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Transcriptome dynamics and metabolite analysis revealed the candidate genes and regulatory mechanism of ganoderic acid biosynthesis during liquid superficial-static culture of *Ganoderma lucidum*

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Summary

Ganoderic acid (GA), an important secondary metabolite of *Ganoderma lucidum*, exhibited many significant pharmacological activities. In this study, the biosynthetic mechanism of GAs was investigated by comparing metabolites and transcriptome dynamics during liquid superficial-static culture (LSSC) and submerged culture (SC). LSSC was a better method to produce GA because thirteen GAs were identified from mycelia by UPLC-QTOF-MS, and the content of all GAs was higher in LSSC than in SC. Ergosterol was accumulated during the SC process in *G. lucidum*. Transcriptome dynamics analysis revealed *CYP5150L8* was the key gene regulating lanosterol flux into GA biosynthesis. Other sixteen CYP450 genes were significantly higher expressed during the culture time in LSSC and could be potential candidate genes associated with the biosynthesis of different GAs. In addition, six of the ten expressed genes in ergosterol biosynthetic pathway shown upregulated at some time points in SC. These results not only provide a fundamental information of the key genes in ergosterol and GA biosynthetic pathway, but also provide directions for future elucidating the regulatory mechanisms of GAs in *G. lucidum* and enabling us to promote the development and utilization of LSSC at the industrial level.

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Introduction

Ganoderma lucidum is a well-known edible and medicinal mushroom in China and also draw more attention in other parts of the world recently (Bishop *et al*., 2015; Pharmacopoeia Commission of People's Republic of China, 2015; Wang *et al*., 2018; Wu *et al*., 2019). Ganoderic acid (GA), classified as a kind of triterpenoid, is well recognized as the main and specific bioactive compound in *G. lucidum* (Bishop *et al*., 2015). More than 170 GAs had been isolated from *Ganoderma* species (Baby *et al*., 2015). According to the carbon skeletons, there were two types of GAs: type I had only one double bond in the lanostane skeleton, and type II possessed two conjugated double bonds in the tetracyclic rings (Hirotani *et al*., 1987; Nishitoba *et al*., 1987; Li *et al*., 2013). It was reported that several individual GAs had important biological and pharmacological activities, such as antitumor, antimetastasis and anti-HIV activities (Li *et al*., 2013; Bishop *et al*., 2015; Gill *et al*., 2016; Wu *et al*., 2019). As a result, the *Ganoderma* bioactive substances are consumed worldwide nowadays as a kind of health tonic and dietary supplement, and resulted in a boom of commercial market needs (Xu *et al*., 2008; Wang *et al*., 2018).

Ganoderic acid biosynthesis could be divided into two modules in *G. lucidum*: lanosterol and GA biosynthesis. As shown in Fig. 1, lanosterol is synthesized via the mevalonate/isoprenoid pathway (MVA) from acetyl-CoA (Xu *et al*., 2010b). There are 11 enzymes encoded by 13 genes in lanosterol biosynthetic pathway in *G.*

lucidum (Chen *et al*., 2012), for that acetyl-CoA C-acetyltransferase (AACT) and farnesyl diphosphate synthases (FPS) each are encoded by two genes in the *G. lucidum* genome. The genes involved in the lanostane skeleton biosynthesis pathway, such as encoding AACT, hydroxymethylglutaryl-CoA (HMGS), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), mevalonate pyrophosphate decarboxylase (MVD), isopentenyl diphosphate isomerase (IDI), FPS, squalene synthase (SQS) and lanosterol synthase (LS), were cloned from *G. lucidum* (Zhao *et al*., 2007; Ding *et al*., 2008; Shang *et al*., 2008, 2010; Shi *et al*., 2012; Fang *et al*., 2013; Ren *et al*., 2013; Wu *et al*., 2013). Overexpression of genes encoding HMGR, FPS, SQS, squalene epoxidase (SE) and LS, which are considered as the major regulatory point, could enhance GA production (Xu *et al*., 2012; Zhou *et al*., 2014; Zhang *et al*., 2017a, b; Fei *et al*., 2019). Nevertheless, details regarding the later steps to biosynthesize the individual GAs, including the cyclization, oxidation and reduction reactions, are unclear. In addition, ergosterol, an important component of fungal membranes, is also synthesized from lanosterol (Dupont *et al*., 2012). However, to the best of our knowledge, the mechanism of lanosterol flux regulation had not been reported in *G. lucidum*, and the elucidation of this mechanism would be an important development in GA research.

Cytochrome P450 monooxygenases (CYP450s) are considered to be responsible for the cyclization step from lanosterol to biosynthesize the individual GAs (Wang *et al*., 2018). A total of 219 CYP450 genes were identified in the released genome information, and 78 CYP450 genes were coexpressed with *LS* during different developmental stages in *G. lucidum* (Chen *et al*., 2012). CYP5150L8 was considered as the first CYP450 that related to the biosynthesis of GA in *G. lucidum*, and it could catalyse lanosterol to form 3-hydroxy-lanosta-8,24-dien-26-al (HLDA) by three successive steps of C-26 oxidation (Wang *et al*., 2018; Lan *et al*., 2019). Yang *et al*. (2018) cloned and characterized the *CYP512U6* gene from *G. lucidum*, and found CYP512U6 could hydroxylate the GAs DM and TR at the C-23 position to produce hainanic acid A and GA Jc respectively. Recently, Xu *et al*. (2019) reported that *CYP5150L8*, *CYP512A2*, *CYP512V2* and *CYP512A13* genes were associated with the oxidative modification of the lanostane skeleton in GA biosynthesis and that the transcription levels of these genes were increased in a GA high-yielding strain.

As a powerful tool to analyse differentially expressed genes (DEGs), transcriptome sequencing, or RNA-Seq, has been used widely in studying specific biological processes of *G. lucidum*, such as heat stress response, nitric oxide response, copper-induced and L-phenylalanine enhancer effects (Gu *et al*., 2017; Tan *et al*., 2018; Ma *et al*., 2019; Jain *et al*., 2020). In addition, many studies also focused on the triterpenoid biosynthesis analysis through transcriptome sequencing in *G. lucidum* and other mushrooms (Xu *et al*., 2013; Gu *et al*., 2017; Wang et al., 2019). However, the transcriptome dynamic behaviour during *G. lucidum* growth, which could reflect specific phenomenon for triterpenoid and GA biosynthesis, was largely unknown. To get deeper insight into GA biosynthesis, here GAs and transcriptomic differences were investigated under liquid superficial-static culture (LSSC) and submerged culture (SC) in *G. lucidum*. The GAs were identified by UPLC-QTOF-MS. The candidate genes and regulatory mechanisms of GA biosynthesis were performed by transcriptome dynamic analysis under the two culture methods.

Results

Effect of culture methods on triterpenoid and ergosterol production in G. lucidum

Liquid superficial-static culture and SC were compared by measuring biomass, triterpenoid and ergosterol production during the culture time (Fig. 2). The mycelia grew faster in LSSC than in SC. The maximal triterpenoid production of 23.75 $\pm$ 0.60 mg g^{-1} was obtained in LSSC on the eighth day, which was 1.87-fold higher than the maximum in SC on the fifth day. The ergosterol content in SC was higher than in LSSC, and the maximum was 9.48 $\pm$ 0.2 mg g^{-1} on the sixth day. The peak value of ergosterol in LSSC was 5.91 $\pm$ 0.15 mg g^{-1} on the fifth day.

GA identification and analysis

To compare the metabolic profiles of *G. lucidum* in different culture methods, six samples at three time points, namely the 4th, 6th and 8th days, in SC (SC-4, SC-6, SC-8) and LSSC (LSSC-4, LSSC-6, LSSC-8) were designated and characterized by UPLC-QTOF-MS analysis (Fig. S1). VIP values were calculated for each altered metabolite and set as the cut-off points for all metabolites obtained from the UPLC-QTOF-MS analysis. The metabolites that had VIP values > 2 and *P*-value < 0.05 were considered the most relevant ones for the two culture methods. In this study, 13 different GAs were detected and identified based on the observed molecular and fragment ions, retention times and UV characteristics (Table 1). Then, these GAs were further confirmed according to the databases and references (Table S2). Among the 13 GAs, there were 10 type I GAs and 3 type II GAs. All 13 GAs were significantly enriched in LSSC mycelia according to the base peak intensity (BPI), VIP and *P*-value (Table 1).

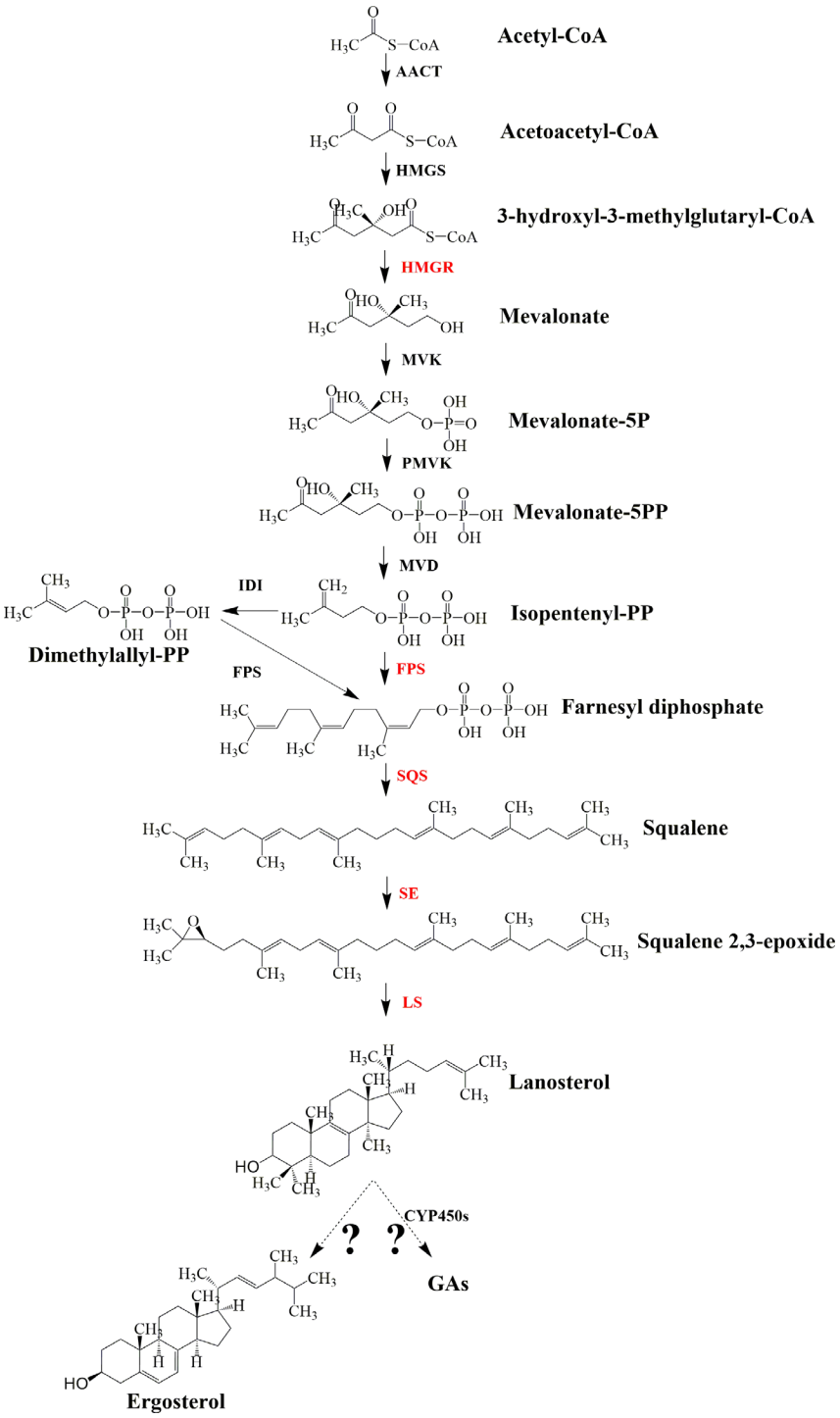

Fig. 1. Lanosterol biosynthetic pathways and the major regulatory point in *Ganoderma lucidum*. The enzymes with red font were reported that overexpression of genes encoding these enzymes could enhance GA production. So they are considered as the major regulatory point.

Transcriptome sequencing and gene expression dynamic analysis of G. lucidum in LSSC and SC

To reveal the candidate genes and regulatory mechanism of GA biosynthesis under the two culture methods, transcriptome sequencing was performed from the 4th to 8th days in LSSC (samples LSSC-4 to LSSC-8) and SC (samples SC-4 to SC-8). Because of a low-quality RNA, the LSSC-6 had only two biological replicates. After redundant and short reads were filtered

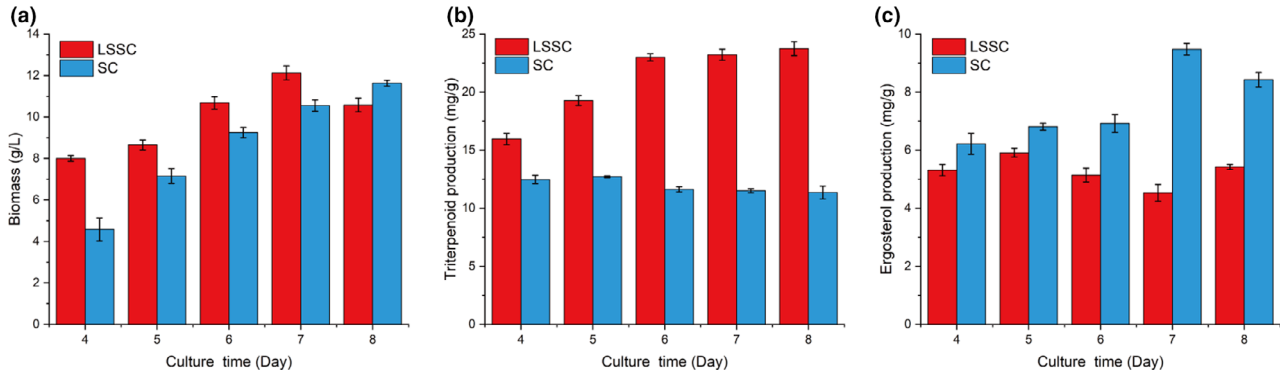

Fig. 2. Effect of the culture method on *Ganoderma lucidum* biomass (A), triterpenoid (B) and ergosterol (C) production.

Table 1. Identification, BPI, VIP values and *P*-values of GAs under the two culture methods of *Ganoderma lucidum*.

NO.	Ret. Time	$[M-H]^-$ *m/z*	UV (254 nm)	BPI						VIP pred	*P*-value	Identification	Type
				SC-4	SC-6	SC-8	LSSC-4	LSSC-6	LSSC-8				
1	14.25	629.2894	+	43.88	2.17	31.41	322.88	197.76	182.01	5.87	1.08E-02	Ganoderic acid Mb/Mc[a]	I
2	15.47	571.2916	+	2.53	0.00	0.00	173.47	180.28	157.52	5.52	1.53E-05	Ganoderic acid Ma/W[a]	I
3	14.71	643.3068	+	2.74	0.00	1.09	185.25	148.72	122.02	5.14	1.20E-03	7-O-methyl ganoderic acid O	I
4	14.93	569.2788	+	0.00	0.00	0.00	125.27	157.62	148.38	5.07	1.17E-04	Ganoderic acid Q	II
5	14.41	587.2913	+	1.22	0.00	0.00	141.82	128.48	123.70	4.85	1.76E-05	Ganoderic acid Mh	I
6	15.65	571.3004	+	0.00	0.00	0.00	94.93	133.16	125.56	4.56	5.43E-04	Ganoderic acid Ma/W[a]	I
7	14.47	629.3060	+	0.00	0.00	1.00	103.77	120.29	115.16	4.50	2.10E-05	Ganoderic acid Mb/Mc[a]	I
8	15.30	611.2816	+	15.91	28.11	4.46	250.62	193.31	169.07	4.26	1.70E-03	Ganoderic acid T	II
9	15.97	585.3181	+	0.00	0.00	0.00	75.90	103.45	89.40	3.99	3.54E-04	Ganoderic acid Md	I
10	16.50	553.2908	+	0.00	14.87	0.00	68.01	110.97	114.65	3.98	4.15E-03	Ganoderic acid R	II
11	14.58	527.2855	+	0.00	0.00	0.00	53.15	85.30	72.61	3.50	1.67E-03	Ganoderic acid V	I
12	12.44	543.2775	+	0.00	0.00	0.00	36.84	62.04	51.93	2.95	2.36E-03	Ganoderic acid Mi	I
13	14.59	601.3106	+	0.00	0.00	0.00	86.85	66.85	22.44	2.60	1.10E-02	Ganoderic acid Mg	I

BPI, base peak intensity; VIP, variable importance for the projection.
a. The two ganoderic acids were indistinguishable, but it did not affect the analysis of the result.

out, the number of reads per library ranged from 40 356 482 to 46 577,572 and from 42 954 950 to 52 910 016 in LSSC and SC respectively (Table S3). All Q20 percentages for the sequences in the 29 libraries were over 98%. All GC levels were between 58.51 and 59.92%. Over 61% of the reads could be mapped to the genome of the *G. lucidum* strain 260125 for each sample.

Differentially expressed gene (DEG) dynamic analysis

Comparative transcriptome analysis was performed to identify the differentially expressed genes (DEGs) between LSSC and SC at different cultural time. Under the threshold value of $|\log_2$ fold change$| > 1$ and *q*-value < 0.005, the DEGs were identified and illustrated in Fig. 3A and Table S4. Comparisons of the samples

showed that there were more upregulated DEGs in SC than in LSSC at the fourth and eighth days. However, more DEGs were upregulated in LSSC from day 5 to day 7 than SC, that was 647, 557 and 51 upregulated DEGs in LSSC. Overall, although the numbers of DEGs were different during the culture time, 303 DEGs were shared at each time point (Fig. 3B). Among all the DEGs, 438 DEGs were common under both conditions, and 2639 DEGs upregulated and 2125 DEGs downregulated in LSSC (Fig. 3C).

KEGG and GO enrichment analysis of the DEGs

All the DEGs were mapped to the KEGG database in order to further understand the putative active biological pathways. The top 20 enriched pathways are shown in Fig. 4. 'Folate biosynthesis' and 'Amino sugar and nucleotide sugar metabolism' pathways were both enriched in LSSC and SC. In addition, the enriched pathways in LSSC were mainly associated with the primary metabolism such as amino acid, sugar and fatty acid metabolism. However, nucleic acid metabolism pathways (including 'Cell cycle - yeast', 'DNA replication', 'Ribosome', 'Meiosis - yeast', 'Aminoacyl-tRNA biosynthesis', 'Mismatch repair') were more active in SC. Interestingly, 'Steroid biosynthesis' pathway was associated with ergosterol biosynthesis.

In addition, the GO enrichment analysis was also performed. As shown in Fig. S2, the upregulated DEGs in LSSC were grouped into three functional categories: biological processes, cellular components and molecular functions. The most significantly enriched GO terms in three functional categories were 'carbohydrate metabolic process' (GO:0005975), 'integral component of membrane' (GO:0016021) and 'hydrolase activity' (GO:0004553) respectively. For downregulated DEGs, the enriched GO terms in three functional categories were 'DNA replication' (GO:0006260), 'organelle' (GO:0043226) and 'DNA binding' (GO:0003677). However, there were no enriched GO terms associated with ergosterol and GA biosynthesis.

DEGs involved in the ergosterol biosynthetic pathway

In fungi, lanosterol is an intermediate metabolite which could produce GAs and ergosterol. According to the gene functional annotation of the *G. lucidum* genome, there were 10 enzymes encoded by 11 genes to synthetize ergosterol from lanosterol. The DEGs involved in the ergosterol biosynthetic pathway during the culture time are shown in Fig. 5. In lanosterol biosynthetic pathway (Fig. 5 left), AACT is encoded by two genes in the *G. lucidum* genome. *AACT-1* was upregulated at days 5 and 6 in LSSC. The *HMGR* gene was upregulated on the

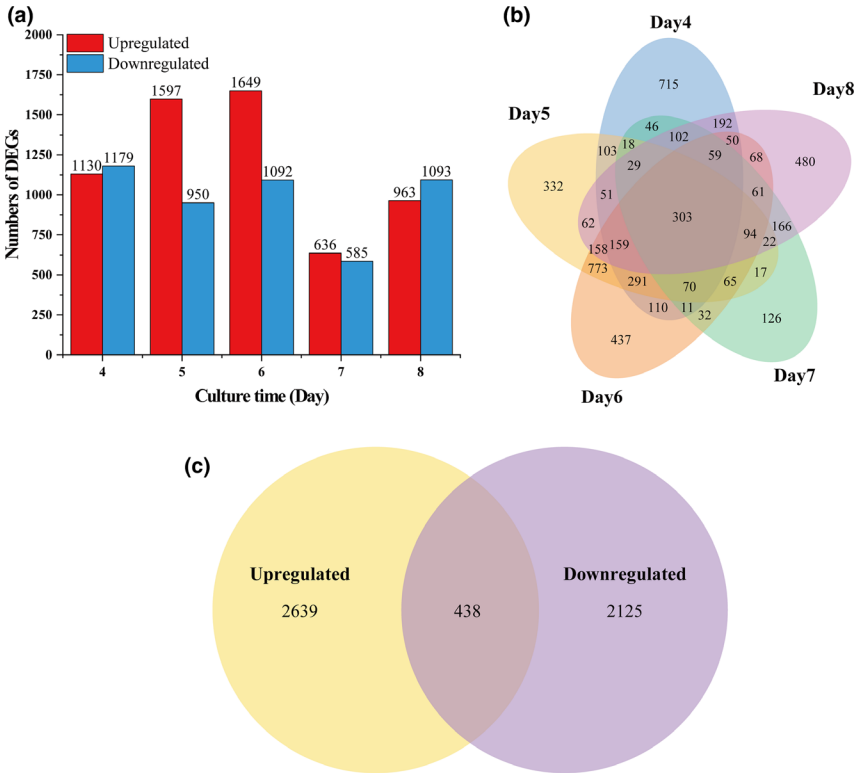

Fig. 3. The differentially expressed genes (DEGs) between LSSC and SC. A: The number of DEGs from day 4 to day 8, B: Venn diagram of the DEG number from day 4 to day 8. C: Venn diagram of all DEG number during the culture time.

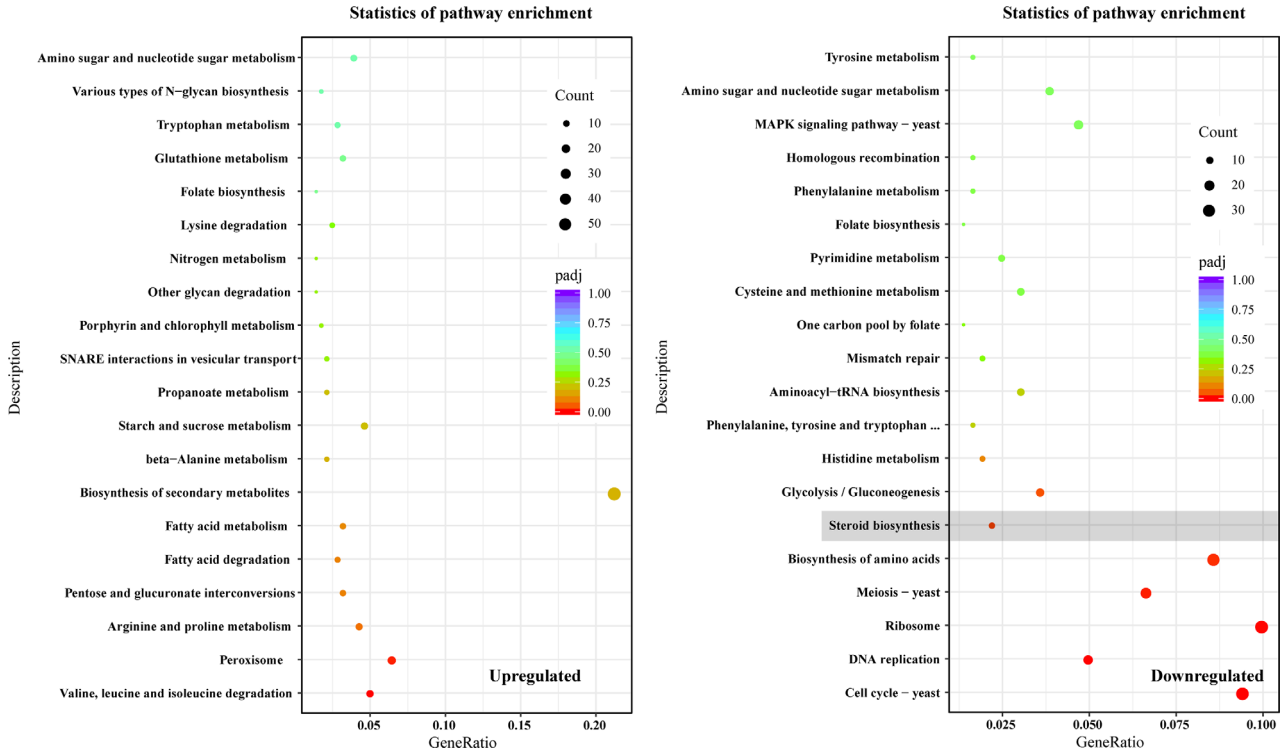

Fig. 4. Statistics of KEGG pathway enrichment. The top 20 enriched pathways were shown. The left is the KEGG analysis of 2639 upregulated genes in LSSC. The right is the KEGG analysis of 2125 downregulated genes in LSSC.

fifth and sixth days and downregulated at the later stage. FPS were also encoded by two genes, and *FPS-1* genes were highly expressed in the LSSC; however, the FPKM (fragments per kilobase of transcript sequence per millions) value of *FPS-1* was lower than that of *FPS-2*. In addition, there were four days when *SE* was downregulated in LSSC. In ergosterol biosynthetic pathway (Fig. 5 right), the first enzyme was ERG11 which is also encoded by two genes in the *G. lucidum* genome. In this study, *ERG11-2* was not expressed (FPKM < 1), and *ERG11-1* gene was higher expressed during the culture period in SC. In addition, *ERG25* and *ERG5* were upregulated at days 4, 7 and 8 in SC. The expression level of *ERG3* was increased during SC process. *ERG6*, *ERG3* and *ERG4* were also upregulated at someday in SC. The other genes were not expressed differently under the two culture methods. The FPKM value of all the genes involved in the ergosterol biosynthetic pathway during culture time is shown in Table S5-1.

DEGs of CYP450s associated with GA biosynthesis

Cytochrome P450 monooxygenases (CYP450s) were considered to be responsible for catalysing the conversion of lanosterol to GAs. According to the literature (Wang *et al.*, 2018), 82 CYP450 genes were selected as candidates for the investigation. In this study, only one gene (*CYP5035U1*) was not expressed. The FPKM values of CYP450 genes associated with GA biosynthesis are shown in Table S5-2. According the DEG numerical statement, twenty-seven CYP450 genes were selected as the significant genes which were differentially expressed for at least three days during the culture time (Table 2). Based on the family classification (Syed *et al.*, 2013), CYP512, CYP5150 and CYP5359 families had 7, 6 and 9 genes respectively. Further, among the twenty-seven CYP450 genes, 10 genes (*CYP5035L2*, *CYP512A13*, *CYP512U5*, *CYP5150J10*, *CYP5150L8*, *CYP5140A3*, *CYP5359A3*, *CYP5359L1*, *CYP5359Q1* and *CYP5359W2*) were upregulated, and 1 gene (*CYP5151A4*) was downregulated from day 4 to day 8 in LSSC. In addition, *CYP5150J1*, *CYP5150K3*, *CYP512U4*, *CYP512U7*, *CYP512V2*, *CYP5359A5* and *CYP5359L3* were significant upregulated genes in LSSC. *CYP5150L4*, *CYP5035K1*, *CYP61A1*, *CYP512X1* and *CYP5359R1* were significant downregulated genes in LSSC. Interestingly, *CYP51F1* and *CYP61A1* were also annotated as *ERG11-1* and *ERG5* respectively.

RT-qPCR analysis

Lanosterol synthase could catalyse the biosynthesis of lanosterol, and reported it was a key gene for GA production (Shang *et al.*, 2010; Xu *et al.*, 2010a; Zhang

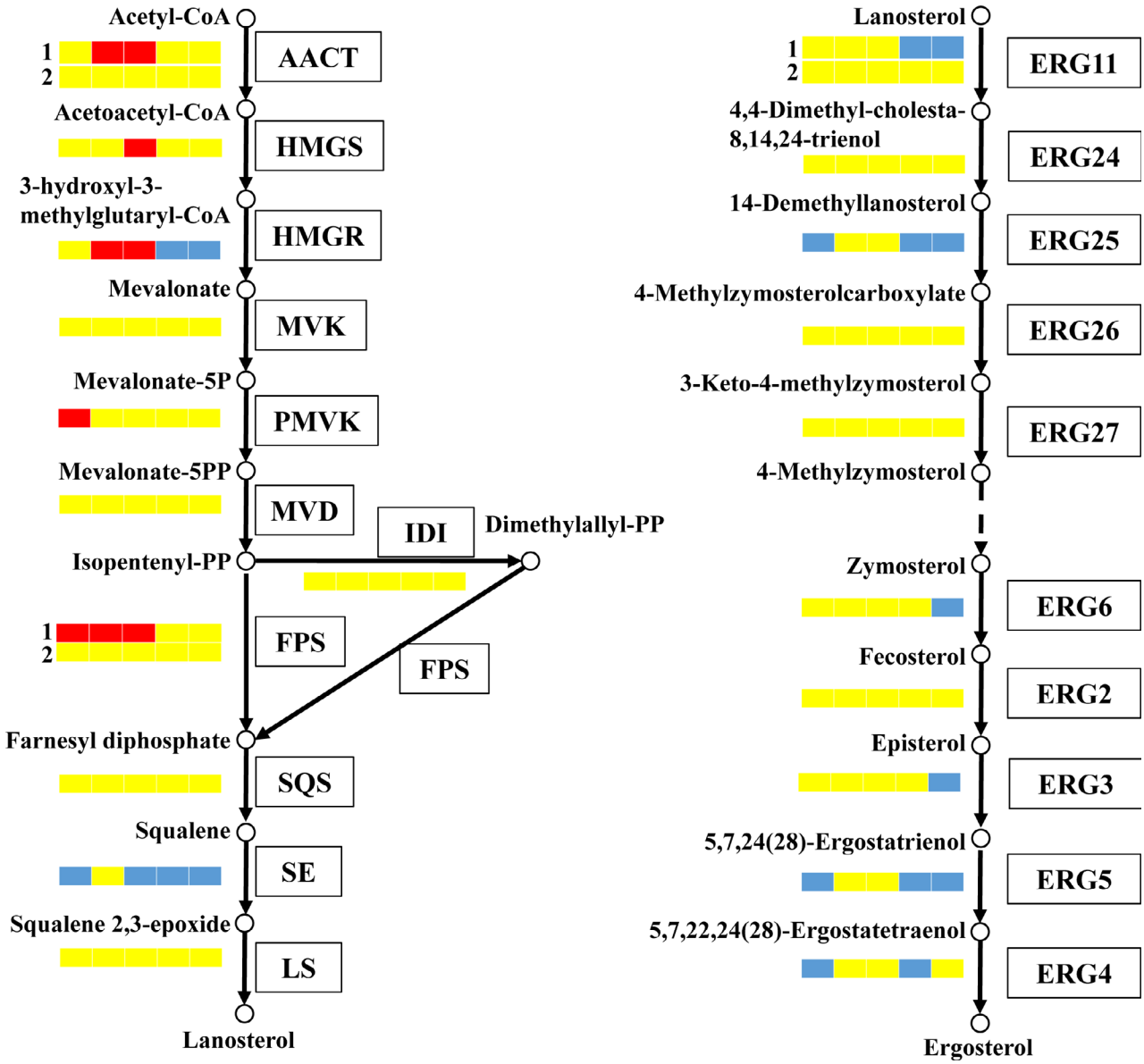

Fig. 5. DEGs involved in the ergosterol biosynthetic pathway in *Ganoderma lucidum* (red: LSSC upregulated; yellow: not DEGs; and blue: LSSC downregulated).

et al., 2017b). However, the expression of *LS* was not different significantly under the two groups in transcriptome. *ERG 11-1* (*CYP51F1*) and *CYP5150L8* are the first gene regulating lanosterol flux into ergosterol and GA biosynthesis respectively. *ERG 11-1* was downregulated on days 7 and 8 in LSSC. Notably, *CYP5150L8* was upregulated from day 4 to day 8 in LSSC. The transcription levels of *CYP512A2*, *CYP512V2* and *CYP512A13* were increased in a GA high-yield *Ganoderma* sp (Xu *et al.*, 2019). *CYP512V2* and *CYP512A13* were significant upregulated genes in LSSC; however, *CYP512A2* was downregulated on fourth day and upregulated on seventh and eighth day. To validate the transcriptome analysis, *LS*, *ERG11-1* (*CYP51F1*), *CYP5150L8* and *CYP512A2* were selected to assess the expression dynamic under the two culture methods by RT-qPCR (Fig. 6). *LS* was upregulated on day 5, and the expression levels on other time points were not significant difference ($P > 0.05$). In addition, *ERG11-1* (*CYP51F1*) was downregulated on days 7 and 8 in LSSC, and *CYP5150L8* was always upregulated during LSSC culture time. *CYP512A2* was downregulated on the sixth day and upregulated on seventh and eighth day in LSSC. For *LS*, *ERG11-1* (*CYP51F1*), *CYP5150L8*

Table 2. The DEGs of CYP450s associated with GA biosynthesis under the two culture methods of *Ganoderma lucidum*.

	Culture time (day)				
CYP450 Gene	4	5	6	7	8
CYP5150J1	/	/	+1.36	+2.62	+1.74
CYP5150J10	+2.14	+3.08	+4.14	+5.76	+5.18
CYP5150K3	+1.30	+2.89	+2.94	/	+1.12
CYP5150L8	+2.91	+4.19	+4.77	+4.44	+5.14
CYP5035L2	+1.73	+2.06	+2.03	+1.24	+1.44
CYP512A13	+1.19	+2.25	+3.98	+1.95	+1.02
CYP512U4	/	/	+1.09	+1.11	+1.24
CYP512U5	+1.89	+1.89	+3.35	+2.82	+2.99
CYP512U7	/	+1.81	+2.29	+2.37	+1.85
CYP512V2	+4.10	+3.47	+4.68	/	+3.04
CYP5140A3	+2.36	+6.95	+6.31	+2.83	+4.05
CYP5359A3	+1.70	+2.91	+2.92	+4.53	+3.98
CYP5359A5	+2.38	+1.97	+2.21	/	+1.41
CYP5359L1	+1.28	+1.48	+1.63	+2.27	+2.75
CYP5359L3	+2.78	+1.55	/	/	+1.45
CYP5359Q1	+2.39	+1.38	+2.01	+2.32	+2.75
CYP5359W2	+1.98	+1.85	+2.29	+2.58	+3.44
CYP5151A4	−1.07	−1.61	−2.23	−1.15	−1.56
CYP5035K1	−2.38	−2.67	−2.38	/	−1.05
CYP5150L4	−2.76	/	/	−1.75	−1.89
CYP61A1	−1.27	/	/	−2.06	−1.87
CYP512X1	/	−1.40	−1.80	−2.05	−2.01
CYP5359R1	−1.06	−1.30	−1.24	/	/
CYP5150L1	−1.48	+1.64	+1.09	/	/
CYP512A2	−1.07	/	/	+2.20	+2.63
CYP5359G1	−1.18	/	−1.04	/	+1.05
CYP5359T1	1.25	/	−1.01	/	+1.07

+, upregulated; −, downregulated; /, not DEG.

and *CYP512A2,* the transcriptional changes obtained by RT-qPCR were in accordance with those obtained in the transcriptome.

Discussion

Liquid superficial-static culture has been shown to be an efficient strategy to produce *G. lucidum* GAs and total triterpenoid. In SC, ergosterol was the major triterpenoid, and the content was higher than that in LSSC. However, the synthesis and regulation mechanisms of ergosterol and GAs remain elusive. In this study, 13 individual GAs were identified, and all of these GAs were accumulated in LSSC compared with SC. Additionally, transcriptome dynamics analysis revealed a set of key factors for ergosterol and GA synthesis and regulation.

Ganoderic acids are synthesized via the MVA pathway in *G. lucidum*. Lanosterol was the common cyclic intermediate of triterpenoids and ergosterol in *G. lucidum*, with different metabolic pathways diverging from this compound. In this study, we investigated the expression levels of the related genes in the lanosterol biosynthesis pathway. AACT played a fundamental role in the lanosterol biosynthesis pathway because it was the first step that AACT could catalyse the formation of acetoacetyl-CoA (Fang *et al*., 2013). However, only *AACT-1* gene expressed differently on fifth and sixth days which was upregulated in LSSC that meant it may be a regulated gene for total triterpenoid biosynthesis. *HMGR* was also recognized as a key point for GA biosynthesis, because the transcription levels of *HMGR* were increased in a GA high-yield *Ganoderma* sp (Xu *et al*., 2010a, 2019), and ganoderic triterpenoid content was increased in the *HMGR* overexpressed strain (Xu *et al*., 2012). In this study, *HMGR* gene was upregulated on days 5 and 6; however, it was downregulated in the next two days. Farnesyl diphosphate is located at the first multiple-branch point in the isoprenoid biosynthetic pathway, and the changes of FPS activity could affect the flux of isoprenoid compounds through these various pathways (Ding *et al*., 2008). FPS was also encoded by two genes, and the FPKM value of *FPS-2* was higher than that of *FPS-1*. In *G. lucidum*, *FPS* gene, actually it is *FPS-2* gene, overexpression was an effective strategy to improve the production of GAs (Fei *et al*., 2019). Although *FPS-1* gene had a low expression level, it was upregulated from day 4 to day 6 in LSSC which meant it need to be further study for the role in triterpenoid biosynthetic pathway. SE, also called squalene monooxygenase, can catalyse the first oxygenation step in lanosterol biosynthesis by converting squalene to 2,3-oxidosqualene (Zhang *et al*., 2017a). In LSSC, the *SE* gene was downregulated during the culture time except for day 5; however, this did not affect triterpenoid production. An earlier reported had found the opposite that overexpression of the SE gene could produce approximately 2 times more GA than the wild type (Zhang *et al*., 2017a). In addition, *SQS* and *LS* genes are associated with GA production (Xu *et al*., 2010a). However, these genes did not show significant differences under the two culture methods. These results indicated that the GA synthesis and regulatory mechanism in LSSC were unlike in other reports, and the *AACT-1* gene and *FPS-1* genes may be the regulatory genes in the lanosterol biosynthetic pathway under the LSSC method to produce triterpenoid and GAs. In addition, the genetic editing with CRISPR has been reported in *G. lucidum*. Wang *et al*. (2020) constructed a CRISPR/CRISPR-associated protein-9 nuclease (Cas9) editing system and investigated the effect of different gRNA constructs with endogenous u6 promoter and self-cleaving ribozyme HDVon ura3 disruption efficiency. Using this genome editing platform, *CYP5150L8* and two *CRZ* genes were disrupted to reveal their function in GA biosynthetic pathway (Li and Zhong, 2020; Wang *et al*., 2020). Liu *et al*. (2020) improved the CRISPR/Cas9-mediated gene disruption frequency in *G. lucidum* by adding an intron upstream of the Cas9 gene and achieved the genomic

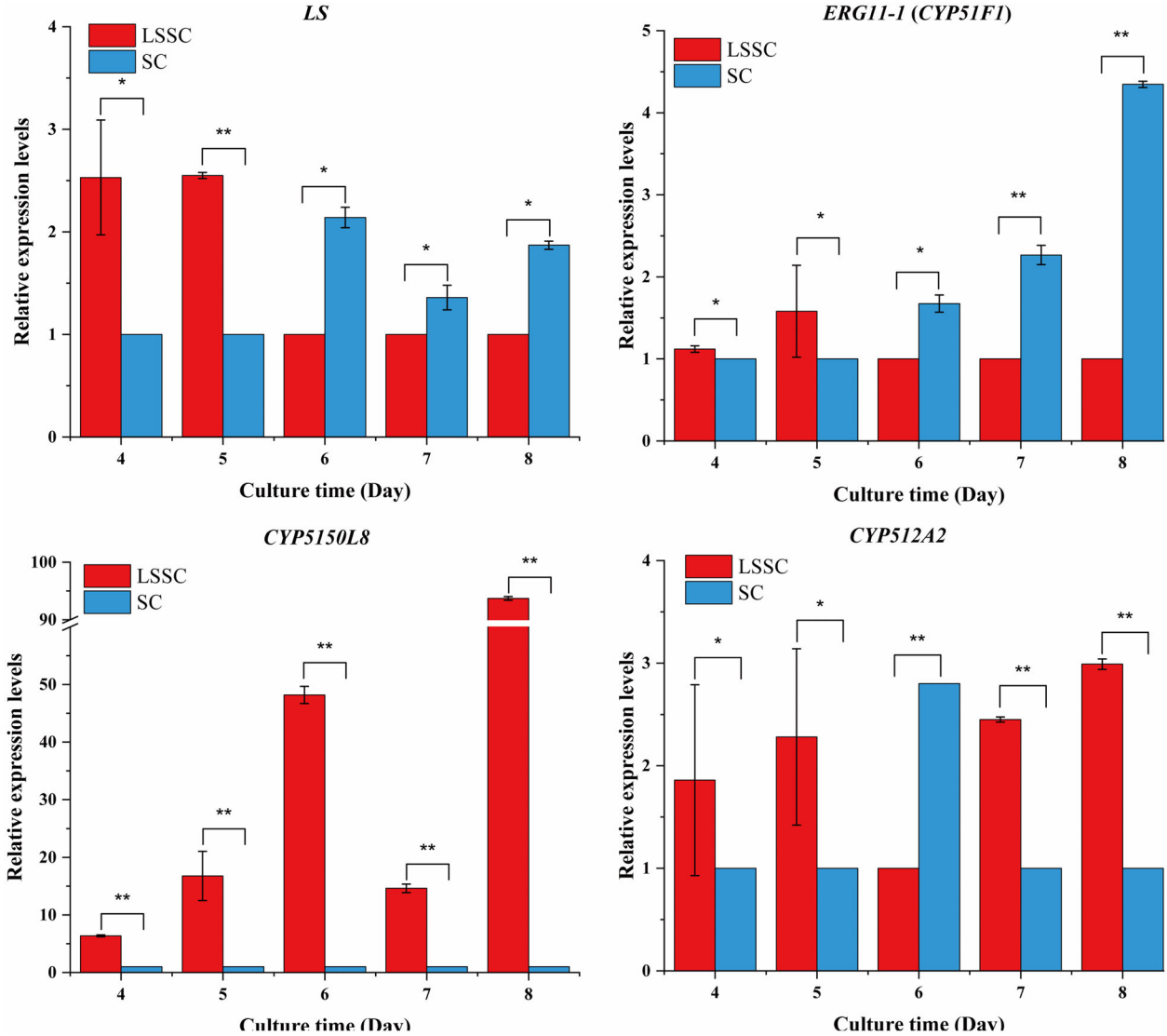

Fig. 6. RT-qPCR analysis of genes associated with GA biosynthesis in *Ganoderma lucidum*. The significantly analysis used the low-expressed group as a reference (**: $|\log_2 \text{fold change}| > 1$ and $P < 0.05$; *: no significance). The fold change is denoted the ratio of the transcription level in the high-expressed group to that in the low-expressed group.

fragment deletions via a dual sgRNA-directed CRISPR/Cas9 system in *G. lucidum*. So the detail regulatory mechanism of the key DEGs which obtained from this study needs to be further investigated by genetic editing.

Ergosterol is an important product of a long multistep biosynthetic pathway and is derived from lanosterol. However, few studies were focused on the ergosterol synthesis pathway in *G. lucidum*. In the *G. lucidum* genome, 11 genes encoding 10 enzymes were identified in the ergosterol biosynthetic pathway. Lanosterol 14-demethylase is the first enzyme encoded by two *ERG11* genes that catalysed the lanosterol to form ergosterol. The encoded protein is a cytochrome P450, which is also known as CYP51F1 (Ceita *et al.*, 2014). In some pathogenic fungi, lanosterol 14-demethylase, as a key enzyme in the ergosterol biosynthetic pathway in fungi, was used as a target for antifungals (Alcazar-Fuoli and Mellado, 2013). Therefore, this protein plays a central role in the regulation of lanosterol flux. In this study, expression analysis of the two *ERG11* genes showed diametrically different results that *ERG-2* gene was not expressed under the different culture methods. The FPKM value of *ERG11-1* was much higher in SC than in LSSC. Furthermore, the *ERG11-1* gene was upregulated in SC on days 7 and 8, and the transcript profile according to RT-qPCR correlated with the transcriptome

profiling data. These results indicated that *ERG11-1* was the key gene regulating lanosterol flux into ergosterol biosynthesis. The *ERG25*, *ERG5* and *ERG4* genes were also upregulated at last two days and *ERG3* had a higher FPKM value in SC during the culture period. Like *ERG11-1*, *ERG5* is a CYP450 family gene (*CYP61A1*) and both of them require the hemoprotein Dap1 for full activity (Hughes *et al*., 2007). *ERG25* and *ERG3* encode oxodiiron enzymes of the fatty acid hydroxylase/sterol desaturase family (Shakoury-Elizeh *et al*., 2010), indicating that the genes had similar regulatory mechanisms. Six of the ten expressed genes in ergosterol biosynthetic pathway shown upregulated at some time points in SC, and the results illustrated that SC was favourable to produce ergosterol.

CYP450s are considered responsible for catalysing the conversion of lanosterol to GA in *G. lucidum* (Wang *et al*., 2018; Yang *et al*., 2018). As the first characterized CYP450, the *CYP5150L8* gene was highly expressed in *G. lucidum* in LSSC during the culture time, and it was also upregulated in RT-qPCT analysis. In the case of a *CYP5150L8* disruptant, amounts of four GAs were significantly reduced which indicated that the *CYP5150L8* gene was essential for the overall pathway of GA biosynthesis from lanosterol in *G. lucidum* (Wang *et al*., 2018, 2020). Additionally, a high number of type I GAs were synthesized in LSSC. The results indicated that the upregulation of the *CYP5150L8* gene directed lanosterol flux towards GA biosynthesis, especially type I GA. Syed *et al*. (2014) reported that CYP5150 family member P450s played a key role both in xenobiotic compound oxidation and fungal primary and secondary metabolism. In *Thanatephorus cucumeris*, CYP5150A family P450 enzymes were steroidal hydroxylases (Lu *et al*., 2019). In this study, *CYP5150J1*, *CYP5150J10* and *CYP5150K3* genes were also highly expressed in LSSC, which clearly suggested that these CYP450 genes in *G. lucidum* might also possess hydroxylase activity and associate with GA biosynthesis. In *G. lucidum*, CYP512 family enzymes were hypothesized to biosynthesize triterpenoids (Chen *et al*., 2012). The *CYP512A2*, *CYP512V2* and *CYP512A13* genes were upregulated in a high-GA-yield strain (Xu *et al*., 2019). Notably, the expression of the *CYP512V2* and *CYP512A13* genes was also higher in LSSC than in SC. Moreover, the *CYP512U4*, *CYP512U5* and *CYP512U7* genes were highly expressed in LSSC, which should be further studied. The differences in expression of CYP512 family genes in *G. lucidum* might explain the variety of individual GAs observed in this study. CYP5140 family has only 1 sequence (*CYP5140A3*) in *Ganoderma* (Kües *et al*., 2015). The CYP5359 family is a newly identified family in *Ganoderma* sp. and is exclusively present and highly enriched in *Ganoderma* sp (Syed *et al*., 2014). Although the detail functions of many CYP450s are unknown, our research narrowed the selections of GA biosynthesis associated CYP450s.

In this work, we identified thirteen GAs from *G. lucidum* mycelia by UPLC-QTOF-MS, and the content of all GAs was higher in LSSC than in SC. By combining the dynamic analysis of DEGs associated with GA biosynthesis, we found *CYP5150L8* was the key gene regulating lanosterol flux into GA biosynthesis, and other sixteen CYP450 genes could be potential candidate genes associated with the biosynthesis of different GAs. In addition, ergosterol was accumulated during the SC process, and six of the ten expressed genes in ergosterol biosynthetic pathway shown upregulated at some time points in SC. This study not only provides a general picture of ergosterol and GA biosynthesis, but also highlights the key CYP450 genes associated with GA biosynthesis. However, the detail regulatory mechanism of the key DEGs obtained from this study needs to be further investigated in the future by gene disruption or chemical inhibitors. Meanwhile, for the utilization of LSSC at the industrial level is also need to use the low-cost complex media. We believe the conclusion obtained from this study is helpful for understanding the GA profile in the future research.

Experimental procedures

Strains and medium

Ganoderma lucidum CGMCC5.26 was purchased from China General Microbiological Culture Collection Center (CGMCC) and maintained on potato dextrose agar (PDA) slants at 4 °C. In order to get consistent metabolites and transcriptome data, a semi-synthetic media was selected in this study. The medium was composed of glucose 20 g l^{-1}, yeast nitrogen base without amino acids (YNB) 5 g l^{-1}, tryptone 5 g l^{-1}, KH_2PO_4 4.5 g l^{-1} and $MgSO_4{\cdot}7H_2O$ 2 g l^{-1} at initial pH. Glucose, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ purchased from Sinopharm (Beijing, China), YNB purchased from BioDee Biotechnology Co., Ltd (Beijing, China), and tryptone purchased from OXOID (Hants, UK). The medium has been used many years to study the secondary metabolism of *G. lucidium* (Peng *et al*., 2015; Ma *et al*., 2018, 2019). The seed was in a 250-ml flask containing 80 ml medium and kept at 30 °C on a rotary shaker (150 rpm) for ten days.

Liquid superficial-static culture and submerged culture

For LSSC, medium was first mixed with *G. lucidum* seed which was homogenized by IKA T10 basic homogenizer (IKA, Königswinter, Germany) and then 4 ml of mixed

fermentation medium was transferred into T25 rectangular canted-neck cell culture flasks with a vent cap (Corning, Oneonta, USA) and cultured statically on an incubator. The control group was SC in a 500-ml flask with 150 ml of medium and cultured on a rotary shaker at 150 rpm. Other culture conditions of LSSC and SC were the same, where the homogenized seed was centrifuged at 10 000 *g* for 5 min, and 3.5 g wet weight of cells l^{-1} was inoculated to the medium, and cultured at 30 °C for 8 days.

Sampling and analysis of biomass, triterpenoid and ergosterol

Mycelia were harvested by centrifugation (10 000 rpm) for 10 min. Because of evaporation, the culture broth volume was restored to the initial value by adding distilled water before centrifugation. The precipitate was washed three times with distilled water and dried by lyophilization. The biomass was determined by the gravimetric method of determining dry cell weight (DCW).

The total triterpenoid content was evaluated according to a protocol described by Oludemi *et al.,* (2018). In brief, the lyophilized *G. lucidum* mycelia were dissolved in methanol and extracted for 2 h by ultrasonication. The extracts were centrifuged, and the supernatants were dried at 100 °C. Vanillin-glacial acetic acid solution (5% w/v) and perchloric acid solution (70%) were added to the dry extracts. Then, the sample solutions were incubated at 60 °C for 20 min and cooled in an ice-water bath. After adding glacial acetic acid, the sample was measured at 550 nm. Ursolic acid was used as a standard.

Ergosterol was analysed according to the methods of Xu *et al.,* (2010a) with some modifications. The supernatant containing the total triterpenoid was evaporated to dryness under N_2. The residue was saponified by a 10% (wt/vol) KOH–75% (vol/vol) ethanol solution at 50 °C for 2 h. The mixture was extracted with hexane three times. The hexane layer was collected and evaporated to dryness under N_2. The residue was dissolved in methanol and subjected to high-pressure liquid chromatography (HPLC) on a Thermo U3000 (Thermo Fisher Scientific, Germering, Germany) apparatus equipped with a Symmetry C18 5.0 column (4.6 mm × 250 mm, Waters, Milford Massachusetts, USA). Separation of ergosterol was achieved using a mobile phase of 100% methanol at a constant flow rate of 1.0 ml min^{-1}. Ergosterol was monitored at a wavelength of 282 nm. Identification and quantification of ergosterol was performed based on retention time, UV spectra and external calibration graphs obtained for an ergosterol standard (Sigma-Aldrich, St. Louis, MO, USA). For the analysis of biomass, triterpenoid and ergosterol, three biological replicates were performed.

UPLC-QTOF-MS conditions and statistical analysis

After extraction of the total triterpenoids, the samples were analysed by an ACQUITY UPLC system coupled with QTOF mass spectrometry (Waters, Milford, MA, USA). The UPLC column was a Waters BEH C-18 column (50 × 2.1 mm, 1.7 μm, Massachusetts, USA). Gradients of solvent A (methanol) and solvent B (0.1% formic acid) were prepared as follows: (i) 0–20 min (A:B, 3:7, v/v), (ii) 20–25 min (A, 100, v/v) and (iii) 25–30 min (A:B, 3:7, v/v). The flow rate was 0.3 ml min^{-1}. The injection volume was 2 μl, and the column temperature was maintained at 45 °C. The mass spectrometer was operated with a capillary voltage of 3.0 kV and a sampling cone of 20 V in negative mode. The other parameters used were as follows: nitrogen gas flow rate of the nebulizer of 50 L h^{-1}; desolvation gas flow rate of 700 l h^{-1}; desolvation temperature of 400 °C; and source temperature of 100 °C. Mass spectra were recorded across an *m/z* range of 50–1500 with a 0.5 s scan time. The collision energy spectrum was recorded at 6.0 and 35 eV. The scanning wavelength of the diode array detector ranged from 200 to 400 nm.

Masslynx (MarkerLynx XS) V4.1 software (Waters, Milford, USA) was used for alignment, deconvolution and data reduction. The basic operational steps were described by Lin *et al.,* (2019). The differences were that the main parameters included a retention time range of 4 ~ 28 min, mass range of 50 ~ 1200 Da, mass tolerance of 0.05, mass window of 0.05, retention time window of 0.20 and noise elimination level of 6. Simca 15.0 software (Umetrics, Malmö, Sweden) was used to visualize the VIP (variable importance for the projection) analysis results.

Transcriptome sequence and assembly

RNA isolation, cDNA library construction, sequencing library preparation and high-throughput sequencing were carried out by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China, http://www.novogene.com). The procedure was summarized in the Supporting Information. The RNA-seq data were obtained in three biological replicates and had been deposited in the NCBI Sequence Read Archive (SRA) database (BioProject: PRJNA643653 and Submission: SUB7707164).

Gene expression analysis by RT-qPCR

RNA isolation and RT-qPCR were performed as previously reported by Zhang *et al*. (2020). The primers were

designed using NCBI Primer Blast and referring to previous research (Table S1) (Xu *et al*., 2010a, 2019). For each gene analysed by RT-qPCR, three biological replicates were performed. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. The representative base peak intensity chromatograms (negative modes) of metabolites from *G. lucidum* in LSSC and SC.

Fig. S2. GO functional classification of differentially expressed genes.

Table S1. Oligonucleotide primers used in this study.

Table S2. The identification of GAs by databases and references.

Table S3. Summary of transcriptome sequencing results.

Table S4. The differentially expressed genes (DEGs) between LSSC and SC during the culture time.

Table S5-1. The FPKM value of all the genes involved in the ergosterol biosynthetic pathway during culture time.

Table S5-2. The FPKM values of CYP450 genes associated with GA biosynthesis.

