Transcriptome Analysis of *Ophiocordyceps Sinensis* Under Low-Temperature Treatment

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Abstract

This study aims to investigate the effect of low temperature on the gene expression in *Ophiocordyceps Sinensis*. To set up the experiments, two groups of *Ophiocordyceps Sinensis* were grown at 4°C and 18°C, respectively, for 60 days. Differential gene expression was analyzed by transcriptome sequencing and the morphology of mycelium was studied under scanning electron microscope for the two groups. The results showed that there were significant differences in hypha morphology between the two groups. Transcriptome analysis indicated that 2799 genes were differentially expressed in the low-temperature treatment group compared to the normal temperature group. Among these genes, 1489 were up-regulated, whereas 1310 were down-regulated. Further analysis revealed that 33 genes were only expressed at low temperature compared to 26 genes which were only expressed at normal temperature. GO enrichment analysis showed that the 33 genes were related to redox enzymes, which have functions in clearing reactive oxygen species and free radicals caused by low-temperature environment. KEGG enrichment analysis showed that the 33 genes were mainly involved in the cyanoamino acid metabolism, biosynthesis of amino acids, carbon metabolism, pantothenate and CoA biosynthesis, biotin metabolism, phenylpropanoid biosynthesis, apoptosis and other metabolic pathways. Our observations provide fundamental clues on the relationship between the low-temperature stimulation and the fruiting body development and further promote the research progress on *Ophiocordyceps Sinensis*.

1. Introduction

The Chinese caterpillar fungus is a parasitic complex composed of the remains of the caterpillar and the fungal sexual stroma (Lo et al., 2013; Zhou et al., 2014; Liu et al., 2015). It is only distributed in the Qinghai-Tibet Plateau and its surrounding high-altitude areas, and has been used in traditional Chinese herbal medicine (Shrestha et al., 2010). Due to the high demand in markets, it has been placed at risk of extinction due to overharvesting. According to the latest IUCN Red List of Threatened Species released in 2020, *O. Sinensis* has been listed as a 'Near Threatened' species (Yang, 2020).

It is widely accepted that *O. Sinensis* is the only asexual type of Chinese caterpillar fungus (Sung et al., 2007; Li et al., 2019; Xie et al., 2011; Li and Huang, 2000; Huang et al., 2001; Chen et al., 2001), Dong et al., 2011; Xia et al., 2016; Xia et al., 2019; Zhang et al., 2010). Most of the current researches focus on the optimal growth temperature of *O. Sinensis* and the selection of the appropriate temperature induced by the fruiting body. By studying different temperatures for colony growth, Wang *et al.* found that the best growth temperature of *O. Sinensis* is 18°C (Wang et al., 2011).

Generally, the transformation from vegetative growth to reproductive growth of fungi needs low temperature for stimulation. It has been shown that the primordium of *Lentinula edodes* culture only differentiates under appropriate low temperature, whereas it cannot be formed under constant temperature (Feng et al., 2005). The low-temperature stimulation triggers the initiation of the sexual development of fungi, makes some specific genes differentially expressed, and induces the start of sexual development and the fruiting body development. For instance, the fruiting body of *Flammulina*

velutips could grow under darkness and low-temperature conditions due to the expression of Pf1 and Pf3 (Sakamoto, 2010). One study showed that in *Pleurotus ostreatus*, the transcription levels of the fourth chitinase gene of immature *Pleurotus ostreatus* fruiting body were higher than that in the mature stage during development, and the transcription levels were significantly increased after low-temperature treatment (Nishihara et al., 2007). In *Flammulina velutips*, the result also showed that the JRL1 gene played significant roles for fruiting body formation under low temperature-treatment (Lu et al., 2016).

It has been reported that the initiation of the *O. Sinensis* sexual development may be related to the unique environmental factors in the Qinghai-Tibet Plateau ecosystem (Xia et al., 2017). *O. Sinensis* generally goes through the frozen time for 5 months. Only in late April that is after the end of the frozen time, the fruiting bodies start to grow. According to this phenomenon, our experiments were designed under low-temperature and normal-temperature treatments (control group) and differential gene expression was studied under these conditions for *O. Sinensis* by transcriptome analysis. Our results provide fundamental clues on the relationship between the low-temperature stimulation and the fruiting body development and promote the research progress on *O. Sinensis*.

2. Materials And Methods

2.1 Experimental strains and temperature treatment

Using the GZC: (Fungal Name registration. FN570054), *O. Sinensis* strain was isolated from the Chinese caterpillar fungus tissues and cultivated under artificial conditions to get its fruiting body. The strain is stored in the Biological Seedling Engineering Institute of Lanzhou Jiaotong University. After 60 days of vegetative growth at 18°C, the strains were randomly divided into two groups: the control group (Group B) incubated at 18°C and the experimental group incubated (group C) at 4°C. After 60 days, the samples from the two groups were collected and stored at -80°C until use.

2.2 Reagents and instruments

2.2.1 The reagents

mirVana™ miRNA ISolation Kit (Ambion-1561; TruSeq Stranded mRNA LTSample Prep Kit (Illumina); Agencourt AMPure XP (BECKMAN COULTER); Qubit RNA Assay Kit (Life Technologies); Qubit dsDNA Assay Kit (Life Technologies); Bioanalyzer 2100 RNA-6000 Nano Kit (Aglient); Bioanalyzer 2100 DNA-1000 Kit (Aglient); SuperScript II Reverse Transcriptase (Invitrogen); HiScript II Q RT SuperMix for qPCR (Vazyme); QuantiFast SYBR Green PCR Kit (Qiagen); Primers (Generay); CTAB ; 0.1mol/L TrIS-Cl buffer (self prepared).

2.2.2The instruments

ST16R Refrigerated centrifuge (Thermo); Tanon 2500 Gel imaging system (Biotanon Co., Ltd.); NanoDrop 2000 UV spectrophotometer (Thermo); Centrifuge 5418R (eppendorf); PCR (Bio-rad); Quantometer (Invitrogen); JSM-5600lv low-vacuum scanning electron microscope (NEC); Jfc-1600 ion sputtering

instrument (Japan Electronics Corporation); Es-2030 freezing vacuum drying plant (Nako Institute of Hitachi High-tech, Japan); Bioanalyzer2100 (Aglient); GeneAmp® PCR System 9700 (Applied Biosystems, USA); LightCycler® 480 Real-time PCR Instrument (Roche, Swiss); Rc-5 +PDF temperature recorder (Elitech).

2.3 The experimental methods

2.3.1 Measurement of the natural ambient temperature of Chinese caterpillar fungus

The prepared temperature recorders were buried to the Chinese caterpillar fungus collection point (Meiren Grassland, near Hezuo city, Gansu Province, China) at a depth of about 5cm. The recording time was set to record once an hour. The power supply of the temperature recorder was replaced every two months. The experiment was recorded for one year with three replicates.

2.3.2 Scanning electron microscope observation method

- 1) After removing the agar, the samples were fixed into 2.5% glutaraldehyde solution over night (at least 12 h);
- 2) After fixation, the samples were rinsed with 0.1 mol/L phosphate buffer for 3 times, each for 15 min;
- 3) Samples dehydration: the samples were treated with different concentrations of ethanol with 15 min for each concentration. After that, the samples were treated twice with pure alcohol, each time for 20 min;
- 4) The samples were treated with alcohol/isoamyl acetate (volume ratio: 1:1) for 30 min, followed by 100% isoamyl acetate for 1-2h;
- 5) The samples were freeze-dried for 15h.
- 6) At the end of drying, the surface was sprayed with gold by an ion sputtering apparatus and then observed by the scanning electron microscope.

The prepared samples were observed under scanning electron microscope by colleagues from the Life Science Research and Experiment Center of Lanzhou University and the State Key Laboratory of Solid Lubrication, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences.

2.3.3 Total RNA extraction and mRNA separation

The total RNA was extracted from the samples with the modified CTAB method. NanoDrop ultraviolet spectrophotometer and Agilent2100 were used for quality check and quantification. After qualify detection, the mRNA was separated by using the magnetic bead for subsequent experiments.

2.3.4 cDNA library construction and sequencing

The library construction and sequencing work was completed by Shanghai OE Biotech. Co., Ltd. These libraries were sequenced on the Illumina sequencing platform (Illumina HiSeq X Ten) and 150 bp paired-end reads were generated.

2.3.5 Sequencing data analysis

Raw sequencing data was filtered to obtain high-quality Clean Data. Trinity (Grabherr et al., 2011) software was used to splicing Clean Reads according to the no-reference genome analysis strategy, and the transcript was obtained for subsequent analysis. The reason for the non-reference genome analysis is that the published genome data of *O. Sinensis* is controversial, and there is no widely recognized reference genome of *O. Sinensis* (Hu et al., 2013; Li et al., 2016; Xia et al., 2017). After splicing, DESeq software (Anders and Huber, 2010) was used to analyze the differential expression of Unigene between sample groups. The screening condition was expression differential multiple $|\log_2\text{FoldChange}| > 1$, significance $P\text{-value} < 0.05$. GO enrichment analysis and KEGG enrichment analysis were performed for the differentially expressed genes obtained from screening to annotate and predict the functions and metabolic pathways of the related genes. Raw Illumina sequencing data of *O. sinensis* were submitted to NCBI as BioProject SUB8768026.

2.3.6 qRT-PCR analysis

cDNA was synthesized from mRNA using Kit (Vazyme). Then the reaction was carried out on the LightCycler® 480 Real-time PCR Instrument using the QuantiFast® SYBR® Green PCR Master Mix (Qiagen, Germany). The results were analyzed by using the calculation method of $2^{-\Delta Ct}$ with 18SrRNA of *O. sinensis* as internal reference gene. The related primer sequences were analyzed by Roche LCPDS2 software and synthesized by Beijing Qingke Xinye Biotechnology Co., Ltd. Primer sequences are listed in Table 1

Table 1 PCR primers used in this study

Gene Symbol	Forward primer	Reverse primer
Gamma-glutamyltranspeptidase	TGAGCAACTCGTTCCGGCTA	GACGATGATGGGCGAGAT
Formamidase	GTATCTGCACAATCGTTGGC	GGTCCAATCCAGGCATTCTA
Hypothetical protein	TTTGACTCGCGCAAAGTG	GACGAGCGAGGAGAAGTTA
Sorbitol dehydrogenase	GTTGGTGCTCAAGGTTGT	TATTGCCCAATGCGCTTC
Nitrate reductase	AAAGGTCATTGTCTCGGC	GACCACCTGACGCTCTTG
NADP-specific glutamate dehydrogenase	GTTTCTTTCTGGCAAGCCAA	GCATGTGCTCGACGTAGTA
Cyanate hydratase	CATCTCAGATACCGGCTTC	GTTCTATTACCGCCAACTC
Ribose 5-phosphate isomerase	TTGGAGTCGGTCCTACAAAC	GTTCAGACTCAGGCTGATG
Ophiocordyceps sinensis 18S	GCAGTGGCATCTCTCAGTC	TCATCGATGCCAGAACC

3. Results And Analysis

3.1 Natural ambient temperature of Chinese caterpillar fungus

Figure 1 shows the ambient temperature of the natural growth of Chinese caterpillar fungus recorded by the author in Meiren Grassland, near Hezuo city, Gansu Province, China. This temperature is the average temperature of each month in a year. As concerning to figure 1, from November to mid-March of every year, there is a frozen time of about 5 months, during these months Chinese caterpillar fungus is always in the corpse state. When the temperature began to rise in April, the fungus started to grow. In late April, it gradually broke through the ground and then entered the harvesting period. Studies have found that the fruiting body of *O. Sinensis* can only be formed after a period of low-temperature induction (Tu et al., 2010; Guo et al., 2015), which is very similar to vernalization of plants. However, the molecular or biochemical mechanisms that regulate this process are not clear until present.

3.2 Effect of low-temperature treatment on the colony and hypha morphology of *O. Sinensis*

Refer to figure 2, there were significant differences in colony morphology under different temperatures conditions. It is obvious signs of growth and development of *O. Sinensis* under two temperature treatments. The *O. Sinensis* is a hypothermic fungus, which resists and continue to develop under low temperature. Genomic investigation showed that *O. Sinensis* has antifreeze protein and can increase the accumulation of lipids and unsaturated fatty acids to adapt to an extremely cold environment (Hu et al., 2013). Besides, the Genome of *O. Sinensis* contains many functional genes, adapted to low temperature (Xia et al., 2017). These reasons enable the *O. Sinensis* to continue its slow growth and development at low temperatures. Compared to the low-temperature environment of 4°C, 18°C is the optimal temperature of the *O. Sinensis* growth. Under this condition, the growth is better and more aerial mycelium.

Figure 3 showed that the hypha morphology of *O. Sinensis* was a significant difference at different temperatures. At the magnification of 500 times, showed that the hypha at the 4°C, low-temperature treatment group is slimmer than at the 18°C, normal temperature control group, and there is a lot of helically curved hypha as shown in the red circle, while there is almost no such hypha in the 18°C normal temperature control group. During the fungi growth and development, hyphae will appear in the phenomenon of bending and kinking, which will form the primordium, and the appearance of the primordium is an important sign of fungal fruit body development. After low-temperature treatment, the helical hypha bending was looked like having a certain connection of hypha kink and primordium formation. When the mycelia were magnified 10,000 times, characteristic verrucous structure, which conidia produce, appeared on the mycelia surface, showed in the red circle in the control group at 18°C, while the mycelia surface appeared smooth and no similar structure in low-temperature treatment at 4°C. It's suggested that no conidia produce under low-temperature treatment.

3.3 Quality control results after total RNA extraction

Table2 Test results of samples

Sample	Concentration[μg/μl]	A260/280	A260/230	Volume (μl)	Total[μg]	Agilent2100	
						28S/18S	RIN
B1	0.5774	2.21	2.19	15	8.66	2.1	9.9
B2	1.0999	2.19	2.10	15	16.50	2.0	10.0
B3	0.6040	2.22	2.16	25	15.10	2.0	9.9
C1	0.4716	2.17	2.13	25	11.79	1.6	9.0
C2	0.6310	2.20	2.11	25	15.78	1.4	8.6
C3	0.3788	2.19	2.15	25	9.47	2.0	10.0

The quality control results after total RNA extraction are shown in Table 2. The quality of RNA samples for transcriptome sequencing requires higher nucleic acid purity when the OD260/OD230 is greater than 2, and RNA Integrity Number (RIN) value is between 1 and 10. The closer the value is to 10, the better the Integrity (Bolger et al., 2014). Refer to Table 1, the RNA purity, concentration, integrity, and other quality indicators of the 6 samples fully meet the requirements of subsequent transcriptome sequencing, so the cDNA library can be constructed for sequencing.

3.4 Quality assessment and analysis of sequencing data

Table 3 List of quality preprocessing results for sequencing data

Sample	Clean_reads	Clean_bases	Valid_bases	Q30	GC
B1	47847626	6930877946	93.73%	96.01%	60.36%
B2	47938792	6945590540	93.18%	96.31%	60.20%
B3	47970638	6960217947	93.84%	95.96%	60.45%
C1	47535504	6906826837	94.13%	96.23%	59.95%
C2	48020666	6964636068	93.78%	96.03%	60.14%
C3	47808510	6938280187	93.85%	96.00%	60.24%

To investigate the mechanism of changes in the low temperature conditions for *O. Sinensis*, the transcriptome sequencing was carried on at first, and then the original pieces been filtered , the data obtained was list in table 3. The group B (18 °C temperature control group, the same below) and group C (4 °C low-temperature treatment group, the same below) of Q30 average 96.09% and 96.08% respectively. Additionally, GC relative contents of group B was 60.34% and group C was 60.11%. Therefore, transcriptome sequencing data is of good quality and high accuracy, which can be used for subsequent analysis.

3.5 Splicing, assembly and length analysis of transcriptome

Transcript and Unigene sequence are counted, and the number of Unigene obtained after splicing was 37601, the maximum length of the sequence was 13572bp, and the N50 is 1524bp, as shown in the following Table 4.

Table 4 General statistics of splicing situation

	Contig	Transcript	Unigene
Total Length (bp)	38531639	67412677	29820756
Sequence Number	99748	62682	37601
Max. Length (bp)	15695	13572	13572
Mean Length (bp)	386.3	1075.5	793.1
N50 (bp)	1239	2031	1524
N50 Sequence No	7372	10440	5658
N90 (bp)	133	394	274
N90 Sequence No	66313	37611	24866
GC%	56.79	58.71	58.22

3.6 Transcriptome functional annotation

3.6.1 Summary of annotation results

The assembly transcripts of *O. Sinensis* were compared in NR, GO, KEGG, eggNOG, and Swissprot databases respectively, and the statistical results were shown in Table 5.

Table 5 Unigene annotation results and ratios in each database

Annotation in Database	Isoform number	Percentage (%)
NR	18294	48.65
GO	11583	30.81
KEGG	3489	9.28
eggNOG	15819	42.07
Swissprot	14260	37.92
In all database	3112	8.2

3.6.2 GO annotation statistics

Using the BLAST2GO software (the default parameters) for GO annotation statistics. After the annotation, the result was completed corresponds to the GO Term and counted the number of Unigene annotated to the secondary classification. The results were shown in Figure 4. The number of Unigene annotated in GO is 11,583, accounting for 30.81% of all Unigene. Most of the Unigene successfully annotated here concentrates on the metabolic process, cellular process, single biological process, etc. in the biological process. The cellular component is mainly reflected in the cell membrane, organelle, cell part, etc. The molecular functions mainly include catalytic activity, binding protein, transport activity, and so on.

3.6.3 eggNOG annotation statistics

During the eggNOG annotation of Unigene, the eggNOG number of the optimal aligned result was assigned to the corresponding gene. Combining the relationship between eggNOG classification and the number, the gene was matched the corresponding eggNOG classification, and the results were shown in Figure 5. A total of 15,819 Unigene were annotated and classified into 25 functional categories of eggNOG, accounting for 42.07% of the total amount of Unigene. The most annotated genes were unknown genes functions, with 5,415. This may indicate that many Unigene spliced have not been studied yet. It is great significant to investigate the functions of these genes further.

3.6.4 KEGG annotated statistics

This part of the annotation was done automatically by KEGG's KAAS system. In the annotation process, the gene set selected the close relationship species, and BBH (bi-directional Best Hit) was the judgment principle of gene KO. After KO annotation was completed, the results were associated with the relevant

metabolic pathways, as shown in Figure 6. A total of 3,468 Unigene were annotated into 35 metabolic pathways of 5 classifications in this pathway, the more typical of which are carbohydrate metabolism and amino acid metabolism in metabolism, translation in genetic information processing, signal transduction in environmental information processing, transport and catabolism in cellular processes, etc.

3.7 Differential gene analysis

In this experiment, the 18°C normal temperature treatment group was used as the control group, and the gene expression difference of *O. Sinensis* was analyzed under 4°C low temperature treatment (Figure 7). The results showed that the total number of differential expressed genes was 2799, among which the number of up-regulated genes was 1489, and the number of down-regulated genes was 1310. After further screening of differential expressed genes, 59 specifically expressed genes were obtained, including 33 differential expressed genes only at low temperature and 26 differential expressed genes that were not expressed at all at low temperature(S1), and cluster analysis was performed for these two types of genes (Figure 7C). The annotation results showed that 34 of these specifically expressed genes were unknown genes without any annotation, 9 were hypothetical protein genes, 1 was unknown functional protein gene, and 15 were known genes with annotation results. It can be seen that unknown genes, hypothetical protein genes and unknown functional protein genes account for 74.6% of the total number of specifically expressed genes. The function of these genes under the stimulation of low temperature should be investigated in future research in this field. Statistical analysis was performed on 15 genes with known functions, and the results were shown in Table 6.

Table 6 Specific expression genes with known functions

ID	Description	B (fpkm)	C (fpkm)	pvalue
TRINITY_DN9987_c0_g1	Chitinase 18-11	0	0.95	0.000765708
TRINITY_DN15069_c0_g5	Ribosomal protein L22/L17	0	6.94	0.001751776
TRINITY_DN15105_c0_g1	Gag polyprotein, partial	0	4.89	0.004255424
TRINITY_DN11747_c0_g1	Metalloprotease 1	0	0.34	0.017059201
TRINITY_DN25953_c0_g1	Matrix protein	0	3.22	0.021485746
TRINITY_DN14003_c1_g2	Triose-phosphate transporter	0	8.58	0.037477241
TRINITY_DN18536_c0_g1	COPII-coated vesicle protein	0	4.12	0.049795154
TRINITY_DN15548_c1_g1	Trichome differentiation protein GL1	2.8	0	8.94798E-15
TRINITY_DN6833_c0_g1	Acetamidase/Formamidase	0.55	0	0.001339126
TRINITY_DN22662_c0_g1	Polyketide synthase	2.92	0	0.003595873
TRINITY_DN11676_c0_g2	Tyrosyl-DNA phosphodiesterase 1	3.38	0	0.012752019
TRINITY_DN22850_c0_g1	Elastinolytic metalloproteinase Mep	5.7	0	0.017174642
TRINITY_DN14860_c0_g1	Glucose-methanol-choline oxidoreductase	1.84	0	0.022876803
TRINITY_DN19095_c0_g1	Lovastatin nonaketide synthase	5.22	0	0.023435381
TRINITY_DN1072_c0_g1	Beta-lactamase domain- containing protein	0.53	0	0.032627589

Analysis of 15 genes of known functions with specific expression revealed that 7 specific expression genes in the low temperature treatment group, among which there are 5 for structural protein gene, corresponding to ribosomal protein L22/L17, gag polyprotein, matrix protein, triose-phosphate transporter, COPII-coated vesicle protein and 2 for enzyme genes, respectively is chitinase 18-11 and metalloprotease 1. There are 8 functional genes known which specific expression in normal temperature group, among which there are 6 for synthetic metabolism related enzyme gene, relating to acetamidase/formamidase, polyketide synthase, tyrosyl-DNA phosphodiesterase 1, elastinolytic metalloproteinase Mep, glucose-methanol-choline oxidoreductase and lovastatin nonaketide synthase, there are 2 structural protein genes, trichome differentiation protein GL1 and beta-lactamase domain-containing protein respectively. It can be seen that the anabolism of *O. Sinensis* is blocked inside at low temperature, and a large number of structural proteins are expressed to prevent cell from damaged and adapt to the low temperature environment.

3.8 Functional annotation of differential expressed genes

3.8.1 GO annotation

The purpose of GO functional enrichment analysis is to obtain the GO functional entries with significant enrichment of the differential expressed genes, to demonstrate the gene functions that may exist in the samples. We first mapped all genes to each Term of the Gene Ontology database and calculated the number of differential genes in each Term. With the whole genome as the background, we used hypergeometric distribution to calculate the terms with significant enrichment of differential genes. The GO classification statistics of the intergroup differential genes in B-vs-C are shown in Figure 8. Divide the enrichment analysis results according to the three functional categories of molecular function, cellular component, and biological process, and order the results of each functional category from small to large according to Corrected P-value. The first 10 categories are selected for drawing, and different colors represent different functional classes.

In the GO classification statistics of the intergroup differential genes of B-vs-C, it was found that the number of the catalytic activity differential genes in the molecular functional classification was the largest, accounting for 58.5%. The proportion of differential genes related to the membrane was the highest among cellular components, which was 37.5%. Under the classification of biological processes, it can be seen that the number of differential genes in the oxidation-reduction process is the largest, accounting for 19.7%.

The maintenance of intracellular environmental balance is particularly important for the formation and normal development of fungal fruiting bodies. As the second messenger, reactive oxygen species (ROS) are involved in physiological processes such as cell signal response and cell proliferation, and the maintenance of its normal level and gradient distribution is very important for sexual reproduction and fruiting body development of fungi (Tudzynski et al., 2012). Studies have proved that temperature can regulate the activity of antioxidant enzymes in plants, thus affecting the tolerance of plants to temperature changes (Kang and Saltveit, 2002). The increase of ROS level in *Cordyceps militaris* cells is usually accompanied by decreasing the ability of fruiting body formation, and the degradation of frustum development in *Cordyceps militaris*, which can be inhibited by the expression of exogenous antioxidant enzyme-glutathione peroxidase A (Xiong et al., 2013). In the term of catalytic activity with the largest number of different genes in GO classification statistics, proteins encoded mainly include enzymes related to antioxidant reduction. This indicates that the low temperature also promotes the overexpression of antioxidant enzyme activity in *O. Sinensis* cells, thus eliminating the adverse effects of ROS and promoting the formation and development of fruiting bodies.

3.8.2 KEGG annotation analysis

Count the number of differential expressed genes in each KEGG Pathway at different levels, and then determine the metabolic pathways and signaling pathways in which the differential expressed genes are mainly involved. With the whole genome as the background, the hypergeometric distribution was used to calculate the pathway of significant enrichment of differential expressed genes. Enrichment analysis of B-vs-C showed that 238 differential expressed genes were successfully annotated into 123 pathways,

selection of significant expression of the top 20 metabolic pathway enrichment results was shown in figure 9, mainly focused on the metabolism of carbon metabolism, biosynthesis of amino acids, nitrogen metabolism, cyanoamino acid metabolism, pantothenate and CoA biosynthesis, biotin metabolism, phenylpropanoid biosynthesis, apoptosis and other metabolic pathways. Among them, the expression of genes in the cyanoamino acid metabolism pathway was the most significant, with 1 up-regulated gene and 3 down-regulated genes, among which the significantly up-regulated gene was gamma-glutamyl transpeptidase(ggt). Details are shown in Table 7, metabolic pathway annotation results are shown in S2.

Table 7 The differential expressed genes in the cyanoamino acid metabolism pathway.

Gene ID	Gene name	Description	Regulate	log2FoldChange
TRINITY_DN10440	ggt	Gamma-glutamyl transpeptidase	up	1.0046
TRINITY_DN11153	fmda	Formaidase	down	-1.3560
TRINITY_DN15394	ghf3	Glycoside hydrolase, family 3	down	-1.4480
TRINITY_DN15752	cel3b	Beta-glucosidase	down	-2.4702

The gamma-glutamyl transpeptidase plays an important role in the metabolism of glutathione and amino acids, catalyzing the reaction of glutathione and amino acids to produce glutamine (Gln) and cysteamine glycine (Cys-Gly) (Figure 10). Studies have found (Xi et al., 2020) that reductive glutathione in *Aspergillus nidulans* can promote the production of its sexual structure. After low-temperature treatment, the expression of ggt gene of *O. Sinensis* was significantly up-regulated. This indicates that low-temperature treatment makes ggt gene expressed in large quantities to synthesize more gamma-glutamyl transpeptidase to regulate the content of glutathione inside, thus affecting the production of the sexual structure of *O. Sinensis*. Therefore, the ggt gene is likely to be a key gene for the response of *O. Sinensis* to the low-temperature environment and may be related to the initiation of fruit body development.

3.9 Transcriptome sequencing expression pattern verification was performed by RT-PCR

In this study, key differential expressed genes were obtained through transcriptomic sequencing technology. In order to further verify the accuracy of the transcriptomic sequencing data, 8 differential expressed genes were selected for Real-time PCR verification. The results are shown in Figure 11, and the verification results are consistent with the transcriptomic sequencing data shown in Table 8. This indicates that the transcriptome data obtained in this experiment have high reliability and biological significance.

Table 8 Transcriptome sequencing results of differential genes

Gene ID	Description	FPKM(B)	FPKM(C)	B-vs-C
TRINITY_DN10440_c0_g1	Gamma-glutamyltranspeptidase	8.43	18.64	Up
TRINITY_DN11153_c0_g1	Formamidase	145.15	59.89	Down
TRINITY_DN15752_c0_g2	Hypothetical protein	41.06	7.77	Down
TRINITY_DN8973_c0_g1	Sorbitol dehydrogenase	13.41	29.29	Up
TRINITY_DN15673_c0_g2	Nitrate reductase	25.9	95.54	Up
TRINITY_DN15159_c2_g2	NADP-specific glutamate dehydrogenase	38.35	369.31	Up
TRINITY_DN12710_c0_g2	Cyanate hydratase	46.74	23.55	Down
TRINITY_DN14699_c0_g2	Ribose 5-phosphate isomerase	52.38	296.29	Up

4. Discussion

4.1 The biological significance of low temperature for *O. sinensis*.

In horticulture, there is a concept called vernalization, which refers to the phenomenon that plants must grow to a certain size and then undergo a period of continuous low temperature before they can transition from vegetative growth stage to reproductive stage (Xi et al., 2020). Similar phenomena have also been found in studies on fungi, such as *lentinus edodes*, *flammulina velutina*, *pleurotus ostreatus* and other common edible fungi, which the low temperature stimulation can promote the formation of its fruiting body (Feng et al., 2005; Sakamoto, 2010; Nishihara et al., 2007; Lu et al., 2016; Mstsumoto, 1988). Both the vernalization of plants and the low temperature stimulation of fungi can be regarded as a kind of adversity that low temperature makes organisms face. This kind of adversity will promote the transformation of the organism from the vegetative stage to the reproductive stage, which is usually closely related to the growth environment of the organism. The low temperature environment is exactly a kind of adversity that *O. sinensis* must face, so the appropriate low temperature treatment is likely to promote the *O. sinensis* to enter the sexual reproduction stage from the nutritional stage.

4.2 Functional analysis of unknown and known genes at low temperature

Statistical analysis of specific differential expressed genes showed that 74.6% of specific expressed genes were unknown genes, hypothetical protein genes and unknown functional protein genes, and the functions of these genes were not clear. In order to fully understand the hypothermia response mechanism of *O. Sinensis*, the function of this part of gene must be clearly understood, and the expression of this part of gene may be closely related to the initiation of sexual development of *O. Sinensis*. Therefore, It might be the breakthrough conten in the future on *O. Sinensis* research area. Analysis of specific differential expressed genes with clear annotations showed that most of the

differential expressed genes in the low-temperature treatment group were structural protein genes and a few were anabolic enzyme genes. On the contrary, most of the differential expressed genes in the normal temperature control group were anabolic enzyme genes, and a few were structural protein genes. Studies have found that the low temperature environment has a significant inhibitory effect on the metabolic activities of organisms (Kubien et al., 2003), and protein play an important role for organisms in the resistance to the low temperature environment (Doxey et al., 2006; Wang et al., 2012). This indicated that the anabolism of *O. Sinensis* was significantly inhibited in the low temperature environment, and other structural proteins were synthesized to adapt to the low temperature environment, enabling *O. Sinensis* to live through the long time in the low temperature environment.

4.3 Attributes and function of differential expressed genes

All the differential expressed genes were annotated by GO and functional cluster analysis was conducted to understand the functional classification of the differential expressed genes between groups. In the GO enrichment analysis between groups of B-vs-C, it was found that the differential expressed genes showed a GO Term of significant enrichment. Further study revealed that the differential gene coding products enriched in this functional classification all had redox activity. This may indirectly confirm that it is the low temperature that creates a kind of adversity, which urges *O. Sinensis* to enter the sexual stage to cope with this adversity. Studies have shown that the content of free radicals in plants increases significantly in cold environments (Hodges et al., 1997), which also leads to the expression of a large number of REDOX enzyme-related genes in the body, so as to eliminate excessive free radicals in the body and maintain the stability of the body (Wenjun et al., 1999; Ball et al., 2004).

4.4 What role does the gene ggt play?

KEGG is a powerful tool for metabolic analysis and metabolic regulatory network research in biological organisms, which can find pathways with significant enrichment in differential expressed genes (Kanehisa et al., 2006). In this study, KEGG pathway enrichment analysis for B-vs-C showed that ggt gene was up-regulated under the influence of temperature, and the amount of gene expression affected the content of gamma-glutamyl transpeptidase in the organism, thereby regulating the content of glutathione in the organism. Some studies have reported that the amount of glutathione may affect the development of fungal sexual structure (Thön et al., 2007). Therefore, low temperature treatment significantly up-regulated the ggt gene expression in *O. Sinensis*, indicating that this gene may be a key gene for the response of *O. Sinensis* to low temperature environment and may be a key gene for its sexual development initiation. At present, ggt gene has been widely studied in animals. For example, in animal cell research, it has been found that ggt gene can affect mitochondrial structure maintenance, membrane integrity, cell differentiation and development (Zhang, 2005). However, there few study on fungi and plant cells until now, so intensive study research on the ggt gene in fungi would be a great significance. Other metabolic pathways with significant differences, such as biosynthesis of amino acids, carbon metabolism, biotin metabolism, phenylpropanoid biosynthesis and apoptosis, have been reported in

transcriptional studies under low temperature stress in plants (Lin et al., 2017; Zhao et al., 2016; Chen et al., 2014; Fan et al., 2016).

Declarations

Author contributions FX designed research; QJS, LH and GZ performed experiments; FX, QH and LH analyzed data; QJS, LH, VU and YXX wrote the paper. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statements

The data used to support the findings of this study are available from the corresponding author upon request

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Figures

Figure 1

Annual variation of the natural ambient temperature of Chinese Caterpillar Fungus

Figure 2

Colony morphology of *O. Sinensis* a: Colony morphology with vegetative growth of 60 days; b: Colony morphology of vegetative growth after 60 days and treated at 4°C for 60 days; c: Colony morphology of vegetative growth after 60 days and treated at 18°C for 60 days;

Figure 3

Scanning electron microscope (SEM) of mycelia of *O. Sinensis* a: Hyphae diagram at 500 times of magnification in the 4°C low-temperature treatment group; b: Hyphae diagram at 10000 times of magnification in the 4°C low-temperature treatment group; c: Hypha diagram at 500 times of magnification in the control group at 18°C; d: Hyphae diagram at 10000 times of magnification in the control group at 18°C;

Figure 4

GO classification annotation results

Figure 5

eggNOG annotation

Figure 6

KEGG annotation

Figure 7

Expression difference; (A) volcanic map; (B) scatter map; (C) Cluster analysis of specifically expressed genes

Figure 8

GO annotation of differentially expressed genes in B-vs-C

KEGG Pathway Enrichment B_vs_C

Figure 9

BvsC KEGG enrichment analysis

Figure 10

The gamma-glutamyl transpeptidase involved in the reaction (1: Glutathione; 2: Amino acids; 3: Cysteamine glycine; 4: Glutamine)

Figure 11

Detection of target gene expression pattern by Real-time PCR (** $P < 0.01$, * $0.01 < P < 0.05$)