Bioresource Technology 101 (2010) 6785–6790

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Methyl jasmonate induces ganoderic acid biosynthesis in the basidiomycetous fungus *Ganoderma lucidum*

Ang Ren, Lei Qin, Liang Shi, Xin Dong, Da Shuai Mu, Yu Xiang Li, Ming Wen Zhao *

College of Life Sciences, Nanjing Agricultural University, Key Laboratory of Microbiological Engineering of Agricultural Environment, Ministry of Agriculture, Nanjing 210095, Jiangsu Province, PR China

ARTICLE INFO

Article history:
Received 16 January 2010
Received in revised form 21 March 2010
Accepted 26 March 2010
Available online 14 April 2010

Keywords:
Methyl jasmonate
Ganoderic acid
Ganoderma lucidum
Uniform design
Quantitative real-time PCR

ABSTRACT

This is the first time study to assess the novel use of methyl jasmonate (MeJA) to elicit ganoderic acid (GA) biosynthesis in *Ganoderma lucidum* and the resulting experiments demonstrated that MeJA was indeed a potent inducer of GA biosynthesis. To maximize GA synthesis, a statistical methodology called uniform design (UD) was used to optimize inducement conditions, which were determined to be 254 μM MeJA solubilized in Tween-20 that was added to the culture on day 6. The resulting GA yield was 4.52 mg/100 mg dry weight (DW), which was 45.3% higher than the untreated control sample. To characterize the effect of MeJA on GA biosynthesis, quantitative real-time PCR was used to measure transcription levels of several genes in the synthesis pathway including hydroxy-3-methylglutaryl-Coenzyme A synthase (*hmgs*), hydroxy-3-methylglutaryl-Coenzyme A reductase (*hmgr*), mevalonate-5-pyrophosphate decarboxylase (*mvd*), farnesyl pyrophosphate synthase (*fps*), squalene synthase (*sqs*) and oxidosqualene cyclase (*osc*). Quantification of transcription levels determined that MeJA significantly induced expression of these genes.

1. Introduction

Ganoderma lucidum is a mushroom with traditional medicinal properties that has been widely used in China and some other Eastern-Asia countries for many centuries. Modern pharmacological research has demonstrated that *G. lucidum* has several functions including the ability to inhibit tumor cells growth, modulate both the immune and nervous systems and the ability to lower blood sugar levels (Mizuno et al., 1995). More than 160 different chemical compounds have already been isolated from *G. lucidum* including ganoderic acids (GAs), which is responsible for some of the most important pharmacological activities attributed to *G. lucidum* including protection of the liver, the elimination of inflammation, inhibition of tumor cells as well as other various androgenic activities (Kohda et al., 1985; Shiao et al., 1994; Liu et al., 2007). Recent research has also shown that triterpene, a type of GA, can inhibit HIV-1 and its protease and reverse transcriptase (El-Mekkawy et al., 1998). Therefore, the GA content within *G. lucidum* is often taken as a measure of the overall quality of the mushroom since many of the beneficial properties of *G. lucidum* can be directly attributed to GA.

Most reports regarding *G. lucidum*-derived GA focus on GA isolation and its pharmacological properties (Komoda et al., 1989; Liu et al., 2007). As a result, relatively little is known about the molecular mechanisms of GA biosynthesis. As a type of terpenoid, GA are synthesized via the mevalonate pathway where acetyl-CoA is converted through a series of chemical reactions to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), to mevalonate (MVA), to isopentenyl-pyrophosphate (IPP), to farnesyl diphosphate (FPP), to squalene and finally to lanosterol. GAs then undergo a series of oxidation and reduction reactions before finally assuming the mature molecule structural (see Fig. 1).

In previous work reported by our group, genes encoding the catalytic enzymes responsible for GA synthesis within the mevalonate pathway were isolated from *G. lucidum*. During the characterization of these enzymes, a positive correlation was noted between the amount of GA produced at different stages of *G. lucidum* development and the expression levels of both squalene synthase (*sqs*) and farnesyl diphosphate synthase (*fps*) (Zhao et al., 2004, 2007; Ding et al., 2008). Further research on the details of the relationship between GA biosynthesis and the expressions of *sqs*, *fps* as well as other genes of the mevalonate pathway will help elucidate the mechanism of GA biosynthesis.

Previous studies focused on the environment conditions that influence GA biosynthesis mainly in *G. lucidum* during fermentation were able to determine the optimal carbon source (sucrose), nitrogen source (soybean powder), mineral ion (calcium chloride), level of dissolved oxygen (25%) and initial pH (4.0) (Tang and

* Corresponding author. Address: Microbiology Department, College of Life Sciences, Nanjing Agricultural University, Key Laboratory of Microbiological Engineering of Agricultural Environment, Ministry of Agriculture, Nanjing 210095, Jiangsu Province, PR China. Tel./fax: +86 25 84395602.
E-mail address: mwzhao@njau.edu.cn (M.W. Zhao).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.03.118

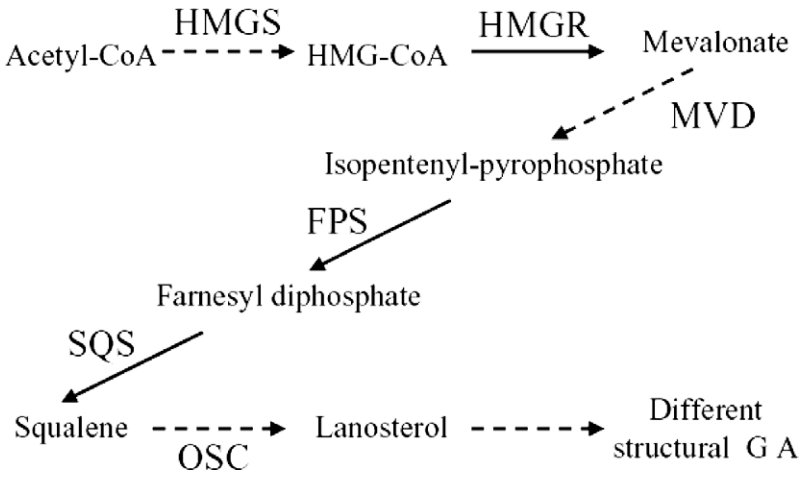

Fig. 1. GA biosynthetic pathway of *G. lucidum*. Dashed lines indicate steps consisting of multiple enzyme reactions. HMG-CoA, hydroxy-3-methylglutaryl-Coenzyme A; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MVD, mevalonate-5-pyrophosphate decarboxylase; IPP, isopentenyl-pyrophosphate; FPS, farnesyl pyrophosphate synthase; FPP, farnesyl diphosphate; SQS, squalene synthase; OSC, oxidosqualene cyclase.

Zhong, 2002; Li et al., 2006; Tang et al., 2009). This information was helpful for further defining the mechanism of GA biosynthesis. However, the interactions between the mineral ion with both the nitrogen source and pH could not be ignored (Li et al., 2006). Additionally, there are no reports on the effect of a single chemical compound on GA biosynthesis in *G. lucidum*, which could help elucidate the expression kinetics and regulation governing key enzymes at each individual step of the biosynthetic pathway.

In fungi, plant hormones have several properties including modulation of fungal growth (Bölker et al., 2008), induction of morphogenetic changes (Prusty et al., 2004), spore germination (Kolattukudy et al., 1995), and secondary metabolism (Goodrich-tanrikulu et al., 1995). Previous work conducted by this group isolated the *fps* gene from *G. lucidum*. Sequence analysis of the *fps* promoter identified two potential methyl jasmonate (MeJA) responsive elements (Ding et al., 2008). This was intriguing because MeJA is a signaling compound that is also involved in the accumulation of secondary metabolites in plants, such as ginsenosides (Hu and Zhong, 2008), paclitaxel (ketchum et al., 1999) and terpenoid (Martin et al., 2002). However, whether MeJA can induce GA biosynthesis in *G. lucidum* was still undetermined.

The concept of Uniform Design (UD) was first proposed by Fang Kai-Tai in the 1980s (Fang, 1980). UD uses design points uniformly scattered on the experimental domain to reduce the number of individual experiments required to optimize the system. Compared to orthogonal design, UD has two main advantages: (1) it greatly reduces the number of experiments without limiting the number or range of parameters tested, and (2) it generates a regression model based on the results allowing it to predict an optimized solution. Therefore, UD is widely used to study and optimize the effect of several variables on a complex system (Chen and Liu, 2007; Yu et al., 2008; Wu et al., 2009).

In this paper, a UD was used to investigate the effects of MeJA on GA biosynthesis in liquid culture at various concentrations, induction times and solvents. Additionally, the transcript levels of GA biosynthesis pathway genes *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc* were assayed in response to MeJA stimulation.

2. Methods

2.1. Mushroom strain

G. lucidum, strain HG, was obtained from the culture collection of the Edible Fungi Institute, Shanghai Academy of Agricultural Science.

2.2. Medium and fermentation conditions

According to the method outlined in Zhao et al. (2004), stock and liquid cultures were both maintained on potato dextrose agar (PDA). Seed cultures were grown in 1000-mL flasks containing 400 mL of a PDA and placed on a rotary shaker incubator at 150 rpm at 28 °C for 6 days. The second set of experiments was performed in 250-mL flasks containing 100 mL of PDA after inoculation with 10% (v/v) of the seed culture. Flasks were then placed on a rotary shaker at 150 rpm at 28 °C for 7 days.

2.3. MeJA stimulation of cultures

As a liquid that is oil miscible, MeJA (Sigma, USA) could not be directly dissolved in the aqueous phase of the culture media. Therefore, MeJA was dissolved in either ethanol or Tween-20 and sterilized through a 0.2-μm Supor Membrane Acrodisc Syringe Filter (PALL, USA) before addition to the medium on day 0. The final ethanol or Tween-20 concentration used was 2 μL/mL and equal volumes of ethanol or Tween-20 were added to all cultures. The cultivation data are displayed as the mean values with the standard deviations from three independent experiments.

2.4. GA measurement

GA content was determined as previously reported (Tang and Zhong, 2002; Li et al., 2006; Tang et al., 2009). Briefly, compounds from the dried and ground mycelia (100 mg) were solubilized with 3 mL of 50% ethanol (v/v) twice for 1 week. After removing the mycelia by centrifugation, the supernatants were dried at 50 °C under a vacuum. The residues were then resuspended in water and extracted with chloroform. The GA in the chloroform was further purified with a 5% $NaHCO_3$ (w/v) extraction. After adding 2 N HCl to adjust the pH of the $NaHCO_3$ phase below 3.0, the GA in the $NaHCO_3$ phase was once again extracted with chloroform. After the chloroform was removed by evaporation at 40 °C, the GA was dissolved in absolute ethanol, and its absorbency measured at 245 nm.

2.5. Optimization of MeJA concentrations on GA biosynthesis

To investigate the influence of MeJA on GA biosynthesis, MeJA was prepared in ethanol as concentrated stock solutions and added to the culture at the beginning of the experiment after filter-steril-

ization at the following concentrations: 2, 5, 10, 50, 100 and 150 μM (Table 1). A control group received equivalent volumes of ethanol (about 2 μL/mL) and another control group remained untreated. All trials were carried out in triplicate and values are displayed as the average of the total amount of GA produced.

2.6. Uniform design

Exposure dosage and time of stimulation are the two main factors that affect the final product yield (Ketchum et al., 1999). The experiment was arranged according to the UD table U7 (7^4) as shown in Table 2 (Fang, 1980) to optimize the MeJA concentration, time of stimulation and solvents used for maximum GA production. Six different concentration levels were tested: 5, 25, 50, 75, 100 and 150 μM. These were compared against seven different stimulation times from day 0 to day 6, and the two solvents treated, ethanol and Tween-20. The two solvents were treated as separate qualitative factors that were designated A_1 and A_2, respectively (Liu, 2005). For example, when the solvent was ethanol, $A_1 = 1$ and $A_2 = 0$; when Tween-20 was the solvent, $A_1 = 0$, $A_2 = 1$.

2.7. Statistical analysis

The results induced by different concentrations of MeJA stimulation were subjected to variance analysis and multiple comparisons. Additionally, the UD experimental data was subjected to step-wise analysis using SPSS software (version 11.5) to calculate a regression equation with an R^2 correlation value greater than 0.95 and the optimal induction conditions were determined with the LSD method (Mu et al., 2009; Liang et al., 2001).

2.8. Transcriptional responses to MeJA

The optimal experimental conditions were determined by UD. Given that exposure dosage and time of stimulation are two of the main factors that affect yield of the final product, the transcript levels of genes in GA biosynthesis pathway, *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc*, were studied by quantitative real-time PCR in response to various MeJA concentrations (0, 10, 50, 100, 150 and 200 μM) and different induction times (0, 12, 24, 36, 48, 60, 72 h), in addition to the optimal conditions of GA yield (254 μM of MeJA in Tween-20 that was added on day 6).

Table 1
The results of single-factor experiment and multiple comparisons.

Treatment	The average of the GA content (mg/100 mg DW)	Standard deviation	Significance[a]	
			$a = 0.05$	$a = 0.01$
Untreated control	3.11	0.09	c	C
Control with ethanol	2.55	0.11	d	D
2 μM MeJA	3.02	0.22	c	C
5 μM MeJA	3.50	0.24	b	B
10 μM MeJA	3.70	0.11	b	B
50 μM MeJA	4.00	0.06	a	A
100 μM MeJA	3.72	0.17	b	AB
150 μM MeJA	3.74	0.13	ab	AB

[a] The multiple comparisons was determined by the LSD method. Different letters stand for significant variances. When $a = 0.05$, $p < 0.05$. When $a = 0.01$, $p < 0.01$.

Table 2
Uniform design U7 (7^4).

Trials	Factors				
	X_1 (solvents)		X_2 (concentrations of MeJA (μM))	X_3 (add-times (day))	GA content (mg/ 100 mg DW)
	Ethanol	Tween-20			
1	1	0	25	2	3.25 ± 0.03
2	1	0	75	5	3.33 ± 0.22
3	1	0	150	1	3.49 ± 0.21
4	1	0	5	4	3.15 ± 0.16
5	0	1	50	0	3.03 ± 0.21
6	0	1	100	3	3.61 ± 0.03
7	0	1	150	6	4.13 ± 0.13

Table 3
Primer sets used for quantitative real-time PCR.

Target gene	Primer	Sequence	Predicted product size (bp)
Gl-hmgs	Forward	5′-CCCATCAACGCTTCCACCA-3′	112
	Reverse	5′-GCTCCTCCTCCGAAATGC-3′	
Gl-hmgr	Forward	5′-GTCATCCTCCTATGCCAAAC-3′	166
	Reverse	5′-GGGCGTAGTCGTAGTCCTTC-3′	
Gl-mvd	Forward	5′-TCGGACTCGCTTGCGGTAGA-3′	160
	Reverse	5′-CGTGCTTGATACGGTGCTG-3′	
Gl-fps	Forward	5′-CCTCATCACCGCTCCAGAA-3′	114
	Reverse	5′-AGGGCGACGGGAAGGTAGAA-3′	
Gl-sqs	Forward	5′-ACAGTTGTCAGCGAAGAGC-3′	170
	Reverse	5′-CGTAGTGGCAGTAGAGGTTG-3′	
Gl-osc	Forward	5′-AGGGAGAACCCGAAGCATT-3′	196
	Reverse	5′-CGTCCACAGCGTCGCATAAC-3′	
Gl-gpd	Forward	5′-GATGAAGGACTGGCGTGGT-3′	102
	Reverse	5′-CCGTTGAGGCTGGGAATGAC-3′	

2.9. RNA isolation and quantitative real-time PCR

Aliquots of 0.2 g mycelia were collected by filtration from the culture media and frozen in liquid nitrogen. Total RNA was extracted using an RNA Isolation Kit (TaKaRa, China), treated with RNase-free DNaseI (TaKaRa) and then reverse-transcribed to cDNA using an oligo $(dT)_{17}$ primer. Afterwards, transcript levels of *gpd*, *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc* were determined by quantitative real-time PCR using SYBR Green I on the PRISM ABI 7300 Sequence Detection System (Applied Biosystems, USA). Primers were designed using Primer Express software (Applied Biosystems), and the sequences are presented in Table 3.

PCR reactions were carried out with the SYBR Green Real-Time PCR Master Mix (TOYOBO, Japan) according to the manufacturer′ s protocol. After an initial denaturation step at 95 °C for 5 min, amplification occurred in three steps: 30 s of denaturing at 95 °C, 60 s of annealing at 60 °C and 30 s of extension at 72 °C for a total of 40 cycles. Identical PCR conditions were used for all targets. Transcript levels were calculated using the standard-curve method and normalized against the *G. lucidum* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene as an internal control because its expression was found to be stable under our experimental conditions (Xu et al., 2006). Untreated mycelia served as the reference sample against which all other genes were compared; expression of the reference sample is defined as 1.0 and the expression of all other genes is displayed as the fold increase over the reference sample. Post-qRT-PCR calculations analyzing the relative gene expression levels were performed according to the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001).

3. Results and discussion

3.1. Effect of MeJA on GA biosynthesis

To investigate the effect of MeJA on GA biosynthesis, GA production was assayed in response to different concentrations of MeJA treatment. The results shown in Table 1 indicate that there is a significant difference in GA production between the control and experimental groups demonstrating that MeJA can potently

stimulate GA biosynthesis. When MeJA was added at the beginning of the experiment, the optimal concentration was 50 μM, which resulted in GA levels that were 28.6% higher than the untreated control group.

Solvents maybe change the culture environment and affect the GA biosynthesis. The variance between untreated control and control with ethanol indicated that ethanol may have a restrictive effect on GA biosynthesis. Therefore, Tween-20 was used as another kind of solvent to investigate the influence of solvent in the subsequent experiments.

3.2. Use of the uniform design method to optimize conditions of MeJA stimulation

To investigate the effect MeJA has on GA biosynthesis over a range of concentrations, stimulation times and solvents, the UD methodology was utilized. Results of UD experiment are shown in Table 2. GA levels are the dependent variable while the factors tested are the independent variables. Using step-wise analysis, the equation relating GA production to the experimental variables was determined to be:

$$Y_{GA} = 3.148 - 5.9 \times 10^{-6} X_2^2 + 0.151 A_2 X_3 - 0.258 A_2 + 0.003 X_2$$
$$(R^2 = 0.998,\ F = 256.038,\ p < 0.05)$$

There are several interesting things that may be noted about this equation. First, this equation does not incorporate A_1 indicating that ethanol may restrict GA biosynthesis; therefore, Tween-20 was used as the solvent ($A_1 = 0$, $A_2 = 1$). Second, the value of X3 is independent of X_2 in the variation intervals. The yield of GA is just linear with X_3 and the relationship was positive, so the maximum value of X_3 (6 day) is the optimal time to begin MeJA treat-

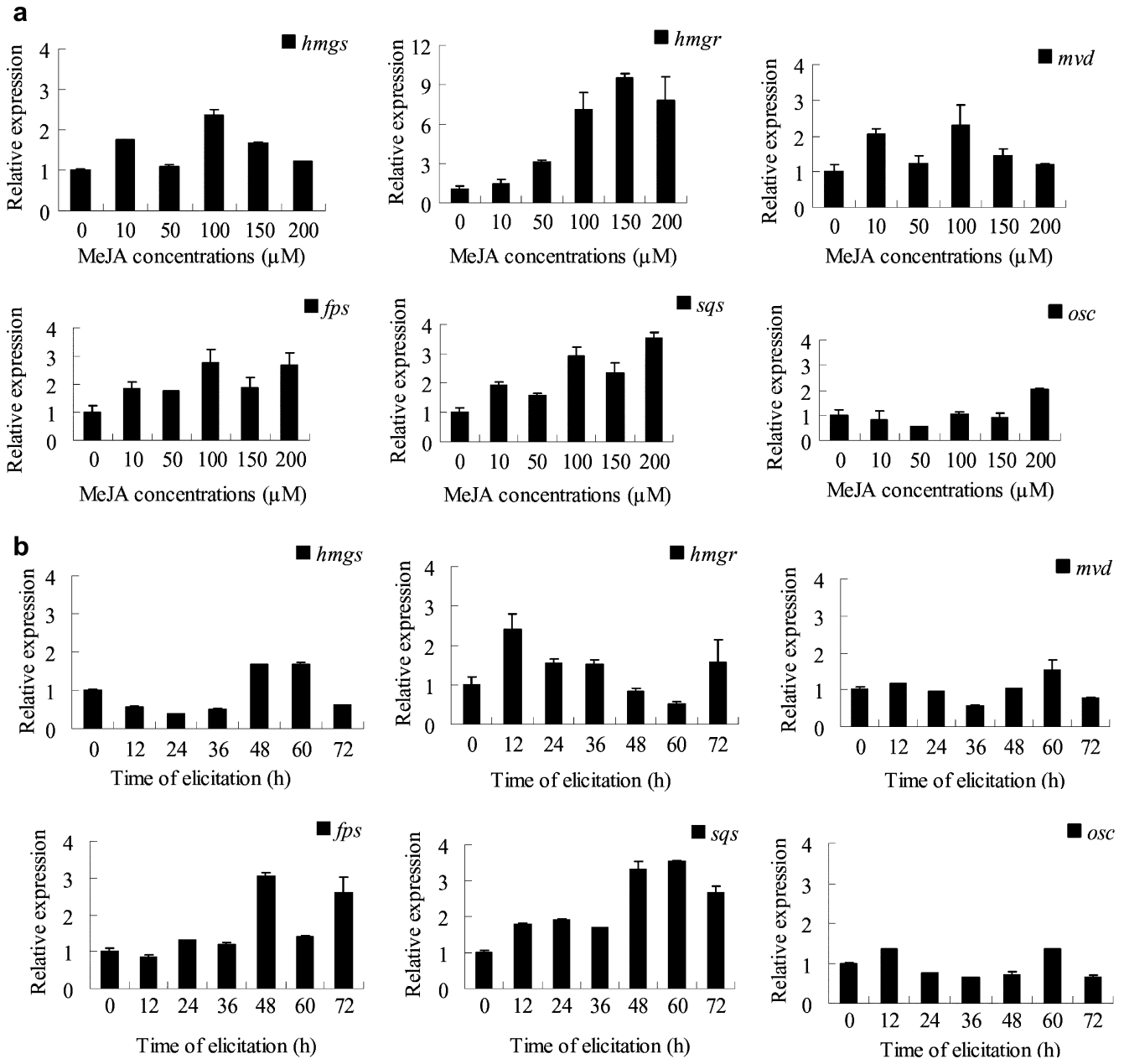

Fig. 2. Quantitative real-time PCR analysis of genes involved in the GA biosynthesis pathway. (a) Transcript levels of the *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc* genes were analyzed by quantitative real-time PCR in response to different concentrations of MeJA. (b) Transcript levels of the *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc* genes were analyzed by quantitative real-time PCR in response to the addition of 50 μM MeJA at the indicated time point. The error bars represent standard deviations of means from three replicate quantitative real-time signal values.

ment. Third, the relationship between X_2 and the yield was characterized by parabolic curve with an optimal value of 254 μM. Therefore, the optimal conditions were calculated to be 254 μM MeJA solubilized in Tween-20 that was added to the culture on day 6. These conditions gave a predicted maximum GA value of 4.178 mg/100 mg dry weight (DW). When these calculated parameters were actually implemented, 4.52 mg/100 mg DW was obtained, which is 45.3% higher than the untreated control. Overall, we found that the experimentally obtained values of GA were in good agreement with those of the predicted values.

Previous work by Tang and Zhong (2002) reported that the GA yield obtained through fed-batch fermentation was 2.05 mg/100 mg DW. Li et al. (2006) investigated the influence of media components on GA yield by the orthogonal design method and reported a yield of 2.63 mg/100 mg DW. The yield of 4.52 mg/100 mg DW obtained in this study is higher than the yield previously reported. However, since Tang and Zhong used a different strains of *G. lucidum* than the one used in this work, it is possible that the inconsistency in GA yield could be attributed to strain-specific differences.

Previous work on optimizing GA yield focused on media components and culture conditions. However, there were the interactions between medium component and culture condition. The interactions make it difficult to study the effect of a single factor on the regulation of the expression of the key enzymes and the GA biosynthesis. Therefore, in this study only a single chemical compound, MeJA, was used to induce GA biosynthesis in *G. lucidum*.

3.3. Transcriptional responses to MeJA stimulation

Previous work by this lab successfully cloned the genes encoding key enzymes involved in *G. lucidum* GA biosynthesis including *sqs* (Zhao et al., 2004, 2007), *fps* (Ding et al., 2008), *hmgs*, *hmgr* (Shang et al., 2008), *mvd* and *osc* (date not published). In this study, the transcript levels of these genes were studied under various conditions of MeJA stimulation. Fig. 2 shows that all of these genes were up-regulated after MeJA induction. However, the highest transcription levels for several genes were not observed at the optimal MeJA concentrations necessary for maximum GA production (Fig. 2a). For instance, transcriptional levels for *hmgs* and *mvd* were highest at 100 μM MeJA where they were approximately 2-fold higher than the control sample. For *hmgr*, the highest transcriptional levels were achieved at 150 μM MeJA where levels were approximately 9-fold higher to the control. The highest transcriptional levels of *sqs* and *osc* were observed at 200 μM MeJA where they were approximately 3.5- and 2-fold greater, respectively, compared to the control. In contrast, the same transcriptional level was observed for *fps* at both 100 and 200 μM MeJA where expression was approximately 2.7-fold higher than the control.

In the second induction experiment, the GA yield was highest when the concentration of MeJA was 50 μM (see Table 3). Therefore, 50 μM MeJA was added at different times for a second induction experiment. The results of the experiment are displayed in Fig. 2b, which shows transcript levels of the different genes in the presence of 50 μM MeJA over time. Although transcript levels of these genes varied as the induction time changed, the precise expression kinetics of these genes in response to MeJA induction varied from gene to gene (see Fig. 2b). For example, when MeJA was added at a time point (12 h), *hmgr* expression increased 2.4-fold compared to the control. In comparison, *osc* expression only increased 1.35-fold at both 12 and 60 h post-induction. This is in contrast to *osc* expression at 24–48 h post-induction where levels changed only slightly. For *fps*, transcription levels increased 3-fold compared to the control 48 h after induction, while the expression of *sqs* and *hmgs* increased 1.5- and 3.5-fold, respectively, at 60 h post-treatment. In contrast, the level of *mvd* transcript changed subtly, which is consistent with *mvd* expression following 50 μM MeJA induction (Fig. 2a).

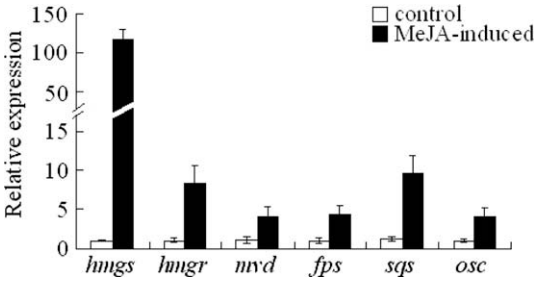

Fig. 3. Transcript levels of genes in the GA biosynthesis pathway in response to optimal MeJA stimulation as determined by UD. The optimal condition for MeJA stimulation as calculated by UD were determined to be 254 μM MeJA solubilized in Tween-20 that was added to the culture on day 6. The transcript levels of the *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc* genes in response to this induction were analyzed by quantitative real-time PCR.

Fig. 3 shows the effect that the optimal MeJA stimulus conditions have on the transcript levels of the genes in GA biosynthesis pathway: *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc*. The transcript levels of *hmgr* and *sqs* were approximately 10-fold higher compared to the control, while *mvd*, *fps* and *osc* were about 4-fold higher. These data indicated that all of these genes were up-regulated in response to MeJA stimulation (Fig. 3), which was consistent with the results of the experiment done with different concentration of MeJA. Compared over various concentrations and different stimulus times (Fig. 2), the optimal conditions significantly improved the degree of transcriptional responses to MeJA induction (Fig. 3), suggesting that the transcriptional responses to MeJA treatment were also affected by the interactions between MeJA concentration and stimulus time. In addition, Fig. 3 shows that not all genes displayed the highest transcriptional level under optimal MeJA conditions when the GA production was the highest. The results indicate that each gene has a different effect on GA biosynthesis.

These results indicate that *hmgr*, *fps* and *sqs* are important genes in the GA biosynthesis pathway in response to MeJA induction. This is consistent with research on terpenoid biosynthesis (Choi et al., 2005; Hu and Zhong, 2008) demonstrating that MeJA induction of gene expression is involved in biosynthesis of secondary metabolites, Suzuki et al. (2002) reported that MeJA could increase the biosynthesis of triterpene glycoside saponins as well as the transcript level of the *sqs* gene in *Medicago truncatula*. Hayashi et al. (2003) reported that MeJA could stimulate soyasaponin biosynthesis and increase the transcript level of the *sqs* gene in *Glycyrrhiza glabra*. Cunillera et al. (1996) reported that FPS is a key enzyme in the biosynthesis of isoprenoid, which has a role in terpenoid synthesis that is widely considered to be rate limiting.

The up-regulation of the genes in GA biosynthesis pathway, *hmgs*, *hmgr*, *mvd*, *fps*, *sqs* and *osc*, was also observed here in *G. lucidum*. Our results are consistent with these reports except for the fact that no obvious effects of 50 μM MeJA on *mvd* were observed (Fig. 2b). This may be attributed to the interactions between these genes in GA biosynthesis pathway. All this work suggests that exogenously supplied MeJA may induce GA biosynthesis via the inducement of enzymes in *G. lucidum* through a very complex system of signals.

4. Conclusions

In this study, we have successfully used uniform design to optimize the conditions of MeJA stimulus for maximum GA yield. The findings in this study show that MeJA could induce the biosynthesis of GA through the transcriptional induction of genes in *G. luci-*

dum. This work may help further elucidate the expression and regulation of the key enzymes and biosynthetic steps of the *G. lucidum* GA biosynthesis pathway.

Acknowledgements

This work was supported by the Chinese National Natural Science Foundation (Nos. 30970042, 30871767 and J0730647), the Chinese National Key Technology R&D Program (No. 2008BADA1B06), and Student Research Training (SRT) Program of Nanjing Agricultural University (No. 0606A17).

References

Bölker, M., Basse, C.W., Schirawski, J., 2008. *Ustilago maydis* secondary metabolism – from genomics to biochemistry. Fungal Genet. Biol. 45, 588–593.

Chen, H.Z., Liu, L.Y., 2007. Unpolluted fractionation of wheat straw by steam explosion and ethanol extraction. Bioresour. Technol. 98, 666–676.

Choi, D.W., Jung, J., Ha, Y.I., Park, H.W., In, D.S., Chung, H.J., Liu, J.R., 2005. Analysis of transcripts in methyl jasmonate-treated ginseng hairy roots to identify genes involved in the biosynthesis of ginsenosides and other secondary metabolites. Plant Cell Rep. 23, 557–566.

Cunillera, N., Arró, M., Delourme, D., Karst, F., Boronat, A., Ferrer, A., 1996. *Arabidopsis thaliana* contains two differentially expressed farnesyl-diphosphate synthase genes. J. Biol. Chem. 271, 7774–7780.

Ding, Y.X., Ou-Yang, X., Shang, C.H., Ren, A., Shi, L., Li, Y.X., Zhao, M.W., 2008. Molecular cloning, characterization, and differential expression of a farnesyl-diphosphate synthase gene from the basidiomycetous fungus *Ganoderma lucidum*. Biosci. Biotechnol. Biochem. 72, 1571–1579.

El-Mekkawy, S., Meselhy, M.R., Nakamura, N., Tezuka, Y., Hattori, M., Kakiuchi, N., Shimotohno, K., Kawahata, T., Otake, T., 1998. Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum*. Phytochemistry 49, 1651–1657.

Fang, K.T., 1980. The uniform design: application of number-theoretic methods in experimental design. Acta Math. Appl. Sin. 3, 363–372.

Goodrich-Tanrikulu, M., Mahoney, N.E., Rodriguez, S.B., 1995. The plant growth regulator methyl jasmonate inhibits aflatoxin production by *Aspergillus flavus*. Microbiology 141, 2831–2837.

Hayashi, H., Huang, P., Inoue, K., 2003. Up-regulation of soyasponin biosynthesis by methyl jasmonate in cultured cells of *Glycyrrhiza glabra*. Plant Cell Physiol. 44, 404–411.

Hu, F.X., Zhong, J.J., 2008. Jasmonic acid mediates gene transcription of ginsenoside biosynthesis in cell cultures of *Panax notoginseng* treated with chemically synthesized 2-hydroxyethyl jasmonate. Process Biochem. 43, 113–118.

Ketchum, R.E.B., Gibson, D.M., Croteau, R.B., Shuler, M.L., 1999. The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. Biotechnol. Bioeng. 62, 97–105.

Kohda, H., Tokumoto, W., Sakamoto, K., Fujii, M., Hirai, Y., Yamasaki, K., Komoda, Y., Nakamura, H., Ishihara, S., Uchida, M., 1985. The biologically active constituents of *Ganoderma lucidum* (Fr.) Karst. Histamine release inhibitory triterpenes. Chem. Pharm. Bull. 33, 1367–1374.

Kolattukudy, P., Rogers, L., Li, D., Hwang, C., Flaishman, M., 1995. Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. USA 95, 4080–4087.

Komoda, Y., Shimizu, M., Sonoda, Y., Sato, Y., 1989. Ganoderic acid and its derivatives as cholesterol synthesis inhibitors. Chem. Pharm. Bull. 37, 531–533.

Li, N., Liu, X.H., Zhou, J., Li, Y.X., Zhao, M.W., 2006. Analysis of influence of environmental conditions on ganoderic acid content in *Ganoderma lucidum* using orthogonal design. J. Microbiol. Biotechnol. 16, 1940–1946.

Liang, Y.Z., Fang, K.T., Xu, Q.S., 2001. Uniform design and its applications in chemistry and chemical engineering. Chemomet. Intell. Lab. Syst. 58, 43–57.

Liu, J., Shimizu, K., Konishi, F., Noda, K., Kumamoto, S., Kondo, R., 2007. Anti-androgenic activities of the triterpenoids fraction of *Ganoderma lucidum*. Food Chem. 100, 1691–1696.

Liu, W.Q., 2005. Design of Experiments. Tsinghua University Press, pp. 122–125 (in Chinese).

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Martin, D., Tholl, D., Gershenzon, J., Bohlmann, J., 2002. Methyl Jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol. 129, 1003–1018.

Mizuno, T., Wang, G.Y., Zhang, J., Kawagishi, H., Nishitoba, T., Li, J.X., 1995. Reishi, *Ganoderma lucidum* and *Ganoderma tsugae*: bioactive substances and medicinal effects. Food Rev. Int. 11, 151–166.

Mu, W., Chen, C., Li, X., Zhang, T., Jiang, B., 2009. Optimization of culture medium for the production of phenyllactic acid by *Lactobacillus* sp. SK007. Bioresour. Technol. 100, 1366–1370.

Prusty, R., Grisafi, P., Fink, G.R., 2004. The plant hormone indoleacetic acid induces invasive growth in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 101, 4153–4157.

Shang, C.H., Zhu, F., Li, N., Ou-Yang, X., Shi, L., Zhao, M.W., Li, Y.X., 2008. Cloning and characterization of a gene encoding HMG-CoA reductase from *Ganoderma lucidum* and its functional identification in yeast. Biosci. Biotechnol. Biochem. 72, 1333–1339.

Shiao, M.S., Lee, K.R., Lin, L.J., Wang, C.T., 1994. Natural Products and Biological Activities of the Chinese Medicinal Fungus *Ganoderma lucidum*. Food Phytochemicals for Cancer Prevention II; ACS Symposium Series, vol. 547. American Chemical Society, Washington, DC. pp. 342–354.

Suzuki, H., Achnine, L., Xu, R., Matsuda, S.P.T., Dixon, R.A., 2002. A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula*. Plant J. 32, 1033–1048.

Tang, Y.J., Zhong, J.J., 2002. Fed-batch fermentation of *Ganoderma lucidum* for hyperproduction of polysaccharide and ganoderic acid. Enzyme Microb. Technol. 31, 20–28.

Tang, Y.J., Zhang, W., Zhong, J.J., 2009. Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and Ganoderma polysaccharides by medicinal mushroom *Ganoderma lucidum*. Bioresour. Technol. 100, 1852–1859.

Wu, R.L., Wang, X.L., Li, F., Li, H.Z., Wang, Y.Z., 2009. Green composite films prepared from cellulose, starch and lignin in room-temperature ionic liquid. Bioresour. Technol. 100, 2569–2574.

Xu, F., Zhao, M.W., Li, Y.X., 2006. Cloning and sequence analysis of a glyceraldehyde-3-phosphate dehydrogenase gene from *Ganoderma lucidum*. J. Microbiol. 44, 515–522.

Yu, J., Liu, Q., Liu, Q., Liu, X., Sun, Q., Yan, J., Qi, X., Fan, S., 2008. Effect of liquid culture requirements on antifungal antibiotic production by *Streptomyces rimosus* MY02. Bioresour. Technol. 99, 2087–2091.

Zhao, M.W., Liang, W.Q., Zhang, D.B., Wang, N., Wang, C.G., Pan, Y.J., 2007. Cloning and characterization of the squalene synthase (SQS) gene from *Ganoderma lucidum*. J. Microbiol. Biotechnol. 17, 1106–1112.

Zhao, M.W., Zhong, J.Y., Liang, W.Q., Wang, N., Chen, M.J., Zhang, D.B., Pan, Y.J., Jong, S.C., 2004. Analysis of squalene synthase expression during the development of *Ganoderma lucidum*. J. Microbiol. Biotechnol. 14, 116–120.