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IAA Biosynthetic Pathway and Related Genes Analysis in *Ganoderma lucidum*

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Key words: *Ganoderma lucidum*; IAA determination; IAA synthesis pathway; gene expression
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Abstract: *Ganoderma lucidum* (*G. lucidum*) is a widely used macro-fungus of traditional Chinese medicine and its demand is increasing. G. lucidum has been regarded as a flagship species for the research on medicinal fungi growth, development and secondary metabolism. Auxin has long been recognized as a hormone

essential for almost every aspect of plant growth and development. Auxin widely presented not only in plants, but also in the endogenous bacteria and mycorrhizal fungi. Different from endogenous bacteria and mycorrhizal fungi for promoting the growth of host plants, IAA secreted by macro-fungi was deduced to be mainly for their growth and development. It is lack of research in IAA synthetic pathway in macro-fungi until now. In the previous research, we discovered and reported the endogenous auxin in the mycelium of *G. lucidum* for the first time. The synthesis pathway of auxin and its effect on *G. lucidum* should be meaningful. In this study, the endogenous auxin IAA was determined by HPLC at different stages of *G. lucidum* fruiting body morphogenesis. The maximum IAA content was 253.313 μg/g and appeared in the second period which was the rapid growth period during *G. lucidum* fruiting body morphogenesis. The synthesis pathway of IAA in *G. lucidum* was predicted based on the genome. The prediction results showed that a complete TAM pathway for IAA biosynthesis pathway might exist in *G. lucidum*. IPA and IAM pathway also were proposed. The relative expression of key enzymes gene in the predicted IAA synthesis pathway at different stages of *G. lucidum* was determined by qRT-PCR. Corresponding to the maximum IAA content, the relative expression of related IAA biosynthetic genes was also the highest in the second period in addition to *ami* gene (GL29633-R1). These results provide insights into the connection between the IAA biosynthesis and the different growth stages in *G. lucidum*. It was significative for further elucidating the relationship between the endogenous auxin and the growth of *G. lucidum*.

Introduction

Ganoderma lucidum (Curtis; Fr.) P. Karst. is one of the best-known medicinal macrofungi in east Asia and its pharmacological activities are widely recognized. Modern pharmacological research has demonstrated that *G. lucidum* exhibits multiple therapeutic activities, including anti-tumor, anti-hypertensive, antiviral and immune modulatory activities[1]. *G. lucidum* has been regarded as a flagship species (model organism) for the research on medicinal fungi growth, development and secondary metabolism with its simple culture condition, short growth cycle and rich active ingredients[2].

Auxin has long been recognized as a hormone essential for almost every aspect of plant growth anddevelopment[3]. Indole-3-acetic acid (IAA) is the main auxin produced by plants, synthesized by tryptophan (Trp)-dependent pathway and Trp-independent pathway. It has been clearly demonstrated that Trp-dependent auxin biosynthesis is essential for embryogenesis, seeding growth, flower development, vascular pattern formation and other development processes[4]. IAA is synthesized from Trp via four possible pathways: the tryptamine(TAM) pathway, indole-3-pyruvic acid (IPA) pathway, indole-3-acetonitrile (IAN) pathway and indole-3-acetamide(IAM)pathway. It was presumed that IPA pathway and TAM pathway are the main types of tryptophan dependent pathway in IAA biosynthesis[5]. The IAM pathway only exists in the microorganism and the IAN pathway was found in few plants[4,6] have defined a simple two-step pathway of auxin from Trp in *Arabidops*: The first chemical step of auxin biosynthesis is the removal of the amino group from Trp by the Tryptophan Aminotransfer of *Arabidopsis* (TAA) family of transaminases to generate indole-3-pyruvate (IPA). IPA then undergoes oxidative decarboxylation catalyzed by the YUCCA (YUC) family of flavin monooxygenases to produce IAA. The two-step conversion of Trp to IAA has been considered a main auxin biosynthesis pathway that plays an essential role in many developmental processes.

Auxin widely presentednot only in plants, but also in the endogenous bacteria and mycorrhizal fungi. The key enzymes genes of auxin synthesis process were reported from *Volvariella volvacea* [7]. In previous studies, we discovered the endogenous auxin in the mycelium of *G. lucidum*[8].

Different from endogenous bacteria and mycorrhizal fungi for promoting the growth of host plants, IAA secreted by macrofungi was mainly for their growth and development. It is lack of research in IAA synthetic pathway in macrofungi until now. To the best of our knowledge, we report the auxin synthetic pathway in *G. lucidum* for the first time. In this study, HPLC were used to detect IAA at different growth stages of *G. lucidum*. The Trp-dependent pathway of IAA in *G. lucidum* was predicted with the premise of the genome. The key enzymes genes in the synthetic pathway of IAA were analyzed and real-time PCR was used to detect the expression of key enzymes genes. The effect of endogenous IAA on the growth and development of *G. lucidum* was to be deduced.

1 Materials and Methods

1.1 Strains, culture conditions and weight determination

The strain of *G. lucidum* S_9 was from the biological fermentation laboratory at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences &Peking Union Medical Collage, originated from the strain CGMCC5.533 (China General Microbiological Culture Collection Center) after spaceflight[9]. The diameter of short-cut wood used was 12 cm, sterilized and then the strain inoculated, cultured at 25 ℃ with dark for 60 days, and moved into greenhouse.

Four different development stages were divided: the first periodwas primordia initiation; the second period stipe development; the third period pileus differentiation; the fourth period the spore ejection. Samples were randomly selected and taken for each period. The fresh and dry weight were determined.

1.2 Determination of IAA by HPLC

10.0 g fresh fruiting body was accurately weighed, grinded with mortar and pestle in liquid nitrogen, added 100mlpre cooled 80% methanol, extracted overnight at 4 ℃. Three independent biological replicates were performed for every period. The solution was centrifuged under 5000 r/min for 15 min, supernatant fluid retained, detritus extracted again for 1 hr, centrifuged as the first time and supernatant fluid combined. The supernatant was evaporated at 40 ℃. After adjusted pH to 8.0 with 1 mol/L disodium hydrogen phosphate, aqueous solution was extracted 3 times with petroleum benzin and ethyl acetate (V:V=1:1), discarded the organic phase. Added 1g polyvinylpyrrolidone to the aqueous solution to adsorption of phenolic compounds, shaked 30 min with shaker, centrifuged and retained supernatant fluid. Adjusted the pH of supernatant fluid to 3.0 with 2 mol/L citric acid, then extracted 3 times with equal volume of ethyl acetate, combined ester phase. The ester phase was evaporated to dryness under 40 ℃ and the residue was dissolved in methanol, transferred to a 10 ml volumetric flask. The samples were filtered through a 0.22um micro-filtration membrane. The chromatographic column was Diamonsil C18 column (4.6 mm×250 mm, 5 μm). The mobile phase consisted of methanol-0.6% acetic acid with a gradient elution from 5%-50% methanol over 10-30 min, 50% methanol. The flow rate was 1.0 ml/min and column temperature was set at 35 ℃ with sample volume 20 μL. The detection wavelength was 254 nm. The chromatogram is shown in Figure 2.

1.3 Preparations of standard solution and calibration curves

The reference substance IAA 0.1 g (purity 98%) was accurately weighed. The chromatographic pure methanol was added to dissolve and constant volume to 100 mL with the concentrations 1 mg/mL. Detect 10 μg/mL, 0.1 μg/mL and 1 ng/mL reference substance by C18 chromatography column. The IAA detection limit was 0.1 μg/mL. According to the content of

IAA in the sample, 5, 10, 20, 30, 40, 50, 100, 200, 300, 400, 500 μg/mL IAA standard solution was prepared. 20 μl standard solution was taken to analyze under the chromatographic conditions respectively. Draw a standard curve and make regression calculation with the mass concentration of the reference as abscissa, peak area as the ordinate. The results showed that the regression equation of IAA was Y = 1E + 06X-89160, R2 = 0.9985.

1.4 Prediction of IAA synthesis pathway

Download thecds sequence of *G. lucidum* on http://www.herbalgenomics.org/galu[9]. Then upload the prediction coding gene to KEGG web site, using KAAS automatic annotation server (http://www.genome.jp/tools/kaas/) to predict the whole genome metabolic map of *G. lucidum*. Best hit bi-directional method was carried out, the selected 38 reference organisms were: hsa, dme, cel, ath, sce, cho, eco, nme, hpy, rpr, bsu, lla, cac, mge, mtu, ctr, bbu, syn, bth, dra, aae, mja, ape, cne, ppl, mpr, uma, mgl, tml, yli, ncr, ssl, ani, aor, ang, osa, cme, tet. (E-value <0.00001), other parameters default. Abbreviations of each species could be queried on http://www.genome.jp/kaas-bin/kaas_org? tax = /bio/user/ideas/kaas/references/taxonomy&genome = /bio/user/ideas/kaas/references/genome.

1.5 Sequence annotation, phylogenetic analysis and motif detection

The theoretical isoelectric point (pI) and molecular weight (Mw) of the retrieved sequences were predicted using the Compute pI/Mw tool on the Protparam (http://web.expasy.org/protparam/) [10]. Conserved domain predicted scan analysis using Pfam (http://pfam.xfam.org/)[11], Motifs were detected using MEME (version 3.0)[12]. Phylogenetic trees were constructed using MEGA 5.0 with the neighbor-joining method[13]. Bootstrap test was replicated 1000 times.

1.6 RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from fruiting bodies using the Quick RNA Isolation Kit (Huayueyang, China). RNA integrity was analyzed on a 1.0% agarose gel. RNA quantity was determined using a NanoDrop 2000C Spectrophotometer (Thermo Scientific, USA). cDNA synthesis was carried out using FastQuant RT Kit (Tiangen, China). qRT-PCR were performed with the SuperReal PreMix Plus (SYBR Green) kit (TIANGEN, China) in triplicate. Gene-specific primers (Table 1) were designed using Primer Premier 5.0. The length of amplicons is between 150 bp and 250 bp. Gpd was selected as a reference gene as described previously. SYBR Green RT-PCR reactions of samples were performed using 2 × SuperReal PreMix (TIANGEN, China) in 10 μl volumes containing 1 μl of cDNA template and 10 μM forward and reverse primers. The program used was: 15min at 95 ℃; 40 cycles of 98 ℃ (15 s), 60 ℃ (30 s); with a melting curve from 60 ℃ to 95 ℃ (increment 0.5 ℃/s). Reactions were run in BioRad CFX96 (BioRad, USA). Three independent biological replicates were performed. Statistical analysis was carried out as described. $2^{-\Delta\Delta Cq}$ was used to achieve results for relative quantification. For statistical analysis, ANOVA (analysis of variance) was calculated using SPSS (Version 17.0, IBM, USA).

2 Result

2.1 Growth and development of *G. lucidum* fruiting body

Figure 1 shows the growth status of the fruiting bodies of *G. lucidum* in four periods. The fruiting bodies were the heaviest at the fourth period and the average dry weight was 101.0 g.

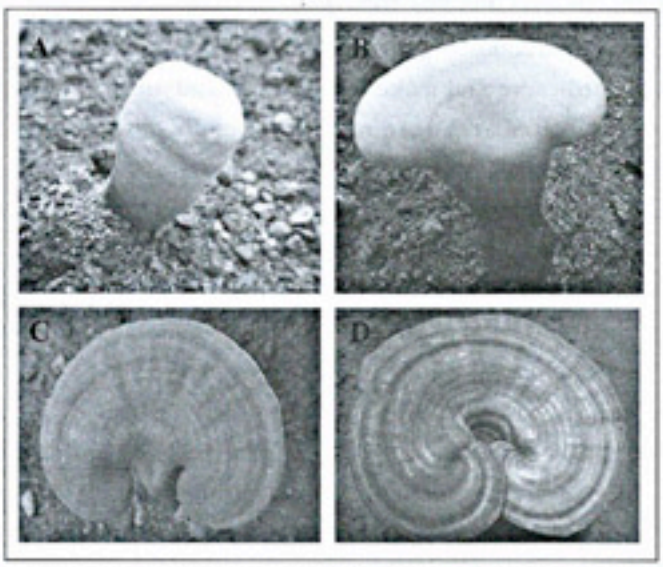

Figure. 1 Fruiting maturity of *G. lucidum* at different stages

2.2 IAA content in different stages of *G. lucidum*

HPLC was used to detect the IAAcontent at different stages. The chromatogram was showed in figure 2. The peaks of IAA appeared in about 16 min, and the retention time was stable among replicates. It has been proved the content of endogenous IAA in *G. lucidum* could be detected by HPLC (Figure 2). The output of IAA of the second stage (253.313 μg/g) was significantly higher than the first (7.677 μg/g), the third (48.716 μg/g) and the fourth stage (6.786 μg/g). This might because the second stage was the fastest growth period (Figure 3).

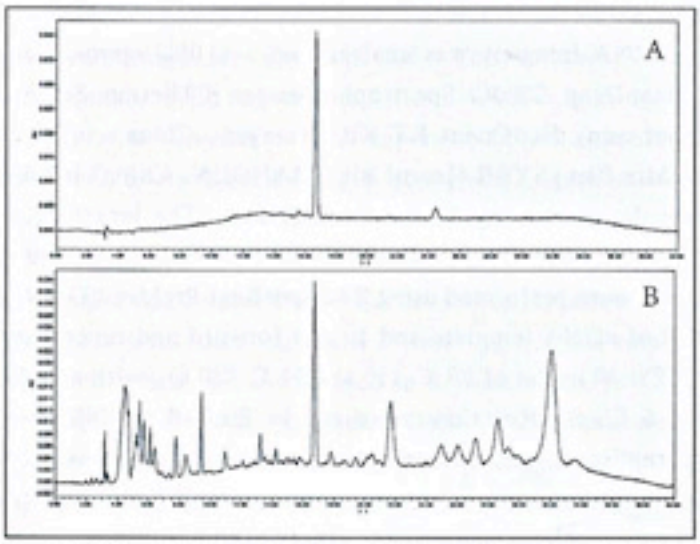

Figure. 2 HPLC chromatograms of reference substance (A) and the third period (B)

2.3 Genomic analyses to predict the IAA biosynthetic pathways in *G. lucidum*

KAAS automatic annotation server was used to predict IAA biosynthesis. IAA biosynthesis was contained in tryptophan metabolism pathway and the number was 00380 on the KEGG. 41 gene sequences were matched to the tryptophan metabolism pathway, and 13 gene sequences related to the synthesis of IAA (Figure 4): two Tryptophan aminotransferase (Tam1), an aromatic-L-amino-acid decarboxylase (DDC), a diamine oxidase (AOC1), six aldehyde dehydrogenase (NAD+)

(Aldh) and three amidase (AMI). Prediction result indicated that the complete TAM pathway was found in *G. lucidum*. Trp was first converted to Trpytamine (TAM) by the DDC of amino decarboxylase and TAM was catalyzed to Indole-3-acetaldehyde (Aldh) by AOC1, subsequently IAA was produced from IALD by the Aldh family of dehyde dehydrogenase. In *G. lucidum*, meanwhile, the IPA pathway and IAM pathway may also existed for the Tam1 in the IPA pathway and the AMI in the IAM pathway also were predicted.

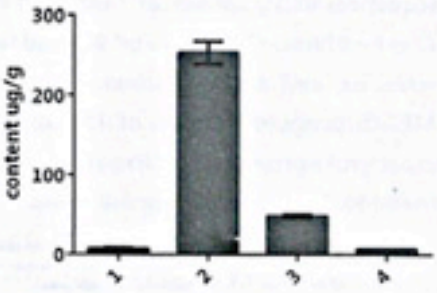

Figure. 3 Variation of IAA in *G. lucidum* at different stages

Table 1 Reference table for enzymes and genes

Gene ID	Name	EC number	CDS (bp)	AA (aa)	PI	MW (Da)	Conserved domain	hmm acc
GL24771-R1	Tam1	2.6.1.27	1200	398	8.51	44053.32	Aminotran-1-2	PF00155.19
GL31258-R1	Tam1	2.6.1.27	1131	376	6.09	41710.42	Aminotran_1_2	PF00155.19
GL21006-R1	DDC	4.1.1.28	1536	510	5.83	56934.83	Pyridoxal_deC	PF00282.17
GL22246-R1	AOC1	1.4.3.22	2808	934	6.55	105013.11	Cu-amine-oxid	PF01179.18
GL30174-R1	Aldh	1.2.1.3	1584	526	6.24	57717.85	Aldedh	PF00171.20
GL24130-R1	Aldh	1.2.1.3	3054	1016	6.05	112172.97	Aldedh	PF00171.20
GL26572-R1	Aldh	1.2.1.3	1602	532	7.48	57823.88	Aldedh	PF00171.20
GL23433-R1	Aldh	1.2.1.3	1659	551	9.19	60799.88	Aldedh	PF00171.20
GL20614-R1	Aldh	1.2.1.3	1437	476	6.53	52510.35	Aldedh	PF00171.20
GL18415-R1	Aldh	1.2.1.3	1695	563	6.30	59213.00	Aldedh	PF00171.20
GL29633-R1	AMI	3.5.1.4	1659	551	4.84	57505.06	Amidase	PF01425.19
GL20521-R1	AMI	3.5.1.4	1662	552	5.50	61006.06	Amidase	PF01425.19
GL18499-R1	AMI	3.5.1.4	1743	579	5.01	62814.48	Amidase	PF01425.19

2.4 Bioinformatics analysis of auxin metabolism related genes

2.4.1 TAM pathway

2.4.1.1 Aromatic-L-amino-acid decarboxylase (DDC)

According to the forecast results of KEGG website, 1Glddc gene (EC 4.1.1.28) existed in *G. lucidum* genome and the number was GL21006-R1. Full length CDS of GL21006-R1 gene was 1536 bp, encoding 510 amino acids. The predicted isoelectric point of the encoded protein was 5.83, and the relative molecular mass was 56934.83 Da. A pyridoxal-dependent decarboxylase conserved domain (71-427 aa) was predicted by Pfam. Pyridoxal-5'-phosphate-dependent amino acid decarboxylases can be divided into four groups based on amino acid sequence[14]. DDC belongs to Group II [15], including glutamate, histidine, tyrosine and aromatic-L-amino-acid decarboxylases. DDC catalyses the decarboxylation of tryptophan to tryptamine. Phylogenetic analysis of DDC amino acid

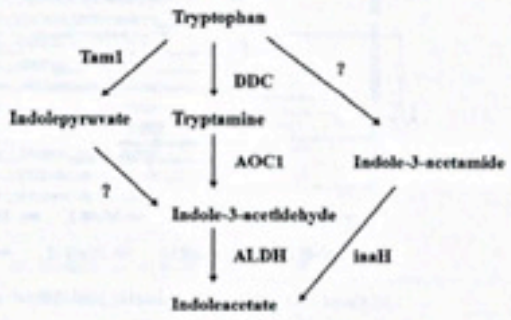

Figure. 4 Prediction of IAA biosynthesis in *G. lucidum*

sequences of 12 species of fungi and 8 species of plants was made (Figure 5), including GLDDC of *G. lucidum*. The results showed that the closest relationships with GLDDC were *Trametes vericolor* and *Obba rivulosa* who belong to Basidiomycota agaricomycetes polypores. With the MEME program, a total of 15 conserved motifs were identified in DDC from the fungi and plants mentioned previously . The result showed DDC was relatively conserved in fungi and plants, with individual species missing 1-2 motif.

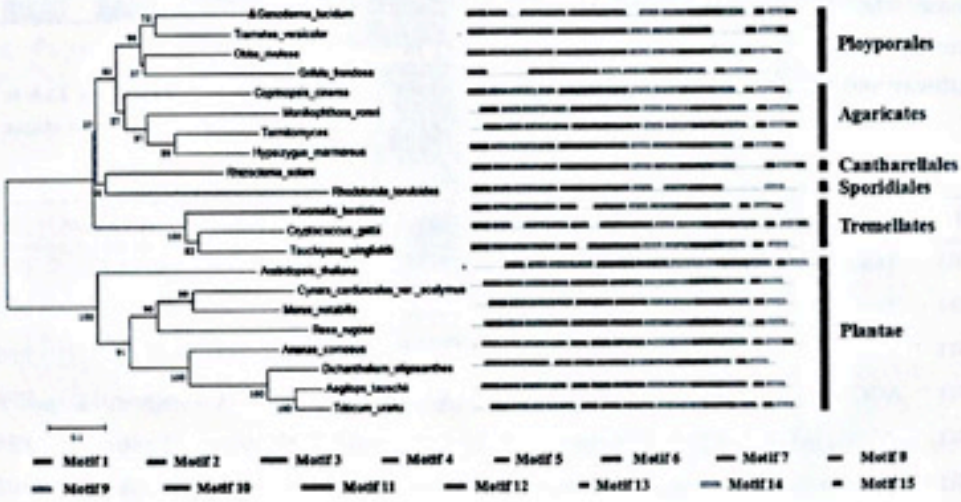

Figure. 5 Phylogenetic and Motif analysis of GLDDC and DDC from other fungi and plants

2.4.1.2 Diamine oxidase (AOCl)

An*aoc*1 gene (GL22246-R1) was found in *G. lucidum* genome, EC 1.4.3.22. Full length CDS of GL22246-R1 gene was 2808 bp, encoding 934 amino acids. The predicted isoelectric point of the encoded protein was 6.55, and the relative molecular mass was 105013.11 Da. GLAOC1 contains a copper amine oxidase characteristic domain (384-441, 481-863 aa) and a transmembrane signals (36-57 aa). AOC1 acts as a disulphide-linked homodimer and catalyses the oxidation of primary amines to aldehydes, with the subsequent release of ammonia and hydrogen peroxide, which requires one copper ion per subunit and topaquinone as cofactor[16]. AOC1 amino acid sequences of 13 species of fungi (Figure 6) showed that the AOC1 sequence was mainly divided into two categories. GLAOC1 was in categories A which includes AOC1 from *agaricales*, *polyporales*, *boletale* etc. belong to *Basidiomycota*. Categories B includes AOC1 from Ascomycota fungi.

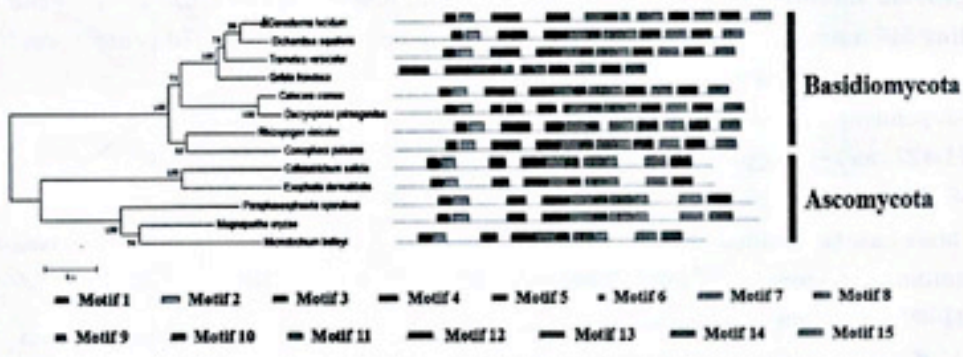

Figure. 6 Phylogenetic and Motif analysis of GLAOC1 and AOC1 from other fungi.

2.4.1.3 Aldehyde dehydrogenase(NAD+)(Aldh)

According to the forecast results of KEGG website, 6GlAldh genes (EC 1.2.1.3) were found

in *G. lucidum* genome, GL30174-R1, GL24130-R1, GL26572-R1, GL23433-R1, GL20614-R1, GL18415-R1, respectively. Full length CDS was between 1437 bp to 3054 bp, coding from 476 aa to 1016 aa. The predicted isoelectric point of the encoded protein were between 6.05 and 9.19, and the relative molecular mass were between 52510.35 Da and 112172.97 Da. All 6 GlAldh contain Aldedh characteristic domain (Table 1), belong to Aldehyde dehydrogenase family, acting on aldehyde substrates with $NADP^+$ as a cofactor[17]. Aldehyde dehydrogenase is a key enzyme in the final step of TAM pathway which catalyzing indole-3-acetaldehyde to produce IAA. Phylogenetic analysis of Aldh amino acid sequences of several kinds of polyporales fungi (Figure 7) showed that, 6 GlAldh were divided into 4 groups. GL30174-R1 and GL26572-R1 belonged to the first group. GL18415-R1 and GL24130-R1 belonged to the second and the third group, respectively. GL23433-R1 and GL20614-R1 belonged to the fourth group. The first group contained motif 1, 2, 4-7, 8, 9, 11-14, but the motif 14 was deleted in GL30174-R1. Compared to the first group of Aldh, the second group of Aldh lacked motif 11 and motif 13, furthermore the motif 2 was replaced by motif 3 at the same location. The motif 1 were deleted in the third and the forth group contained motif 6 and motif 10 instead of motif 11. Motif 14 existed in almost all of the third group except for KZT70410.1 which belonged to *Daedalea quercia*. Motif order of GL24130-R1 in third group was different from other sequences (Figure 7).

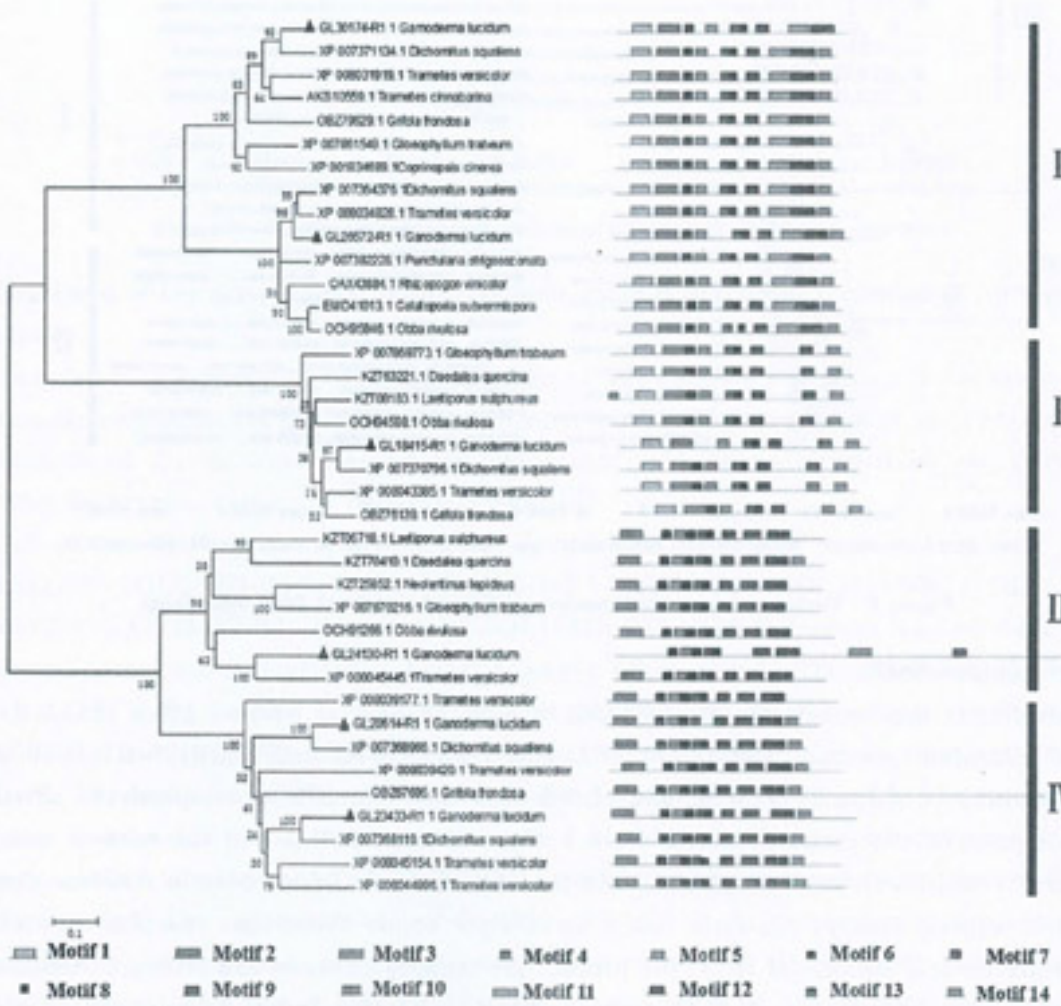

Figure. 7 Phylogenetic and Motif analysis of GlAldh and Aldh from other fungi

2.4.2 IPA pathway and IAM pathway

2.4.2.1 Tryptophan amiotransferase(Tam1)

Full length CDS of *Tam* 1 (GL24771-R1) gene was 1200 bp, encoding 398 amino acids. Predicted isoelectric point of the encoded protein was 8.51, and the relative molecular mass was 44053.32 Da. The other Tam1 (GL31258-R1) gene full length CDS was 1131 bp, encoding 376 amino acids. Predicted isoelectric point of the encoded protein was 6.09, and the relative molecular mass was 41710.42 Da. The two GlTam1 containing Aminotransferase class I and II domain (Table 1), belongs to the PLP-dependent enzymes superfamily[18]. L-tryptophan and 2-oxoglutarate were catalyzed to produce indole-3-pyruvate and L-glutamate under the action of tryptophan aminotransferase[19].

The two GlTam1 sequences in *G. lucidum* were compared with several polyporales fungi Tam1 and phylogenetic tree was constructed. The results showed that GlTam1 were divided into two groups. GL24771-R1 belonged to the first group, the closest relationship with GL24771-R1 were OCH85707.1 and OCH88806.1 from *Obba rivulosa*. GL31258-R1 belonged to the second group, with the closest genetic relationship of XP007365008.1 from *Dichomitus squalens*. Motif analysis found that the first group contained motif 3, lacked motif 2 and motif 5, compared with the second groups, motif 14 and motif 15 were present in both groups (Figure 8).

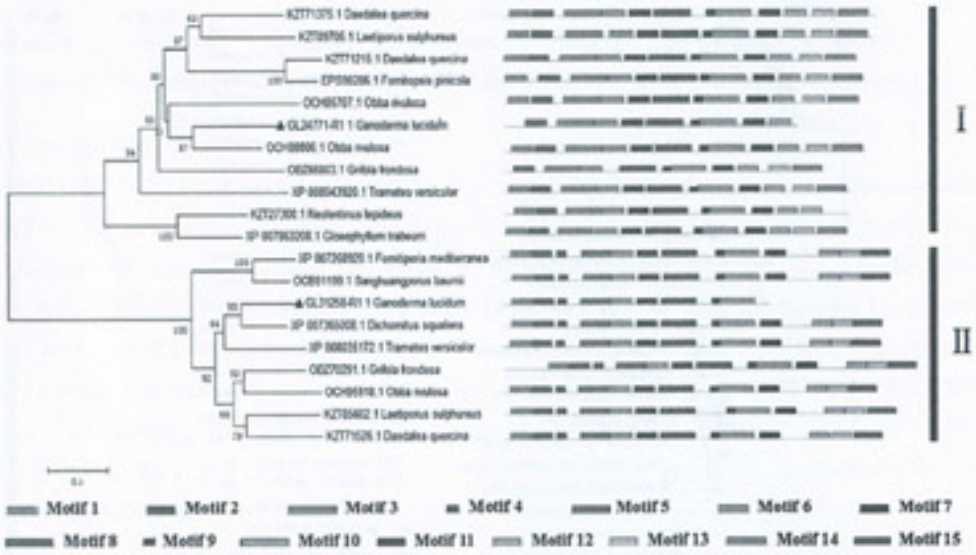

Figure. 8 Phylogenetic and Motif analysis of GlTam1 and Tam1 from other fungi

2.4.2.2 Amidase(AMI)

According to the forecast results of KEGG website, there were 3 *Glami* genes (EC 1.2.1.3) in the *G. lucidum* genome, namely GL29633-R1, GL20521-R1 and GL18499-R1. Full length CDS were 1659, 1662 and 1743 bp and coded 551, 552 and 579 aa, respectively. Predicted isoelectric point of the encoded protein were 4.84, 5.50 and 5.01, and the relative molecular mass were 57505.06, 61006.06 and 62814.48 Da. All of the 3 GlAMI contain Amidase domain, belong to hydrolase family, the main role is to catalyze amide hydrolysis, reaction substrate are monocarboxylic acid amide and H_2O, the product are monocarboxylate and NH_3[20]. Amidase is a key enzyme in the final step of IAM pathway which catalyzing indole-3-acetamide to produce IAA.

Sequence alignment ofGlAMI with AMI of other fungi and the phylogenetic tree was

constructed. 3 GlAMI were divided into 3 groups, the closest relationship with GL20521-R1 and GL29633-R1 of *G. lucidum* were respectively XP007367351.1 and XP007368473.1 sequence from *D. squalens*, while GL18499-R1 had the closest genetic relationship with AMI sequence of *Neoectria dittissima*. Motif analysis showed that the first half of AMI sequences of fungi were very conservative, motif 1-6, 13 and 14 were common to three groups, in addition, motif 8 exists in the first group, motifs 9-11 commonly existed in group 2, while motif 12 existed in group 3 (Figure 9).

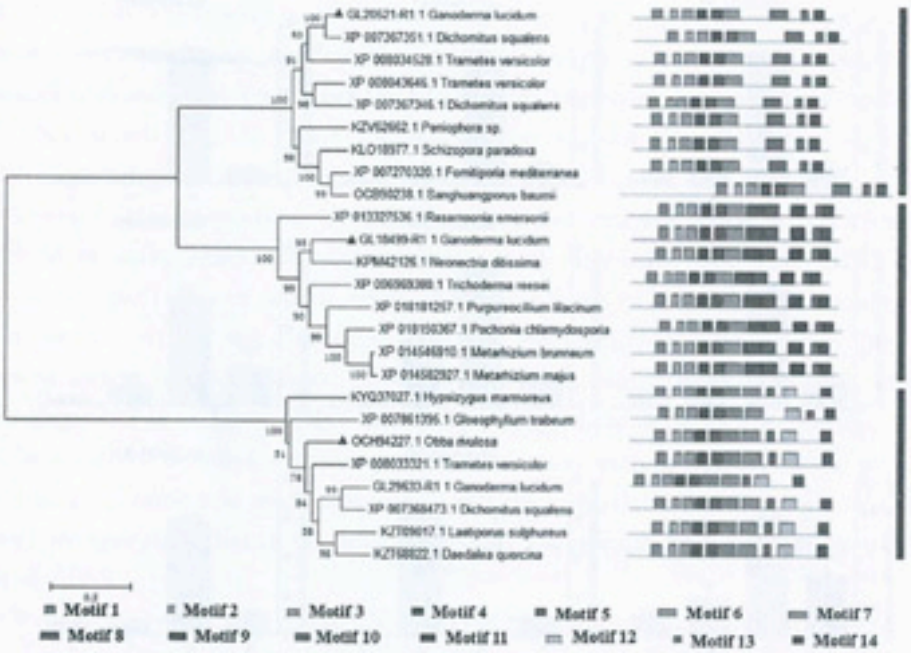

Figure. 9 Phylogenetic and Motif analysis of GlAMI and AMI from other fungi

2.5 Expression of key enzyme genes of IAA synthesis pathway during the formation of fruiting body of *G. lucidum*

In order to preliminarily understand the roles of key enzymes of IAA synthesis pathway in*G. lucidum* development, the qRT-PCR was utilized to analyze the expression of key enzyme genes in four periods of *G. lucidum*. All of the genes identified were expressed in four periods and exhibited differential expression patterns (Figure 10).

All of the TAM pathway key enzyme genes identified were expressed in four periods, including*ddc* (GL21006-R1), *aoc*1 (GL22246-R1) and *Aldh* (GL30147-R1, GL24130-R1, GL26572-R1, GL23433-R1, GL20614-R1, GL18415-R1). The expression level of three genes in the second period were significantly higher than the other period. The expression of two *Tam* 1 genes (GL24771-R1 and GL31258-R1) in IPA pathway and three *ami* genes (GL18499-R1, GL20521-R1 and GL29633-R1) in IAM pathway were all identified in four periods of *G. ludicum*. The highest expression level of two *Tam* 1 genes were appeared in the second period, then the first period. The three enzyme genes of AMI family showed different expression patterns, the relative expression of GL18499-R1 was the highest in the second period, followed by the third period. The expression level of GL20521-R1 gene in the second period were significantly higher than the other period, and the relative expression level of GL29633-R1 was the highest in the third period. The results showed that the transcription of the GlAMI gene was mainly concentrated in the second and the third period, and GlAMI gene transcription at the different stages of *G. lucidum* was different.

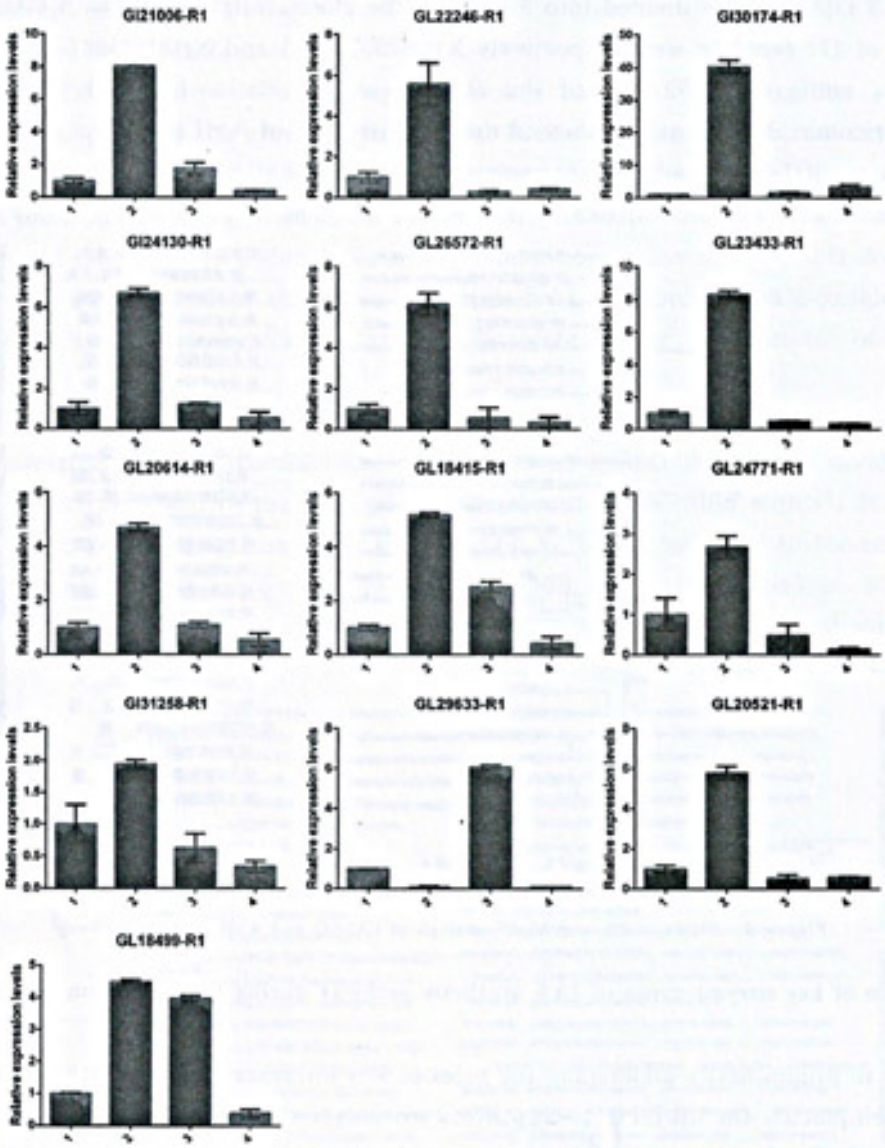

Figure. 10 The expression of key enzyme genes of IAA synthesis pathway at different stages

3 Discussion

In this study, endogenous hormone IAA in *G. lucidum* was detected by HPLC and the maximum IAA value was obtained in the second period. KAAS automatic annotation server was used to predict the whole genome metabolic map of *G. lucidum* and 13 sequences related to IAA synthesis were screened, including 2 GlTam1, 1 GlDDC, 1 GlAOC1, 6 GlAldh, and 3 GlAMI. The results showed that there was a complete TAM pathway, and might have IPA and IAM pathway in *G. lucidum* (Figure 5). Subsequently, the bioinformatics were used to predict and analyze the function of 13 gene sequences and phylogenetic tree was constructed so as to preliminarily judge the function of auxin synthesis enzyme genes in *G. lucidum*. Finally, the relative expression level of each gene in auxin synthesis pathway was detected by qRT-PCR. The results showed that all of the 13 genes identified were expressed in four periods in addition to a *Glami* gene (GL29633-R1). The relative expression of the other 12 genes was the highest in the second period, and significantly higher than the other three periods. It is consistent with the change of auxin in *G. lucidum*.

The second period was the fastest growing period during*G. lucidum* fruiting body morphogenesis. In this period the expression level of key enzyme genes of IAA synthesis pathway increased. We deduced that the fruiting bodies synthesized a great deal of IAA with the need for promoting the cell differentiation and growth. In the third period, the IAA had fallen to the one fifth of the second period and the lowest in the fourth period. This may be that IAA synthised in the second period activate its signal transduction to further regulate the *G. lucidum* growth. In addition, free IAA was readily oxidized and broken down in organisms and IAA would be combined with other compounds such as sucrose, inositol, amino acids, peptides and proteins[21]. The formation of IAA conjugates was also one of the most important regulation methods to maintain the level of IAA activity[22]. So after the second period, IAA might have been converted into conjugates, resulting in free IAA content reduced.

According to the annotations on KEGG, the 3 key enzyme genes in the TAM pathway were also involved in many other synthetic pathways and metabolic processes. DDC was involved in many metabolic pathways of amino acids and biosynthesis ofisoquinoline alkaloids, betalains and other compounds. AOC1 was also present in the metabolism of arginine, proline and histidine. Aldh existed widely in cells involved in many important biosynthetic pathways, such as glycolysis pathway, degradation of fatty acids, metabolism of multiple amino acids and so on; AMI was also involved in a variety of other anabolic pathways in cells, such as the metabolic pathways of amino acids (arginine, proline and phenylalanine), and the degradation of benzaminic acid and styrene. Therefore, we speculate that in the second period, the growth and metabolism of G. lucidum are the most vigorous.

Tam1 only involved in the production of TPA by tryptophan, which indicated the formation of IPA the second period may be higher than other periods. As mentioned, the recently study have defined a simple two-step pathway of auxin from tryptophan in *Arabidops*[4]. Tam1 and YUCCA will be used to convert tryptophan into IAA. Unfortunately, we have not found YUCCA family members or other enzymes that catalyze indole-3-pyruvate to produce indole-3-acetaldehyde or directly generate IAA in *G. lucidum* genome. One of the hallmarks of fungal genetic organization, similar to bacteria, is the physical linkage between biosynthetic genes in a given pathway, it is helpful to explore the natural biosynthetic pathways of fungal[23]. Therefore, based on the existing genes, we will amplify the genes may exist in IAA synthesis pathway, so that to improve the IAA synthesis in *G. lucidum* pathway.

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Table 2 PCR primer used in this study

Primer	The sequence(5'-3')
21006-F	AGCACGCTTAAGGAGACACT
21006-R	CGCATCCAGTTTTCCTACCG
22246-F	ACAACACTTTACCGCCACAC
22246-R	CGGCGGTTAGATTGAGGTTG
30174-F	GCGTATCTTTGTGCAGGAGG
30174-R	CTTGCCAGAGTCGATGAAGC
24130-F	AACTGCTCGCCGTTAACATC
24130-R	GGAAGTGAGTGAACGTGTCG
26572-F	CTACGGCCAAGCAGATTTCC
26572-R	CCCAGGAGGTTTTGAATGCC
23433-F	CTTCAAGGAGGCTCTTCGGA
23433-R	CGGGATGCTTTGTGGCTTAA
20614-F	CGCAGCGTATCAAGAAGCTT
20614-R	GAAGCACGGGTCCAAAGATC
18415-F	TATAGCCCGCGTTTCTCAGT
18415-R	GAGAGGGCGAGGTTCCTATC
24771-F	GCAAGCCTAACATGGTGTCC
24771-R	GCATGGGTCGTGTACTGATG
31258-F	TCGAGAGGAAGAAGGCGATC
31258-R	TGACCCGTCCCTGATAATCG
29633-F	TGTCCAAACCCTTCTCGACA
29633-R	TGTAAAGGTCCGATGGGCTT
20521-F	CTCTTCACCAGTGTCGACCT
20521-R	CTGGCGAGAGTTGCAATGTT
18499-F	CATCGTGCAGTACAACCTCG
18499-R	CCAGCCTCTTTCCCTCGTAA
gpd-F	GATGAAGGACTGGCGTGGT
gpd-R	CCGTTGAGGCTGGGAATGAC