Bioresource Technology 102 (2011) 8185–8190

Contents lists available at ScienceDirect

Bioresource Technology

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

Enhanced production of ganoderic acids in static liquid culture of *Ganoderma lucidum* under nitrogen-limiting conditions

Wei Zhao, Jun-Wei Xu, Jian-Jiang Zhong *

State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, 800 Dong-Chuan Road, Shanghai 200240, China
Laboratory of Molecular Biochemical Engineering, Department of Bioengineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dong-Chuan Road, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 25 April 2011
Received in revised form 10 June 2011
Accepted 14 June 2011
Available online 26 June 2011

Keywords:
Glutamine
Nitrogen limitation
Ganoderic acids
Gene expression
Medicinal mushroom fermentation

ABSTRACT

The effect of nitrogen limitation on the production of the antitumor compounds, ganoderic acids (GAs), by *Ganoderma lucidum* and on transcription levels of triterpene biosynthesis genes in this mushroom was investigated. At 3 mM glutamine, a maximal content of GA-Mk, GA-T, GA-S, and GA-Me was 2.16, 11.76, 31.09, and 7.04 μg/mg cell dry weight, respectively, which was 2.8-, 5.8-, 8.3-, and 5.1-fold that obtained at 60 mM glutamine. The transcription levels of biosynthetic genes encoding 3-hydroxy-3-methylglutaryl-CoA reductase, farnesyl pyrophosphate synthase, squalene synthase, lanosterol synthase, and a putative nitrogen regulator, AreA, were up-regulated by 37-, 18-, 4.5-, 3.2-, and 13-fold, respectively, in nitrogen limitation conditions, suggesting that increased GAs biosynthesis may result from higher expression of those genes. This study demonstrated a useful strategy for enhancing GAs production and provided useful information for further investigation on its biosynthesis regulation.

1. Introduction

Mushrooms can produce a large number of bioactive compounds, among them some are promising antitumor triterpenoid secondary metabolites. *Ganoderma lucidum*, a traditional Chinese medicinal mushroom, has been used for the prevention and treatment of various human diseases for several thousand years in East Asia. *Ganoderma* species produce ganoderic acids (GAs), a type of triterpene compounds, that have a number of biological functions such as cytotoxicity to hepatoma cells, antitumor effect, and anti-HIV-1 activities (Zhong and Tang, 2004; Xu et al., 2010b). Recent studies on GAs showed that different individual GAs exhibit different bioactivities. For instance, GA-T induces apoptosis of lung cancer cells and has higher cytotoxicity to 95-D cell line than to normal cell lines (Tang et al., 2006); GA-S stimulates platelet aggregation and induces HeLa cell apoptosis (Wang et al., 1989; Liu and Zhong, 2011); and GA-Me effectively inhibits tumor growth, invasion and lung metastasis (Chen et al., 2008).

Like other sterol-related compounds, GAs are synthesized via the mevalonate/isoprenoid pathway. The steps after lanosterol formation including a series of oxidation, reduction, and acylation reactions to form different individual GAs are yet unclear (Xu et al., 2010b). So far, several genes involved in early steps of the GAs biosynthetic pathway have been cloned (Shi et al., 2010). The enzymes 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMGR), farnesyl-diphosphate synthase (FPS), squalene synthase (SQS), and lanosterol synthase (LS) are reported to catalyze key-step reactions in this pathway (Shi et al., 2010).

Because solid cultivation of this mushroom is a time-consuming and labor-intensive process, submerged fermentation of *G. lucidum* is viewed as a promising alternative for efficient production of ganoderic acids (Zhong and Tang, 2004; Wagner et al., 2003). The yields of GAs by *G. lucidum* have being increased through manipulation of fermentation conditions, addition of inducers and development of new bioprocessing strategies (Tang and Zhong, 2002, 2003; Fang and Zhong, 2002a; Gong and Zhong, 2005; Tang et al., 2009; Ren et al., 2010). For example, a two-stage culture process that combines liquid fermentation and static culture was developed (Fang and Zhong, 2002b; Xu et al., 2010a). However, to satisfy the need for high-quality individual GAs for large-scale (pre-)clinical trials and ultimate commercialization, further increases in the yield of individual GA are required.

In a few filamentous fungi and plants, nitrogen limitation is an efficient strategy for increasing production of secondary metabolites (Brückner and Blechschmidt, 1991; Hsieh et al., 2006; Zhou and Zhong, 2009). Glutamine and ammonium, the primary nitrogen source in fungi, are the key compounds involved in nitrogen regulation of both primary and secondary metabolism (Marzluf, 1997; Muñoz and Agosin, 1993). The biosynthesis of many secondary metabolites of fungi is transcriptionally regulated by the nitrogen status of the cells (Kohut et al., 2009; Mihlan et al., 2003;

* Corresponding author at: State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, 800 Dong-Chuan Road, Shanghai 200240, China. Tel.: +86 21 34206968; fax: +86 21 34204831.
E-mail address: jjzhong@sjtu.edu.cn (J.-J. Zhong).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.06.043

Marzluf, 1997; Rodríguez-Ortiz et al., 2009), and regulation is usually mediated by the zinc finger transcription activator AreA/NIT-2 (Marzluf, 1997; Mihlan et al., 2003; Haas and Marzluf, 1995). However, it is currently not known if and how nitrogen supply influences transcription of triterpene biosynthetic genes in mushrooms.

In the current study, accumulation of GA-Mk, GA-T, GA-S, and GA-Me in response to different nitrogen concentrations was measured in static liquid cultures of *G. lucidum*, and the transcription profiles of four genes encoding the enzymes, HMGR, SQS, FPS and LS, involved in ganoderic acid biosynthesis and of the gene encoding putative nitrogen-regulated activator AreA were established.

2. Methods

2.1. Strain and culture conditions

G. lucidum CGMCC 5.616 from the China General Microbiological Fermentation Center was maintained on potato dextrose agar slants and cultured at 30 °C for 7 days.

The details of preculture (shake flask culture) medium and conditions were described by Fang and Zhong (2002b). The mycelia from shake flask culture were isolated by centrifugation (10 min, 10,000*g*), washed with sterile water, and resuspended in basal medium containing microelements supply (Geissman et al., 1966) and 2% glucose (Rodríguez-Ortiz et al., 2009). Ammonium sulfate, L-glutamine, L-asparagine or glycine served as nitrogen sources and their concentration varied according to the experiment requirement. The suspended cells were transferred to plates for liquid static culture study (Xu et al., 2010a).

2.2. Sampling, analyses of dry weight, residual sugar in medium and individual GAs

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 residual sugar was measured according to the method described by Miller (Miller, 1959). For determining individual GAs, high-performance liquid chromatography (HPLC) was performed on an Agilent 1200 series (5 μm Agilent Zorbax SB-C18 column, 250 × 4.6 mm). Details on the HPLC analysis method are described elsewhere (Xu et al., 2010a).

2.3. Total RNA extraction and cDNA synthesis

The total RNA from fresh cell was extracted using TriZol (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined using a Biophotometer Plus (Eppendorf, Germany). After DNase treatment, 1 μg of total RNA from each sample was reverse-transcribed with RevertAid™ M-MuLV Reverse Transcriptase system for RT-PCR following the vendor's instructions.

2.4. Isolation of cDNA of putative nitrogen catabolite regulatory protein AreA from G. lucidum

The putative *G. lucidum* AreA partial cDNA was isolated by PCR amplification using degenerate primers designed on the basis of the regions conserved among fungi in the previous reported sequences (Genebank accession number: XM_003029814, AB04 3467, XM_002911566): forward, 5′-ACBCAGGTKTGGMRGATGTA-3′, B(G, T, C), K(G, T), M(A, C), R(A, G); reverse, 5′-ACVGTGTGRTCR AARCTCGT-3′, V(G, A, C). The amplified fragments were cloned into the pMD19-T vector with a TA cloning system (Takara, Dalian, China) and sequenced (Invitrogen). The AreA amino acid sequence showed 70% and 72% identity with *Aspergillus nidulans* AreA amino acid sequence (XP_681936.1) and *Gibberella fujikuroi* AreA amino acid sequence (P78688.1), respectively. The AreA nucleotide sequence was submitted to Genbank and its accession number was JF720040.

2.5. Measurement of hmgr, fps, sqs, ls, and areA gene expressions by real-time quantitative PCR (qRT-PCR)

The transcription levels of the *hmgr*, *fps*, *sqs*, *ls*, and *areA* genes (which encode the enzymes HMGR, FPS, SQS, LS, and AreA, respectively) were analyzed by real-time quantitative PCR (qRT-PCR). The sequences of the primer for amplification of *hmgr*, *sqs*, and *ls* were those described by Xu et al. (2010a). For *fps* and *areA*, the following primer sets were used: *fps*-forward, 5′-CCTCATCACCGCTCCAGAA-3′ and *fps*-reverse, 5′-GGCGACGG GAAGGTAGAAG-3′; *areA*-forward, 5′-CAGGACGGAGCAGACGAAA-3′ and *areA*-reverse, 5′-CCG AGCCAGGTATGGGAAT-3′. qRT-PCR was performed as described by Xu et al. (2010a). The 18S rRNA gene was used as the internal control gene because its expression was found to be stable under our experimental conditions. The expression level of the different genes was normalized with respect to the 18S rRNA expression level. For each gene, an expression level of 1 was assigned to the samples from the culture grown in the presence of 60 mM glutamine, and expression levels under other conditions are presented as fold changes relative to this reference level.

2.6. Statistical analysis

Data are the average of three independent sample measurements. The error bars indicate the standard deviation (SD) from the mean of triplicates. Data were analyzed with Student's *t*-test. The difference between contrasting treatments was considered significant when $P < 0.05$ in a two-tail analysis.

3. Results and discussion

3.1. Effect of nitrogen source concentration on GAs production

As shown in Table 1, limitation of the nitrogen sources led to an increase in GAs content. The increases under conditions of ammonium sulfate, glutamine and asparagine limitation were higher than those under glycine limitation, and this observation suggests that these compounds may be directly involved in nitrogen repression of GA synthesis in *G. lucidum*. In static culture media with different glutamine concentrations, some significant differences ($P < 0.05$) in the production of GA-Mk, GA-T, GA-S and GA-Me were observed (Fig. 1). The highest production of GA-Mk, GA-T, GA-S, and GA-Me was 23.5 ± 1.83, 121.6 ± 16.9, 268.1 ± 17.5, and 57.3 ± 2.24 mg/L, respectively, which was 2.11, 5.08, 5.13, and 4.0 times higher than that obtained at 60 mM glutamine. Further reduction in the glutamine concentration led to a decreased yield.

Table 1
Effect of limitation of different nitrogen sources on GA biosynthesis.

Nitrogen source	Cell dry weight (g/L)	GA content (μg/mg)			
		Mk	T	S	Me
60 mM $(NH_4)_2SO_4$	6.51	0.29	5.21	3.93	1.72
3 mM $(NH_4)_2SO_4$	7.12	1.13	17.34	16.81	2.35
60 mM asparagine	9.61	0.66	1.63	2.90	0.88
3 mM asparagine	8.06	1.76	11.67	27.46	5.34
60 mM glycine	9.73	0.99	2.69	8.31	1.75
3 mM glycine	8.45	0.68	4.74	9.45	1.89
60 mM glutamine	10.83	0.92	2.08	4.74	1.58
3 mM glutamine	8.88	1.26	7.31	19.25	3.52

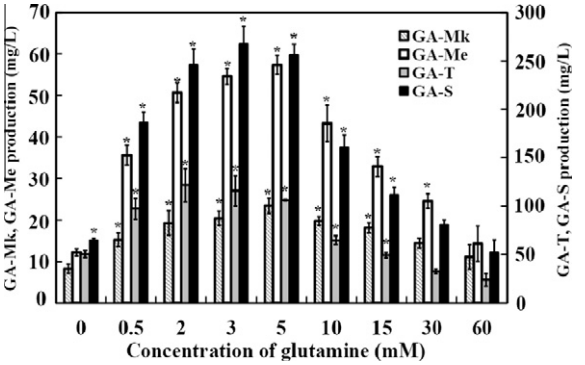

Fig. 1. Effect of glutamine concentration on GAs production. Precultured cells were transferred to liquid static culture with different glutamine concentration at 30 °C for 12 days. The error bars in the figure indicate the standard deviations from three independent samples. * indicates statistic significance ($p < 0.05$) compared to 60 mM glutamine culture.

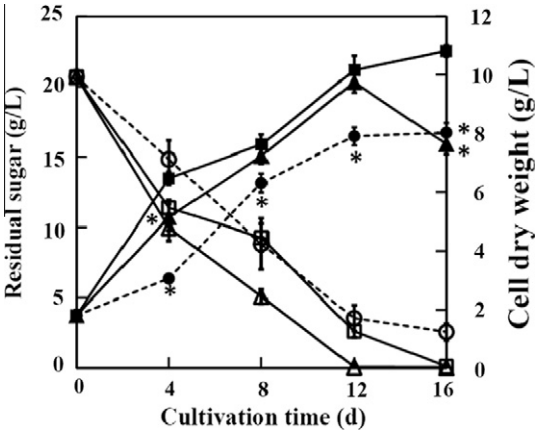

Fig. 2. Time course of cell growth (solid squares, 60 mM glutamine culture; solid triangles, 3 mM glutamine culture; solid circles, nitrogen free culture) and residual sugar (open squares, 60 mM glutamine culture; open triangles, 3 mM glutamine culture; open circles, nitrogen free culture). * indicates statistic significance ($p < 0.05$) compared to 60 mM glutamine culture.

3.2. Kinetic profiles of cell growth and GAs accumulation at different glutamine concentrations

To examine the effect of glutamine concentration on GAs biosynthesis by *G. lucidum* in detail, the kinetic profiles of cell growth, sugar consumption, and ganoderic acids accumulation were studied in cultures with 60 mM glutamine (control), 3 mM glutamine (nitrogen limitation), or without nitrogen supply (complete nitrogen starvation). As shown in Fig. 2, the maximum dry cell weight at 60 mM, 3 mM or 0 mM of glutamine was 10.16, 9.75, and 8.02 g/L, respectively. Compared with the control, nitrogen limitation only slightly affected cell growth, suggesting that 3 mM glutamine was almost sufficient for cell growth, while complete nitrogen starvation reduced dry cell weight significantly. Time profiles of the accumulation of four major individual ganoderic acids are shown in Fig. 3. While the contents of GA-Mk, GA-T, GA-S, and GA-Me showed increases until day 12 in cultures containing an initial concentration of 60 mM glutamine, the contents remained well below the contents observed in the cultures

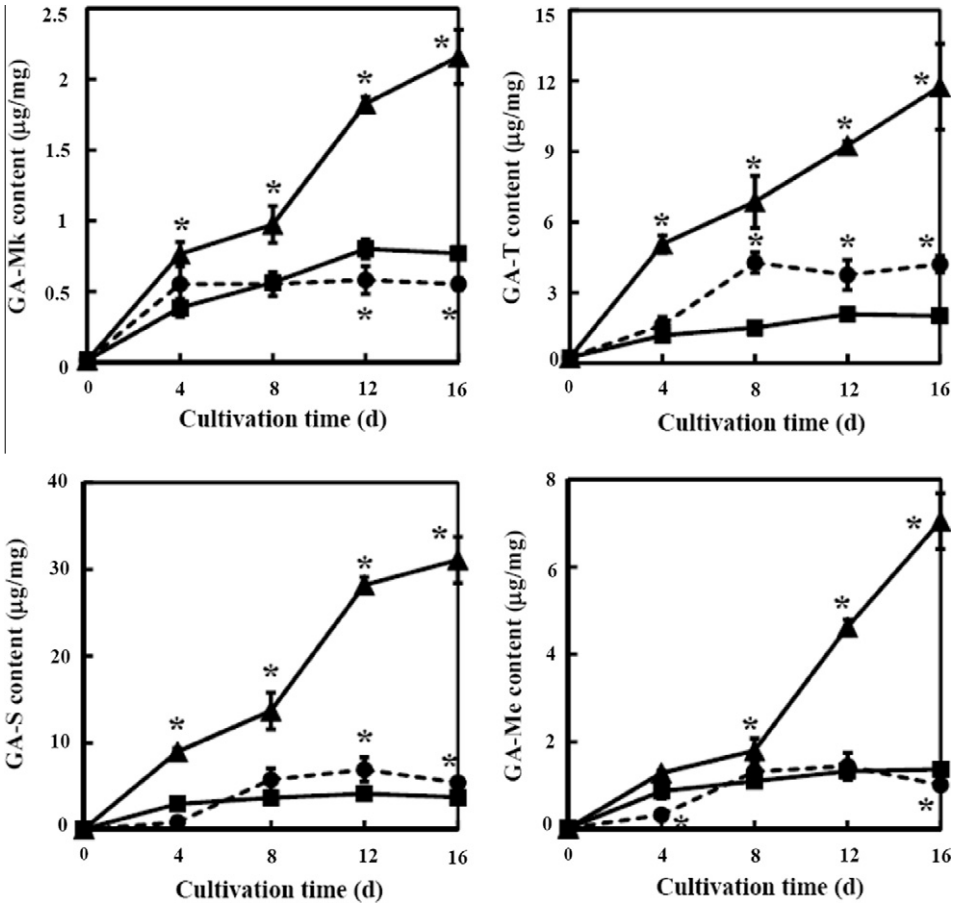

Fig. 3. Time courses of GA-Mk, GA-T, GA-S, and GA-Me content in 60 mM glutamine (squares), 3 mM glutamine (triangles), and nitrogen free (circles) culture. * indicates statistic significance ($p < 0.05$) compared to 60 mM glutamine culture.

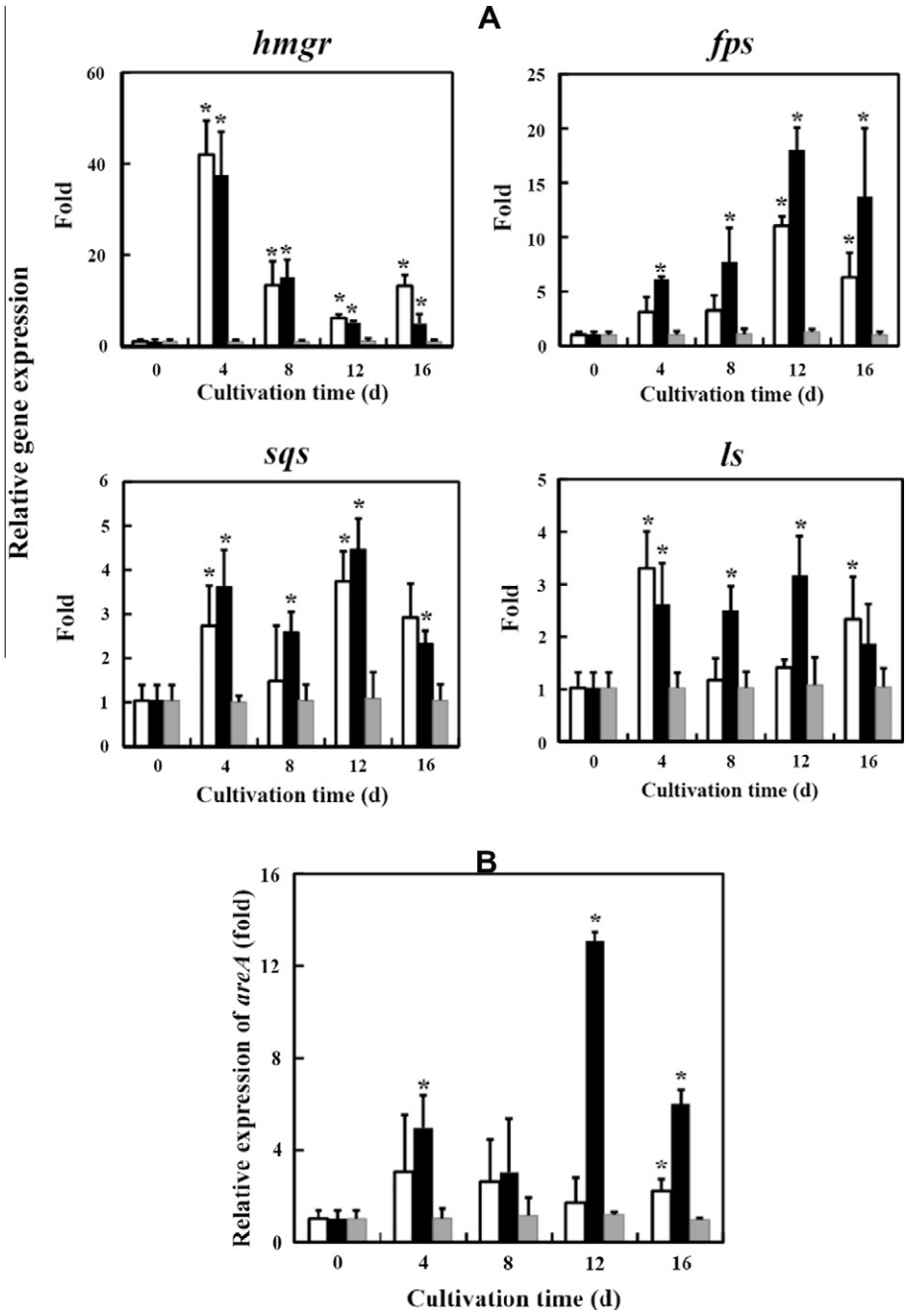

Fig. 4. Transcriptional level of biosynthetic genes (A) and the putative transcriptional factor *areA* (B) in 60 mM glutamine (gray), 3 mM glutamine (dark), and nitrogen free (blank) culture. Expression of the samples from the culture grown with 60 mM glutamine is defined as 1.0 and expression levels under other conditions are displayed as the fold increase over the reference sample. * indicates statistic significance ($p < 0.05$) compared to 60 mM glutamine culture.

containing only 3 mM of glutamine. The maximum GA-Mk, GA-T, GA-S, and GA-Me levels measured at 3 mM glutamine were 2.16 ± 0.19, 11.76 ± 1.82, 31.09 ± 2.67, and 7.04 ± 0.64 μg/mg cell dry weight, respectively, which were 2.81, 5.84, 8.33, and 5.12 times higher than those obtained at 60 mM glutamine. Under nitrogen-free condition, the content of most individual GAs increased as well, although the increase was lower than that in the culture with 3 mM glutamine. GA-S and GA-Me reached their maximum level on day 12 and then declined slightly on day 16, while GA-Mk and GA-T increased until day 4 and day 8, respectively, and then showed a nearly constant level. The total GAs showed a similar trend as the individual GAs. The maximal levels in 3 mM glutamine and nitrogen free culture were 4.91- and 1.45-fold that obtained in the presence of 60 mM glutamine, respectively. The maximal contents of the four individual GAs were 1.93, 4.51, 25.26, and 8.87 times higher than those reported for liquid static cultures of *G. lucidum* in the present of phenobarbital, a cytochrome P450 inducer (Liang et al., 2010).

3.3. Gene expression response to different glutamine concentrations

As shown in Fig. 4A, the transcription level of *hmgr* in 3 mM glutamine culture and nitrogen free culture on day 4 was about 37 times and 42 times higher than that in 60 mM glutamine culture. It decrease afterwards, but still remained at a much higher level than the control. In the case of *fps*, the highest transcription level in 3 mM glutamine culture was about 18 times higher than that of the control on day 12. For *sqs*, the largest changes in

transcription profiles were observed on day 12 when it was up-regulated 4.5- and 3.7-fold in the nitrogen limitation and complete nitrogen starvation culture, respectively. The expression level of *ls* increased 3.2-fold in 3 mM glutamine culture and 3.3-fold in nitrogen free culture. Up-regulation of the four biosynthetic genes began on day 4 in glutamine limitation-cultured cells, which parallels the increase in content of four individual GAs. The mRNA levels remained much higher throughout the later fermentation process in nitrogen limitation cultured cells than in control. These trends were consistent with those observed for GAs accumulation levels, suggesting that the rapid increase in the GAs contents in the nitrogen limited cells could be due to increased transcription of biosynthetic genes as was observed for transcriptional regulation by nitrogen levels of bikaverin and gibberellin biosynthetic genes in *Fusarium fujikuroi* (Wiemann et al., 2009; Mihlan et al., 2003). When the fungus *Elsinoë fawcettii* was grown on a medium without nitrogen, the elsinochrome phytotoxin biosynthetic genes were also up-regulated (Chung and Liao, 2009).

As AreA/NIT-2 is reported to be a transcription factor involved in nitrogen regulation in fungi, the expression profile of the *areA* gene in *G. lucidum* was also investigated. As shown in Fig. 4B, the transcription of this gene was higher when nitrogen was limited or depleted in medium than when 60 mM glutamine was available. Similar observations were reported for *A. nidulans* and *Fusarium oxysporum* (Langdon et al., 1995; Morozov et al., 2001; Divon et al., 2006). Bioinformatics analysis of the promoter sequence of our four biosynthetic genes predicted several putative binding sites of NIT2/AreA (Supplementary data), implying the potential role of this global transcription factor in regulating GA biosynthesis. Further work such as knocking down the putative *areA* gene in *G. lucidum* or deletion/mutation analysis of the promoters of biosynthetic genes will be necessary to clarify the role of AreA in GA biosynthesis regulation.

4. Conclusions

The production of the antitumor agents, ganoderic acids, in static liquid culture of *G. lucidum* was shown to be higher under nitrogen-limiting than nitrogen-sufficient conditions. Nitrogen limitation appears to induce GA biosynthesis through transcriptional induction of its biosynthetic genes. The findings may help to further understand the mechanism of nitrogen regulation of GA biosynthesis as well as to develop a more efficient fermentation process.

Acknowledgements

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 No. B203) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.06.043.

References

Brückner, B., Blechschmidt, D., 1991. Nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*. Appl. Microbiol. Biotechnol. 35, 646–650.

Chen, N.H., Liu, J.W., Zhong, J.J., 2008. Ganoderic acid Me inhibits tumor invasion through down-regulating matrix metalloproteinases 2/9 gene expression. J. Pharm. Sci. 108, 212–216.

Chung, K.R., Liao, H.L., 2009. Determination of a transcriptional regulator-like gene involved in biosynthesis of elsinochrome phytotoxin by the citrus scab fungus, *Elsinoë fawcettii*. Microbiology 154, 3556–3566.

Divon, H.H., Ziv, C., Davydov, O., Yarden, O., Fluhr, R., 2006. The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during *Fusarium oxysporum* pathogenesis. Mol. Plant Pathol. 7, 485–497.

Fang, Q.H., Zhong, J.J., 2002a. Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. Process Biochem. 37, 769–774.

Fang, Q.H., Zhong, J.J., 2002b. Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderma lucidum*. Biotechnol. Prog. 18, 51–54.

Gong, H.G., Zhong, J.J., 2005. Hydrodynamic shear stress affect cell growth and metabolite production by medicinal mushroom *Ganoderma lucidum*. Chin. J. Chem. Eng. 13, 426–428.

Geissman, T.A., Verbiscar, A.J., Phinney, B.O., Cragg, G., 1966. Studies on the biosynthesis of gibberellins from (−)-kaurenoic acid in cultures of *Gibberella fujikuroi*. Phytochemistry 5, 933–947.

Haas, H., Marzluf, G.A., 1995. NRE, the major nitrogen regulatory protein of *Penicillium chrysogenum*, binds specifically to elements in the intergenic promoter regions of nitrate assimilation and penicillin biosynthetic gene clusters. Curr. Genet. 28, 177–183.

Hsieh, C., Tseng, M.H., Liu, C.J., 2006. Production of polysaccharides from *Ganoderma lucidum* (CCRC 36041) under limitations of nutrients. Enzyme Microb. Technol. 38, 109–117.

Kohut, G., Ádám, A.L., Fazekas, B., Hornok, L., 2009. N-starvation stress induced *FUM* gene expression and fumonisin production is mediated via the HOG-type MAPK pathway in *Fusarium proliferatum*. Int. J. Food Microbiol. 130, 65–69.

Langdon, T., Ravagnani, A., Gielkens, M., Caddick, M.X., Arst, H.N., 1995. Mutational analysis reveals dispensability of the N-terminal region of the *Aspergillus* transcription factor mediating nitrogen metabolite repression. Mol. Microbiol. 17, 877–888.

Liang, C.X., Li, Y.B., Xu, J.W., Wang, J.L., Miao, X.L., Tang, Y.J., Gu, T.Y., Zhong, J.J., 2010. Enhanced biosynthetic gene expressions and production of ganoderic acids in static liquid culture of *Ganoderma lucidum* under phenobarbital induction. Appl. Microbiol. Biotechnol. 86, 1367–1374.

Liu, R.M., Zhong, J.J., 2011. Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. Phytomedicine 18, 349–355.

Marzluf, G.A., 1997. Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev. 61, 17–32.

Morozov, I.Y., Galbis-Martinez, M., Jones, Meriel.G., Caddick, M.X., 2001. Characterization of nitrogen metabolite signalling in *Aspergillus* via the regulated degradation of *areA* mRNA. Mol. Microbiol. 42, 269–277.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31, 426–428.

Mihlan, M., Homann, V.D., Liu, T.W., Tudzynski, B., 2003. AREA directly mediates nitrogen regulation of gibberellin biosynthesis in *Gibberella fujikuroi*, but its activity is not affected by NMR. Mol. Microbiol. 47, 975–991.

Muñoz, G.A., Agosin, E., 1993. Glutamine involvement in nitrogen control of gibberellic acid production in *Gibberella fujikuroi*. Appl. Environ. Microbiol. 59, 4317–4322.

Ren, A., Qin, L., Shi, L., Dong, X., Mu, D.S., Li, Y.X., Zhao, M.W., 2010. Methyl jasmonate induces ganoderic acid biosynthesis in the basidiomycetous fungus *Ganoderma lucidum*. Bioresour. Technol. 101, 6785–6790.

Rodríguez-Ortiz, R., Limón, M.C., Avalos, J., 2009. Regulation of carotenogenesis and secondary metabolism by nitrogen in wild-type *Fusarium fujikuroi* and carotenoid-overproducing mutants. Appl. Environ. Microb. 75, 405–413.

Shi, L., Ren, A., Mu, D.S., Zhao, M.W., 2010. Current progress in the study on biosynthesis and regulation of ganoderic acids. Appl. Microbiol. Biotechnol. 88, 1243–1251.

Tang, W., Liu, J.W., Zhao, W.M., Zhong, J.J., 2006. Ganoderic acid T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. Life Sci. 80, 205–211.

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.

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., Zhong, J.J., 2003. Role of oxygen supply in submerged fermentation of *Ganoderma lucidum* for production of *Ganoderma* polysaccharide and ganoderic acid. Enzyme Microb. Technol. 32, 478–484.

Wagner, R., Mitchell, D.A., Sassaki, G.L., De Almeida Amazonas, M.A.L., Berovič, M., 2003. Current techniques for the cultivation of *Ganoderma lucidum* for the production of biomass, ganoderic acid and polysaccharides. Food Technol. Biotechnol. 41, 371–382.

Wang, C.N., Chen, J.C., Shiao, M.S., Wang, C.T., 1989. The aggregation of human platelet induced by ganodermic acid S. Biochim. Biophys. Acta 986, 151–160.

Wiemann, P., Willmann, A., Straeten, M., Kleigrewe, K., Beyer, M., Humpf, H.U., Tudzynski, B., 2009. Biosynthesis of the red pigment bikaverin in *Fusarium fujikuroi*: genes, their function and regulation. Mol. Microbiol. 72, 931–946.

Xu, J.W., Xu, Y.N., Zhong, J.J., 2010a. Production of individual ganoderic acids and expression of biosynthetic genes in liquid static and shaking cultures of *Ganoderma lucidum*. Appl. Microbiol. Biotechnol. 85, 941–948.

Xu, J.W., Zhao, W., Zhong, J.J., 2010b. Biotechnological production and application of ganoderic acids. Appl. Microbiol. Biotechnol. 87, 457–466.
Zhong, J.J., Tang, Y.J., 2004. Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. Adv. Biochem. Eng. Biotechnol. 87, 25–59.
Zhou, X., Zhong, J.J., 2009. Effect of initial ammonium concentration on taxoid production and biosynthesis genes expression profile in suspension cultures of *Taxus chinensis* cells. Eng. Life Sci. 3, 261–266.