Comparative transcriptome analysis revealed genes involved in the fruiting body development of *Ophiocordyceps sinensis*

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Ophiocordyceps sinensis is a highly valued fungus that has been used as Traditional Chinese Medicine (TCM). This fungus is one of the most important sources of income for the nomadic populations of the Tibetan Plateau. With global warming and excessive collection, the wild *O. sinensis* resources declined dramatically. The cultivation of *O. sinensis* hasn't been fully operational due to the unclear genetic basis of the fruiting body development. Here, our study conducted pairwise comparisons between transcriptomes acquired from different growth stages of *O. sinensis* including asexual mycelium (CM), developed fruiting body (DF) and mature fruiting body (FB). All RNA-Seq reads were aligned to the genome of *O. sinensis* CO18 prior to comparative analyses. Cluster analysis showed that the expression profiles of FB and DF were highly similar compared to CM. Alternative splicing was also analyzed to provide additional insights. Functional enrichment analyses showed that differentially expressed genes (DEGs) were enriched in protein synthesis and baseline metabolism during fruiting body development, revealing that more protein and energy might be required for fruiting body development. Besides, some fruiting body development-associated genes impacted by ecological factors were up-regulated in fruiting body stages, such as nucleoside diphosphate kinase gene (*ndk*), β subunit of fatty acid synthase gene (*cel-2*) and superoxide dismutase gene (*sod*). Moreover, the expression levels of several cytoskeletons genes were altered during all growth stages, suggesting that these genes play crucial roles in both vegetative growth and the fruiting body development. Quantitative PCR (qPCR) was used to validate the gene expression profile and the results supported the accuracy of the RNA-Seq and DEGs analysis. Our study offers a novel perspective to understand the biology of *O. sinensis* fruiting body development and the underlying growth stage-specific molecular differences.

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Abstract: *Ophiocordyceps sinensis* is a highly valued fungus that has been used as Traditional Chinese Medicine (TCM). This fungus is one of the most important sources of income for the nomadic populations of the Tibetan Plateau. With global warming and excessive collection, the wild *O. sinensis* resources declined dramatically. The cultivation of *O. sinensis* hasn't been fully operational due to the unclear genetic basis of the fruiting body development. Here, our study conducted pairwise comparisons between transcriptomes acquired from different growth stages of *O. sinensis* including asexual mycelium (CM), developed fruiting body (DF) and mature fruiting body (FB). All RNA-Seq reads were aligned to the genome of *O. sinensis* CO18 prior to comparative analyses. Cluster analysis showed that the expression profiles of FB and DF were highly similar compared to CM. Alternative splicing was also analyzed to provide additional insights. Functional enrichment analyses showed that differentially expressed genes (DEGs) were enriched in protein synthesis and baseline metabolism during fruiting body development, revealing that more protein and energy might be required for fruiting body development. Besides, some fruiting body development-associated genes impacted by ecological factors were up-regulated in fruiting body stages, such as nucleoside diphosphate kinase gene (*ndk*), β subunit of fatty acid synthase gene (*cel-2*) and superoxide dismutase gene (*sod*). Moreover, the expression levels of several cytoskeletons genes were altered during all growth stages, suggesting that these genes play crucial roles in both vegetative growth and the fruiting body development. Quantitative PCR (qPCR) was used to validate the gene expression profile and the results supported the accuracy of the RNA-Seq and DEGs analysis. Our study offers a novel perspective to understand the biology of *O. sinensis* fruiting body development and the underlying growth stage-specific molecular differences.

Keywords: *Ophiocordyceps sinensis*; comparative transcriptome analysis; fruiting body development

1. Introduction

Ophiocordyceps sinensis (Berk.), belonging to Ascomycetes, is endemic to the Tibetan Plateau and the Himalayas with high latitudes from 3000 - 5000 m [1]. The Chinese name for *O. sinensis* is "Dong Chong Xia Cao", meaning "winter worm, summer plant". The name derived from an unusual lifestyles: entering and growing inside the ghost moth caterpillars (*Thitarodes spp.*), protruding of fruiting body from the caterpillar shell, forming a parasitic fungus-insect complex that comprises a mature fruiting body with cleistothecia[1]. Besides, the life cycle of *O. sinensis* is very long. In the

wild, this fungus requires 3-5 years to fully mature [2]. At present, the molecular basis of fruiting body development is poorly understood.

O. sinensis has been widely used as Traditional Chinese Medicine (TCM) to treat asthma, bronchitis, lung inflammation, nocturnal emissions, night sweats, and other diseases for over 2000 years in some Asian countries [3]. This medicinal fungus is one of the most important sources of income for the native tribes of the Tibetan Plateau [4-6]. Due to the high medical value and huge market demand, the price was as high as approximately USD 60,000 / kg for the top grade in 2015 and the natural populations of *O. sinensis* were overexploited, inevitably impairing the sustainability of the regional economy of Tibet and endangering this precious species [2]. Even though under favorably artificial cultivation, fruiting body costs 1-2 years to mature [2]. To date, the large-scale cultivation of *O. sinensis* hasn't been realized. A better understanding the biology of the fruiting body development would be helpful for cultivating *O. sinensis* to meet the medicinal demand and ease the exploitation of wild *O. sinensis* populations

Fruiting body development of *O. sinensis* requires special environmental factors and has been demonstrated to be initiated by the ecological factors specific to the alpine ecosystem [6]. These years, the influence of environmental factors on fruiting body development has been investigated in several *O. sinensis* like fungi, such as *Neurospora. crassa*, *Aspergillus. Nidulans,* and *Pestalotiopsis microspore* [7-9]. Moreover, some genes involved in the perception of some environmental factors (e.g. light, temperature and gravity, etc.) have been identified. These results provided good references for researches in *O. sinensis*.

With the development of high throughput sequencing technology, genomes and transcriptomes have been increasingly obtained for further researches [10, 11]. Recent research detected the signals of the positive selection for genes involved in peroxidase and hypoxia to enable its highland adaptation by sequencing 31 whole genomes of *O. sinensis* from different distributions [6]. Li Xiang et al. constructed an *O. sinensis* EST database by using Roche/454 GS-FLX (Titanium) pyrosequencing technology, 4 mating type genes and 121 genes putatively associated with fruiting body development were identified [12]. Moreover, the transcripts of three developmental stages (mycelia, sclerotium and fruiting body)were sequenced and compared [13]. However, the sclerotium samples were artificially-cultivated, while mature fruiting body samples were naturally-cultivated. These different growth environment might have a potential effect on the dynamic expression patterns of different growth stages. Transcriptome analysis of *O. sinensis* before and after infection of *Thitarodes* offers insights into the infection biology by which the fungus interacts with its host partner [14].

Built on prior results, in this study, samples of *O. sinensis* were harvested from artificial cultivation in lab, which offers a convenience in sampling, including the asexual mycelium, the developed stroma samples and mature fruiting body. With the state-of-art Illumina Hiseq 2000, transcriptome profiles of the three developmental stages were sequenced and compared. Our study reveals significant differences in expression among genes involved in protein biosynthesis and baseline metabolism for different growing periods of *O. sinensis*. Moreover, several fruiting body development associated genes influenced by environmental factors were identified. And the expression levels of cell cytoskeleton genes were found to be altered during the growth stages for the first time in our study, indicating that they may be critical for the fruiting body maturation of *O. sinensis*. Besides, the stage – specific alternative splicing (AS) genes may also play roles in the process of fruiting body development of this fungus. Together, Our study provided novel resources for further studies into the genetic basis of fruiting body development and aids the large-scale artificial cultivation of *O. sinensis*.

2. Results and discussion

2.1. Differential gene expression and functional analyses of O. sinensis

To investigate the profile of gene expression during the development process, duplicate samples from three stages were submitted for RNA-Seq, including the mature fruiting body (FB), the developed fruiting body (DF) and mycelium (CM) were submitted for the following RNA-Seq study. After cleaning and quality control, more than 63 million paired-end, reads per replicate, were generated with Illumina HiSeq. Reads of CM, DF and FB were aligned to the *O. sinensis* CO18 genome using HISAT2 [15-17], and were assembled by StringTie [15,18], resulting in 7518 protein coding genes and 233 putative novel genes (Table S1). The number of aligned reads per gene was normalized by the STAR-RSEM based algorithm. In a previous study, Li Xiang et al. constructed an *O. sinensis* EST database using Roche / 454 GS-FLX (Titanium) pyrosequencing technology and they obtained a total of 1,743,676 high-quality reads with an average length of 426 bp, and assembled these reads into 34,289 unique sequences with an average length of 612 bp. BLAT comparison was performed between the 11,497 genes in this study and 34,289 sequences in Li's study[12], which revealed that more than 90.00% of the 34,289 sequences can be found in the 11,497 genes with an identity of 90%, while only 78.01% of the 11,497 genes can be found in the 34,289 sequences, indicating that our assembly identified more gene than the previous study.

2.2. Differential gene expression and functional enrichment analysis

Differentially expressed genes (DEGs) were considered statistically significant if the $|\log_2 FC$ ($\log_2$ fold-change)$|$ is more than 2 and the FDR is less than 0.001. This threshold resulted in a total of 1234 gene as significant DEGs in FB vs. CM, 503 in FB vs. DF, 1153 in DF vs. CM (Figure 1A, Table S1). These results revealed that the expression patterns of the fruiting body developmental stages (FB and DF) were more similar to each other compared to the vegetative CM stage. There are 19 shared DEGs among all three comparisons of the different growth stages (Figure 1B, Table S2). Most of the shared DEGs were functionally unknown proteins and required to be further studied. However, among them, one gene encoding ribosomal structure protein (*MSTRG.3745, $p < 0.001$*) was significantly up-regulated in FB compared to that in both CM and DF. Two genes were identified to encode cytochrome p450 (*MSTRG.9555, MSTRG.4151,* both *$p < 0.001$*), the express levels of which were remarkably up-regulated in CM compared to fruiting body stages (FB and DF). Cytochrome p450 has been prove to be required for fungal development and virulence in the plant pathogen *Fusarium graminearum* [19, 20]. In 2019, one cytochrome p450 was identified to be a hub gene in *O. sinensis* [21]. Taken together, cytochrome p450 would be associated with the vegetative growth of *O. sinensis*.

Based on both KEGG and GO databases, we analyzed the enrichment of the DEGs with fold change $|\log_2 FC| \geq 2$ and $p < 0.005$ (Table S2) during the three growth stages. In FB vs. DF and DF vs. CM comparisons, GO categories including 'structural constituent of ribosome', 'proteasome core complex', 'MCM complex', 'ribonucleoprotein complex', 'peptide biosynthetic / metabolism', 'nucleoside metabolic process'. 'ATP catabolic process', 'ATPase activity', etc. were enriched (Figure 2, Table S2), suggesting the importance of these terms for the fruiting body development. Comparing the FB vs. CM transcripts, the significant enriched terms mainly included 'carbohydrate metabolic process', 'pyridine-containing /organic hydroxy compound biosynthetic process' and 'iron ion binding', etc., in which the annotation percentage of up-regulated genes in CM were higher than in FB (Figure 2, Table S3). This analysis suggested that the carbohydrate metabolism and secondary metabolites biosynthesis might be more active during mycelium growth stage, which might be utilized for rapid growth as well as a preparation for the following fruiting body development.

129

130 *2.3. Clustering of gene expression profiles across three growth stages*

131 Hierarchical clustering analysis revealed that the three growth stages with two biological replicates
132 could be assigned into two groups with, FB and DF as one group and CM as another. The principle
133 component analysis (PCA) also showed that the mature fruiting body (FB) shared more similar expression
134 profile with the developed fruiting body (DF) than the undifferentiated mycelium (CM) (Figure 3A). The
135 expression patterns of all genes were further examined from CM to FB. There are seven gene clusters with
136 visible different expression profiles (Figure 3B). Cluster 1 and cluster 3, with 712 and 72 transcripts,
137 respectively, had a sudden decrease in expression upon transition from CM to FB, and a steady trend from
138 FB to DF, indicating that these genes were up-regulated and might play crucial roles in CM. Cluster 4, with
139 188 transcripts, had a sudden decrease in expression patterns upon transition from CM to FB, and then a
140 sudden increase in expression from FB to DF, illustrating that these genes mainly were expressed in CM
141 and DF. Cluster 1, 3 and 4 mainly consisted of genes that are involved in terms of 'carbohydrate metabolic
142 process', 'oxidoreductase activity' and 'protein kinase activity', which might contribute to this fungus
143 vegetative growth ($p < 0.01$, Table S4). Cluster 2, with 139 transcripts, had a gradual decrease from CM to
144 FB, and cluster 6, with 280 transcripts, had a gradual increase from CM to FB, which indicated that these
145 genes have different roles during the three stages. Cluster 5, with 471 transcripts, had a sudden increase in
146 expression patterns upon transition from CM to FB, and then a sudden decrease in expression from FB to
147 DF. Cluster 5 consisted of genes involved in terms of 'ribosomal structure constituent', 'translation', 'starch/
148 sucrose metabolic process', 'polysaccharide catabolic process', etc. ($p < 0.01$, Table S4). Starch/ sucrose
149 metabolism is associated with energy production. Three genes involved in starch metabolic process
150 (*MSTRG.2946, MSTRG.3495, MSTRG.5107,* all $p < 0.001$) were at significant and the highest expression level
151 in FB, revealing that ATPs produced from starch/ sucrose were more important in the fruiting body
152 development stages compared to the asexual mycelium stage. Besides, polysaccharides have been reported
153 to be important for the growth of fungi like *Tuber melanosporum* [22] and *Trichoderma reesei* [23]. And
154 polysaccharides have been proven to have antioxidant activity of the fruiting body in *Cordyceps militaris*
155 [24], immune-stimulating activity [25] and antitumor activity in some other medicinal mushrooms [26].
156 Therefore, polysaccharide catabolic processes might contribute to the medicinal value of the fruiting body
157 of *O. sinensis.* Cluster 7, with 280 transcripts, had a sudden increase in expression patterns upon transition
158 from CM to FB, and then remained a steady trend between FB and DF, indicating that these genes may be
159 involved in the structural formation and sexual maturation of the fruiting body. Cluster 7 mainly consisted
160 of genes involved in terms of 'ribosome', 'ATP catabolic process' and 'protein metabolism process' ($p < 0.01$,
161 Table S4), indicating that the process of fruiting body development is energy and nutrient intensive,
162 consistent with the analysis of DEGs.
163
164

165 *2.4. Alternative Splicing Prediction*

166 Alternative splicing (AS) events increase the complexity and diversity of genes. Some basic AS events
167 include exon skipping (SKIP), retention of single/multiple intron (IR / MIR), alternative transcript start/
168 termination (A3SS / A5SS), and alternative exon (AE). Our data identified that 19038 AS events in DF, 18137
169 in FB and 20247 in CM (Figure 4A). A3SS and A5SS are the major classes which accounted for over 80% of
170 all AS events in *O. sinensis,* whereas IR / MIR is the major class involved in AS in other fungi, such as
171 *Cordyceps militaris* [19], *Aspergillus oryzae* [27] and *Tuber melanosporum* [28]. When comparing AS events
172 between different developmental stages of *O. sinensis*, we found that the number of AS events was the
173 highest in CM, followed by in DF, and then in FB. Moreover, 937, 946 and 652 AS genes were found to be

significantly different between in FB vs. CM, in DF vs CM and in FB vs DF, respectively (Figure 4B). Retention of intron event (IR) is the major class in these stage specific AS genes during the growth stages (Figure 4B, Table S5). Between FB and DF, AS genes were enriched in 'damaged DNA binding', 'oxidoreductase activity/ reduction of molecular oxygen',' heme binding' and 'tryptophan/ tyrosine/ L-phenylalanine biosynthetic process ', etc. ($p <= 0.01$,Table S5). Previous studies found that mutants of oxidoreductase led to sterile in *N. crassa* with respect to sexual differentiation [29], consistent with an increased demand of energy and metabolites for the maturation of fruiting body. In DF vs. CM, the different AS genes were enriched in GO terms, including 'tryptophan/ tyrosine/ L-phenylalanine biosynthetic process'. In FB and DF comparison, AS genes were enriched in terms of 'leucine and valine biosynthetic process' ($p <= 0.01$, Table S5). This analysis revealed that more varieties of amino acids /proteins might be required during the fruiting body development than the vegetative mycelium growth. Moreover, there are all 173 shared AS genes among the three growth stages (Table S6), including genes encoding Hsp 90 binding chaperon (*mod-E*, *MSTRG.8149*, $p < 0.001$) and eukaryotic translation initiation factor (*MSTRG.6510*, $p < 0.001$), both of which were significantly up-regulated in FB compared to CM. Previous study showed that a mutation of *mod-E* affected the sexual cycle and suppressed vegetative incompatibility in *Podospora anserine* [30]. Hence, our results revealed that AS genes in different developmental stages may play a significant role in the development of *O. sinensis* and require further investigation.

2.5. Analysis of Cell Cytoskeleton Genes

The fruiting body formation is accompanied by many morphologically distinct cellular differentiations. Most of specialized cell types were found in fruiting body [8]. Moreover, genes for cell cytoskeleton structure and organizations have been shown to contribute to sexual department in some Ascomycetes species [8]. Our comparative transcriptome analysis showed that the expression level of cell cytoskeleton genes were altered during these three growth stages. One actin gene (*MSTRG.3317*, $p < 0.005$, Table S1) was remarkably down-regulated by 3.43-fold in FB than in DF, and two tubulin / FtsZ protein genes (*MSTRG.3823* and *MSTRG.6894*, both $p < 0.005$, Table S1) were up-regulated by 3.04- and 2.90- fold in FB than in DF, respectively. One actin related protein gene (*MSTRG. 3090*, $p < 0.0001$, Table S1) was up-regulated by 3.66- fold in FB compared to DF, whereas in DF vs. CM, the gene was down-regulated by about 2.86- fold in DF compared to CM. Previous studies found that actin and actin-binding protein fimbrin are critical in the growth of hyphae in *A. nidulans* [31]. Two genes encoding α-tubulin, tubA and tubB, tub A is essential for mitosis and nuclear migration, and tubB is required for ascospore formation [32, 33]. Thus, our results suggested that genes for cell cytoskeleton organization may be important in both vegetative growth and the fruiting body maturation of *O. sinensis*.

2.6 Analysis of development-related genes regulated by environmental conditions

Fruiting body development is a complex cellular differentiation process, which is regulated by special environmental conditions. In general, the mycelia of *O. sinensis* grows in the dark, while fruiting body grows under the light to maturity [13], suggesting that light is required for fruiting body development of this fungus. In our study, some putatively development-related genes putatively regulated by light were identified (Table S7). For examples, the nucleoside diphosphate kinase gene (*ndk* , *MSTRG. 2107*, FDR < 0.01), was up-regulated by 6.23-fold in FB compared to DF. In *N. crassa*, NDK was essential for light-dependent neck positioning on the perithecia and possibly involved in a light signal transduction pathway[34]. Three of superoxide dismutase gene (*sod*, *MSTRG. 2218*, *MSTRG. 5954* and *MSTRG.6951*, all FDR < 0.01, Table S7) were remarkably up-regulated in FB compared to DF. Previous study displayed that SOD-1 was required for correct fruiting body morphology and it could be due to that SOD-1 is involved in

generating a light-dependent ROS gradient that controls neck positioning [4]. The expression level of the cytochrome oxidase (COX) gene (*MSTRG.3789*, FDR < 0.001, Table S7) was altered during the growth period, at much higher expression level in CM and FB compared to DF. Mutants of COX showed the delayed perithecial formation and reduced ascus production [35]. COX as well as some superoxide dismutases were involved in the generation of ROS [35]. Moreover, mutants of the NADPH oxidase gene, *noxA*, are also sterile. A *noxA* mutant, ortholog *PaNox1*, no longer developed to mature fruiting bodies in *P. anserine*[36]. The expression level of the NADPH oxidase gene (*MSTRG.7071*, FDR < 0.001, Table S7) was the significantly highest in FB. Moreover, both *paNox1* and *paNox2*, orthologs of *nox* were required for controlled production of superoxide as well as peroxide during sexual development [35]. These results revealed that ROS level would impact fruiting body development of *O. sinensis.* Further study is essential to investigate how ROS regulates fruiting body development of this fungus.

In addition, *O. sinensis* adapts to low temperature, which is another physical factor influencing fruiting body development. Some genes influence by cold were identified, such as β subunit of fatty acid synthase (*cel-2* , *MSTRG.6193*, FDR < 0 .001, Table S7) [37] and acyl -CoA desaturase gene (*MSTRG.3201*, FDR < 0.001, Table S7) [38]. The expression level of *cel-2* and acyl -CoA desaturase gene were remarkably up-regulated in FB compared to CM. In FB vs. DF, acyl -CoA desaturase gene has a much higher expression level in FB. *Cel-2* mutant led to fewer peritecia or rare ascospores. Besides, two genes (*MSTRG.4136, MSTRG.10796*, FDR < 0.001, Table S7) encoding low-temperature enzyme were potentially identified to be heat-shock protein (Hsp) 70 chaperone, which was supported by the evidence that, Mod-E, a Hsp 90 homology, was reported to be involved in the temperature-dependence of sexual development in *Podospora anserine* [30]. Moreover, four heat shock protein 101 genes (*MSTRG.11220, MSTRG.10780, MSTRG.10779* and *MSTRG.1122*, FDR < 0.001, Table S7) were found to be remarkably up-regulated in both FB and DF compared to CM. However, few studies have been reported. It remains to be determined whether heat-shock proteins are involved in temperature-dependence of fruiting-body development and the underlying mechanism.

2.7. Analysis of signal transduction pathway

The fruiting body development was a complex cellular differentiation process regulated by the specific environmental stimuli, and subsequently regulated by signal transduction pathways [8].

Mitogen-activated protein kinase (MAPK) modules have been identified in *N. crassa* by genome analysis. MAPK module includes three kinases that establish a sequential activation pathway comprising a MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK [39], among which three different MAPKs and two different MAPKKKs have been shown to be involved in the fruiting body development in different mycelial ascomycetes[8].

In our study, over ten genes encoding MAPKKK , MAPKK and MAPK were identified in the transcriptome during the growth period of *O. sinensis*. MAPKKK cascade protein kinase regulators, similar to *S. cerevisiae* Ste 11p, which regulates conidiophore development and is required for cleistothecial development [40]. In plant pathogenic fungus, *M. grisea* and *Fusarium graminearum*, the homologs of MAPKKK protein genes, are essential for plant infection and female fertility [41, 42]. Our data found that four out of five MAPKKK encoding genes (*MSTRG.10919, MSTRG.5562, MSTRG. 8422*, and *MSTRG.3582*, Table S1), were expressed at higher levels in DF and FB stages compared to CM. Two of them (*MSTRG.5562* and *MSTRG.3582*, both $p < 0.001$, Table S7) are significantly up-regulated in DF compared to CM. Besides, several MAPK protein genes were identified. *MSTRG.6508* was annotated as mitogen protein- activated protein kinase, and *MSTRG.3890* as WD domain containing protein, which is a MAPK binding protein. Both *MSTRG.6508* and *MSTRG. 6508* were expressed at higher levels in FB compared to CM (both FDR < 0.001, Table S7). *Cpc 2* encoding WD proteins were proved to control sexual development as well as amino acid biosynthesis [43].Genes encoding serine / threonine-protein kinase, which act upstream of MAK-2

during fruiting-body development by regulating conidiophore development are essential for cleistothecial development [44]. These genes (e.g. *MSTRG.9127*, *MSTRG.6087*, *MSTRG.3872* and *MSTRG.3872*, Table S1) were at relatively higher expression levels in fruiting body stages than in mycelium growth stage. Afterwards, these activated MAPK protein would activate the downstream Transcript Factors (TFs) to regulate the fruiting body development.

In addition, PKA pathway has been demonstrated to lead to ascocarp formation and be associated with the asexual development, hyphal growth, and pathogenicity in several Ascomycetes species[8]. One cAMP-dependent protein kinase encoding gene (*MSTRG. 2256*, $p < 0.05$, Table S1) is up-regulated in CM compared to DF, suggesting that the PKA signal pathway might play roles in vegetable growth of *O. sinensis*. Taken together, our results indicated that fruiting body development process in *O. sinensis* might be more depend on MAPK cascade signaling pathway than on PKA pathway, consistent with *C. militaris*. Further studies were needed to investigate the sexual regulation network.

2.8. Validation oftTranscriptome data by quantitative RT-PCR analysis

To confirm the reliability of RNA-Seq analysis, the expression level of 10 genes were randomly selected and analyzed by qPCR during FB and CM stages, compared to DF. In FB vs. DF, except for three genes (*MSTRG.7313*, *MSTRG.5396* and *MSTRG.5638*, Table S1), the qPCR results of seven genes were similar to the Illumina sequencing results including five genes up-regulated in FB and two genes up-regulated in DF (Figure 5A). In DF vs. CM comparison, except for two genes (*MSTRG.5638*, *MSTRG.5297*, Table S1) , the qPCR results of eight genes were in accordance with the transcriptome data, including four genes up-regulated in CM, two gene up-regulated in DF and two genes were similarly expressed between the two stages (Figure 5B). These results showed that our transcriptome data are reliable.

3. Conclusion

To conclude, Illumina sequencing was performed to investigate the transcriptomes from three growth stages of the artificially cultivated *O. sinensis* (CM, DF and FB). Functional annotations and enrichment analysis showed that 'structural constituent of ribosome', 'peptide biosynthetic process', 'ATPase activity', 'DNA replication' and 'MCM complex' were more active in fruiting body developmental stages compared to CM. Furthermore, several fruiting body development associated genes that are regulated by ecological factors (e.g. solar ultraviolet radiation, low temperature) were identified, such as *csnD*, *ndk*, *sod*, *cox*, *nox A* and *mod-E*. Cluster analysis showed that the transcriptome profiles of FB and DF were more similar that that of the undifferentiated CM stage. Alternative splicing (AS) analysis enriched the known knowledge of *O. sinensis*. Besides, the expression levels of several cytoskeleton genes were found to be altered between the different growth stages for the first time. The results indicated that cytoskeleton genes may contribute to the fruiting body development. Besides, fruiting body development process may be more dependent on MAPK-cascade signaling pathway than PKA pathway. Therefore, the comparative transcriptome analysis of the three growth stages enriches resources and valuable information to understand the genetic basis of the fruiting body development of *O. sinensis*, as well as paving a way for successful artificial cultivation of this valuable fungus in the future.

4. Materials and Methods

4.1. Specimen collection, RNA extraction and Sequencing

Three developmental stages of *O. sinensis* were artificially cultivated and collected in our lab. *O.*

sinensis strain was deposited in the China General Microbiological Culture Collection center, accession No. CGMCC 3.14243, and cultured on potato dextrose agar (PDA) plate. The mycelium from PDA plate was designated as CM and harvested for RNA isolation. The samples of stroma without ascus forming, was designated as the developed fruiting body (DF). The samples of fruiting body with mature perithecium were designated as FB and harvested for RNA isolation. All fresh specimen collected were stored in RNAlater® (Ambion, Austin, Texas, USA) according to the manufacturer's instruction until ready for RNA extraction.

Total RNA was isolated by using TRIzol (Invitrogen, USA) according to the manufacturer's instruction. Genomic DNA was digested by DNase I (Fermentas, USA). Purified RNA were electrophoresed in a 1% agarose gel, and the purity and the quality of RNA were assessed by OD260 and OD230. At least 20 μg total RNA was then submitted to Biomarker Technology Co., Ltd (Beijing, China) for quality control using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA).

The mRNA was enriched from total RNA using poly (T)+ oligo attached magnetic beads, eluted with Tris-HCl buffer and fragmented in fragmentation buffer using an RNA fragmentation kit (Ambion, Austin, TX, USA). First-strand cDNAs were reverse transcribed with reverse transcriptase and random hexamer-primers, and then second-strand cDNAs were synthesized by DNA Polymerase I.A single 'A' base was added to the ends of these dscDNA, and then the cDNA was ligated with Illumina sequencing adaptors and separated in an agarose gel. The 200 bp cDNA fragments were then purified from the gel using Gel Extraction Kit (Axygen, Biosciences, Central Avenue Union City, CA, USA) for further library preparation using NEB Next Ultra RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacture's instruction. The fragmented cDNA libraries of each sample were then submitted to an Illumina Hiseq 2000 platform at Biomarker Technology Co., Ltd. (Beijing, China) for transcriptome sequencing and the 125 bp paired-end (PE) chemistry. Adapter sequence and low quality bases were removed from the raw reads.

4.2. Read mapping, annotation and differential expression analysis

O. sinensis genome was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/assembly/GCA_000448365.1/). Trimmed paired-end reads of FB, DF and CM were aligned to the reference genome by the fast, splice-aware HISAT2 aligner (version 2.0.4) [15]. Reads that have been aligned to the genome from each sample were then assembled by StringTie (ve1.3.3b) using default parameters (version 2.0.6) [15-17]. Assembly quality (max length, average length, N50 number and unigene number with length ≥ 1000 bp) was generated by perl scripts. To globally characterize the expression patterns of diverse RNA-Seq samples, paired-end reads were aligned back to the assembled scaffold using Bowtie 2.0 as the aligner [17].

Comparative analysis was performed in a pairwise manner for duplicates assemblies of each sample by BLAT with different identity threshold. The matched unigene number between each paired assemblies were counted using Shell command in the Linux system. The mapping results of *O. sinensis* transcriptome were shown in Table S1. Coding sequences were predicted by Transdecoder (version 2.0.1). Functional annotation of assembled sequences was retrieved from the databases of Kyoto Encyclopedia of Genes [45] and Genomes (KEGG) [46], Cluster of Orthologous Groups (COG) [47], NCBI's non-redundant protein (NR) and SwissProt

Gene expression patterns were quantified using STAR-RSEM algorithm (version 4.1) based on the read numbers that were mapped to each gene. RSEM results of each replicate of the sample were merged as one matrix for following analyses. Differentially expressed genes (DEGs) were identified

based on TMM algorithm with a threshold of |log2FC (log2 fold-change)| ≥ 2 and false discovered rate (FDR) < 0.001[14] as statistically significant. Enrichment was determined by a hypergeometric test with a threshold value of $p_{adj} \leq 0.05$ for GO functions and KEGG pathways. The mapped read numbers of each assembled contig were calculated, and were normalized by RESM-based algorithm to get FPKM values for each RNA-Seq sample using perl scripts in the Trinity package [15]. Differentially expressed transcripts (DETs) were identified with a threshold of |log2 fold-change (log2FC)| ≥ 1 and $p_{adj} \leq 0.05$ [14] that has been integrated in the Trinity package [15]. Similarly, enrichment was determined by a hypergeometric test with a threshold value of $p_{adj} \leq 0.05$ for GO functions and KEGG pathways. Hierarchical clustering was used to test the statistical enrichment of differentially expressed genes in GO functions.

Raw Illumina sequencing results of *O. sinensis* were submitted to NCBI Sequence Read Archive (SRA) with the accession numbers: SRR5282569, SRR5282570, SRR5282574, SRR5282575, SRR5282577and SRR5282578.

4.3. Alternative Splicing Prediction

Alternative splicing (AS) events were classified into the following classes: SKIP (exon skipping), MSKIP (cassette exons), IR (retention of single intron), MIR (retention of multiple introns), AE (alternative exon), A3SS (alternative transcript start) and A5SS (alternative transcript termination). The 'X' prefix before each class indicates an imprecise pairing of exon boundaries. ASprofile (version b-1.0.4, http://ccb.jhu.edu/software/ASprofile/) was used to detect the splice junction sites and provided information about boundaries and combinations of different exons in a transcript, then total splice junction sites of the same gene were used to distinguish the type of its AS event. The number of each AS type were compared between different developmental stages and Q value (FDR analogue of the p-value) less than 0.05 was the threshold. TopGO was used to perform function enrichment analysis of AS genes between different growth stages [48] and the corrected p-value no more than 0.01 (p <= 0.01) was used to judge the significant enriched GO terms.

4.4. Quantitative RT-PCR

Ten genes that was statistically significant different between three growth stages according to the transcriptome sequencing analysis were selected and validated by qPCR experiments in triplicate. Primers were designed according to the sequences acquired from Illumina sequencing results with Primer Premier 5.0. The specific primer sequences are listed in Table S8. Aliquots of cDNA template submitted for Illumina HiSeq sequencing were used for qPCR analysis.. cDNA amplification was performed in a total of 25 μL reaction system containing 12.5 μL 2 × UltraSYBR qPCR Mix (Cwbiotech, Co., LTD, Beijing, China), 2 μL of diluted cDNA mix, 0.5 μL of each primer (10 mM) .The cycling conditions were as follows: 95 ℃ for 10 min, 40 cycles of 95 ℃ for 10 s, 57 ℃ for 15 s and 72 ℃ for 20 s. Relative gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method. The histone 2A gene (*MSTRG. 2474*) was used to normalize the expression level. Graphpad Prism program (version 5.0, GraphPad Software, Inc.) was used for statistical analysis.

Abbreviations: FB: mature fruiting body of *O. sinensis*, CM: the mycelium of *O. sinensis;* DF: developed fruiting body of *O. sinensis* ; NGS: next-generation sequencing; RNA-seq: RNA-sequencing; log2FC: log2 fold-change; DEG: differentially expressed genes; SEG: specifically expressed genes; EC: enzyme codes. PDA: potato dextrose agar; SRR: clean reads of *O. sinensis* from the Sequence Read Archive; AS: Alternative splicing; SKIP: including exon skipping; IR/MIR: retention of single/multiple intron; A3SS / A5SS: alternative transcript start/ termination; AE: alternative exon.

Authors' contributions: Jinlin Guo conceived this study, designed the experimental plan. Cheng Peng participated in designing the experiment. XinxinTong performed data analysis, writing-original draft. Han Zhang participated in data analysis and performed qPCR experiment. Zhengyao Xue revised and reviewed the manuscript. Jing Cao and Fang Wang participated in sample preparing, sample processing and total RNA extracting. All authors read and approved the final manuscript.

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Competing interests: The authors declare that they have no competing interests.

Supplementary Materials: Table S1 Sequence annotations and gene expression profiling, Table S2 GO enrichment of differentiated expressed genes. Significant, Table S3 Differentially expressed genes involved in enriched GO terms, Table S4 GO enrichment of DEGs clustered, Table S5 GO enrichment of different AS genes between sample comparisons, Table S6 All shared AS genes among all different comparisons of the three growth, Table S7 Fruiting-body development candidate genes, Table S8 Primers used for quantitative RT-PCR.

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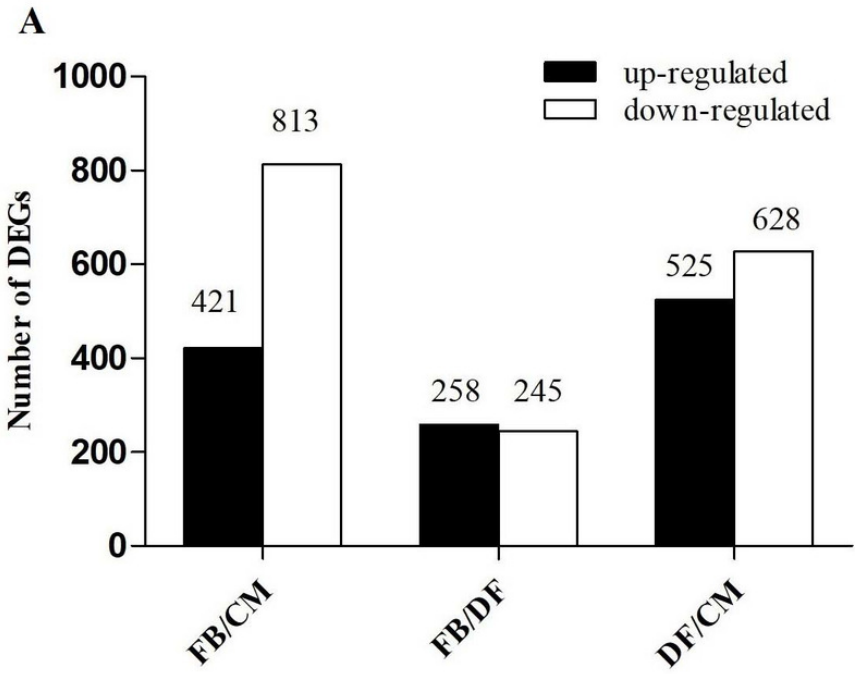
518

Figure 1

Analysis of differentially expressed genes between different growth stages.

A.The number of genes differentially expressed (DEGs) is shown on the top of histograms. statistics of DEGs from *O. sinensis* between different developmental stages. B. Venn diagram of DEGs comparing between different developmental stages from *O. sinensis*. FB represents fruiting body with mature perithecium. CM represents the asexual mycelium. DF represents the developed fruiting body.


A
1000
800
600
400
200
0
Number of DEGs
up-regulated
down-regulated
421
813
258
245
525
628
FB/CM
FB/DF
DF/CM

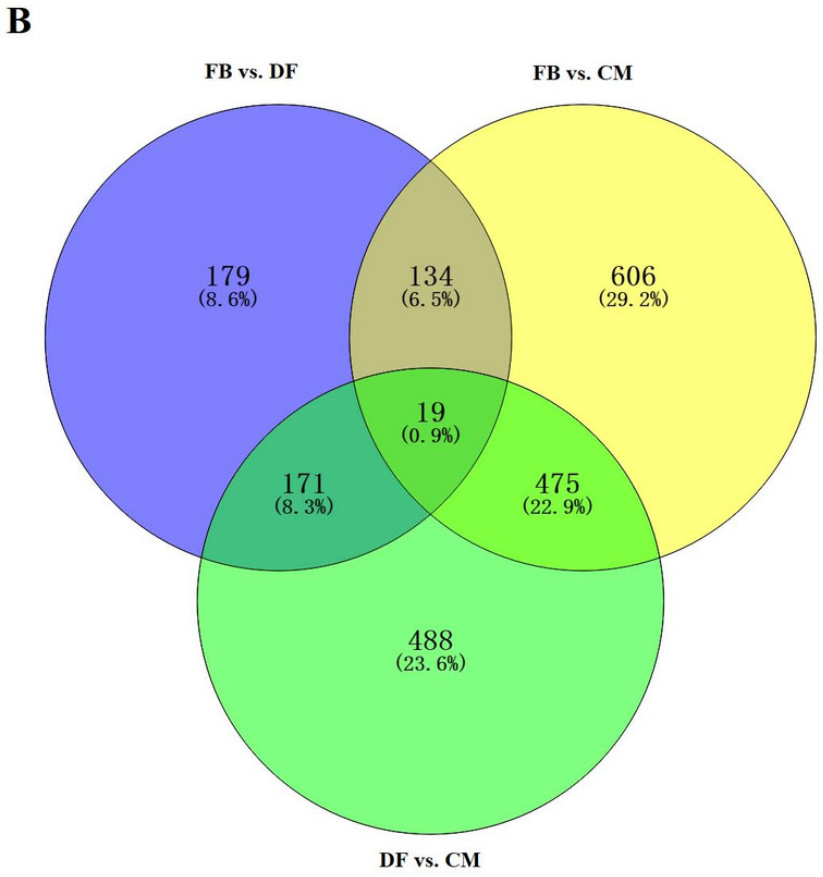

B
FB vs. DF
FB vs. CM
179
(8. 6%)
134
(6. 5%)
606
(29. 2%)
19
(0. 9%)
171
(8. 3%)
475
(22. 9%)
488
(23. 6%)
DF vs. CM

Figure 2

The most enriched GO functional classification of DEGs between different growth stages.

Only the significant GO terms ($p < 0.005$) were shown. The green bars represent cellular components; The orange represent biological processes; the blue bars represent molecular functions. FB represents fruiting body with mature perithecium. CM represents the asexual mycelium. DF represents the developed fruiting body.

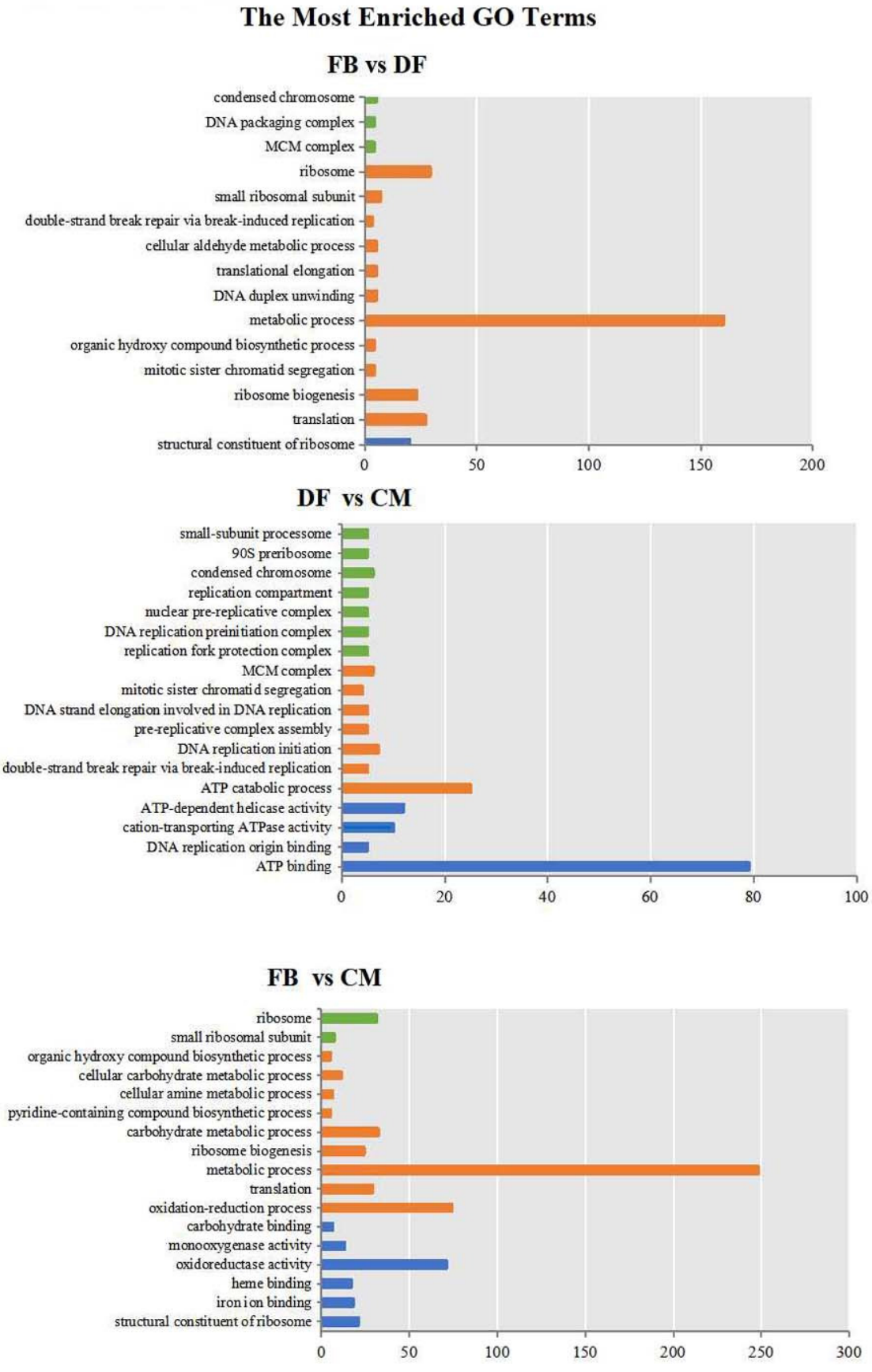
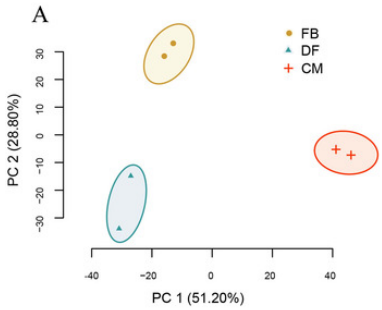

The Most Enriched GO Terms
FB vs DF
condensed chromosome
DNA packaging complex
MCM complex
ribosome
small ribosomal subunit
double-strand break repair via break-induced replication
cellular aldehyde metabolic process
translational elongation
DNA duplex unwinding
metabolic process
organic hydroxy compound biosynthetic process
mitotic sister chromatid segregation
ribosome biogenesis
translation
structural constituent of ribosome
0
50
100
150
200
DF vs CM
small-subunit processome
90S preribosome
condensed chromosome
replication compartment
nuclear pre-replicative complex
DNA replication preinitiation complex
replication fork protection complex
MCM complex
mitotic sister chromatid segregation
DNA strand elongation involved in DNA replication
pre-replicative complex assembly
DNA replication initiation
double-strand break repair via break-induced replication
ATP catabolic process
ATP-dependent helicase activity
cation-transporting ATPase activity
DNA replication origin binding
ATP binding
0
20
40
60
80
100
FB vs CM
ribosome
small ribosomal subunit
organic hydroxy compound biosynthetic process
cellular carbohydrate metabolic process
cellular amine metabolic process
pyridine-containing compound biosynthetic process
carbohydrate metabolic process
ribosome biogenesis
metabolic process
translation
oxidation-reduction process
carbohydrate binding
monooxygenase activity
oxidoreductase activity
heme binding
iron ion binding
structural constituent of ribosome
0
50
100
150
200
250
300

Figure 3

Clustering of gene expression profiles across the three growth stages.

A. Principle component analysis of the RNA-Seq data. B. six gene clusters with different expression profiles. Overlaying curves of all genes within the cluster were shown. FB represents fruiting body with mature perithecium. CM represents the asexual mycelium. DF represents the developed fruiting body.


A
FB
DF
CM
PC 2 (28.80%)
PC 1 (51.20%)

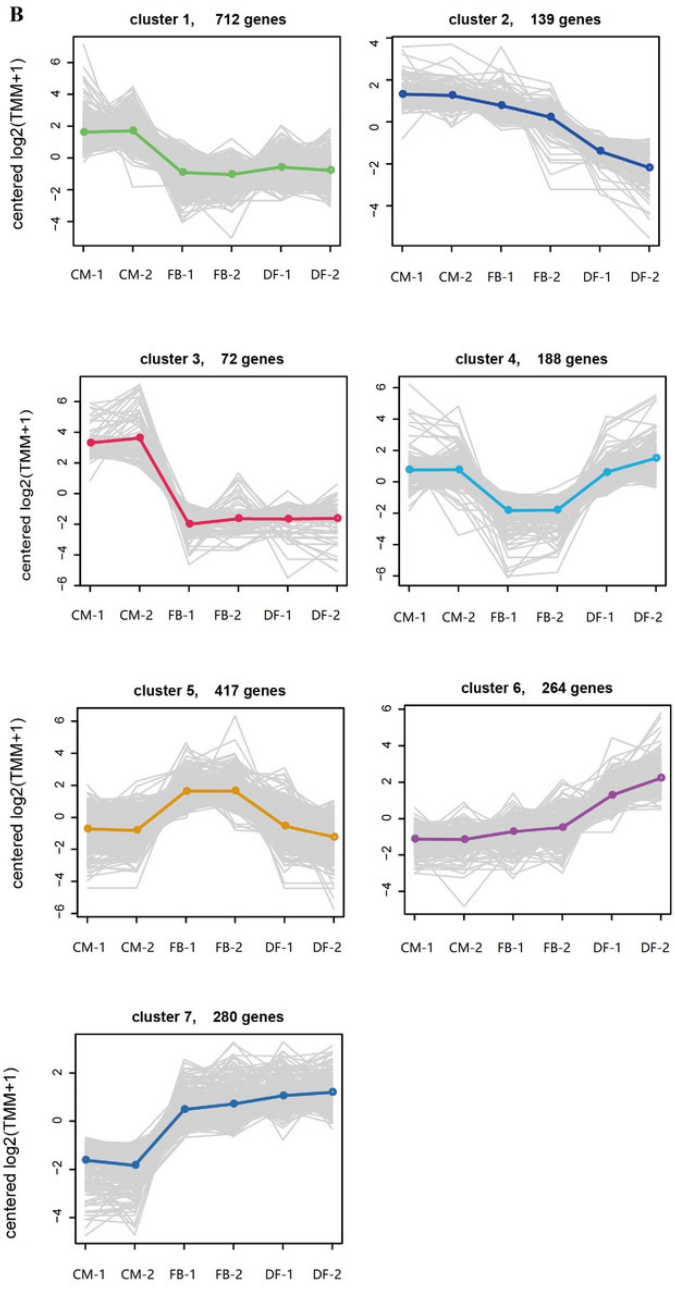

B
cluster 1, 712 genes
cluster 2, 139 genes
cluster 3, 72 genes
cluster 4, 188 genes
cluster 5, 417 genes
cluster 6, 264 genes
cluster 7, 280 genes
centered log2(TMM+1)
CM-1 CM-2 FB-1 FB-2 DF-1 DF-2

Figure 4

AS events in the *O. sinensis* transcriptome.

A. Numbers of alternative splicing events in different developmental stages of *O. sinensis*. B. Different numbers of genes involved in AS in different developmental stages of *O. sinensis*. The x-axis represents types of alternative splicing events. FB represents fruiting body with mature perithecium. CM represents the asexual mycelium. DF represents the developed fruiting body.

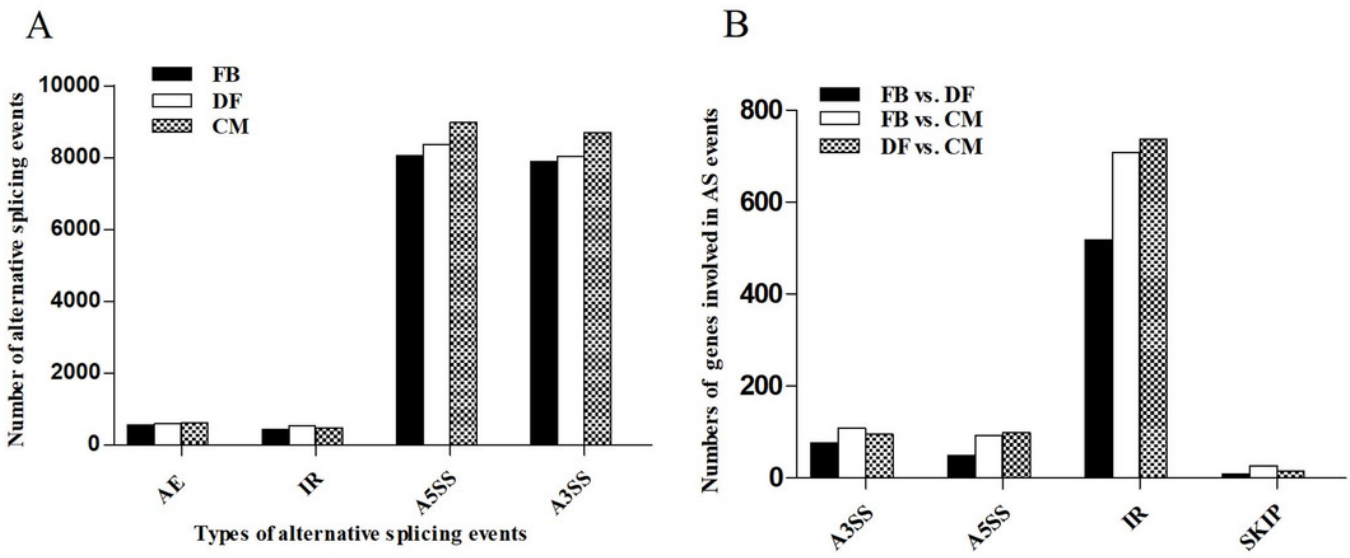


Figure 5

qPCR validation of the 10 expressed genes in Illumina sequencing.

Histone H2A gene (MSTRG. 2474) was the internal reference. The genes expression levels in DF were set as control samples and those in FB (A) and CM (B) were normalized to the control. The values are the results of three technical repetitions (mean 3 ± SD).one-way analysis of variance (ANOVA) was used to test for difference, $p < 0.05$. FB represents fruiting body with mature perithecium. CM represents the asexual mycelium. DF represents the developed fruiting body.