Combining transcriptomics and metabolomics to characterize ergosterol biosynthesis in *Flammulina velutipes* during the fruiting process

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Abstract

Background: Flammulina velutipes (F. velutipes) is one of the most important mushrooms in Japan and China because of its potential medicinal and nutritional value. Ergosterol is an important precursor of vitamin D2, progesterone, hydrocortisone, brassinolide, and novel anticancer and anti-HIV drugs. One of the main methods for obtaining ergosterol is extraction of its natural products from fungi. However, because of the low production of ergosterol due to its inefficient biosynthesis, its approach cannot meet human needs. Therefore, increasing the ergosterol level in fungi is an important goal to be pursued. Although genes encoding key enzymes in the biosynthesis pathway of ergosterol have been identified in Saccharomyces cerevisiae, the mechanism and regulation of ergosterol biosynthesis in F. velutipes remain unclear, and are the focus of this study.

Results: In this work, nine cDNA libraries produced from the three stages of F. velutipes were sequenced by using the Illumina HiSeqTM 4000 platform, resulting in at least 6.63 Gb of clean reads from each library. De novo sequence assemblers were used to generate 220,523 transcripts and 28,330 unigenes, which were annotated according to seven protein databases. Here, we combined transcriptomics and metabolomics approaches to investigate the biosynthesis of bioactive ergosterol from F. velutipes. The contents of 16 intermediates or metabolites from different stages (young fruiting body stage and mycelium stage) were found to be significantly different. Then, transcriptional profiling of the genes involved in ergosterol biosynthesis was carried out, and the transcripts of key genes involved in the metabolism were analysed. A total of 51 key unigenes (12 upregulated unigenes and 39 downregulated unigenes) were identified as differentially expressed. Furthermore, four genes (i.e., ERG10s, ERG1s, ERG11s and ERG26s) were identified as the most important genes that played roles in the regulation of the pathway flux towards ergosterol synthesis in F. velutipes. Combining transcriptomics and metabolomics, we explored the regulatory relationship between ergosterol biosynthesis genes and metabolites in F. velutipes.

Conclusions: The data obtained in this work provide useful information for understanding the biosynthesis, metabolism and regulation of sterols in F. velutipes and for channelling the metabolic flux towards ergosterol at different growth stages.
Edible fungi are the sixth largest crop in China with a total output of 33 million tons in 2015 [1]. *F. velutipes* as one of the most edible fungi, and an excellent source of vitamins, amino acids, polysaccharides, fibers, terpenoids, phenolic acids, steroids, fatty acids and other metabolites, has been widely cultivated all over the world [2-4]. In this study, the compounds with pharmaceutical value were isolated from the fruiting body or mycelium of *F. velutipes* including anti-inflammatory and immunomodulatory proteins [5], antitumor, antioxidant and acetylcholinesterase inhibitory polysaccharides [6-9], antitumor agglutinin and immunomodulatory [10], antimicrobial terpenoids [11], and antitumor and antioxidant sterols [12, 13]. The extracted active antitumor sterols from *F. velutipes* contained ergosterol, 22,23-dihydroergosterol, ergosta-5,8,22-trien-3-ol, ergo-8(14)-ene-3-ol, etc. Ergosterol is the most abundant sterol in *F. velutipes*, so its content could serve as an important indicator for assessing the quality of sterol-related products of *F. velutipes* [14, 15].

Ergosterol (C_{28}H_{43}OH) is a typical sterol of fungi and it is an important constituent of various membrane structures of fungal cells. It displayed multifaceted physiological functions in cells, such as ensuring cell viability, membrane fluidity, membrane integrity, and cellular material transport [16]. Therefore, once ergosterol is lacking, it would cause abnormal cell membrane function and even cell rupture [17-19]. In recent years, a variety of fungicides, collectively called sterol biosynthesis inhibitors (SBIs), have been successfully developed to target some enzymes or end products of ergosterol biosynthesis pathway, and have been widely used in medicine and agricultural production [20]. Ergosterol level is also one of quality indicators for some traditional Chinese medicines and Chinese medication preparations. More importantly, ergosterol and some of its biosynthetic intermediates are important metabolites of great economic value. In the pharmaceutical industry, ergosterol is an important precursor for vitamin D2, progesterone, hydrocortisone, and brassinolide, and almost all steps of its biosynthetic process represent potential drugs [21-25].

Ergosterol and its derivatives are mainly obtained by the methods of chemical synthesis, genetic engineering and metabolic engineering [26]. Because of the various steps, long route, low efficiency and high cost, chemical synthesis of ergosterol and its derivatives is not the preferable way to obtain them. One of the main approaches to producing ergosterol and its derivatives is through metabolic
engineering of yeast, but the common problem with the strains used in production is the low content of ergosterol in cells [27]. The biosynthesis of ergosterol is an extremely complicated process. Transcriptional regulation of expression of related genes is one of the main means to control ergosterol biosynthesis, or feedback regulation can play an important role in regulating ergosterol production [28, 29]. Sterol regulatory element-binding proteins (SREBPs) are transcriptional factors which bind to the sterol regulatory element DNA sequence. Therefore, manipulation of biosynthesis genes by genetic engineering may be an effective way to change sterol biosynthesis and intracellular sterol components. Although metabolic engineering or genetic engineering has shed light on synthetic pathways in Saccharomyces cerevisiae, how the ergosterol biosynthesis genes regulate ergosterol production has not yet been fully understood.

The ergosterol biosynthetic pathway can be divided into the two parts: the mevalonate pathway and the post-squalene pathway (Fig. 1). The former has nine steps (Fig. 1A) including the synthesis of farnesyl pyrophosphate from acetyl-CoA [30]. The latter produces acetoacetyl-CoA by using the two acetyl-CoA molecules catalyzed by acetoacetyl-CoA thiolase (ERG10). Then, ERG13, HMG, ERG12, ERG8, ERG19, IDI1 and ERG20 successively catalyzed eight reactions to produce farnesyl pyrophosphate from acetoacetyl-CoA. The enzymes in the mevalonate pathway are essential genes which are conserved in eukaryotes [31-33]. The second part comprises 14 steps from farnesyl pyrophosphate to ergosterol (Fig. 1B). The first step forms farnesyl pyrophosphate for squalene which will then be converted into lanosterol by squalene cyclization. Ergosterol is produced from lanosterol through a sequence of reactions by ERG7, ERG11, ERG24, ERG25, ERG26, ERG27, ERG6, ERG2, ERG3, ERG5 and ERG4 [34].

Nowadays multiomics has been a common biological approach for systemic genomes [35-38]. In this paper, we prepared the first RNA-seq libraries from F. velutipes samples at three developmental stages, that is, mycelium liquid culture stage (FrI), young fruiting body stage (FrII) and mature fruiting body stage (FrIII). The transcriptome technique was used to identify the DEGs involved in the biosynthesis of ergosterol in F. velutipes at different developmental stages. Afterwards, the differential metabolites were completely scanned by non-targeted metabolomic techniques.
Differentially expressed genes (DEGs) combined with differential metabolites were used to assist in identifying genes associated with ergosterol biosynthesis. The results showed that a large number of ergosterol biosynthesis-related genes were confirmed in the transcriptome of *F. velutipes*. The results revealed 19 DEGs related to ergosterol biosynthesis of *F. velutipes* at different developmental stages. After transcriptomic and metabolomic analysis of the ergosterol biosynthesis, four genes (ERG1s, ERG10s, ERG11s, ERG26s) were identified as the most important genes in the regulation of the pathway flux towards ergosterol biosynthesis in *F. velutipes*. And the results showed that there might be 17 differential metabolites from the ergosterol biosynthesis in *F. velutipes* between different developmental stages. All the data and results had a vital significance for understanding the metabolic pathway of ergosterol biosynthesis in *F. velutipes*.

Methods

Materials and sample collection

This article used *F. velutipes* samples at three different developmental stages as experimental materials. They were mycelium liquid culture stage (FrI), young fruiting body stage (FrII) and mature fruiting body stage (FrIII) (Fig. 2). Fresh mycelium, young fruiting body and mature fruiting body were acquired from a *F. velutipes* farm in Xianyang City, Shaanxi Province, China in June 2017. Mycelium fluid culture and the culture conditions could be consulted in literature [3]. These *F. velutipes* samples at three developmental stages were collected under sterile conditions, immediately frozen and stored in liquid nitrogen at -80 °C before the metabolic content determination and RNA isolation [2].

RNA extraction, library preparation and mRNA-Seq

Total RNA was extracted with RNA pure Plant kit (Tiangen Biotech Co., Ltd., Beijing, China). The total RNA concentration, RIN value, 28S/18S and fragment size were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Co. Ltd., Santa Clara, CA, USA) with Agilent RNA 6000 Nano Kit. The purity of the samples was measured using an ultraviolet spectrophotometer NanoDrop™. After the
isolation and fragmentation of the total RNA, the eukaryotic mRNA was enriched by using Oligo (dT) coupled to magnetic beads. The preparation of cDNA libraries was based on the method of PCR described in the following procedure. The first strand cDNA was manufactured from the fully spliced mRNA as a template, and then a second strand reaction system was used to make double-stranded cDNA. The second strand cDNA was obtained and purified by a system kit. When the cDNA ends were repaired, an ‘A’ nucleobase hybridized to the 3’ ends of the cDNA. Then the cDNA fragments of different sizes were selected and then amplified. Then the cDNA fragments of different sizes were selected and then amplified. The qualities of amplified cDNA libraries were checked and tested. At last, the sequencing was run on an Illumina HiSeq™ 4000 platform.

**De novo assembly and functional annotation**

First, we filtered out low-quality sequences, contaminated adaptors and unknown N base (N percentage > 5%). The resultant data is called clean reads. We used Trinity platform (http://trinityrnaseq.github.io/) for de novo transcriptome assembly to obtain the clean reads (removing PCR repeats to improve assembly efficiency). Then we assembled the clean reads using Tgicl to get unigenes, and performed functional annotation on unigenes. These unigenes were aligned by BLASTx against 7 protein databases including the NCBI non-redundant protein database (NR), non-redundant nucleic acid database (NT), the Swiss-Prot protein database, the Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG), the eukaryotic Orthologous Groups of proteins database (KOG), and the Gene Ontology database (GO). The best result from alignment determined the annotation of the unigenes.

**FPKM calculation and DESeq2 differential expression analysis**

We used alignment package Bowtie2 (v 2.2.5) to map clean reads back to unigenes. Then the gene expression levels of every sample were calculated by RSEM (v1.2.12) (RNA-Seq by expectation maximization) [39, 40] which is a software package for RNA-seq reads to calculate the expression of
its genes and transcript isoforms. This paper used the hclust function in the R software for hierarchical clustering analysis. Fragments Per Kilobase of transcript per Million (FPKM) values were calculated by mapping the reads to fragments, which could be used to quantify the abundance of the transcripts and so to analyze differential gene expression. Differential expression analyses between genes at different developmental stages were performed with DESeq2 package [41]. Differentially expressed genes (DEGs) were revealed by adjusted value (AP ≤ 0.05) and fold change analysis (FC ≥ 2.00).

**GO and KEGG enrichment analysis of DEGs**

According to the official classification categories and based on the GO and KEGG annotation results, the DEGs are annotated to functional and biological pathways, and the phyper of Rstatistics package is used for enrichment analysis. A p-value of 0.05 (p ≤ 0.05) implies that the accepted false discovery rate (FDR) is 5%.

**RT-qPCR validation and DEGs analysis**

In order to validate internal control genes for expression analysis, ABI StepOnePlusTM RT-PCR System (Applied Biosystems, USA) was used to perform RT-qPCR and analyze the expression of DEGs related to ergosterol biosynthesis. Primer Premier 5.0 software was adopted to build sequencing primers, presented in Additional file 1: Table S1. The acquisition of cDNA and RT-qPCR were carried out by PrimeScript™ RT reagent Kit and SYBR Green™ Premix Ex Taq™ II (Takara Biotech Co., Ltd., Japan). GAPDH was used as an internal control. Three independent technical replicates and three biological replicates for each sample were run to measure and assess the performance of RT-qPCR.

**Measurement of metabolites**

In this article, we also performed a metabolomic analysis. The tests were performed on six biological replicates for each metabolome. Tissue samples stored at -80 °C were placed in a freezer at -20 °C for 30 min and then thawed in a 4 °C freezer. 25 mg tissue was weighed, and placed in the EP tube; 800 ul of a cooled solution of methanol/water (1:1) was added to each EP tube and two small steel balls
were frozen and put to each EP tube; the sample was put in the tissue lyser and the parameters was set to 35 Hz for 4 min. After grinding, the ball was removed and the tube was placed in a -20 °C refrigerator for 2 h. the sample was centrifuged at 30,000 g for 15 min at 4 °C. The EP tube was carefully removed from the centrifuge and 550 ul of each sample was transferred into a new EP tube; the sample was placed on the rack of the centrifuge and a picture was taken according to the order of samples on the task sheet; The sample will be handed over to be analyzed by LC-MS, and the remaining original sample will be handed over to the sample manager for storage.

All samples were made and processed by following protocols for LC-MS data analysis. Firstly, all chromatographic isolation was performed by using UPLC system (Waters, UK). The column type, column temperature, mobile phase flow rate, mobile phase solvent ratio and program settings are the same as the reference [42]. The injection volume was 10 μl. The Q-TOF was run with the capillary tube and sampling voltages set at 3 kV and 40 V in the positive ion mode and at 1 kV and 40V in the negative ion mode. MS data was generated by Xevo G2 XS QTOF with TOF mass ranging from 50 to 1200 Da and scanned 0.2 s. The MS/MS was conducted to select, separate and detect precursor ions using 20 and 40 eV in different steps and with scan time 0.2 s. When processing the data, the LE signal was received at a 3 s interval to measure the mass accuracy. And a control sample was picked up from every 10 samples for evaluation of the performance of the LC-MS on the data acquisition.

Results

**De novo assembly and analysis of RNA-seq data**

To provide insight into the ergosterol biosynthesis in *F. velutipes* at three different developmental stages, nine libraries (FrI_1, FrI_2, FrI_3, FrII_1, FrII_2, FrII_3, FrIII_1, FrIII_2 and FrIII_3) were prepared and the libraries for each stage were repeated 3 times. The number 1, 2, and 3 stood for the times of repetition. We used an Illumina HiSeq™ 4000 platform to sequence them. After data filtering, about 60.29 Gb clean reads were obtained, and every library generated clean reads not less than 6.63 Gb. Q30 percentage were 92.88%, 92.91%, 92.89%, 91.38%, 91.63%, 92.22%, 92.75%, 92.72% and 92.46%, respectively. These results demonstrated that the transcriptional profiling datasets had
satisfactory reliability for further analysis (Additional file 2: Table S2).

The de novo assembly produced 220,523 transcripts and 28,330 unigenes. The length distribution of all-unigenes was shown in Additional file 3: Fig. S1. In total, sequence size of 4383 (15.47%) unigenes was between 200 and 500 nt; sequence size of 4511 (15.92%) unigenes was between 500 and 1000 nt; 9474 (33.44%) unigenes ranged from 1000 to 2000 nt; 5261 (18.57%) unigenes ranged between 2000 and 3000 nt; 4701 (16.00%) unigenes were longer than 3000 nt. The profile of 28,330 unigenes about the total number, total length, mean length, N50 and GC content was shown in Table 1.

Functional annotation

Because of lack of a complete profile of genome sequences for F. velutipes, only 28,330 unigenes were annotated against 7 databases by alignment. Among them, 21,638 unigenes (76.38% of all annotated unigenes) had annotation to NR database, meanwhile 3,380 (11.93%), 12,611 (44.51%), 12,427 (43.87%) and 14,834 (52.36%) were annotated to NT, SwissProt, KOG and Interpro databases, respectively. At last, we annotated 14,433 (50.95%) and 5,910 (20.86%) unigenes to KEGG and GO databases.

Assignments of KOG were used to predict and classify the possible functions of the unique sequences, and describe gene evolutionary processes. In this study, 12,427 unigenes (43.87% of all assembled unigenes) had KOG annotation functions and the KOG-annotated putative proteins were classified into 25 functional groups. 24.23% of the proteins were annotated to General functional prediction only the largest group, which is thought to be responsible for processing the processors of storage proteins into mature proteins, followed by signal transduction mechanisms (18.29%) and posttranslational modification, protein turnover, chaperones (13.24%). Just a small part of proteins was assigned to cell motility (0.22%). It is noteworthy that a great deal of proteins was annotated to secondary metabolites biosynthesis, transport and catabolism (8.57%), carbohydrate transport and metabolism (9.99%), transcription (7.44%), lipid transport and metabolism (7.23%) (Fig. 3).

GO enrichment analysis and DEGs annotation and assignment
This article also dealt with enrichment analysis on gene sets, and investigated how GO terms were represented using annotations for that gene set. The GO enrichment analysis tool Blast2GO package (v 2.5.0) was used to perform enrichment analysis. The results were interpreted and displayed. It was determined that a corrected FDR was the threshold. De novo transcriptome assembly and analysis revealed 10,266 DEGs between FrII and FrI group, 10,467 DEGs between FrIII and FrI group and 2,677 DEGs between FrIII and FrII group (Additional file 4: Fig. S2). 9,265, 10,254 and 2,384 genes from the three DEG sets were assigned at least one GO term. 49 significant shared terms were displayed in Fig. 4. The results showed that metabolic process, cellular process, single-organism process, localization, biological regulation, cellular component organization or biogenesis and regulation of biological process were significantly shared GO terms in biological process category. Cell, cell part, membrane, organelle, membrane part, macromolecular complex and organelle part were the most shared in cellular component category. Catalytic activity, binding, transporter activity, structural molecule activity and enzyme regulator activity were markedly shared in molecular function category.

**KEGG function enrichment of DEGs**

The genes were divided into 7 categories according to the KEGG Orthology classification for KEGG pathway maps. Metabolic pathway analysis provided insight into the biological functions and gene interactions and we found the most represented category was metabolism (Additional file 5: Fig. S3). From the bubble map of the DEGs pathway enrichment analysis (only the top 20 metabolic pathways shown) (Fig. 5), we found that the two metabolic pathways related to ergosterol biosynthesis with significant enrichment were terpenoid backbone biosynthesis pathway (ko00900, 32 DEGs, 59 orthologs annotated) and steroid biosynthesis pathway (ko00100, 43 DEGs, 78 orthologs annotated).

**DEGs related to ergosterol biosynthesis**

We analyzed the DEGs involved in terpenoid backbone biosynthesis pathway and steroid biosynthesis pathway in *F. velutipes*, which were related to ergosterol biosynthesis. Venn diagram and heatmap showed the differentially expressed genes of *F. velutipes* between three different developmental stages (Fig. 6). The results between FrII group and FrI group showed that 51 key unigenes (12 up-
regulated and 39 down-regulated) were significantly differentially expressed. In addition, we analyzed transcriptional profiles of the differential genes between FrIII group and FrI group, to find a total of 56 key significantly differentially expressed unigenes (14 up-regulated and 42 down-regulated) shown in Table 2 and Fig. 6C. The results for the analyses showed strong similarity in differential expression between FrIII - FrI DEGs and FrII - FrI DEGs. Likewise, we found that the both metabolic pathways had only 8 DEGs (4 up-regulated and 4 down-regulated) between FrIII and FrII group (Fig 6A and 6B). These results indicated that during the formation of fruiting body, the difference of gene expression between the first and second stage was very distinct in ergosterol biosynthesis, but was quite small between the second and third stage. The distinctive gene expression difference may be due to the dramatic morphological changes in the first two stages of *F. velutipes*, while the gene expression similarity could be explained that change in size did not significantly affect gene expression at all. Among them, more than one unigenes were identified through annotation as the same enzyme. The results in Fig. 6C demonstrated that the four genes (*ERG10s, ERG1s, ERG11s, ERG26s*) could be the most important genes for ergosterol biosynthesis in *F. velutipes*. The information of ergosterol biosynthesis in *F. velutipes* concerning the two pathways in the three different developmental stages was obtained. Then, we could learn more about the ergosterol biosynthesis pathway in *F. velutipes*. To validate the reliability of transcriptome sequencing data, the sequences of 15 core differentially expressed unigenes were analyzed by RT-qPCR primers. The results of RT-qPCR analysis showed a close similarity to the results of RNA-Seq, as shown in Fig. 7.

Analysis of biosynthesis intermediates and ergosterol from *F. velutipes* at the three different stages

In this article, we performed metabolome sequencing of *F. velutipes* at three different developmental stages. PCA analysis of *F. velutipes* metabolome at different developmental stages was shown in Fig. 8A and 8B. Metabolic pathway analysis was based on the KEGG database. To understand the ergosterol biosynthesis, we compared the metabolic profiles of *F. velutipes* groups at different developmental stages (Fig 8C). Differential ions and identification results for different developmental groups were shown in Additional file 7: Table S3. LC-MS revealed 1,742 differential metabolites between FrII and FrI group, of which 732 were found up-regulated respectively and 1,010 down-
regulated in the positive ion modes; and revealed 2,154 differential metabolites between FrII and FrI group, of which 925 were found up-regulated respectively and 1229 down-regulated in the negative ion modes. Similarly, between FrIII and FrII group, revealed were 751 and 944 differential metabolites respectively in positive and negative ion modes, of which 246 and 197 were up-regulated, and 505 and 747 were down-regulated.

We identified 17 differential intermediates or metabolites involved in ergosterol biosynthesis as mevalonate, mevolonate-5-phosphate, isopentenyl pyrophosphate, dimethylallyl pyrophosphate, farnesyl pyrophosphate, squalene-2-3-epoxide, lanosterol, 4,4-dimethyl-cholesta-8,14,24-trienol, 4-methylzymosterol -carboxylate, 14-demethyl lanosterol, 3-keto-4-methylzymosterol, 4-methylzymosterol, fecosterol, episterol, ergosta-5,7,24(28)-trienol, ergosta-5,7,22,24(28)-tetaenol and ergosterol, which were listed in Additional file 8: Table S4. By comparing FrII against FrI group, 16 out of 17 abovementioned intermediates or metabolites except farnesyl pyrophosphate had significantly differential expression. Among them, the content of isopentenyl pyrophosphate, dimethylallyl pyrophosphate, lanosterol, 4-methylzymosterol, episterol and ergosterol were significantly increased, and the content of 4-methylzymosterol-carboxylate, 4,4-dimethyl-cholesta-8,14,24-trienol, squalene-2-3-epoxide and ergosta-5,7,22,24(28)-tetaenol were significantly reduced (the differential fold is more than 2). The LC-MS profile of each metabolite between FrIII and FrII group was listed in Additional file 9: Table S5. A total of 12 metabolites or intermediates were significantly different in their content, and they were mevalonate, mevolonate-5-phosphate, farnesyl pyrophosphate, squalene-2-3-epoxide, lanosterol, 14-demethyl lanosterol, 3-keto-4-methylzymosterol, 4-methylzymosterol, ergosta-5,7,24(28)-trienol, ergosta-5,7,22,24(28)-tetaenol, episterol and ergosterol. Among them, the content of squalene-2-3-epoxide, 3-keto-4-methylzymosterol, lanosterol, ergosta-5,7,22,24(28)-tetaenol, 4-methylzymosterol, episterol and ergosterol were significantly increased, and the content of mevalonate and lanosterol were significantly decreased (the differential fold is more than 1.5). The results revealed that the content of the intermediates varied significantly in the different stages of the fruiting bodies.

To determine the metabolomic performance, we measured the end product ergosterol in the
ergosterol biosynthesis. The results were shown in Additional file 10: Fig. S5. It was found that the m/z and retention time of the metabolomic results were consistent with the validation measurement, indicating that the metabolomic results were reliable.

Combined analysis of transcriptome and metabolome of ergosterol biosynthesis in *F. velutipes* at different developmental stages

Systematic analyses of metabolites and genes were intended to investigate the relationship between genetic control of metabolite levels and metabolic impact on gene expression [43]. This article makes a comparison between the profiles of metabolites and gene expression in *F. velutipes* in different developmental stages using Pearson’s correlation coefficient (Additional file 11: Table S6 and Additional file 12: Table S7). Metabolite datasets of FrII vs. FrI group and FrIII vs. FrII group were extracted and clustered using correlativeity. And the cluster of gene expression profiles was made using the same method. Then, we combined the metabolite and gene expression profiles by calculating correlation coefficients. The heatmap in Fig. 9A and 9B illustrated the combined results. The horizontal axis represents metabolites and the vertical axis represents genes with the distance between each metabolite or gene reflecting similarity between their profiles. Red indicates positive correlation between metabolites and genes, and blue negative correlation. The metabolite-gene heatmap helps us understand the correlations between them.

Discussion

Dynamic changes of metabolites in *F. velutipes* at different developmental stages

*F. velutipes* is one of the most medicinal and edible fungi, which is secure and has been well received by consumers. Since the successful cultivation of *F. velutipes* in 1980s, its outputs and consumptions have rapidly increased cross the world [2, 44]. As the yield of *F. velutipes* increases, its nutritional and medicinal values become the focus of interest. In this study, by using metabonomic analysis, we could find metabolites involved in the ergosterol biosynthesis underwent significant change in their content during the three developmental stages (Additional file 8: Table S4 and Additional file 9: Table S5). In Fig. 8A and 8B, the results indicated that the biological replicates of *F. velutipes* in three different developmental stages were good and significantly different. In the course of three different growth
stages (Fig. 8C), the expression level of the only metabolite i.e. lanosterol increased in the first two stages and then decreased in the latter two stages, and the expression level of the metabolites, including ergosta-5,7,22,24-tetraenol, squalene-2-3-epoxide and 14-demethyl lanosterol, decreased in the first two stages and then decreased in the latter two stages. We found that some metabolites such as mevalonate-5-phosphate, 4-methylzymosterol, episterol, ergosta-5,7,24(28)-trienol and ergosterol were gradually increasing during the three growth stages, and the only metabolite mevalonate was gradually decreasing during the three growth stages. These results indicated that a portion of the sterols in *F. velutipes* were accumulated in the young fruiting body, and a portion of sterols were accumulated in the mature fruiting body.

Among the 17 intermediates or metabolites of FrIII vs FrII group, the level of mevalonate and lanosterol were decreased from developmental stage II to developmental stage III; the level of isopentenyl pyrophosphate, dimethylallyl pyrophosphate, 4,4-dimethyl-cholesta 8,14,24-trienol, 4-methylzymosterol-carboxylate and fecosterol remained stayed unchanged during the process; and the level of other metabolites increased. The results indicated that the content of sterols in the fruiting bodies tended to increase in mature fruiting body stage. These results might be closely related to the expression of genes and the substrate content in their metabolic processes in *F. velutipes* [45]. Based on the above results, we found that the nutritional and medicinal values of *F. velutipes* were higher in the mature fruiting stage. Our results provide scientific grounds for health and food guidance, but more definite conclusion takes more effort and research.

**Ergosterol biosynthesis pathway in *F. velutipes***

At present, effective approaches to efficient ergosterol production from the mycelium or fruiting body of fungus cannot be devised until the genetic engineering based on the metabolic pathway is well understood. Although the biosynthesis pathway of ergosterol in *S. cerevisiae* has been well characterized, few efforts have explored the ergosterol biosynthesis in *F. velutipes* [33]. In this paper, the extracts of *F. velutipes* samples in different developmental stages were analyzed by LC-MS, and
the sterols were studied thoroughly. At the same time, the expression of key enzymes in the ergosterol biosynthesis in *F. velutipes* indifferent developmental stages was established by RNA-Seq. This could provide insight into improving production of ergosterol and its derivatives. We found that these genes such as *ERG10s, HMG, ERG8, ERG19s, ERG20s, ERG9s, ERG1s, ERG7s, ERG11s, ERG24s, ERG25s, ERG26s, ERG27s, ERG6s, ERG2, ERG3s and ERG4* were differentially expressed in FrII vs. FrI group (Fig. 6C). Most unigenes was down-regulated. The results showed that the four genes (*ERG10s, ERG1s, ERG11s and ERG26s*) might be the most important genes involved in the biosynthesis of ergosterol. In previous studies, *ERG1* and *ERG11* are identified as the key regulators for the post-squalene biosynthesis and in the feedback regulation of ergosterol biosynthesis in *S. cerevisiae* and *Trichoderma harzianum* [46, 47], which our results were consistent with and whose conclusion our results supported. In *S. cerevisiae*, the deletion of the *ERG26* was lethal and disrupt the synthesis of ergosterol [48, 49]. These results indicated that *ERG26* was essential for cell growth, impacting the synthesis of ergosterol. The *ERG10* gene encodes an acetoacetyl-CoA thiolase that catalyzed the formation of acetoacetyl-CoA by two acetyl-CoA molecules. Studies have found that acetoacetyl-CoA reduced the activity of Erg10p when sterols were more than required [50], which our study supported. Others’ work confirmed that this regulation occurred during the transcriptional process. When the levels of some sterols in the cell are low, *ERG10* gene is expressed to a higher level and then regulates the activation pathway [51]. The efficiency of ergosterol biosynthesis was determined by rate-limiting enzymes and more crucially by optimal coordination of all enzymes [52]. Transcription factor *UPC2* was reported to upregulate target genes involved in the biosynthesis of sterol through activating the sterol response elements in their promoter regions [53, 54]. The core motifs of sterol-response elements had been identified in nine responsive *Candida albicans ERGs* (*ERG1, ERG2, ERG5, ERG6, ERG10, ERG11, ERG24, ERG26 and ERG27*). Our results revealed that the expression levels of most of these genes had differed, which was consistent with the regulatory effect of *UP2C* on these genes. And most of these genes were found to be related to the post-squalene pathway, which promised to improve the sterol biosynthesis in *F. velutipes* [26, 54-56]. However, over-expression of specific enzymes could result in imbalance of sterol intermediate accumulation, and significantly
reduced total cell biomass yield and repressed end-products. Therefore, it was necessary to avoid the over-expression of some specific enzymes and control the accumulation of some cytotoxic intermediates [57]. In summary, a balance between precursor supplies and catalytic activities of enzymes should be struck to achieve optimal production of ergosterol.

In Fig. 9, combining analyses of differential metabolites and genes were expected to find regulation relationships between them. We made a comparison between profiles of metabolites and gene expression between three different stages using Pearson’s correlation coefficient (Additional file 11: Table S6 and Additional file 12: Table S7). The horizontal axis and the vertical axis respectively represent metabolites and genes. With the distance between each metabolite or gene reflecting the similarity between their profiles [43]. Red indicates positive correlation between metabolites and genes, and blue negative correlation. This could be a useful method for comparing correlations of metabolites or genes between different groups. The heatmap of metabolites and genes helps us to understand the correlations between them. Thus, the systematic analysis in this study can be used for further research of metabolic and gene functions for bioproduction. The heatmap in Fig. 9A illustrated the results of FrII vs. FrI group. The results indicated that ERG2, ERG3, ERG9, ERG10, ERG19, ERG24 and ERG25 had a positive regulatory effect on mevalonate, 3-keto-4-methylzymosterol, ergosta-5,7,22,24-tetraenol, squalene-2,3-epoxide, 4-methylzymosterol-carboxylate, fecosterol, 14-demethyl lanosterol and 4,4-dimethy-cholesta 8,14,24-trienol, and a negative regulatory effect on lanosterol, ergosta-5,7,24(28)-trienol, mevolonate-5-phosphate, episterol, 4-methylzymosterol, ergosterol, farnesyl-pyrophosphate, dimethylallyl pyrophosphate and isopentenyl pyrophosphate. However, ERG4, ERG7 and ERG8 had negative regulatory effect. These data can provide useful information for engineered ergosterol biosynthesis in pharmaceutical industrial production for future studies.

In-vivo biosynthesis of ergosterol is a complex metabolic process requiring involvement of a variety of enzymes (at least 20 in this case), reactions and genes. Due to the different positions of each reaction in the metabolic pathway, the role of metabolic regulation is different, so the effect of high expression of related genes are not the same. Some genes, when properly expressed, get well along with ergosterol synthesis, while high expression of them may inhibit ergosterol biosynthesis. ERG1s,
ERG10s, ERG11s and ERG26s were the most important genes in the regulation of the pathway flux towards ergosterol biosynthesis of *F. velutipes*. These results will shed light on the molecular mechanisms responsible for ergosterol biosynthesis in *F. velutipes*.

**Conclusion**

In this work, a high-quality database for *F. velutipes* transcriptome was created based on NGS technology to identify genes related to ergosterol biosynthesis. Transcriptome analysis of extracts of *F. velutipes* in three different developmental stages resulted in 28,330 unigenes. Comparing FrII against FrI group, we identified 51 (12 up-regulated and 39 down-regulated) unigenes that encoded enzymes related to ergosterol biosynthesis. The results showed that ERG1s, ERG10s, ERG11s and ERG26s were the most important four genes in the regulation of the pathway flux towards ergosterol biosynthesis in *F. velutipes*. We used RT-qPCR method to check the reliability of transcriptomics of *F. velutipes*. The profiles of sterols in *F. velutipes* from three different development stages were listed. In this article, we performed metabolomics with LC-MS approach to characterize the ergosterol biosynthesis of *F. velutipes*, and identified 17 important differential intermediates or metabolites related to ergosterol biosynthesis. Combining transcriptomics and metabolomics, we explored a regulation relationship between ergosterol biosynthesis genes and metabolites in *F. velutipes*. To sum up, this study is instrumental for further research of the biosynthesis of sterol-related intermediates or metabolites in *F. velutipes*.

**Abbreviations**

*Flammulina velutipes*: *F. velutipes*; FVS: *F. velutipes* sterols; FrI: Mycelium liquid culture stage; FrII: Young fruiting body stage; FrIII: Mature fruiting body stage; BIs: Sterol biosynthesis inhibitors; NGS: Next generation sequencing; COG: Clusters of Orthologous Groups of proteins; DEGs: Differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOG: Eukaryotic Orthologous Groups of proteins; N50: Covering 50% of all the nucleotide sequences of the largest unigene length; FDR: False discovery rate; FPKM: Fragments Per Kilobase of transcript per Million mapped reads; AP: Adjusted P value; FC: Fold change; UPLC: Ultra-performance liquid chromatography; NR: Non-redundant; Q30 percentage: Percentage of bases with sequencing error
rate lower than 1‰; RNA-Seq: RNA sequencing; RT-qPCR: Real-time quantitative polymerase chain reaction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included with in the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

RHW drafted the manuscript and was responsible for the sequence data analysis. JED, PDM and ZSL assisted to manuscript revision. CL arranged the manuscript and help to collect the sample. LGX assisted with the sample collection. All authors read and approved the final version of the manuscript.

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References

1. Li HP, Yang WJ, Qu SX, Pei F, Luo X, Mariga AM, Ma L. Variation of volatile terpenes in the edible fungi mycelia *Flammulina velutipes* and communications in fungus-mite interactions. Food Res Int.2018;103:150-55.

2. Cai H, Liu X, Chen Z, Liao S, Zou Y. Isolation, purification and identification of nine chemical compounds from *Flammulina velutipes* fruiting bodies. Food Chem.2013;141(3):2873-9.

3. Liu JY, Chang MC, Meng JL, Feng CP, Zhao H, Zhang ML. Comparative Proteome Reveals Metabolic Changes during the Fruiting Process in *Flammulina velutipes*. J Agric Food Chem.2017;65(24):5091-100.

4. Smiderle FR, Carbonero ER, Sassaki GL, Gorin PAJ, Iacomini M. Characterization of a heterogalactan: Some nutritional values of the edible mushroom *Flammulina velutipes*. Food Chemistry.2008;108(1):329-33.

5. Chang YC, Hsiao YM, Wu MF, Ou CC, Lin YW, Lue KH, Ko JL. Interruption of lung cancer cell migration and proliferation by fungal immunomodulatory protein FIP-fve from *Flammulina velutipes*. J Agric Food Chem.2013;61(49):12044-52.

6. Ma Z, Cui F, Gao X, Zhang J, Zheng L, Jia L. Purification, characterization, antioxidant activity and anti-aging of exopolysaccharides by *Flammulina velutipes* SF-06. Antonie Van Leeuwenhoek.2015;107(1):73-82.

7. Yin H, Wang Y, Wang Y, Chen T, Tang H, Wang M. Purification, characterization and immunomodulating properties of polysaccharides isolated from *Flammulina velutipes* mycelium. Am J Chin
8. Yang W, Fang Y, Liang J, Hu Q. Optimization of ultrasonic extraction of *Flammulina velutipes* polysaccharides and evaluation of its acetylcholinesterase inhibitory activity. Food Research International. 2011;44(5):1269-75.

9. Wu DM, Duan WQ, Liu Y, Cen Y. Anti-inflammatory effect of the polysaccharides of golden needle mushroom in burned rats. Int J Biol Macromol. 2010;46(1):100-3.

10. El Enshasy HA, Hatti-Kaul R. Mushroom immunomodulators: unique molecules with unlimited applications. Trends Biotechnol. 2013;31(12):668-77.

11. Xiao H, Zhong JJ. Production of Useful Terpenoids by Higher-Fungus Cell Factory and Synthetic Biology Approaches. Trends Biotechnol. 2016;34(3):242-55.

12. Shao S, Hernandez M, Kramer JK, Rinker DL, Tsao R. Ergosterol profiles, fatty acid composition, and antioxidant activities of button mushrooms as affected by tissue part and developmental stage. J Agric Food Chem. 2010;58(22):11616-25.

13. Yi C, Zhong H, Tong S, Cao X, Firempong C, Liu H, et al. Enhanced oral bioavailability of a sterol-loaded microemulsion formulation of *Flammulina velutipes*, a potential antitumor drug. Int J Nanomedicine. 2012;7:5067-78.

14. Tong S, Zhong H, Yi C, Cao X, Firempong CK, Zheng Q, Feng Y, Yu J, Xu X. Simultaneous HPLC determination of ergosterol and 22,23-dihydroergosterol in *Flammulina velutipes* sterol-loaded microemulsion. Biomed Chromatogr. 2014;28(2):247-54.

15. Yi C, Sun C, Tong S, Cao X, Feng Y, Firempong CK, Jiang X, Xu X, Yu J. Cytotoxic effect of novel *Flammulina velutipes* sterols and its oral bioavailability via mixed micellar nanoformulation. Int J Pharm. 2013;448(1):44-50.

16. Ma L, Chen H, Dong P, Lu X. Anti-inflammatory and anticancer activities of extracts and compounds from the mushroom *Inonotus obliquus*. Food Chem. 2013;139:503-8.

17. Sharma M, Sasvari Z, Nagy PD. Inhibition of sterol biosynthesis reduces tombusvirus replication in yeast and plants. J Virol. 2010;84(5):2270-81.

18. Hui KP, Kuok DI, Kang SS, Li HS, Ng MM, Bui CH, Peiris JS, Chan RW, Chan MC. Modulation of sterol
biosynthesis regulates viral replication and cytokine production in influenza A virus infected human alveolar epithelial cells. Antiviral Res.2015;119:1-7.

19. Blanc M, Hsieh WY, Robertson KA, Watterson S, Shui G, Lacaze P, et al. Host defense against viral infection involves interferon mediated down-regulation of sterol biosynthesis. PLoS Biol.2011;9(3):e1000598.

20. Palmie-Peixoto IV, Rocha MR, Urbina JA, de Souza W, Einicker-Lamas M, Motta MC. Effects of sterol biosynthesis inhibitors on endosymbiont-bearing trypanosomatids. FEMS Microbiol Lett.2006;255(1):33-42.

21. Tan W, Pan M, Liu H, Tian H, Ye Q, Liu H. Ergosterol peroxide inhibits ovarian cancer cell growth through multiple pathways. Onco Targets Ther.2017;10:3467-74.

22. Rhee YH, Jeong SJ, Lee HJ, Lee HJ, Koh W, Jung JH, Kim SH, Sung-Hoon K. Inhibition of STAT3 signaling and induction of SHP1 mediate antiangiogenic and antitumor activities of ergosterol peroxide in U266 multiple myeloma cells. BMC Cancer.2012;12:28.

23. Shimizu T, Kawai J, Ouchi K, Kikuchi H, Osima Y, Hidemi R. Agarol, an ergosterol derivative from Agaricus blazei, induces caspase-independent apoptosis in human cancer cells. Int J Oncol.2016;48(4):1670-8.

24. Li X, Wu Q, Xie Y, Ding Y, Du WW, Sdiri M, Yang BB. Ergosterol purified from medicinal mushroom Amauroderma rude inhibits cancer growth in vitro and in vivo by up-regulating multiple tumor suppressors. Oncotarget.2015;6(19):17832-46.

25. Pluchino LA, Liu AK, Wang HC. Reactive oxygen species-mediated breast cell carcinogenesis enhanced by multiple carcinogens and intervened by dietary ergosterol and mimosine. Free Radic Biol Med.2015;80:12-26.

26. Ma BX, Ke X, Tang XL, Zheng RC, Zheng YG. Rate-limiting steps in the Saccharomyces cerevisiae ergosterol pathway: towards improved ergosta-5,7-dien-3beta-ol accumulation by metabolic engineering. World J Microbiol Biotechnol.2018;34(4):55.

27. Souza CM, Schwabe TM, Pichler H, Ploier B, Leitner E, Guan XL, Wenk MR, Riezman I, Riezman H. A stable yeast strain efficiently producing cholesterol instead of ergosterol is functional for tryptophan
uptake, but not weak organic acid resistance. Metab Eng.2011;13(5):555-69.

28. Zhang K, Tong M, Gao K, Di Y, Wang P, Zhang C, Wu X, Zheng D. Genomic reconstruction to improve bioethanol and ergosterol production of industrial yeast Saccharomyces cerevisiae. J Ind Microbiol Biotechnol.2015;42(2):207-18.

29. Yuan J, Ching CB. Dynamic control of ERG9 expression for improved amorpha-4,11-diene production in Saccharomyces cerevisiae. Microb Cell Fact.2015;14:38.

30. Hu Z, He B, Ma L, Sun Y, Niu Y, Zeng B. Recent Advances in Ergosterol Biosynthesis and Regulation Mechanisms in Saccharomyces cerevisiae. Indian J Microbiol.2017;57(3):270-77.

31. Alcazar-Fuoli L, Mellado E, Garcia-Effron G, Lopez JF, Grimalt JO, Cuenca-Estrella JM, Rodriguez-Tudela JL. Ergosterol biosynthesis pathway in Aspergillus fumigatus. Steroids.2008;73(3):339-47.

32. Layer JV, Barnes BM, Yamasaki Y, Barbuch R, Li L, Taramino S, Balliano G, Bard M. Characterization of a mutation that results in independence of oxidosqualene cyclase (Erg7) activity from the downstream 3-ketoreductase (Erg27) in the yeast ergosterol biosynthetic pathway. Biochim Biophys Acta.2013;1831(2):361-9.

33. Long N, Xu X, Zeng Q, Sang H, Lu L. Erg4A and Erg4B Are Required for Conidiation and Azole Resistance via Regulation of Ergosterol Biosynthesis in Aspergillus fumigatus. Appl Environ Microbiol.2017;83(4):e02924-16.

34. Wriessnegger T, Pichler H. Yeast metabolic engineering--targeting sterol metabolism and terpenoid formation. Prog Lipid Res.2013;52(3):277-93.

35. Wu Q, Wu J, Li SS, Zhang HJ, Feng CY, Yin DD, Wu RY, Wang LS. Transcriptome sequencing and metabolite analysis for revealing the blue flower formation in waterlily. BMC Genomics.2016;17(1):897.

36. Wei G, Tian P, Zhang F, Qin H, Miao H, Chen Q, et al. Integrative Analyses of Nontargeted Volatile Profiling and Transcriptome Data Provide Molecular Insight into VOC Diversity in Cucumber Plants (Cucumis sativus). Plant Physiol.2016;172(1):603-18.

37. Szymanski J, Brotman Y, Willmitzer L, Cuadros-Inostroza A. Linking gene expression and membrane lipid composition of Arabidopsis. Plant Cell.2014;26(3):915-28.
38. Chen S, Xu J, Liu C, Zhu Y, Nelson DR, Zhou S, et al. Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat Commun. 2012;3:913.

39. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3):25.

40. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323.

41. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10):106.

42. Huang JF, Shen ZY, Mao QL, Zhang XM, Zhang B, Wu JS, Liu ZQ, Zheng YG. Systematic Analysis of Bottlenecks in a Multibranched and Multilevel Regulated Pathway: The Molecular Fundamentals of L-Methionine Biosynthesis in *Escherichia coli*. ACS Synth Biol. 2018;7(11):2577-89.

43. Hsu HH, Araki M, Mochizuki M, Hori Y, Murata M, Kharar P, Yoshida T, Hasunuma T, Kondo A. A Systematic Approach to Time-series Metabolite Profiling and RNA-seq Analysis of Chinese Hamster Ovary Cell Culture. Sci Rep. 2017;7:43518.

44. Kang LZ, Zeng XL, Ye ZW, Lin JF, Guo LQ. Compositional analysis of the fruiting body of transgenic *Flammulina velutipes* producing resveratrol. Food Chem. 2014;164:211-8.

45. Wang Y, Bao L, Yang X, Li L, Li S, Gao H, Yao XS, Wen H, Liu HW. Bioactive sesquiterpenoids from the solid culture of the edible mushroom *Flammulina velutipes* growing on cooked rice. Food Chem. 2012;132(3):1346-53.

46. Veen M, Stahl U, Lang C. Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res. 2003;4(1):87-95.

47. Cardoza RE, Vizcaino JA, Hermosa MR, Sousa S, Gonzalez FJ, Llobell A, Monte E, Gutierrez S. Cloning and characterization of the erg1 gene of *Trichoderma harzianum*: effect of the erg1 silencing on ergosterol biosynthesis and resistance to terbinafine. Fungal Genet Biol. 2006;43(3):164-78.

48. Gachotte D, Barbuch R, Gaylor J, Nickel E, Bard M. Characterization of the *Saccharomyces cerevisiae* ERG26 gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol
biosynthesis. Proc Natl Acad Sci U S A. 1998;95(23):13794-9.

49. Swain E, Baudry K, Stukey J, McDonough V, Germann M, Nickels JT, Jr. Sterol-dependent regulation of sphingolipid metabolism in Saccharomyces cerevisiae. J Biol Chem. 2002;277(29):26177-84.

50. Trocha PJ, Sprinson DB. Location and regulation of early enzymes of sterol biosynthesis in yeast. Arch Biochem Biophys. 1976;174(1):45-51.

51. Dimster-Denk D, Rine J. Transcriptional regulation of a sterol-biosynthetic enzyme by sterol levels in Saccharomyces cerevisiae. Mol Cell Biol. 1996;16(8):3981-9.

52. Ghodasara A, Voigt CA. Balancing gene expression without library construction via a reusable sRNA pool. Nucleic Acids Res. 2017;45(13):8116-27.

53. Yang H, Tong J, Lee CW, Ha S, Eom SH, Im YJ. Structural mechanism of ergosterol regulation by fungal sterol transcription factor Upc2. Nat Commun. 2015;6:6129.

54. Dunkel N, Liu TT, Barker KS, Homayouni R, Morschhauser J, Rogers PD. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryot Cell. 2008;7(7):1180-90.

55. MacPherson S, Akache B, Weber S, De Deken X, Raymond M, Turcotte B. Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother. 2005;49(5):1745-52.

56. Silver PM, Oliver BG, White TC. Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell. 2004;3(6):1391-7.

57. Zhou P, Xie W, Li A, Wang F, Yao Z, Bian Q, Zhu Y, Yu H, Ye L. Alleviation of metabolic bottleneck by combinatorial engineering enhanced astaxanthin synthesis in Saccharomyces cerevisiae. Enzyme Microb Technol. 2017;100:28-36.

Supplementary Legends

Additional files

Additional file 1: Table S1. Primer sequences of DEGs related to ergosterol biosynthesis of F. velutipes in different developmental stages. (DOC 47 kb)

Additional file 2: Table S2. The statistics of RNA-Seq data of F. velutipes in different developmental
stages. (DOC 34 kb)

Additional file 3: Figure S1. Length distribution of all unigenes of transcriptome in three different stages of *F. velutipes*. (PDF 707 kb)

Additional file 4: Figure S2. Statistics of DEGs in different stages of *F. velutipes*. (PDF 673 kb)

Additional file 5: Figure S3. KEGG classified into six largest categories pathways. (PDF 848 kb)

Additional file 6: Figure S4. Heatmap analysis of DEGs related to terpenoid backbone biosynthesis pathway and steroid biosynthesis pathway in *F. velutipes*. (PDF 782 kb)

Additional file 7: Table S3. The results of differential ions and identification of *F. velutipes* in different developmental stages. (DOC 31 kb)

Additional file 8: Table S4. Differential metabolites in the ergosterol biosynthesis of FrII vs. FrI in *F. velutipes*. (DOC 46 kb)

Additional file 9: Table S5. Differential metabolites in the ergosterol biosynthesis of FrIII vs. FrII in *F. velutipes*. (DOC 45 kb)

Additional file 10: Figure S5. Verification results of the end product ergosterol in metabolomics. (A) LC-MS spectrum of ergosterol standard. (B) LC-MS spectrum of ergosterol from *F. velutipes*. Their corresponding m/z and retention time (RT) are indicated in the figure. (PDF 312 kb)

Additional file 11: Excel S1. Pearson’s correlation coefficient of metabolites and gene expression between FrII and FrI group. (XLS 45 kb)

Additional file 12: Excel S2. Pearson’s correlation coefficient of metabolites and gene expression between FrIII and FrII group. (XLS 33 kb)

**Tables**

Table 1 Transcript quality standards of the RNA-Seq analysis of *F. velutipes*

Table 2 The unigenes related to ergosterol biosynthesis of *F. velutipes* in different developmental stages

**Figures**
Figure 1

The biosynthesis pathway of ergosterol in S. cerevisiae. Biosynthesis intermediates, end
products, and enzymes involved in ergosterol biosynthesis are indicated. (A) The mevalonate pathway is the first part in blue color. (B) The post-squalene pathway is the second part in yellow. Enzymes’ name are shown at the side of each step. This figure was modified from Hu et al. [30]
Figure 2
Different developmental stages of F. velutipes. (A) mycelium solid culture stage; (B) mycelium liquid culture stage; (C) young fruiting body stage; (D) mature fruiting body stage.

The scale bar of each figure was shown in the lower right corner.
Figure 3
KOG categories of the annotated all unigenes of *F. velutipes* in different developmental stages
Figure 4
GO functional annotation and classification of DEGs between different developmental stages of F. velutipes
Figure 5

The bubble map of the DEGs pathway enrichment analysis of FrII vs. FrI group of F. velutipes. The red font shows two metabolic pathways related to ergosterol biosynthesis
Figure 6

Venn diagram and heat map showing differentially expressed genes obtained between three different developmental stages of F. velutipes. (A) Down-regulated genes and (B) Up-regulated between three different developmental stages of F. velutipes. (C) Heatmap showing differentially expressed genes between three different developmental stages of F. velutipes. Red color represents up-regulated genes and blue color down-regulated expressed genes.
Differential expression profiles of fifteen unigenes between three different developmental stages of F. velutipes. GAPDH was used as an internal control. Each unigene has three biological replicates and three technical replicates.
Figure 8

PCA and differential expression analysis of F. velutipes metabolome of different developmental groups. (A) PCA analysis of positive ions. (B) PCA analysis of negative ions. (C) Differential expression analysis of biosynthesis intermediates and ergosterol in F. velutipes between different developmental stages.
Figure 9
Heatmap analysis of metabolic and gene expression profiles of F. velutipes in different developmental stages. (A) Heatmap analysis of metabolic and gene expression profiles of FrII vs. FrI group. (B) Heatmap analysis of metabolic and gene expression profiles of FrIII vs. FrII group. Correlativity of FrII vs. FrI and FrIII vs. FrII profiles of metabolites (vertical axis) and gene expression (horizontal axis) were calculated by Pearson’s correlation coefficient. Correlativity of every metabolite and gene between groups was calculated to generate the heatmap using programming language R. Red represents positive correlations and blue represents negative correlations.

Supplementary Files
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