Appl Microbiol Biotechnol (2010) 86:1367–1374
DOI 10.1007/s00253-009-2415-8

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Enhanced biosynthetic gene expressions and production of ganoderic acids in static liquid culture of *Ganoderma lucidum* under phenobarbital induction

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Received: 18 August 2009 / Revised: 12 December 2009 / Accepted: 16 December 2009 / Published online: 14 January 2010

Abstract Static liquid culture of *Ganoderma lucidum*, a traditional Chinese medicinal mushroom, is a proven technology for producing ganoderic acids, which are secondary metabolites that possess antitumor properties. In this work, the addition of phenobarbital, a P450 inducer, was used to enhance the production of total and individual ganoderic acids in a two-stage cultivation involving a period of initial shake flask culture followed by static liquid culture of *G. lucidum*. The dosage and time of phenobarbital induction were critical for the enhanced production of ganoderic acids. The addition of 100 μM (final concentration) phenobarbital on day 5 after the shake flask culture was converted to the static liquid culture was found to be optimal, resulting in a maximal amount of total ganoderic acids of 41.4±0.6 mg/g cell dry weight and increases in the levels of ganoderic acid-Mk, -T, -S, and -Me in the treated cells by 47%, 28%, 36%, and 64%, respectively. Meanwhile, the accumulation of lanosterol, a key intermediate, was found to decrease and transcriptions of three key genes encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase, squalene synthase, and lanosterol synthase in the triterpene biosynthetic pathway were up-regulated under phenobarbital induction. This work demonstrated a useful strategy for the enhanced production of ganoderic acids by *G. lucidum*.

Keywords *Ganoderma lucidum* · Ganoderic acid · Phenobarbital induction · Gene expression · Mushroom fermentation technology

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Introduction

Some secondary metabolites from higher fungi are unique bioactive compounds, and their discovery and production through fermentation of higher fungi, including medicinal mushrooms, have received much attention in recent years (Hsieh et al. 2006; Wagner et al. 2003; Zhong and Xiao 2009). *Ganoderma lucidum*, a well-known traditional Chinese medicinal mushroom, has been used as a tonic and home remedy in the Far East since ancient times. Lanostane-type triterpenes, especially ganoderic acids, are the most important bioactive compounds produced by *G.*

lucidum. They possess various important pharmacological activities such as antitumor and anti-HIV activities (Chen et al. 2008; El-Mekkawy et al. 1998; Tang et al. 2006).

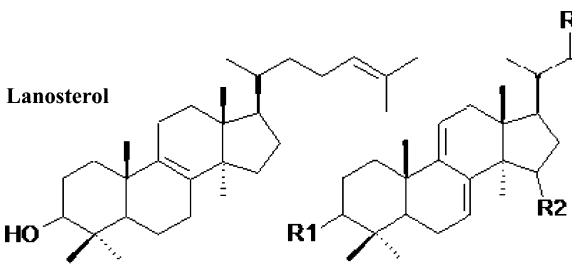
Submerged fermentation of *G. lucidum* is a promising technology for the production of ganoderic acids (Wagner et al. 2003; Zhong and Xiao 2009). However, yields of ganoderic acids in *G. lucidum* mycelia and the fermentation throughput of the mycelia are still low, in spite of the improvements made in recent years, and this presents a major hurdle in the commercialization of the production of ganoderic acids. A two-stage cultivation strategy for the efficient production of ganoderic acids by *G. lucidum* was first reported by our research group (Fang and Zhong 2002). More recently, hyperproduction of individual ganoderic acids, compared to conventional shake flask culture, was demonstrated in a two-stage culture of *G. lucidum* (Xu et al. 2010).

Induction using an elicitor is an effective method of enhancing the production of secondary metabolites such as ginseng saponin (Yue and Zhong 2008) and other plant cell products (Contin et al. 1999; Hu and Zhong 2008). Recently, Zhu et al. (2008) reported the enhanced production of ganoderic acids under induction by a microbial polysaccharide in the submerged culture of *G. lucidum*. Unfortunately individual ganoderic acids were not analyzed, and no information on the expression of related biosynthetic genes was reported. Different ganoderic acids exhibit different bioactivities (Tang et al. 2006; Wang et al. 2007; Chen et al. 2008), although they share the same chemical structure (Fig. 1). Thus, it is important to investigate the accumulation of individual ganoderic acids during the fermentation (Xu et al. 2010). It is also worthwhile to study the effect of the elicitor on gene transcription, because there has been a lack of understanding of the mechanism of induction of ganoderic acid biosynthesis.

The detailed ganoderic acid biosynthetic pathway is still unclear. Triterpenes in *G. lucidum* are thought to be synthesized via the mevalonate pathway (Yeh et al. 1989; Hirotani et al. 1990; Shiao 1992; Zhao et al. 2007). It is believed that 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMGR) catalyzes the first step specific to isoprenoid biosynthesis (Shang et al. 2008) and that squalene synthase (SQS) catalyzes the first enzymatic step from the central isoprenoid pathway toward sterol and triterpenoid biosyntheses (Abe et al. 1993). Jennewein et al. (2001) reported that lanosterol synthase (LS) catalyzes the cyclization of 2, 3-oxidosqualene to form lanosterol, which is the lanostane ring skeleton of ganoderic acids (Shiao 1992). Although lanosterol is subsequently converted to ganoderic acids in *G. lucidum*, as indicated by ^{13}C NMR experiments (Hirotani et al. 1990; Shiao 1992), the later steps in ganoderic acid biosynthetic pathway remain unknown.

A comparison of chemical structures of lanosterol and ganoderic acids (Fig. 1) suggests that ganoderic acid biosynthesis from lanosterol may involve a series of reactions including hydroxylation, oxidation, and demethylation, most of which are presumed to be catalyzed by P450-type enzymes (Contin et al. 1999; Shibuya et al. 2006; Mitsuguchi et al. 2009). Mitsuguchi et al. (2009) reported the first fungal P450 triterpene hydroxylase. It catalyzes the oxidation of the C-29 methyl group into carbinol and then to carboxylic acid in the biosynthesis of helvolic acid by *Aspergillus fumigatus*. Given the importance of P450 in the regulation of biosyntheses of secondary metabolites such as triterpenoids, it is reasonable to consider the addition of a P450 inducer as a potential strategy to enhance the production of ganoderic acids. Phenobarbital is a typical P450 inducer that can increase transcription of several plant cytochrome P450 enzymes (Contin et al. 1999). To the best of our knowledge, there have been no reports on the use of phenobarbital induction to enhance the production of ganoderic acids.

In this work, phenobarbital was added during the static liquid culture step of a two-stage cultivation of *G. lucidum* to improve the production of ganoderic acids. The levels of four individual ganoderic acids, namely ganoderic acid-Mk, -T, -S, and -Me, were analyzed quantitatively. Transcription levels of the three genes encoding key enzymes HMGR, SQS, and LS in the isoprenoid and ganoderic acid biosynthetic pathways (Xu et al. 2010) were also investigated.



Compounds	R1	R2	R3
Ganoderic acid-Mk	-OAc	-OH	-OAc
Ganoderic acid-T	-OAc	-OAc	-OAc
Ganoderic acid-S	-OH	-H	-OAc
Ganoderic acid-Me	-OAc	-OAc	-H

Fig. 1 Chemical structures of lanosterol and ganoderic acids (*GAs*)

Materials and methods

Chemicals

Phenobarbital (purity >99%) was purchased from Kelong Chemical Reagent Factory (Chengdu, China), and HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Ultrapure water from a Milli-Q purification system (Millipore, Bedford, MA, USA) was used throughout this study.

Maintenance of mycelial culture of *G. lucidum*

G. lucidum CCGMC 5.616 from China General Microbiological Fermentation Center was maintained on potato–dextrose-agar slants. Details of the culture medium and procedures of the two-stage culture of *G. lucidum* were reported earlier (Fang and Zhong 2002). In the two-stage culture method, 45 mL of medium in a 250-mL shake flask was inoculated with 5 mL of a second preculture broth. The flask was incubated on a rotary shaker for 4 days. After this initial shake flask culture period, it was converted to static liquid culture (second stage).

Induction

Phenobarbital was dissolved in dimethyl sulphoxide (DMSO) and sterilized by filtering through 0.22-μm polyvinylidenedifluoride syringe filters (Millipore, Germany) before it was added to *G. lucidum* cultures at three different final concentrations (10, 100, and 1,000 μM) for induction. Three different induction times, corresponding to the early, middle, and late growth stages, were tested. They were days 2, 5, and 7 after the shake flask culture was converted to static liquid culture in an incubator. Each 250-mL flask contained 50 mL broth. The DMSO concentration in the broth was 0.05% (*v*/*v*) following the addition of phenobarbital. DMSO was also added to some cultures at the same concentration but without phenobarbital induction as a control. Cells were harvested on days 2, 5, 7, and 10 in the static liquid culture stage. In order to observe better the effects of phenobarbital induction, a set of tests was carried out in which 100 μM phenobarbital induction on day 5 was used, and the cells were harvested every day afterward from duplicate flasks. Each flask was harvested only once and then stored in a freezer for analysis.

Measurements of cell dry weight and residual sugar in the medium

Each culture flask was duplicated, and the data were averaged from the three samples taken from the triplicate flasks. Mycelia floating on the liquid surface in each flask were harvested and washed with a large amount of distilled water and then dried at 50°C to constant weight. The broth left in each flask was stored at −20°C and later thawed for the analysis of residual sugar using the conventional phenol-sulfuric acid assay.

Determination of ganoderic acids

Total crude ganoderic acids in the harvested mycelia were measured as previously described (Fang and Zhong 2002). For individual ganoderic acids, HPLC analysis was performed on an Agilent 1200 series HPLC system equipped with a vacuum degasser, a quaternary gradient pump, and a diode array detector. An Agilent Zorbax SB-C18 column (250×4.6 mm, 5 μm) was used with methanol as the mobile phase at a flow rate of 1 mL/min. Each sample volume was 100 μL. Chromatographic peaks detected at 210 nm wavelength were identified by comparing the retention times and spectra against lanosterol standards (97%, Sigma, St. Louis, MO, USA). The purities of all ganoderic acids were higher than 98% based on HPLC results. Details on the HPLC analysis method are described elsewhere (Xu et al. 2010).

Extraction and analysis of lanosterol

The extraction and analysis of lanosterol from the harvested mycelia followed the methods described by Yuan et al. (2006) and Xu et al. (2010). The dry cell powder from each flask was extracted three times with a methanol and ethanol (60:40 *v*/*v*) mixture at 1:3 (*w*/*v*), and the three extracts were pooled and saponified with 0.1 M methanolic NaOH at 50°C for 2 h. Each sample was then mixed with 2 mL of deionized water and extracted twice with 5 mL petroleum ether (with boiling point range of 60–90°C). The two petroleum ether fractions were pooled and evaporated to dryness under a nitrogen stream. Each dried sample was redissolved in ethanol and then analyzed by HPLC.

Total RNA extraction and cDNA synthesis

The total RNA of 0.1 g fresh weight cells was extracted with 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) following Invitrogen's procedure. The RNA concentration was determined using a Biophotometer Plus (Eppendorf, Germany) instrument. The residual genomic DNA was removed by incubating the Trizol solution with RNase-free DNase I (MBI Fermentas, Canada) according to the vendor's protocol. One microgram of DNase-treated RNA was reverse-transcribed with RevertAid™ M-MuLV Reverse Transcriptase system for RT-PCR following the vendor's instructions.

Measurement of *hmgr*, *sqs*, and *ls* gene expressions by real-time quantitative PCR

The expression of the *hmgr*, *sqs*, and *ls* genes (which encode the enzymes HMGR, SQS, and LS, respectively) was measured by real-time quantitative PCR (qRT-PCR) using the procedure reported by Xu et al. (2010). Transcription levels were calculated using the standard-curve method and were normalized against the *G. lucidum* 18S gene (an internal control) with fifth day untreated cells as a reference. For each gene, the reference sample was viewed as having an expression level of 1.0, and the results of other samples were expressed as the fold increase of mRNA level over the reference sample.

Results

Effects of induction time and dosage on cell growth and production of ganoderic acids

The induction time and dosage of an elicitor are two main factors affecting cell growth and metabolite yields for a specific culture system (Wang et al. 2006). Thus, tests for phenobarbital induction time and concentration were carried out first.

For the study of induction time, *G. lucidum* cells were transferred to static culture after 2 days of shake flask culturing and then treated with 100 μM (final concentration) phenobarbital on days 2, 5, and 7 during the static liquid culture stage after transference, respectively. The cell dry weight (DW) and yields of individual ganoderic acids measured on day 10 are summarized in Table 1. *G. lucidum* cell growth was inhibited slightly by DMSO and more severely by phenobarbital when they were added on day 2 (early growth stage). Ganoderic acids showed different stimulatory responses to phenobarbital induction at different growth stages. The total ganoderic acid content in the sample treated with phenobarbital on day 2 decreased by about 75% compared to the control. On the other hand, the cell dry weight and yields of individual ganoderic acids showed very little change compared to the control when DMSO was added on day 5 or 7. Higher yields of individual ganoderic acids were observed when the cells were exposed to phenobarbital on day 5 (middle growth stage) and day 7 (late growth stage). Phenobarbital induction on day 5 led to better yields than on day 7 as shown in Table 1. Thus, day 5 was the optimal induction time.

To determine the optimal phenobarbital dosage, 5-day-old static cultures of *G. lucidum* were treated with different concentrations of phenobarbital (10- to 1,000-μM final concentrations). The results are summarized in Table 2. Phenobarbital had almost no or only a minor impact on the cell dry weight measured on day 10 when it was added at the optimal induction time (i.e., day 5). Induction with 10 μM of phenobarbital had little effect, while 100 μM phenobarbital increased the yields of individual ganoderic acids by around 1.3- to 1.6-fold. Increasing the concentration to 1,000 μM decreased the yields slightly compared to 100 μM. Results in Table 2 indicate that the optimal phenobarbital dosage for induction was 100 μM.

Kinetic profiles of cell growth, total ganoderic acids, and lanosterol under phenobarbital induction

Examining the kinetic effects of phenobarbital induction on cell growth and on the production of ganoderic acids is essential for a better understanding of the mechanism of its effect on ganoderic acid biosynthesis by *G. lucidum*. Figure 2a shows the time courses of cell growth and residual sugar, with and without phenobarbital induction. The results are consistent with those in Table 1. Phenobarbital induction had no significant impact on cell growth or residual sugar when it was added on day 5. The cell dry weight on day 10 reached a maximum of 19.2 g/L in the 100 μM phenobarbital-treated culture, which is close to that of the control (18.6 g/L) and that treated with only DMSO (18.2 g/L). The sugar consumption patterns in all cases are also similar, showing a continuous decline, a behavior

Table 1 Effects of phenobarbital addition time on cell dry weight and yields of individual ganoderic acids on day 10 of static liquid culture

Cultivation conditions	DW (g/L)	Ganoderic acids (μg/g DW)			
		Mk	T	S	Me
Control	18.4±0.6	788±37	2,045±50	821±42	517±34
DMSO (0.05% (*v*/*v*)) added on day 2	17.3±0.7	301±34	905±43	426±32	222±21
Phenobarbital added on day 2	15.6±0.1	202±17	500±51	221±4	136±22
DMSO (solvent) added on day 5	18.2±0.2	756±50	2,007±33	856±22	496±28
Phenobarbital added on day 5	19.4±0.5	1,044±40	2,480±78	1,168±38	774±27
DMSO (solvent) added on day 7	18.2±1.1	758±23	2,095±49	854±4	520±50
Phenobarbital added on day 7	18.8±0.28	862±97	2,336±124	971±56	715±54

Table 2 Effects of phenobarbital concentration on cell dry weight and yields of individual ganoderic acids on day 10 of static liquid culture

Culture Conditions	DW (g/L)	Ganoderic acids (μg/g DW)			
		Mk	T	S	Me
Control	18.4±0.6	788±37	2,045±50	821±42	517±34
DMSO (solvent) at 0.05% (*v*/*v*)	18.2±0.2	756±50	2,007±33	856±22	496±28
10 μM phenobarbital	18.5±0.5	778±14	1,916±42	798±57	614±45
100 μM phenobarbital	19.4±0.5	1,044±40	2,480±78	1,168±38	774±27
1,000 μM phenobarbital	18.7±0.3	1,024±29	2,269±56	1,102±19	756±53

which is in agreement with the cell growth profiles in Fig. 2a. On day 10, residual sugar was almost exhausted. Figure 2b shows that the total crude ganoderic acids increased sharply in the phenobarbital-treated culture on day 5 when phenobarbital induction occurred. It continued to increase rapidly until day 8 and then leveled off at 41.4± 0.6 mg/g DW. In both the control and DMSO-treated cultures, the total crude ganoderic acids also increased sharply on day 5. However, in both cases, the values leveled off at lower levels (28.3±0.16 and 27.2±2.1 mg/g DW, respectively).

Time courses of the four individual ganoderic acids are shown in Fig. 3. The control and DMSO-treated cultures exhibited similar individual ganoderic acid profiles, indicating that DMSO had no significant impact. However, the four individual ganoderic acids all reached much higher peak levels under phenobarbital induction. The maximum individual ganoderic acid-Mk, -T, -S, and -Me levels were 1,119±76, 2,609±126, 1,231±102, and 794±79 μg/g DW, respectively. Ganoderic acid-Mk reached its maximum level on day 8, as did the total ganoderic acid content (Fig. 2b), while the other three ganoderic acids had a 1-day delay in reaching their maxima. The levels of ganoderic acid-Mk and other three ganoderic acids started to decrease on days 8 and 9, respectively (Fig. 3) while cell growth continued. Under phenobarbital induction, ganoderic acid-Mk, -T, -S, and -Me levels increased by 47%, 28%, 36%, and 64% compared to the control. The ganoderic acid-Me level had the highest gain, indicating that phenobarbital not only enhanced ganoderic acid biosynthesis but also slightly altered the distribution (heterogeneity) of individual ganoderic acids in the static liquid culture of *G. lucidum*.

Lanosterol with its lanostane ring skeleton is an important precursor of ganoderic acids (Shiao 1992). We investigated how this intermediate product responds to phenobarbital induction. Figure 4 shows the time courses of lanosterol under different conditions. In both the control and DMSO-treated cultures, lanosterol levels oscillated between 4 and 6 μg/g DW between days 5 and 10. However, upon phenobarbital induction on day 5, the lanosterol level decreased sharply from 6.0 on day 5 to 2.9 μg/g DW on day 7. It then returned to the lanosterol levels of the control and DMSO-treated cultures by day 8, remaining similar to these two treatments until day 10, possibly indicating that the effects of phenobarbital induction diminished.

Gene expression response to phenobarbital induction

It is known that ganoderic acids as triterpenoids are synthesized through the isoprenoid pathway (Yeh et al.

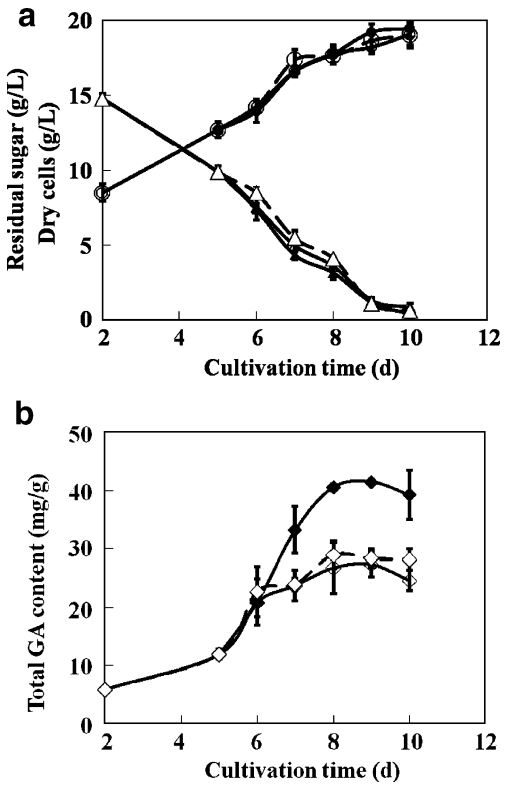


Fig. 2 Time courses of cell growth (**a**), residual sugar (**a**), and total ganoderic acid (*GA*) content (**b**) of the cells treated with phenobarbital on day 5 of the static liquid culture of *G. lucidum*. Data are the means of three independent samples, and *vertical bars* show standard errors. *Empty circles with dashed line* cell growth of control, *empty circles with solid line* cell growth with 25 μL DMSO, *filled circles* cell growth with 100 μM phenobarbital, *empty triangles with dashed line* residual sugar of control, *empty triangles with solid line* residual sugar with 25 μL DMSO, *filled triangles* residual sugar with 100 μM phenobarbital addition, *empty diamonds with dashed line* GA of control, *empty diamonds with solid line* GA with 25 μL DMSO, *filled diamonds* GA with 100 μM phenobarbital

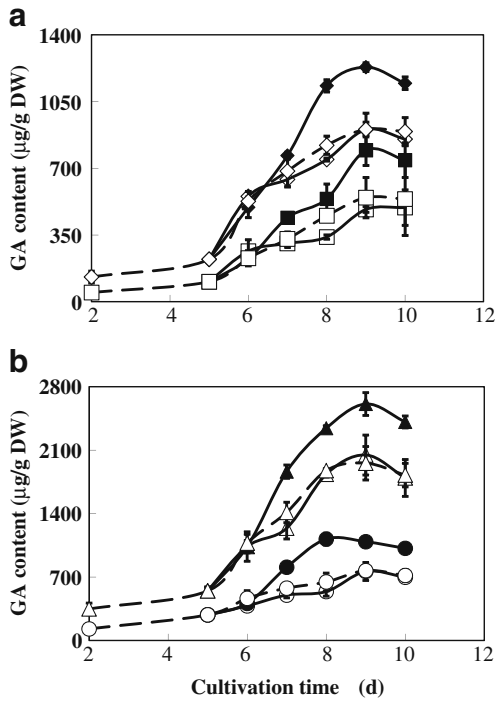


Fig. 3 Time courses of ganoderic acid (*GA*)-Mk and GA-T (**a**), and GA-S and GA-Me (**b**) in cultures treated with 100 μM phenobarbital or 25 mL DMSO on day 5. Data are the means of three independent samples, and *vertical bars* show standard errors. *Empty circles with dashed line* GA-Mk of control, *empty circles with solid line* GA-Mk with 25 μL DMSO, *filled circles* GA-Mk with 100 μM phenobarbital, *empty triangles with dashed line* GA-T of control, *empty triangles with solid line* GA-T with 25 μL DMSO, *filled triangles* GA-T with 100 μM phenobarbital, *empty diamonds with dashed line*, GA-S of control, *empty diamonds with solid line* GA-S with 25 μL DMSO, *filled diamonds* GA-S with 100 μM phenobarbital, *empty squares with dashed line* GA-Me of control, *empty squares with solid line* GA-Me with 25 μL DMSO, *filled squares* GA-Me with 100 μM phenobarbital

1989; Hirotani et al. 1990; Shiao 1992; Zhao et al. 2007), which includes steps catalyzed by HMGR, SQS, and LS (Jennewein et al. 2001; Abe et al. 1993; Shang et al. 2008). However, it is unclear how the genes encoding these enzymes would respond to phenobarbital induction. To understand the mechanism underlying the enhanced production of ganoderic acids through phenobarbital induction, the expression kinetics of the three corresponding genes, i.e., *hmgr*, *sqs*, and *ls*, were examined using qRT-PCR. Gene expressions were normalized against the expression level of the 18S rRNA gene in fifth day untreated cells (control). Figure 5 shows that DMSO treatment had no effect on the gene expressions compared to the control. In both DMSO-treated and control cultures, as the cells grew from days 5 to 9, the expression levels of *hmgr* and *ls* genes peaked on day 7, while the expression level of *sqs* continued to increase. All three genes were up-regulated in phenobarbital-treated cells. The mRNA levels of *hmgr*, *sqs*, and *ls* in the seventh day cells were increased 3.26-, 7.24- and 3.22-fold those in the fifth day cells, respectively,

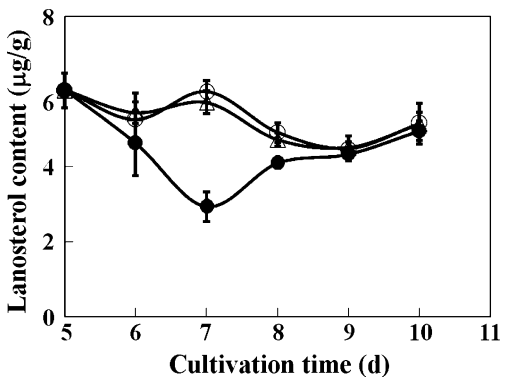


Fig. 4 Time courses of lanosterol level during fermentation treated with 100 μM phenobarbital (*filled circles*) or 25 μL DMSO (*empty triangles*) on day 5. Data are the means of three independent samples, and *vertical bars* show standard errors. *Empty circles* control

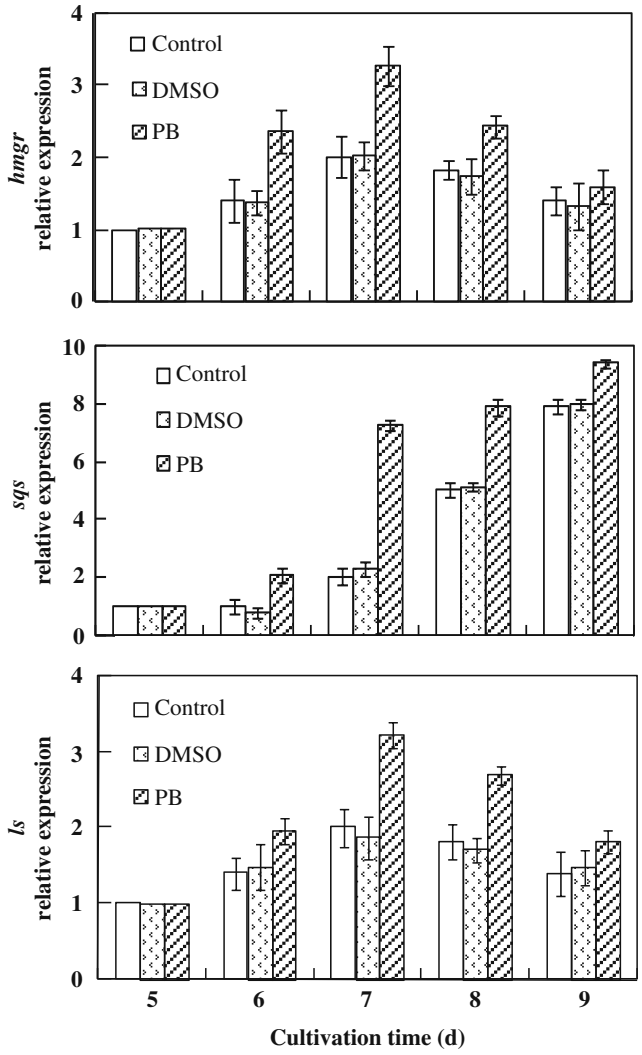


Fig. 5 Time courses of biosynthetic gene expressions in *G. lucidum* treated with 100 μM phenobarbital or 25 mL DMSO on day 5. Data are the means of three independent samples, and *vertical bars* show standard errors

under phenobarbital induction. In comparison, the mRNA levels in DMSO-treated cells were increased only 2.01-, 2.32- and 1.87-fold, respectively. These trends corresponded with those observed for individual ganoderic acid levels in Fig. 2, proving that higher expression levels of the three genes in the cells led to increased production of individual ganoderic acids.

Discussion

The induction time is critical to the manipulation of secondary metabolite accumulation (Yue and Zhong 2008; Wang et al. 2006; Qian et al. 2004). In this work, the addition of phenobarbital at the early growth stage (day 2) inhibited cell growth and greatly reduced the production of individual ganoderic acids (Table 1). In contrast, its addition at the middle growth stage (day 5) significantly increased the production of individual ganoderic acids while the cell growth was not inhibited. This indicated that the response of ganoderic acid biosynthesis to induction time was closely related to the *G. lucidum* growth stage, namely its cellular physiological state. This system gave similar results to those obtained with suspension cultures of *Panax notoginseng* cells for the production of Rg-type ginsenosides in which the highest yield was achieved by administering phenobarbital at a suitable growth stage of cultivation (Yue and Zhong 2008). In that work, phenobarbital induction increased the activity of protopanaxadiol 6-hydroxylase, an important enzyme involved in the biosynthesis of Rg-type ginsenosides, by up to more than three times compared to the control.

In the present work, 100 μM phenobarbital was the optimal induction dosage. This kind of dose-dependent induction was also observed in phenobarbital-induced ginsenoside production (Yue and Zhong 2008) and for the production of other terpenoids (Rijhwani and Shanks 1998; Qian et al. 2004). Though the exact mechanism of phenobarbital induction in fungal and plant cells is still unclear, it is thought to be mediated by phenobarbital interaction with the nuclear receptor, constitutive androstane receptor, and the pregnane X receptor in mammalian cells (Blättler et al. 2007; Lambert et al. 2009). In the *G. lucidum* system, it is speculated that when a lower than optimal elicitor dose is used, the elicitor-interaction sites in cells are not sufficiently utilized for activating the secondary metabolite synthesis, whereas an excessive dose causes cytotoxic effects (Lambert et al. 2009). This theory requires further experimental investigations.

The time courses of total crude ganoderic acids and the individual ganoderic acids in this work showed that their levels all started to increase sharply on day 5 with and without induction by phenobarbital. This is consistent with the increased expressions of *hmgr*, *sqs*, and *ls* starting on day 5 (Fig. 5). The levels of total crude ganoderic acids and the four individual ganoderic acids under phenobarbital induction leveled off at much higher peak values compared to the control and DMSO-treated cases, indicating that phenobarbital was an effective elicitor in stimulating the *G. lucidum* secondary metabolism. The increased expressions of *hmgr*, *sqs*, and *ls* on days 6 and 7 following phenobarbital induction on day 5 correlated with enhanced accumulation of ganoderic acids in phenobarbital-treated cells. Shang et al. (2008) and Zhao et al. (2007) reported that the increased expressions of the three genes also enhanced the production of ganoderic acids in their *G. lucidum* cultures without an elicitor. The drop of lanosterol level between days 5 to 7 in phenobarbital-treated *G. lucidum* cells suggests that more lanosterol was being converted to ganoderic acids than in untreated cells when transcription levels of *hmgr*, *sqs*, and *ls* were increased due to phenobarbital induction. These results agreed with our previous work (Xu et al. 2010) without an elicitor, in which increased production of ganoderic acids accompanied up-regulated expressions of *hmgr*, *sqs*, and *ls* and a decreased lanosterol level in static liquid cultures of *G. lucidum* compared to conventional shake flask culture. As the lanosterol to ganoderic acids biosynthetic steps are still unclear and some enzymes involved in the steps have not been identified yet, more investigations, such as the elucidation of P450 enzymes and their corresponding genes, are required to further understand ganoderic acid biosynthesis and its regulation.

SQS is generally considered a crucial branch-point enzyme and a potential regulatory point controlling the carbon flux into the terpenoid synthesis (Robinson et al. 1993; Lee et al. 2002). In the *G. lucidum* system, among the three genes monitored in this work, *sqs* showed a continual increase in expression between days 5 and 9 and relatively higher transcription levels compared to the other two. Under phenobarbital induction, production of individual ganoderic acids continued into days 8 and 9 after the levels of expression of *hmgr* and *ls* started to decrease on day 7 (Fig. 3). This suggests that SQS is more critical than HMGR and LS to ganoderic acid biosynthesis in *G. lucidum*, as reported recently (Zhao et al. 2007; Shang et al. 2008; Xu et al. 2010).

This work has, for the first time, presented experimental data on the up-regulation of *hmgr*, *sqs*, and *ls* genes in *G. lucidum* cells under phenobarbital induction. It provides a clear example of increased expression of the three key biosynthetic genes in the *G. lucidum* secondary metabolism in response to treatment with a P450 inducer. This information is useful to the further understanding and manipulation of ganoderic acid biosynthesis. It also provides a simple and effective strategy for enhancing the

production of total and individual ganoderic acids by *G. lucidum* fermentation.

Acknowledgments Financial support from the National Natural Science Foundation of China (NSFC Project No. 20776084, No. 30821005), the National High Technology R&D Program (863 Project No. 2007AA021506), Shanghai Science & Technology Commission (Project No. 08DZ1971900), and the Shanghai Leading Academic Discipline Project (Project Nos. B203 and B505) is gratefully acknowledged. We also thank the reviewers for their time and helpful comments.

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