Comparative analysis of transcriptomes revealed the molecular mechanism of development of *Tricholoma matsutake* at different stages of fruiting bodies

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Abstract The purpose of the study is to investigate the molecular mechanisms of development of *Tricholoma matsutake* fruiting body at the primordial stage (TM-1), the intermediate stage (TM-2) and the mature stage (TM-3) using RNA-Seq sequencing technology. The analysis of gene expression level revealed that the *Spn2* and *Eef1a1* gene were the key genes in the primordial stage of *T. matsutake* by regulating cytokinesis, protein synthesis, and cell growth. And the *Ubc*, *Atp6*, *Cytb*, and *Pth2* gene were the key genes in the mature stage of *T. matsutake* by regulating energy metabolism and protein synthesis. Differential expression genes (DEGs) analysis results showed that *Cdc28*, *Rad53*, *Dun1*, *Pho85* and *Pho81* were the key DEGs regulating cell cycle genes of *T. matsutake* from primordial stage to intermediate stage. And *APC*, *Cyr1*, *Cdc45*, *Spo11* and *Rec8* genes were the key DEGs for the meiosis and sporogenesis of *T. matsutake* from the intermediate stage to the mature stage.

Keywords *Tricholoma matsutake* · Molecular mechanism · Development · RNA-sequencing · Transcriptome

Introduction

The molecular mechanism of the formation and development of edible fungus fruiting body is critical to their yield and quality. It is a complex process for the fruiting bodies of edible fungi differentiate and develop. With the rapid development of transcriptome sequencing technology, high-throughput sequencing has been used for transcriptome analyses of mushrooms, such as *Lentinula edodes* (Wang et al., 2018), *Agrocybe aegerita* (Wang et al., 2013), *Pleurotus eryngii* (Xie et al. 2018), *Cordyceps guangdongensis* (Zhang et al., 2018) and *Coprinopsis cinerea* (Plaza et al., 2014), and the molecular mechanism of macrofungus development has gradually been revealed. At the same time, some genes and transcription factors related to the growth and development of fruiting bodies of macrofungus have been excavated. For instance, differential expression of *fvh1* (Ando et al., 2001), *nox5* (Mu et al., 2014), *hsp9* (Kurahashi et al., 2014), *dst* (Kuratani et al., 2010; Tera-shima et al., 2005), *eln3* (Arima et al., 2004), *ppo* (Li et al., 2011) and *pro1* (Steffens et al., 2016) were found in the fruiting body of macrofungus formation.

Tricholoma matsutake is an ectomycorrhizal fungus which belongs to the subgenus *Tricholoma* and coexists with pine trees (Liu et al., 2010). *T. matsutake*, as a traditionally edible fungus, has long been regarded as a delicious and nutritious food (Yoon et al., 2016). *T. matsutake* requires extremely harsh growth environment, and its growth process is very slow, usually takes 5 to 6 years. At present, there is no successful precedent for artificial

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cultivation in the world (Yamada et al., 2006). *T. matsutake*, which is a secondary endangered species in China, is an endemic species in Asia and China is the main exporter of *T. matsutake* (Xu et al., 2010). *T. matsutake* varieties exist in the United States and Europe, but their yield is low and their quality is poor. In recent years, due to the deterioration of the environment and large-scale predatory collection, wild *T. matsutake* resources are depleting day by day, the global production of *T. matsutake* is decreasing while its economic value is rising year by year. In addition, *T. matsutake* is a natural medicinal fungus. *T. matsutake* contains polysaccharides, proteins, minerals, fatty acids, amino acids, aromatic compounds and volatile components (Cho et al., 2006; Ding and Hou, 2012; Li et al., 2019). The relevant studies showed that *T. matsutake* has good biological activity and medicinal value, which can play the role of anti-tumour, anti-virus, anti-microorganism, anti-oxidant, improvement of immunity, etc. (Ding et al., 2019; Hou et al., 2013; You et al., 2013). With the constant discovery of the value of *T. matsutake*, its market demand has also increased rapidly, but the *T. matsutake* has not been successfully cultivated (Oh et al., 2018; Park and Ka, 2010). The present researches on the growth of *T. matsutake* mainly focus on fairy ring bacteria and effects of bacterial community on the growth of *T. matsutake* (Oh and Lim, 2018a, b; Yamaguchi et al., 2016). However, the molecular mechanism of development of *T. matsutake* fruiting body has not been reported.

There are four major producing areas of *T. matsutake* in China: Shangri-la in Yunnan, Yajiang in Ganzi Prefecture in Sichuan, Starna in Danba County in Ganzi Prefecture in Sichuan and Xiaojin in Aba Prefecture in Sichuan. In this study, we performed a transcriptome analysis of three developmental stages (the primordia stage, the intermediate stage and the mature stage) of *T. matsutake* from Xiaojin in Aba Prefecture in Sichuan. Illumina sequencing technique was used to explore the transcriptome during maturation of the fruiting body at the genome level. It provides foundation for further research on the development of *T. matsutake* fruiting bodies and its artificial cultivation.

Materials and methods

Materials

The fresh fruiting bodies of *T. matsutake* were collected from Xiaojin County, which lies in the Sichuan Aba Tibetan and Qiang Autonomous Prefecture. According to the shape of the fruiting bodies, the *T. matsutake* fruiting bodies were divided into three stages: the primordial stage (TM-1) [Fig. 1A], the intermediate stage (TM-2) [Fig. 1B]

Fig. 1 *T. matsutake* fruiting bodies in the early stage (TM-1) (A); *T. matsutake* fruiting bodies in the intermediate stage (TM-2) (B); *T. matsutake* fruiting bodies in the mature stage (TM-3) (C); Gene ontology (GO) function annotation classification for the transcriptome sequences of *T. matsutake* (D); KOG function annotation classification for the transcriptome sequences of *T. matsutake* (E); KEGG annotation for the transcriptome sequences of *T. matsutake* (F)

and the mature stage (TM-3) [Fig. 1C]. Fresh fruiting bodies were decontaminated, and all samples immediately

transferred to liquid nitrogen for freezing and preservation at -80°C .

RNA extraction, cDNA library construction and Illumina sequencing

Samples of total RNA were extracted in triplicate from the fruiting bodies of *T. matsutake* as described previously by TRIzol reagent (Invitrogen, Burlington, ON, Canada). The quality and quantity of total RNA were assessed in four methods: NanoPhotometer[®] spectrophotometer (Implen, Inc., Westlake Village, CA, USA), the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA), Qubit[®] RNA Assay Kit in Qubit[®] 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and 1% agarose gels. After the total RNA was qualified, it was sent to Novogene (Beijing, China) for construction of the DNA library and RNA-sequencing.

Transcriptome sequencing of *T. matsutake* and transcriptome assembly

To explore transcriptomic changes in three stages [the primordial stage (TM-1), the intermediate stage (TM-2) and the mature stage (TM-3)] of *T. matsutake* fruiting bodies, three cDNA libraries were constructed for RNA-Seq. The clean reads were obtained by removing the adapter reads, low quality reads, and reads containing poly-N from sequenced reads. The transcript was obtained by de novo assembly clean reads via Trinity. And the longest transcript in each gene was selected as Unigene. All the downstream analyses were based on clean data with high quality.

Gene functional annotation

In order to obtain comprehensive information about sequence annotation and gene function, Unigene was annotated in three databases: Gene Ontology (GO), euKaryotic Ortholog Groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Differential expression analysis

The quantitative analyses of gene expression were performed using the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) method by RSEM for each sample. Clean data were mapped back onto the assembled transcriptome and read counts for each gene was obtained from the mapping results.

According to the RSEM results, DESeq R package (1.10.1) was used to analyze the differential expression

genes (DEGs). Benjamini and Hochberg's approach were used to adjust the *P* value for controlling the false discovery rate (FDR). Genes with an adjusted *p* value < 0.05 found by DESeq were assigned as differentially expressed.

KEGG pathway enrichment analysis

KEGG is a database resource for understanding high-level functions and utilities of the biological system from molecular-level information by genome sequencing and other high-throughput experimental technologies. For pathway enrichment analysis, differential expression genes (DEGs) were enrichment in the KEGG database by using the KOBAS software.

Results and discussion

Transcriptome sequencing of *T. matsutake* and transcriptome assembly

In total, 56479601, 50222435, and 51558689 the Sequence reads were obtained from TM-1, TM-2 and TM-3, respectively. After filtering low-quality reads, the Clean reads obtained from TM-1, TM-2 and TM-3, were 54213854, 47888858, and 49127819, respectively, which corresponded to a total size of 8.13G, 7.69G and 7.33G. The Q20 (%) of the three samples were 97.90%, 97.91% and 97.89%, the GC content (%) were 49.85%, 50.08% and 49.92, and the base error rate was 0.01%. The mean length was 1001 bp and 1383 bp respectively, and the N50 were 1712 bp and 1904 bp respectively. The above results indicated that the sequencing results were highly reliable.

Gene functional annotation

The GO (Gene Ontology) annotation was performed using Blast2GO to analyze the gene functions. A total of 70,631 unigenes were successfully annotated and classified into biological processes (1064), cellular components (338), and molecular functions (165) [Fig. 1D]. The GO function annotation results showed that in the key gene categories involving the development of *T. matsutake* fruiting bodies in the three stages (the primordial stage (TM-1), the intermediate stage (TM-2) and the mature stage (TM-3)), the most abundant genes in the biological process categories were metabolic process, cellular process and single—organism process, the most abundant genes in the cellular component categories were cells and cell parts, and the most abundant genes in the molecular function categories were binding and catalytic activity.

The KOG (euKaryotic Ortholog Groups) database was used to further analyze the annotations and classify

functions of genes. A total of 46201 unigenes were assigned and classified into 25 KOG categories in genes of *T. matsutake* [Fig. 1E], which mainly contained general function prediction only (6532; 14.14%), posttranslational modification, protein turnover, chaperones (5721; 12.38%), signal transduction mechanisms (3882; 8.40%), translation, ribosomal structure and biogenesis (3874; 8.39%), cell motility (29; 0.06%), and other categories. The results showed that the KOG functional categories involved in the development of *T. matsutake* were mainly including protein synthesis, signal transduction etc.

The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was used to identify biological pathways in the development of *T. matsutake*. A total of 39,029 unigenes were assigned into 5 KEGG categories [Fig. 1F], including cellular processes (4603; 11.79%), environmental information processing (2789; 7.15%), genetic information processing (8737; 22.39%), metabolism (17,135; 43.90%), organismal Systems (5765; 18.73%). The results showed that in the development of *T. matsutake*, the most unigenes participated in metabolic pathways and genetic information processing.

Gene expression of three stages of *T. matsutake* fruiting bodies

All gene abundances were calculated and normalized by the FPKM method using uniquely mapped reads. Genes with FPKM > 0.3 were considered to be expressed, and genes with FPKM > 60 were considered to be expressed at a high level. The results showed that 74,082 genes (57.71%) were expressed in TM-1, of which 4091 genes (3.19%) were highly expressed, and 45,249 genes (39.25%) were expressed in TM-2, of which 2915 genes (2.27%) were highly expressed, and 51,338 genes (39.99%) were expressed in TM-3, of which 3147 genes (2.45%) were highly expressed.

In recent years, with the wide application of high-throughput sequencing technology in the field of life science, many important genes related to the growth and development of edible fungi have been analyzed and reported. Transcriptome comparison between the primordium and fruiting body of *Pleurotus eryngii* showed that blue light could up regulated most of carbohydrate-active enzymes (CAZymes) genes, such as families *gh16*, *gh3*, *gh 61*, *gh15*, *gh47*, *gh18* and *gh28*, during the differentiation from primordium to fruiting body (Xie et al., 2018). The hydrophobic genes such as *sc1* and *sc3* were related to the development of mycelium browning in *Lentinula edodes* (Yoo et al., 2019). The mads-box genes, including *c2h2*, *hom*, *fst3*, *fst4* and *gata*, were up-regulated in the cold-stimulated mycelium, primordia and fruiting body of *Pleurotus tuoliensis* (Fu et al., 2017). The most

abundant transcription factors associated with the development of *Ophiocordyceps sinensis* fruiting body included the C2H2 zinc finger, Zn2Cys6, bZIP and GATA-type Zinc finger (Xiang et al., 2014). The genome sequence of Tibetan Plateau *Agaricus bisporus* identified key genes related to the cell wall and membrane system and gene family associated with adaptation to the harsh environment such as cold, hypoxia, and strong ultraviolet rays (Sun et al., 2019).

In our research, further analysis revealed that 11 genes (*Rps26*, *Spn2*, *Rps15*, *Eef1a1*, *Rps25*, *Rpl40*, *Rplp2*, *Rpl12*, *Rpl36*, *Rpl11*, and *Rpl29*) were extremely high expressed (FPKM > 2000) in the primordial stage (TM-1) (Table 1), 10 genes (*Rps27Ae*, *Rpl44*, *Rpl37A*, *Rps11*, *H4*, *H3*, *Rpl34*, *Rps23*, *Rps19* and *Rpl32*) were extremely high expressed (FPKM > 2000) in the intermediate stage (TM-2) (Table 2), whereas 8 genes (*Rpl37A*, *H2B*, *Ube2D/E*, *Atp6*, *Cytb*, *H4*, *H3* and *Pth2*) were extremely high expressed (FPKM > 2000) in the mature stage (TM-3) (Table 3). Most of the extremely high expressed genes in TM-1 and TM-2 were ribosome subunits, which indicated that protein metabolism was vigorous in the primordial stage (TM-1) and the intermediate stage (TM-2) of *T. matsutake* fruiting body development.

The *Spn2* gene and *Eef1a1* gene were extremely highly expressed only in TM-1 and the FPKM of the *Spn2* and *Eef1a1* were 6696.97 and 3375.08, respectively. The *Spn2* gene encodes the septin (Addi et al., 2018), a conserved GTP-binding protein, which extremely highly expressed in TM-1, indicating that *Spn2* gene is the key gene in cytokinesis in the primordial period of *T. Matsutake*. The *Eef1a1* gene, encodes the eEF1A1 (Migliaccio et al., 2016), which also extremely highly expressed only in TM-1, indicating that this gene plays an important role in prolonging amino acid chains on ribosomes and regulating protein synthesis, cell growth and morphology in the primordial period of *T. Matsutake*. The FPKM of the *H4* gene was 2728.25 in TM-2 and 3932.39 in TM-3, the FPKM of the *H3* gene was 2316.75 in TM-2 and 5108.19 in TM-3, and the *H2B* gene was extremely highly expressed only in TM-3 and the FPKM was 3407.76. The *H4*, *H3* and *H2B* gene encode the subunit of histone, which composed of 5 subunits in eukaryotes (Clément et al., 2018; Gurard-Levin et al., 2014). The *H4* gene, *H3* gene and *H2B* gene were extremely highly expressed in TM-2 and TM-3, indicating that these genes play an important role in the process of chromosome formation in the intermediate stage and the mature stage of *T. matsutake*. Furthermore, the *Ubc* gene, *Atp6* gene, *Cytb* gene, and *Pth2* gene were extremely highly expressed only in TM-3 and the FPKM of the *Ube2D/E*, *Atp6*, *Cytb*, and *Pth2* were 2265.16, 2413.79, 3016.19 and 5307.39, respectively. The *Ubc* gene which was extremely highly expressed in TM-3 encodes the

Table 1 Quantification of gene expression in TM-1 (FPKM > 2000)

Gene ID	FPKM	Gene length	Symbol	Description	KO ID
Cluster-5908.46431	6696.97	260	<i>Rps26</i>	ribosomal protein S26e	K02976
Cluster-5908.46580	2103.38	568	<i>Spn2</i>	septin 3/9/12	K16938
Cluster-5908.46619	2124.97	296	<i>Rps15</i>	ribosomal protein S15e	K02958
Cluster-5908.46632	3375.08	214	<i>Eef1a1</i>	eukaryotic elongation factor 1-alpha	K03231
Cluster-5908.46740	3960.83	328	<i>Rps25</i>	ribosomal protein S25e	K02975
Cluster-5908.47045	2165.16	535	<i>Rpl40</i>	ribosomal protein L40e	K02927
Cluster-5908.47340	2008.19	488	<i>Rplp2</i>	ribosomal protein LP2	K02943
Cluster-5908.48094	2100.58	632	<i>Rpl12</i>	ribosomal protein L12e	K02870
Cluster-5908.48305	2397.14	426	<i>Rpl36</i>	ribosomal protein L36e	K02920
Cluster-5908.48516	2022.61	329	<i>Rpl11</i>	ribosomal protein L11e	K02868
Cluster-5908.49927	2405.81	353	<i>Rpl29</i>	ribosomal protein L29e	K02905

Table 2 Quantification of gene expression in TM-2 (FPKM > 2000)

Gene ID	FPKM	Gene length	Symbol	Description	KO ID
Cluster-5908.37971	2972.04	460	<i>Rps27A</i>	ribosomal protein S27Ae	K02977
Cluster-5908.44129	2130.5	515	<i>Rpl44</i>	ribosomal protein L44e	K02929
Cluster-5908.44804	3468.19	412	<i>Rpl37A</i>	ribosomal protein L37Ae	K02921
Cluster-5908.49057	2042.67	721	<i>Rps11</i>	ribosomal protein S11e	K02949
Cluster-5908.50406	2728.25	328	<i>H4</i>	histone H4	K11254
Cluster-5908.51062	2316.75	312	<i>H3</i>	histone H3	K11253
Cluster-5908.51158	2171.97	557	<i>Rpl34</i>	ribosomal protein L34e	K02915
Cluster-5908.54564	2200.54	665	<i>Rps23</i>	ribosomal protein S23e	K02973
Cluster-5908.57621	2276.38	320	<i>Rps19</i>	ribosomal protein S19e	K02966
Cluster-5908.70058	2436.28	531	<i>Rpl32</i>	ribosomal protein L32e	K02912

Table 3 Quantification of gene expression in TM-3 (FPKM > 2000)

Gene ID	FPKM	Gene length	Symbol	Description	KO ID
Cluster-5908.44804	2432.18	412	<i>Rpl37A</i>	ribosomal protein L37Ae	K02921
Cluster-5908.46036	3407.76	813	<i>H2B</i>	histone H2B	K11252
Cluster-5908.46390	2265.16	739	<i>Ubc</i>	ubiquitin-conjugating enzyme E2 D/E	K06689
Cluster-5908.46998	2413.79	1234	<i>Atp6</i>	F-type H + -transporting ATPase subunit A	K02126
Cluster-5908.49482	3016.19	1509	<i>Cytb</i>	cytochrome b	K00412
Cluster-5908.50406	3932.39	328	<i>H4</i>	histone H4	K11254
Cluster-5908.52388	5108.19	620	<i>H3</i>	histone H3	K11253
Cluster-5908.8478	5307.39	1149	<i>Pth2</i>	peptidyl-tRNA hydrolase, PTH2 family	K04794

ubiquitin-conjugating enzyme (E2), which together with ubiquitin-activating enzymes (E1) and ubiquitin ligases (E3) (Morreale and Walden, 2016) indicated that labeling proteins that need to be decomposed to regulate vital movement is the key function in the mature fruiting bodies (TM-3). The *Pth2* gene encodes peptidyl-tRNA hydrolase (Sharma et al., 2014) which also extremely highly expressed in TM-3 indicated that catalyzes the breakdown of peptidyl-tRNA into peptides and tRNA to recycle tRNA is the most active biological processes in in the mature

stage (TM-3). The *Ubc* gene and *Pth2* gene extremely high expressed in TM-3 indicated that protein metabolism is vigorous in the mature stage of *T. Matsutake* development. The *Atp6* gene encodes the ATPase subunit a, and the *Cytb* gene encodes the cytochrome b, which extremely highly expressed in TM-3, indicating that energy metabolism is significance in mature stage of *T. Matsutake*. The high expression genes screened above provided a scientific basis for revealing the key genes of *T. Matsutake* in different growth and development stages.

Fig. 2 Volcano plot of di-erentially expressed genes (DEGs) between TM-1 and TM-2 (A); GO annotation of di-erentially expressed genes (DEGs) between TM-1 and TM-2 (B); Volcano plot of di-erentially

expressed genes (DEGs) between TM-2 and TM-3 (C); GO annotation of di-erentially expressed genes (DEGs) between TM-2 and TM-3 (D)

Pathway enrichment analysis of differentially expressed genes (DEGs)

EdgeR program was used to analyze the DEGs of TM-1, TM-2 and TM-3 ($\text{padj} < 0.05$ and $|\log_2\text{foldchange}| > 2$), then the DEGs were analyzed by GO annotation method, which were divided into three categories: biological process, cellular component and molecular function. A total 54,311 DEGs were identified between TM-1 and TM-2, of these, 42,700 genes were up-regulated, whereas 7111 genes were down-regulated [Fig. 2A]. And interestingly, the GO annotation results of DEGs between TM-1 and TM-2 showed that the majority DEGs were annotated in the molecular functional [Fig. 2B]. The results suggested that the catalytic activity in molecular function is a key differential gene category for the development of *T. matsutake* from primordial (TM-1) to the intermediate stage (TM-2). At the same time, a total of 6061 DEGs were identified between TM-2 and TM-3, of these, 2245 genes were up-regulated and 3816 genes were down-regulated [Fig. 2C]. It is worth noting that most of DEGs between TM-2 and TM-3 were annotated in biological processes and cell component, and several DEGs were annotated in molecular function [Fig. 2D]. These results suggested that biological processes and cell component are key differential gene categories for the development of *T. matsutake* from the intermediate stage (TM-2) to the mature stage (TM-3).

The metabolic pathway and signal pathway related to different growth and development stages of edible fungi have attracted more and more attentions. The transcriptome analysis of *Volvariella volvacea*, *Morchella importuna* and *Agrocybe aegerita* showed that the genes of fruit body were significantly involved in carbohydrate metabolism and energy metabolism (Bao et al., 2013; Hao et al., 2019; Wang et al., 2013). Blue light regulated carbon metabolism, glycolysis/gluconeogenesis and biosynthesis of amino acids pathways during *Pleurotus eryngii* fruiting body formation and growth (Xie et al., 2018). DEGs, in light-induced brown film formation in *Lentinula edodes*, were significantly enriched in starch, sucrose, fatty acid metabolism and the two-component phosphorelay system. (Tang et al., 2013). During the mature mycelia of *Pleurotus tuoliensis*, genes involved in nucleotide synthesis and energy metabolism pathways were significantly upregulated, meanwhile, oxidation reduction activities pathways are very active (Du et al., 2019).

In our study, to further evaluate the key pathway involved in the development of *T. matsutake* fruiting bodies, the DEGs of TM-1 vs TM-2 and TM-2 vs TM-3 were matched to the KEGG database, respectively. Via analyzing the top 20 pathways in TM-1 vs TM-2, it was found that the DEGs up-regulated in TM-2 were involved in DNA replication, base excision repair, cell cycle,

ribosome, steroid biosynthesis and oxidative phosphorylation [Fig. 3A], and the DEGs down-regulated in TM-2 were involved in homologous recombination, nucleotide excision repair, biosynthesis of unsaturated fatty acids and glycerophospholipid metabolism [Fig. 3B]. These represent pathways suggested that cell division was vigorous and a large amount of energy was required in TM-1 and TM-2. It is worth noting that the orderly division of eukaryote cell cycle is the basic guarantee of genetic material transmission, and cell division is important for the growth and development of edible fungi. The cell cycle contains cell growth, proliferation and development and is divided into four phases (G1 phase, S phase, G2 phase and M phase). Cell cycle signaling pathway gene plays a key role in controlling the normal transformation of the cell cycle. In our research, the expression of genes in cell cycle pathway, such as *Cdc28*, *Pho81*, *Pho85*, *Orc4*, *Cdh1*, *Mad2*, *Rad53*, *Dam1*, *Dun1*, *Grr1*, *Cdc55*, *Cyc8*, and *Cdc14*, were higher in TM-1 than in TM-2 [Fig. 3C].

The *Cdc28* gene encodes cyclin-dependent kinase CDC28 (Koltovaya, 2013). The *Cdc28* gene expression was higher in TM-1 indicated that CDC28 function in a genetic network which could support cellular activity in DNA damage is very important in the primordial stage (TM-1). The *Rad53* gene and *Dun1* gene encode serine/threonine/tyrosine protein kinase RAD53 and serine/threonine-protein kinase CHEK2, respectively. RAD53 and CHEK2 belong to the Chk2 family kinases, which are responsible for cellular responses to DNA damage (Chen et al., 2007, 2017). The *Rad53* gene and *Dun1* gene were also up-regulated in TM-1, indicating that the *Rad53* gene and *Dun1* gene are also key genes for cellular to DNA damage in the primordial stage of *T. matsutake*. The *Pho85* gene encodes the CDK Pho85 (Mirallas et al., 2018) and the *Pho81* gene encodes the CDK inhibitor Pho81 (Wanke et al., 2014). These two genes up-regulated in TM-1, indicating that *pho85* controls the cell cycle by regulating phosphate metabolism when *pho85* binds to the *pho80* family of cyclins, and the CDK inhibitor Pho81 regulated cell cycle by inhibiting the *pho80-pho85* complex involved in phosphate-responsive pathway in the primordial stage of *T. matsutake*. The *Cdh1* gene encodes the cell division cycle 20-like protein 1 (Cdh1) (Oakes et al., 2014), which up-regulation in TM-1, indicating that the *Cdh1* gene is the key gene in the cell cycle of the primordial stage of *T. matsutake* from S phase to G2 phase. This process would explain the mechanism of cell cycle in the primordial stage of *T. matsutake*. These high expression genes screened above in the primordial stage of *T. matsutake* provided a scientific basis for revealing the key genes in the cell cycle of *T. matsutake* from the primordial stage (TM-1) to the intermediate stage (TM-2).

Fig. 3 The representative top 20 pathways of differential expressed genes of TM-1 vs TM-2. Up-regulated in TM-2 (A); Down-regulated in TM-2 (B); KEGG annotation of the DEGs involved in cell cycle. (C) (Note: The DEGs between TM-1 and TM-2 are shown in the cell cycle pathway at G1, S, G2, and M phases. The genes in green represent down-regulated genes in TM-2, the genes in red represent up-regulated genes in TM-2, the genes in yellow represent that those genes are annotated by different contigs, which may be either up-regulated or down-regulated.); The representative top 20 pathways of differential expressed genes of TM-2 vs TM-3.

Up-regulated in TM-3 (D); Down-regulated in TM-3 (E); and KEGG annotations of the DEGs involved in meiosis. (F) (Note: The DEGs between TM-2 and TM-3 are shown in the meiosis pathway at G1, S, G2, and M phases. The genes in green represent down-regulated genes in TM-2, the genes in red represent up-regulated genes in TM-3, the genes in yellow represent that those genes are annotated by different contigs, which may be either up-regulated or down-regulated.)

carotenoid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis [Fig. 3E]. The expression of some genes in meiosis pathway was very interesting. The expression of some genes in meiosis pathway was very interesting. Meiosis, closely related to sexual reproduction,

is a special mode of division in sexual reproduction and an essential step in sexual life cycle. In the transcriptome sequencing of *Lentinula edodes*, the expression of genes related to meiosis such as *Mcm5*, *Orc2*, *Orc4* and *Dmc1* was higher in the fully developed stage than in the early bud stage (Wang et al., 2018). In the model mushroom *Schizophyllum commune*, *fst4* was found to be involved in the transition between the vegetative and reproductive stages of the mushroom (Ohm et al., 2011). In the transcriptome analysis of *Pleurotus tuoliensis*, genes differentially expressed in the cell cycle and meiosis pathway, such as *Cdc28*, *Mps1*, and *Rad53* (Fu et al., 2017). KEGG pathway analysis of *Ophiocordyceps sinensis* transcriptome showed that DEGs between anamorph mycelia and teleomorph sclerotium were enriched in meiosis, cell cycle and ABC transporter, and the DEGs between the teleomorph stroma and teleomorph sclerotium were also enriched (Zhong et al., 2018). In our research, the expression of *APC/C*, *Cyr1*, *Msn2/4*, *Cdc28*, *Cdc45*, *Dbf4*, *Spo11*, *Cdc5*, *Esp1*, *Rec8*, *Scm3* and *Rst1* in TM-3 was higher than TM-2. These differentially expressed genes and pathways related to the cell cycle and meiosis suggested that *T. matsutake* spore production is a key process in TM-3.

The *APC* gene encodes the anaphase-promoting complex (APC), and the *Esp1* gene encodes the separate protein (Esp1) (Luo and Tong, 2018; Zhang et al., 2016). These genes were up-regulated in TM-3, indicating that APC is a key complex formed by *esp1p* and *pds1* which could promote the dissociation and separation of sister chromatids by releasing sister separation ESP1 and its inhibitor Pds1p in the mature stage (TM-3). The *Cyr1* gene encodes adenylate cyclase (AC), which up-regulates cAMP level to activate PKA. PKA was important in regulating cell growth, differentiation, proliferation and cell cycle (Scheib et al., 2018). The *Cyr1* genes were also up-regulated in TM-3, indicating that the *Cyr1* genes are also the key genes in regulating cell cycle and cytokinesis in the mature stage of *T. matsutake*. The *Cdc45* gene encodes cell division control protein 45 (CDC45), which is a component of the Cdc45-MCM-GINS (CMG) helicase (Simon et al., 2016). The *Cdc45* gene was up-regulated in TM-3, suggesting that the Cdc45 is essential in the process of DNA replication which works with ORC and MCM proteins to initiate the replication of the whole genome in the mature stage of *T. matsutake*. The *Spo11* gene encodes meiotic recombination protein SPO11 (Vrielynck et al., 2016) and the *Rec8* gene encodes meiotic recombination protein REC8 (Yan and McKee, 2013). The *Spo11* gene and *Rec8* gene were up-regulated in TM-3, suggesting that they were key genes which catalyzed the formation of meiotic DNA double strand breaks (DSBs) and were essential for ensure homologous chromosome segregation to proceed correctly in meiosis in the mature stage of *T. matsutake*. These high

expression genes screened above in the mature stage of *T. matsutake* provided a scientific basis for revealing the key genes in the process of meiosis and spore formation of *T. matsutake* from the intermediate stage (TM-2) to the mature stage (TM-3).

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