Communication to the Editor

Biotechnology and Bioengineering
DOI 10.1002/bit.25288

Induction of ganoderic acid biosynthesis by Mn^{2+} in static liquid cultivation of *Ganoderma lucidum*†

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Running title: Induction of ganoderic acid biosynthesis by Mn^{2+}

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/bit.25288]

Received February 14, 2014; Revision Received May 05, 2014; Accepted May 06, 2014

Abstract

Metal ions affect cell physiology and metabolism significantly, but the role of Mn^{2+} in the secondary metabolism of mushrooms is yet unclear. In static liquid cultivation of *Ganoderma lucidum* for producing antitumor ganoderic acids (GAs), the Mn^{2+} addition was performed. Addition of 10 mM Mn^{2+} at the start of the static liquid cultivation resulted in 2.2-fold improvement of total GAs production. The expression levels of GA biosynthetic and Ca^{2+} sensors' genes were up-regulated with Mn^{2+} induction while down-regulated by adding cyclosporin A (calcineurin inhibitor), suggesting that higher GA production might result from calcineurin signal regulation. Intracellular Ca^{2+} imaging and calcineurin inhibitor study revealed that addition of Mn^{2+} led to Ca^{2+} influx from medium to the cells to trigger calcineurin signals. Mn^{2+} addition was therefore an efficient induction strategy for improving GAs production, whose regulation mechanism was *via* calcineurin signaling transduction.

Key words: *Ganoderma lucidum*; antitumor triterpenoid; calcineurin signal; secondary metabolism; medicinal mushroom fermentation

Medicinal mushrooms are a treasury of therapeutically bioactive agents (Zhong and Xiao, 2009). Ganoderic acids (GAs) produced by *Ganoderma lucidum*, a traditional medicinal mushroom, have important pharmacological activities such as anti-tumor and anti-metastasis (Tang et al., 2006; Chen et al., 2010). GAs, a class of oxygenated C30 lanostane-type triterpenoids, is synthesized through mevalonate pathway, but the detailed later steps in their biosynthetic pathway are unclear (Shiao, 1992; Xu and Zhong, 2012). The fact implies that construction of heterologous biosynthetic pathway of GAs in other hosts is yet not feasible.

The low yield of GAs from traditional field cultivation is a major bottleneck for commercial application (Xu et al., 2010). Recently, liquid fermentation was developed as an alternative to meet the need for individual GAs in clinical trials. Bioprocess engineering strategies such as medium optimization (Wagner et al., 2003), inducer addition (Liang et al., 2010) and fermentation process improvement (Wagner et al., 2003) as well as separation technology innovation (Wang et al., 2012) were explored to enhance GAs yield. However, further increase in GAs production is expected.

Metal ions play important roles in the cellular physiology and metabolism of various organisms. For example, Cd^{2+}, Zn^{2+} and Pb^{2+} increased the biosynthesis of fungal emulsifier in filamentous fungus *Curvularia lunata* (Paraszkiewicz et al., 2007). However, the mechanism how these metal ions worked on the cellular metabolism requires in-depth studies. Recently, our group reported that addition of Ca^{2+} and Na^{+} enhanced GAs production in *G. lucidum* liquid cultures through inducing calcineurin signal pathway (Xu and Zhong, 2012; Xu et al., 2013).

In the present work, impact of Mn^{2+}, another important metal ion in mushrooms, on the GAs production was studied. The positive effect of Mn^{2+} addition on GAs production was observed. The levels of total crude GAs and four individual GA-Mk, -T, -S, and -Me were detected. Transcription profiles of genes involved in GA biosynthesis and calcineurin signals were examined to understand how Mn^{2+} affected the metabolism of *G. lucidum* and what was the relation between Ca^{2+} and Mn^{2+} acting mechanism.

Since an addition dosage is generally critical to the cellular growth and metabolism (Liang et al., 2010; Huang and Zhong, 2013; Xu et al., 2013; Xu and Zhong, 2012), 1, 10 and 100 mM $MnCl_2$ were added at the start of the static liquid culture to study its impact on GA production. As

a result, 10 mM Mn^{2+} was found best to the toal crude GAs production (Xu YN, Ph.D thesis, SJTU, 2013). The production titer (GA content multiplied by biomass concentration) of individual GAs, i.e., GA-Mk, GA-T, GA-S and GA-Me under 10 mM Mn^{2+} was 211.25±0.73, 164.35±1.11, 59.19±0.30 and 72.11±0.95 mg/L, respectively, which was 2.13, 2.73, 2.76 and 1.78 times higher than that of control (refer to supplementary file Table SI). Mn^{2+} addition did not affect the cell growth under the tested conditions.

To find the suitable addition time of Mn^{2+}, 10 mM Mn^{2+} was added to the fermentation every two days. The results (Table SII) indicated that Mn^{2+} stimulation on the 2nd day (beginning of the static liquid culture) led to the highest GAs yield, while addition time did not influence the cell growth. The optimal time for Mn^{2+} induction was the 2nd day, implying that the response of GAs biosynthesis was closely related to cell physiology. The results demonstrated that Mn^{2+} supplementation was efficient in enhancing GAs production.

To examine the Mn^{2+} effect on GAs biosynthesis in detail, the kinetic profiles of biomass (Fig. 1A), sugar consumption (Fig. 1B), spore number (Fig. 1C) and GA accumulation (Fig. 1D-H) with or without 10 mM Mn^{2+} were analyzed during *G. lucidum* fermentation. No significant differences were observed for biomass (Fig. 1A) and sugar consumption profiles (Fig. 1B), suggesting that Mn^{2+} addition did not affect cell growth. Spore number (Fig. 1C) and the content of total crude GAs (Fig. 1D) and four individual GAs (Fig. 1E-H) were enhanced obviously by adding Mn^{2+}. They increased sharply from the 8th to 12th day and descended slightly from the 12th to 16th day by Mn^{2+} stimulation. It was also noted that different from antibiotics fermentation (Mehmood et al., 2011), which are produced in stationary phase, the GAs were synthesized from the beginning of cultivation, which coincided with our previous work (e.g., Xu et al., 2013). The trend of the time course of spore number (Fig. 1C) was similar to that of GA content (Fig. 1D-H), indicating that Mn^{2+} might regulate GA biosynthesis by affecting the cellular asexual sporulation. As previously mentioned (Zhang et al., 2010), asexual sporulation was important to GA biosynthesis. The results agreed with what we observed when adding Ca^{2+} and Na^{+} to induce GAs biosynthesis (Xu and Zhong, 2012; Xu et al., 2013).

To understand the impact of Mn^{2+} addition on the cellular physiology and metabolism further, the expression of genes in GA biosynthesis and calcineurin signaling regulation was investigated

by qRT-PCR. Signals generally influence the biosynthesis of secondary metabolites by mediating their biosynthetic genes expression (Peebles et al., 2009). Therefore, the transcriptional levels of GA biosynthetic genes *hmgr*, *sqs* and *ls* were detected firstly. As shown in Fig. 2A-C, the highest transcription levels of *hmgr*, *sqs* and *ls* reached on the 4th day under Mn^{2+} stimulation and kept higher than that of control during the entire fermentation. This coincided with the increase in total GAs which was moderate at the 8th day and became evident at the 12th day under Mn^{2+} stimulation (Fig. 1D), because the induced expression of biosynthetic genes generally is much earlier than secondary metabolite accumulation as also reported in cases of the diterpenoid taxoid and triterpenoid ginsenoside (Hu et al., 2006; Huang and Zhong, 2013).

The transcriptional levels of sensor genes *cam*, *cna* and *crz1* of calcineurin signals were detected to investigate the molecular events involved in the enhanced GAs production (Fig. 2D-E). When intracellular Ca^{2+} level increases, calmodulin (encoded by *cam*) will bind with Ca^{2+} to form Ca^{2+}/calmodulin complex and then interact with the catalytic subunit of calcineurin (encoded by *cna*) to activate the transcription factor calcineurin-responsive zinc finger (encoded by *crz1*). This transcription factor will act subsequently as a positive regulator of downstream genes with CDRE (calcineurin-dependent responsive element) motif in their promoter regions (Soriani et al., 2010). Notably, a putative CDRE motif was found in the promoter regions of GA biosynthetic genes *sqs* and *ls* by bioinformatics analysis (Xu and Zhong, 2012). In this study, the expression of the three sensor genes was significantly increased by Mn^{2+} addition, suggesting participation of calcineurin signals in GA biosynthetic regulation by Mn^{2+} at the transcriptional level.

To clarify the relationship between medium Mn^{2+} and calcineurin signals, the transcription level of *pmr1*, encoding the Ca^{2+}/Mn^{2+} P-type ATPase, was assessed (Fig. 2G). PMR1 was reported to be involved in control of intracellular Mn^{2+} concentration, and a higher transcription level of *pmr1* might be important to trigger calcineurin signals by Mn^{2+} to regulate ion homeostasis (Maeda et al., 2004). The gene expression of *pmr1* was apparently raised by adding Mn^{2+}on the 4th and 8th day. The results indicated that Mn^{2+} addition might be through PMR1 to induce calcineurin signals to regulate GA biosynthesis. In order to cope with the toxicity caused by the relatively higher level of intracellular Mn^{2+}, extracellular Ca^{2+} might transport into the cells through PMR1 to increase intracellular Ca^{2+} (Maeda et al., 2004).

To further demonstrate that the enhanced GA biosynthesis by Mn^{2+} was through calcineurin signal regulation, the extracellular and intracellular Mn^{2+} concentrations were assayed during cultivation. The levels of extracellular and intracellular Mn^{2+} (Fig. 3A) kept almost constant in control. In contrast, upon Mn^{2+} addition, the extracellular Mn^{2+} dropped at the beginning, reaching its minimum on the 8th day and then increased gradually, while intracellular Mn^{2+} (Fig. 3A) significantly increased initially, reaching its maximum on the 4th day and then declined gradually, indicating that medium Mn^{2+} transported into cells at the beginning to respond to higher Mn^{2+} concentration (stress). After that, intracellular Mn^{2+} gradually effused to the medium from the 4th day (Fig. 3A).

Under salt stress, cytosolic Ca^{2+} burst and further induced Ca^{2+} signals as cellular response regulation (Ariño et al., 2010). To prove the relationship of Mn^{2+} addition with Ca^{2+} signals, the time profiles of extra- and intracellular Ca^{2+} levels were investigated. As revealed in Fig. 3B, upon Mn^{2+} induction, extracellular Ca^{2+} decreased sharply, reaching its minimum on the 4th day, and then kept at a low level. On the contrary, intracellular Ca^{2+} ascended significantly, reaching its maximum on the 4th day, and then declined gradually. Compared with that of Mn^{2+} addition, extracellular and intracellular Ca^{2+} in the control kept almost constant during the entire cultivation. Results suggested that high medium Mn^{2+} would lead to the influx of Ca^{2+} in order to cope with the toxicity caused by the relatively high level of intracellular Mn^{2+}. The time course of extracellular and intracellular Mn^{2+} and Ca^{2+} was in good agreement with the results of the gene expression of *pmr1*. This gene expression was notably enhanced by adding Mn^{2+} on the 4th and 8th day. Similar phenomenon was also claimed in fission yeast *Schizosaccharomyces pombe* (Maeda et al., 2004), and calcineurin signaling system was reported to function as a Mn^{2+} sensor and homeostasis regulator there. However, the diffeence between the reported yeast case and this study is that the former was a very rapid (seconds or minutes) and transient effect, opposed to the much delayed (hours or days) changes seen here. This may be due to the difference in biological systems. Similar slow responses like this work were also claimed in other cases, e.g., in plant cell cultures for production of taxoid (Hu et al., 2006) and ginsenoside (Huang and Zhong, 2013).

To further demonstrate the involvement of calcineurin signaling system in the medium Mn^{2+} regulation of GA biosynthesis, a calcineurin inhibitor (cyclosporine A) was adopted. The kinetic

profiles of biomass (Fig. 1A), sugar consumption (Fig. 1B), spore number (Fig. 1C) and GA accumulation (Fig. 1D-H) with 15 μM cyclosporine A or 15 μM cyclosporin A plus 10 mM Mn^{2+} were analyzed. There were no significant differences in biomass (Fig. 1A) and sugar consumption (Fig. 1B) between the control and addition groups, suggesting that calcineurin signals did not affect cell growth. Spore number (Fig. 1C) and contents of total crude GA (Fig. 1D) and four individual GAs (Fig. 1E-H) declined significantly by adding cyclosporine A and partially relieved by Mn^{2+} supplementing, implying that Mn^{2+} might stimulate GA biosynthesis through calcineurin signaling pathway.

To more visibly investigate the changes of cytosolic Ca^{2+} in the calcineurin inhibitor study, Fluo-3/AM fluorescent dye was used as before (Xu et al., 2013),whose intensities represent relative amounts of free intracellular Ca^{2+}. As shown in Fig. 4, strong green fluorescence was observed in the cells under Mn^{2+} induction on the 4^{th} day. The fluorescence decreased significantly when the cells treated with cyclosporine A, but it became comparatively stronger when supplementing Mn^{2+}, indicating that calcineurin signals might be involved in regulating the intracellular Ca^{2+} levels under Mn^{2+} induction. Similar phenomenon was also observed in our recent report on Na^{+} induction (Xu et al., 2013).

To support our hypothesis, the transcriptional levels of *pmr1*, *cam*, *cna*, *crz1*, *hmgr*, *sqs* and *ls* were also investigated in the calcineurin inhibitor study (Fig. 2A-G). As shown in Fig. 2, the expression levels of those genes were significantly down-regulated on the 4^{th} day by adding cyclosporine A, while the inhibiting impact of cyclosporine A was partially relieved by Mn^{2+} supplementation. The facts supported that GA biosynthesis was induced by calcineurin signals at the transcriptional level under Mn^{2+} addition. Further work such as RNAi knocking-down of *cam*, *cna*, *crz1* and *pmr1* genes or using mass-spectrometry approach to identify signal-inducible proteins (Chen et al., 2005) would help to clarify the specific role of calcineurin signal targets in GA biosynthetic regulation.

In another aspect, since Mn^{2+} is part of the structure of an important antioxidant (superoxide dismutase) that protects the cells by deactivating free radicals which can destroy the organism, the induction process might also be under the regulation of redox network. Our previous work indicated (Zhang et al., 2010) that reactive oxygen species (ROS) generated during fermentation

were positive to biosynthetic gene expression and GAs production by *G. lucidum*. The effect of Mn^{2+} addition as shown here might also be related to the ROS signaling pathway, which needs further confirmation.

In conclusion, medium Mn^{2+} manipulation significantly improved the antitumor GAs production *via* calcineurin signal transduction. Here, the direct effect of CRZ1 on GA biosynthetic genes is not yet proved, which requires further study. The work indicated that Mn^{2+} addition enhanced cytosolic Ca^{2+} concentration through PMR1, thus inducing calcineurin signals to up-regulate GA biosynthetic genes. It provided another successful approach to enhance valuable secondary metabolite production by signal transduction engineering in mushroom cultures. The information obtained may be also helpful to understand the regulatory mechanism of divalent metal ions on the cellular metabolism of other higher fungi.

Materials and Methods

Strain, culture conditions, and sampling, analyses of cell weight, medium sugar, total crude GAs and individual GAs

The strain, basic culture conditions, and sampling and analyses of cell weight, medium sugar, total crude GAs and individual GAs were the same as reported elsewhere (Xu and Zhong, 2012; Xu et al., 2013). Briefly, the total crude GAs were extracted from the dried mycelia of *G. lucidum*, then after several treatment steps they were detected at 245 nm by spectrophotometer; and the individual GAs were quantified by HPLC using their pure standard samples as controls (Liang et al., 2010; Xu and Zhong, 2012; Xu et al., 2013).

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

Similar to previous reports (Xu and Zhong, 2012; Xu et al., 2013), qRT-PCR was used to analyze the transcriptional levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*hmgr*), squalene synthase (*sqs*), lanosterol synthase (*ls*), calmodulin (*cam*), calcineurin subunit A (*cna*), calcineurin-responsive zinc finger transcription factor 1 (*crz1*) and plasma membrane-related Ca^{2+}-ATPase 1 (*pmr1*) by the standard-curve method. Their GenBank accession numbers are EU263989, DQ494674.1, GQ169528.1, HQ456914.1, HQ456915.1, JN052923.1 and JX912719, respectively. The sequences of the primers applied to analyze *hmgr*, *sqs*, *ls*, *cam*, *cna* and *crz1*

were described elsewhere (Xu and Zhong, 2012; Xu et al., 2013). For *pmr1*, the following primer set was used: *pmr1*-forward, 5'-TGTGCTGCGTTGACCCTGAT-3' and *pmr1*-reverse, 5'-AGACGCTGGCTGATGATTGG-3'. Gene expression levels were normalized by that of *G. lucidum* 18S rDNA gene of untreated cells, which was found stable under our experimental conditions (Xu and Zhong, 2012). For each gene, the reference sample was viewed as having an expression level of 1.0, and the results of samples under other conditions were presented as the fold change of mRNA level over the reference sample.

Determination of extracellular and intracellular Mn^{2+} and Ca^{2+} concentrations

Extracellular and intracellular Mn^{2+} and Ca^{2+} were measured with inductively coupled plasma emission spectrometer (Thermo fisher, iCAP6300, USA) by standard procedures. Fluo-3/AM (Sigma, USA), a Ca^{2+}-specific probe, was applied to assay the cytoplasmic calcium, and as described elsewhere (Xu et al., 2013), images of intracellular Ca^{2+} green fluorescence were observed under a microscope using a 450- to 490-nm excitation filter and a 520-nm barrier filter.

Statistical analysis

Data are averaged by the measurement of three independent samples. The error bars showed the standard deviation (SD) from the mean of triplicates. The significance of samples was determined by analysis of variance, and sample means were separated by the Student paired t test ($P < 0.05$).

Financial support from the National Basic Research Program of China (973 program No. 2012CB721006) and the National High Technology R&D Program (863 project No. 2012AA021701) is gratefully acknowledged. XXX appreciates the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning (No. ZXDF080005), and JJZ appreciates SJTU University Distinguished Professorship.

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Figure Legends

Figure 1. Time profiles of biomass (A), residual sugar (B), spore number (C), and the content of total crude GAs (D), individual GA-Mk (E), GA-T (F), GA-S (G) and GA-Me (H) in static liquid cultures of *G. lucidum*. Effects of addition of Mn^{2+} and calcineurin inhibitor (cyclosporine) were investigated. Symbols: filled diamond, control; filled triangle, adding 10 mM $MnCl_2$; blank diamond, 15 μM cyclosporine A; blank triangle, 15 μM cyclosporine A plus 10 mM $MnCl_2$. The error bars in the figure indicate standard deviations from three independent samples, * $p < 0.05$.

Figure 2. Transcriptional levels of *hmgr* (A), *sqs* (B), *ls* (C), *cam* (D), *cna* (E), *crz1* (F) and *pmr1* (G) genes. The mean fold of gene transcriptional level over control was presented with/without addition of $MnCl_2$ and cyclosporine A. Symbols: blank bar, adding 10 mM $MnCl_2$; black bar, 15 μM cyclosporine A; grey bar, 15 μM cyclosporine A plus 10 mM $MnCl_2$. The error bars in the figure indicate the standard deviations from three independent samples,* $p < 0.05$.

Figure 3. Concentrations of extracellular and intracellular Mn^{2+} (A) and Ca^{2+} (B) in static liquid of *G. lucidum.* Symbols: blank square, intracellular Mn^{2+} of control; blank round, intracellular Mn^{2+} of adding 10 mM $MnCl_2$; filled square, extracellular Mn^{2+} of control; filled round, extracellular Mn^{2+} of adding 10 mM $MnCl_2$; blank square, intracellular Ca^{2+} of control; blank round, intracellular Ca^{2+} of adding10 mM $MnCl_2$; filled square, extracellular Ca^{2+} of control; filled round, extracellular Ca^{2+} of adding 10 mM $MnCl_2$. The error bars in the figure indicate the standard deviations from three independent samples, * $p < 0.05$.

Figure 4. Cytosolic Ca^{2+} images of *G. lucidum* observed by light microscopy (A, C, E, G) and fluorescence microscopy (B, D, F, H) (original magnification ×400). $MnCl_2$ and cyclosporine A were added during fermentation. Fluo-3/AM (150 μM) was added to the small pieces (1cm×1cm) of *G. lucidum*. Intensities of green fluorescence represent relative amounts of free cytosolic Ca^{2+}.

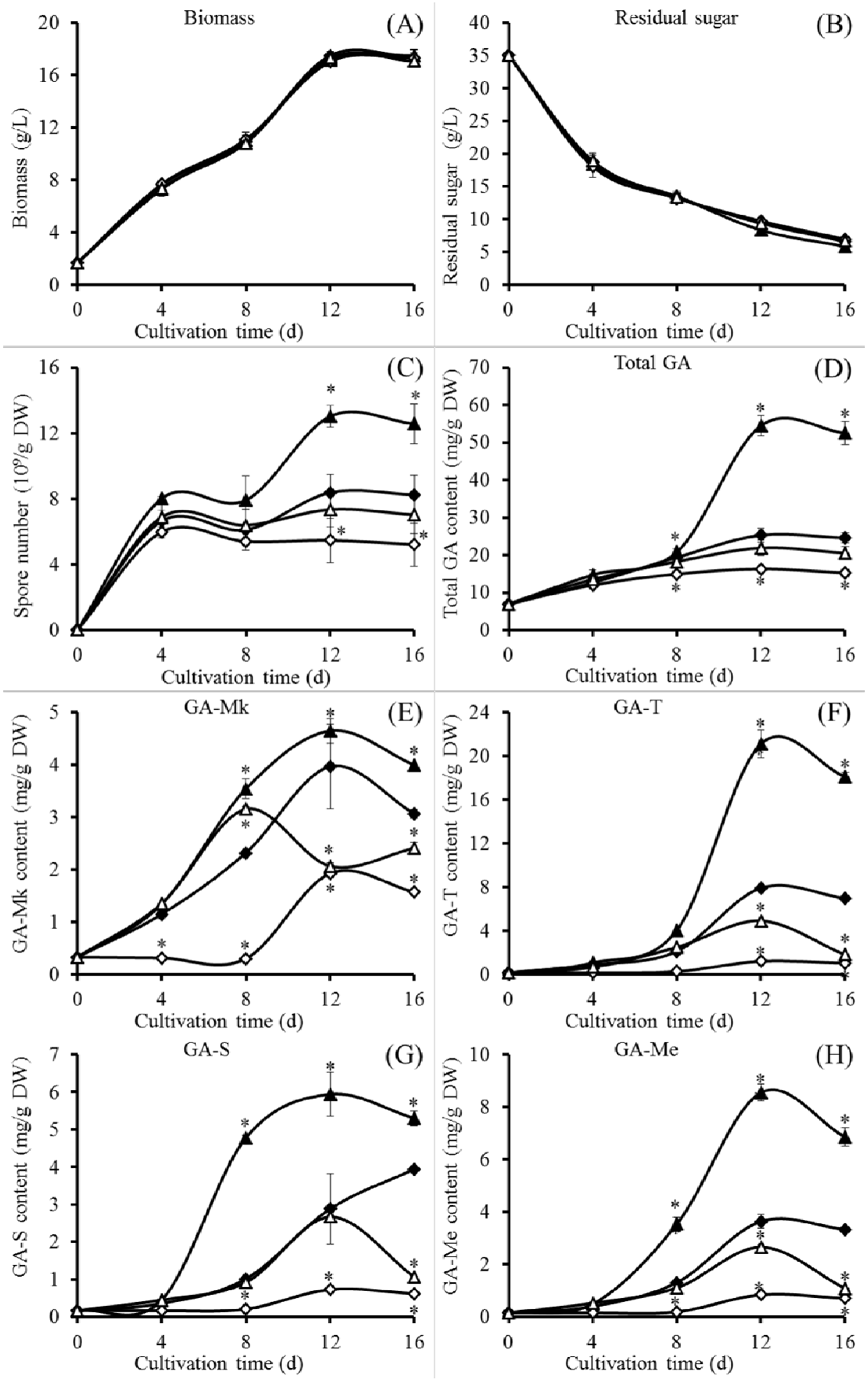
Biomass
(A)
Biomass (g/L)
Cultivation time (d)
Residual sugar
(B)
Residual sugar (g/L)
Cultivation time (d)
(C)
Spore number (10⁹/g DW)
Cultivation time (d)
Total GA
(D)
Total GA content (mg/g DW)
Cultivation time (d)
GA-Mk
(E)
GA-Mk content (mg/g DW)
Cultivation time (d)
GA-T
(F)
GA-T content (mg/g DW)
Cultivation time (d)
GA-S
(G)
GA-S content (mg/g DW)
Cultivation time (d)
GA-Me
(H)
GA-Me content (mg/g DW)
Cultivation time (d)

Fig 1

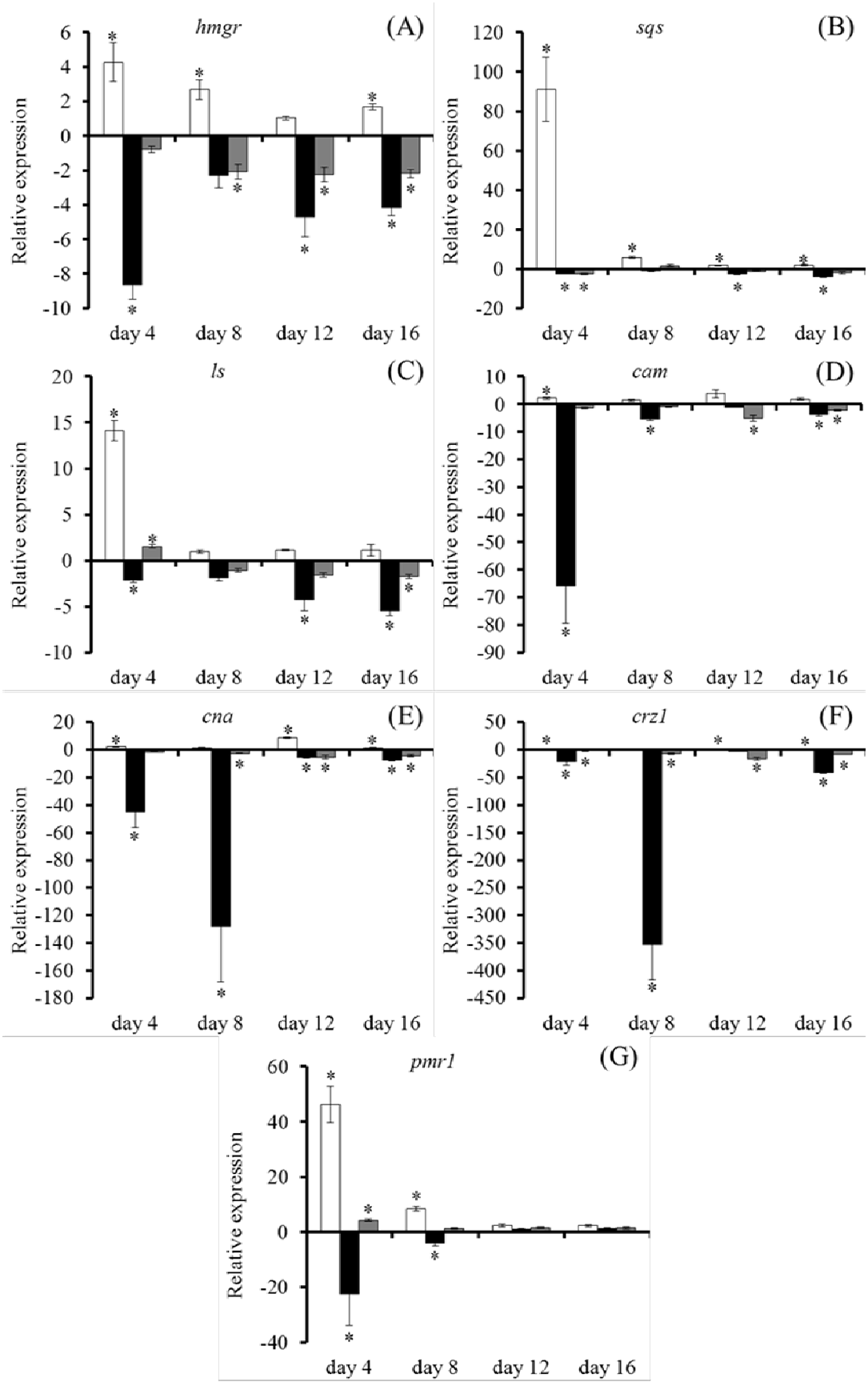
hmgr
(A)
sqs
(B)
ls
(C)
cam
(D)
cna
(E)
crz1
(F)
pmr1
(G)
Relative expression
day 4
day 8
day 12
day 16

Fig 2

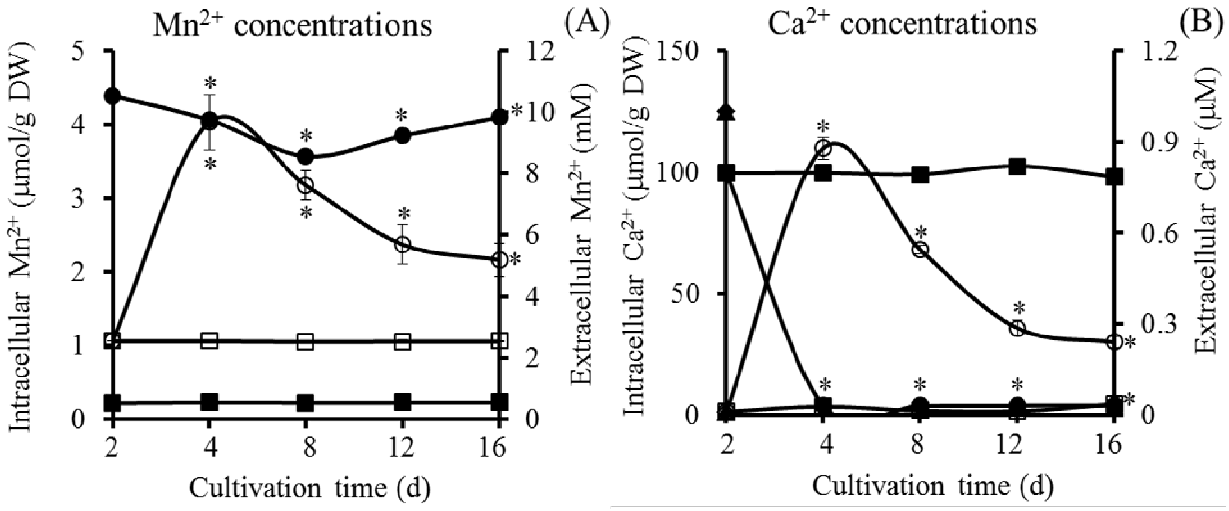
Mn2+ concentrations
(A)
Intracellular Mn2+ (μmol/g DW)
Extracellular Mn2+ (mM)
Cultivation time (d)
Ca2+ concentrations
(B)
Intracellular Ca2+ (μmol/g DW)
Extracellular Ca2+ (μM)
Cultivation time (d)

Fig 3

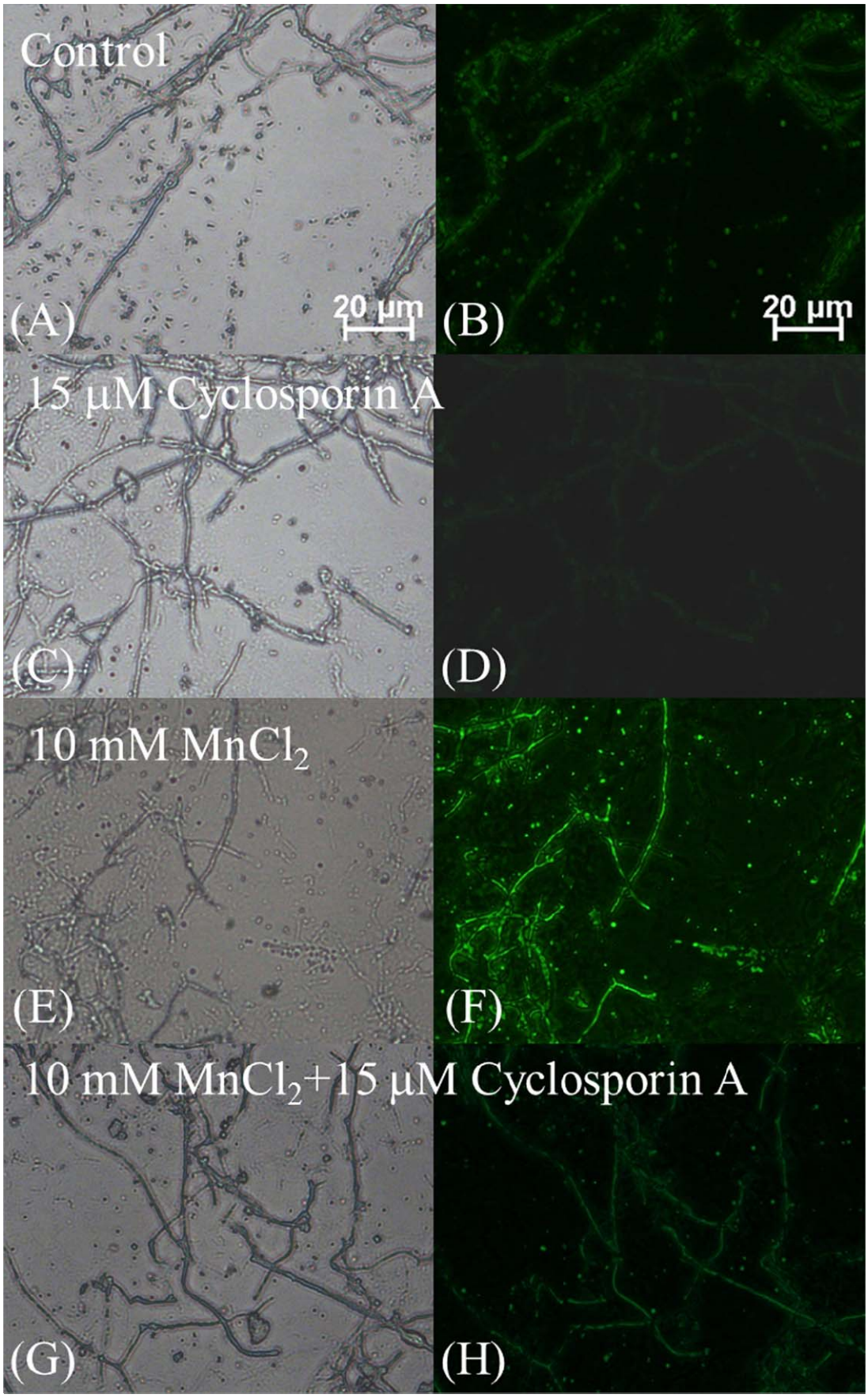
Control
(A)
20 μm
(B)
20 μm
15 μM Cyclosporin A
(C)
(D)
10 mM MnCl2
(E)
(F)
10 mM MnCl2+15 μM Cyclosporin A
(G)
(H)

Fig 4