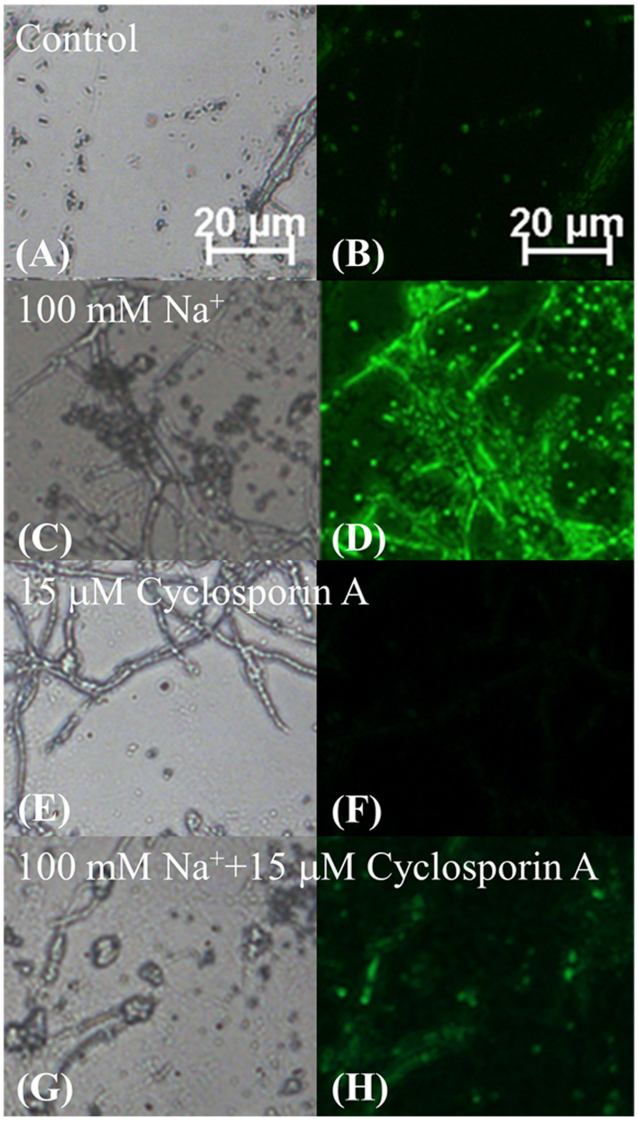

Figure 4. Cytosolic Ca^{2+} images of *G. lucidum* (original magnification 400×) observed by light microscopy (**A**, **C**, **E**, and **G**) and fluorescence microscopy (**B**, **D**, **F**, and **H**) when NaCl and cyclosporine A were added as shown in the pictures. Fluo-3/AM (150 μM) was added to the small pieces (1 cm × 1 cm) of G. lucidum. The intensities of green fluorescence represent the relative amounts of free cytosolic Ca^{2+}. Bar, 20 μm.

sharply from Day 8 to Day 12 by Na^+ elicitation, coincident with the significant increment of gene expression from day 4. The results indicated that higher transcription level of GA biosynthetic genes led to a higher GA accumulation, which agreed with other reports on GA biosynthesis (Liang et al., 2010; Ren et al., 2010; Takahashi et al., 1999; Xu et al., 2010a). In *S. cerevisiae* (Dai et al., 2012), *Arabidopsis* (Ohyama et al., 2007) and *Eleutherococcus senticosus* (Seo et al., 2005), a positive relationship between the transcription level of *hmgr*, *sqs*, and *ls* and the content of terpenoids was also reported.

The expression levels of three sensor genes (*cam*, *cna*, and *crz1*) were quantitatively detected to probe the molecular events underlying the enhanced GA biosynthesis. For *cam*, *cna*, and *crz1* (Fig. 5D–F), their transcription level was remarkably raised by Na^+ addition but significantly depressed by cyclosporine A. The inhibiting effect of cyclosporine A was partially moderated by Na^+ supplementation, implying that calcineurin signal was important to regulate GA biosynthesis at the transcriptional level under Na^+ stimulation. Our earlier work by Ca^{2+} elicitation demonstrated that calcineurin signal would induce GA biosynthesis at the transcription level and suggested the potential role of the transcription factor CRZ1 in GA biosynthesis (Xu and Zhong, 2012). The mechanism of Na^+ induction for GA biosynthesis was considered similar to that of Ca^{2+} stimulation (Xu and Zhong, 2012). Previously it was also reported that calcineurin signals were involved in the responses to environmental signals such as salt stress, heat shock and pheromone response by *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *S. cerevisiae* (Stie and Fox, 2008).

The transcription levels of *ena1* and *Ca^{2+}-ATPase* were assayed to clarify the relationship between medium Na^+ and calcineurin signals (Fig. 5G and H). They were remarkably upregulated when adding Na^+ on Day 4 and Day 8, agreeing with the time course of extracellular and intracellular levels of Na^+ and Ca^{2+}. From the study of Ca^{2+} flux (Fig. 1A) and Na^+ flux (Fig. 1B), medium Na^+ flew into cells when adding Na^+, then the intracellular Na^+ streamed out to the medium in accompanying with the inflow of medium Ca^{2+} to increase intracellular Ca^{2+} level. The role of Na^+ gradient in facilitating extracellular Ca^{2+} influx might result from the high gradient of extracellular and intracellular Na^+, which might cause the medium Na^+ influx to enhance intracellular Na^+ level. In order to cope with the toxicity caused by the relatively higher level of intracellular Na^+, extracellular Ca^{2+} might flow into the cells through Na^+/Ca^{2+} exchanger to increase intracellular Ca^{2+} (Ozaki et al., 1991). *Ena1* encoding Na^+-ATPase, combining with Ca^{2+}-ATPase, was responsible for the exchange of Na^+ and Ca^{2+} (Guerini, 1998; Park et al., 2001). Higher transcription levels of *ena1* and *Ca^{2+}-ATPase* might help for ion homeostasis to respond to high Na^+ stress (Nakayama et al., 2004) and probably further activate signal pathway. Furthermore, a CDRE motif in the promoter of *ena1* was found in yeast and demonstrated to be regulated by calcineurin signals (Ruiz et al., 2003; Withee et al., 1998). The decreased transcription levels of *ena1* and *Ca^{2+}-ATPase* by adding cyclosporine A in this work also supported the hypothesis on the signal transduction (Fig. 6). Further work such as knocking down *cam*, *cna*, *crz1*, *ena1*, and *Ca^{2+}-ATPase* genes in *G. lucidum* with RNAi or using a mass spectrometry-based approach to identify the signal-inducible proteins (Chen et al., 2005) would help clarify the more detailed role of each calcineurin signal target in GA biosynthesis regulation. In addition, as reported in *Rubia cordifolia* plant cells for production of anthraquinone (Shkryl et al., 2011), heterologous expression of calcium sensors may improve cellular secondary

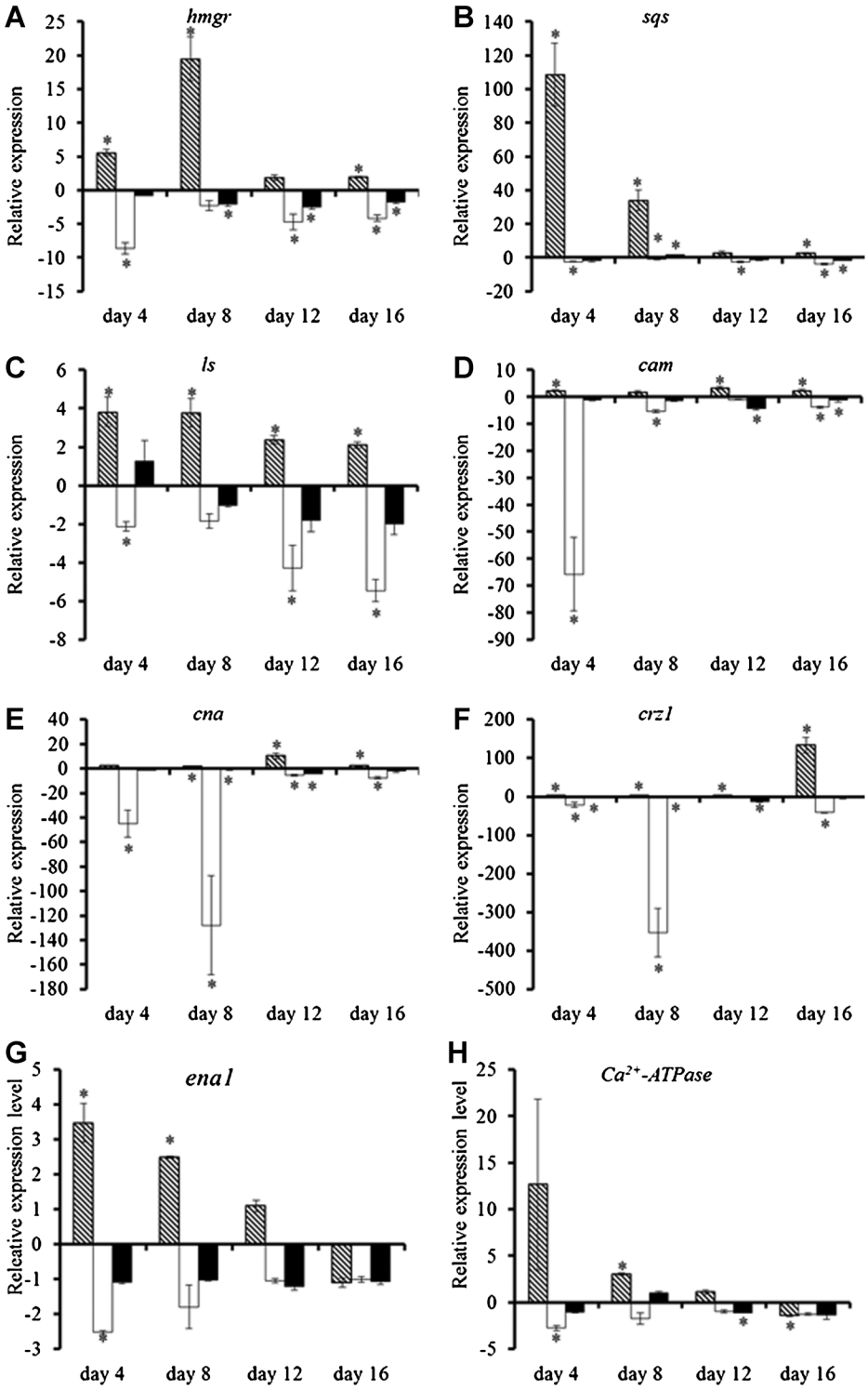

Figure 5. Transcriptional levels (mean fold of gene transcriptional level over control) of *hmgr* (**A**), *sqs* (**B**), *ls* (**C**), *cam* (**D**), *cna* (**E**), *crz1* (**F**), *ena1* (**G**), and Ca^{2+}*-ATPase* (**H**) when NaCl and cyclosporine A were added. Symbols for 100 mM NaCl (hatched bar), 15 μM cyclosporine A (blank bar) and 15 μM cyclosporine A plus 100 mM NaCl (dark bar). Control: without addition of NaCl or cyclosporine A. The error bars in the figure indicate the standard deviations from three independent samples, $^{*}P < 0.05$.

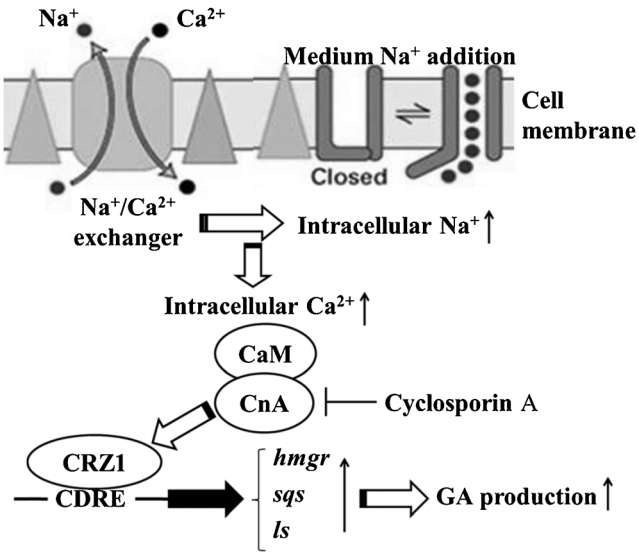

Figure 6. Proposal of Na^+ induction mechanism on GA biosynthesis via calcineurin signaling. CaM, calmodulin; CnA, calcineurin subunit A; CRZ1, calcineurin-responsive zinc finger (transcription factor); CDRE, calcineurin-dependent response element; *hmgr*, 3-hydroxy-3-methyglutaryl coenzyme A reductase gene; *sqs*, squalene synthase gene; *ls*, lanosterol synthase gene; cyclosporin A, inhibitor of calcineurin. Symbols: ⟶, promoter regions; ⇨, increasing effect; ⊣, inhibition effect.

metabolism, which could be an interesting future topic for the GA applied research.

Conclusions

The findings in this study provided a successful 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 Na^+, which proved to be a simple and efficient strategy. The results indicated that Na^+ induction would enhance cytosolic Ca^{2+} to induce the GA biosynthesis through calcineurin signal pathway to upregulate its biosynthetic genes in the transcriptional level (Fig. 6). The work would be helpful to the large-scale GA production (applied research) as well as to the further research on secondary metabolism regulation (basic research) in mushrooms.

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