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Transcriptomic Analysis of Permafrost Mucormycete Stress on Fennel Thin-Winged Borer

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Abstract: In this study, a comprehensive transcriptome analysis was performed on Evergestis extimalis Scoli under the stress of Mucor permafrost by transcriptome sequencing technology, aiming to reveal the regulatory mechanism and biological characteristics of gene expression under pathogenic fungal stress. The experiment used high-throughput sequencing technology to annotate Unigenes using multiple databases, and found that Asian corn borer was the species with the most homologous sequences. A total of 357, 444, 198 and 890 differentially expressed genes were detected by differential expression analysis, which were mainly enriched in cell components, molecular functions and biological processes, involving polysaccharide degradation, metabolic processes, and antibacterial humoral reaction regulation. KEGG enrichment analysis revealed multiple pathways related to insect metabolism and signaling, such as "oxidative phosphorylation", "glycolysis/gluconeogenesis" and "MAPK signaling pathway", among which the environmental information processing-signal transduction pathway enriched the most differential genes. In addition, real-time PCR verified that the expression trends of 9 genes with large differences were basically consistent with the transcriptome sequencing results, indicating that the transcriptome data had high accuracy and reliability. The results of this study provide an important theoretical basis for understanding the gene expression regulation mechanism and biological characteristics of F. fennel under permafrost Mucormycete stress.

Keywords: Fennel thin-winged borer, Environmental stress, Transcriptome, qRT-PCR

1 INTRODUCTION

Evergestis extimalis Scopli, belonging to the Lepidoptera family Pyralidida, also known as fennel borer and rape borer. It is mainly distributed in North Korea, Japan, the United States, and Siberia, and is mainly distributed in northern regions such as Qinghai, Gansu, Ningxia, and Heilongjiang in China, and the insect is mainly harmful to plants such as sugar beet, cabbage, rapeseed, cabbage, and mustard greens [1]. The larvae harm spring rapeseed by drilling horn fruits and feeding on the seeds [2]. In Qinghai Province, one generation occurs a year, and the larvae overwinter in the soil as cocoons, with a pupal duration of 10~35 days, and the longest can reach 40 days, and the duration gradually shortens with the increase in temperature. Adults in the field first appeared in mid-June, and the peak of adult eclosion occurred in mid-to-late July [3]. After the adults emerge, they like to move among the weeds that grow vigorously, and when the rapeseed blooms to the horn hanging stage, the adults migrate to the rapeseed field to start laying eggs. The first appearance period of eggs in the field is late June, and the peak period is mid-to-late July, and the duration of eggs is 5~15 days, which shortens with the increase of temperature [4,5]. The larvae began to appear in mid-July, and the field was damaged for more than 60 days, and they continued to feed on the horn fruit after the rapeseed was harvested. After the larvae mature, they overwintered in soil cocoons under the topsoil, and 97.77% of the overwintering larvae were in the soil layer of 0~4 cm [6,7].

Mucor hiemalis is a fungus that is widely distributed in nature, widely distributed in soil and air. In the alpine ecological region of the Qinghai-Tibet Plateau, Mucor mycomyces is one of the 11 genera [8], and its distribution is regional, only in Taiwan, Gansu, Qinghai, Xinjiang, and other places [9]. Recent studies have shown that permafrost mucor has certain biocontrol potential in the control of animal and plant diseases [10,11]. The results of Ma Youhua et al. [12] showed that the cumulative mortality rates of 2nd, 3rd, 4th, and 5th instar larvae were 71.78%, 49.44%, 46.51%, and 28.89%, respectively, after 9 days of treatment with the larvae of Mucormycetes fennel, and

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the infection rate increased with time, which had a good effect on the biological control of F. fennel. In addition, biogenic pesticides such as Chlorella chinensis [13] and Coccidioide albicane [14,15] have been reported.

When insects are subjected to adverse environmental stresses, they will cause a series of physiological and biochemical reactions to resist the adverse effects caused by stress [16]. This is due to the gradual development of a set of mechanisms for sensing and responding to environmental changes in the long evolutionary process [17]. They are able to rapidly recognize stress signals and translate these signals into physiological and biochemical responses within cells through complex signal transduction pathways. These reactions involve multiple levels such as the regulation of gene expression. These behavioral and physiological adaptation strategies allow insects to maintain their ability to survive and reproduce in adverse environments.

The development of transcriptome sequencing technology is becoming more and more rapid, and the transcriptome data obtained based on this is quite rich, which can be used to mine important functional genes for species with lack of genetic information, further promote the functional genome analysis of non-model organisms, and provide data support for the study of insect species classification, development, evolution and other issues. In order to further understand the expression of genes related to the signaling pathway of Mucor fennel after permafrost mucor infestation, the transcriptome of the 3rd instar larvae of Mucor fennel treated with Mucor fista was analyzed.

2 Materials and Methods

2.1 Insects for Test

Fennel thin-winged borer eggs were collected from Huangjiazhai Town, Datong County, Xining City, Qinghai Province (36°53′N, 101°46′E, 2 289 m above sea level), and the collected eggs were placed in a square insect breeding box (size: 17 cm long) lined with fresh baby cabbage (Brassica pekinensis leaves) × 12 cm wide × 8 cm high), incubated in an artificial climate incubator, and raised in fresh baby cabbage after hatching, the relative humidity was (65±5)%, the photoperiod was L:D=18 h:6 h, and the light intensity was 9 000 lx. The 3rd instar larvae with the same size and healthy growth were selected for follow-up experiments.

2.2 Test Method

The permafrost mucor suspension is configured by laboratory separation and purification of the preserved permafrost mucor. The medium plates were eluted with sterile distilled water (containing 0.05% Tween 80) and filtered with four layers of gauze to make a spore suspension.

The 3rd instar larvae of healthy fennel thin-winged borer were selected for experiment, inoculated by impregnation method, and soaked in 1. 0×107, 1. 0×106, 1. 0×105, 1. 0×104, 1. 0 × 103 spores/mL suspension, 30 larvae in each group, and repeated 3 times, taken out and dried naturally, moved into a self-made insect breeding box (8 cm×14 cm×5 cm), and put in (T=22 ° C± Fresh baby cabbage was raised in an incubator at 1 ° C, RH=75% ±5%). The 3rd instar larvae of F. fennel treated with sterile water under stress were used as controls. The number of deaths was observed and recorded every day, and the dead insects were picked out in time to count the mortality rate at each time point in different groups.

2.3 Transcriptome Sequencing Analysis

2.3.1 Sequencing Sample Preparation

Using the above method, the spore suspension with the highest lethality rate was selected for experimental treatment on the 3rd instar larvae of healthy fennel thin-winged borer, and sterile water was used as a control. Samples were taken at 24, 48, 72, and 96 h, placed in a cryopreservation tube, quick-frozen with liquid nitrogen, and frozen in a freezer at -80 °C to ensure the integrity and stability of RNA for subsequent experiments.

2.3.2 Sample Sequencing, Assembly and Functional Annotation

After passing the quality test of the fennel thin-winged borer sample library, high-throughput sequencing operations were carried out with the help of the Illumina NovaSeq 6000 platform. After the sequencing task is completed, the obtained data is preprocessed using Fastp software, and the low-quality reads (sequencing fragments) and linker sequences are eliminated according to the base mass value. Get preprocessed clean data. Trinity software was used to perform transcriptome splicing, and after splicing, the redundant sequences were further removed using CD-HIT software, resulting in high-quality Unigenes (single gene clusters). These Unigenes are integrated with NR, SwissProt, KEGG, KOG via BLASTx software , eggNOG, GO, and Pfam to realize the functional annotation information of Unigenes.

2.3.3 Differentially Expressed Genes and Functional Annotations

Unigenes expression was calculated using Bowtie2 and eXpress software, followed by DEG (differentially expressed gene) analysis using DESeq 2 software. During the analysis, genes that meet the threshold conditions of P-valμe≤0.05 and |log2fold change|≥1 are defined as differentially expressed genes, so that those genes with significant differential expression are screened out.

2.3.4 Real-Time PCR Validation

Based on previous studies, we screened out differentially expressed genes related to signaling pathways: DN795, DN6, DN68, DN266, DN164, and so on DN1270、DN1072、DN374、DN72 、 actin1. In order to verify the accuracy of the transcriptome data, 9 genes were screened from the sequencing results for real-time PCR detection, and Actin1 was selected as the internal reference gene. The DEGs screened according to the enrichment results of GO and KEGG were designed using Primer Quest Tool (http://sg.idtdna.com/Primerq μest/Home/Index).

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TABLE 1 PRIMER SEQUENCE

Gene Name	Forward sequence	Reverse sequence
DN795	TACCTGAAGTGGCTCCTATTAACCTC	CCACAGGCAAGGCGGACTC
DN6	GCAGGCTCAGCTCAAGAACG	GGCAATGTAGGTGACAGTGTAGAC
DN68	ACCGCCGCCCTTCTTGAG	GCAATGGATGGTGTCTGGTAGG
DN266	ACGAGAACGGTGAACTAAAACAGAC	ACGGACTCCTGCTTGCCTTC
DN164	CGTGGCTTCCGCTTCAATCG	TCAATGAGCATTTGTTCGAGTAGGG
DN1270	CGCCGATGACGCAAAGGAATG	CATGATGACGCCGTGAACTTCG
DN1072	TTGCTCCGACCTCCGTGATG	TAAACGACCCAGGCTAGGAATCC
DN374	AGAAGCGTGCTGTCATCAACTAC	CATGTCATCCTCGTGCCTGAAC
DN72	ATCCACGAAACCACATACAACTCC	TGCGGTCGGCGATTCCAG
actin1	TCAAGGAAGATGAGGCTGAACAATC	CTGGAGCATTACTCAAGACGAGAC

3 RESULTS AND ANALYSIS

3.1 EXPLORATION OF THE MOST SUITABLE INFECTION CONDITIONS FOR FENNEL THIN-WINGED BORER

It can be seen from Fig. 1 that the number of deaths of Mucor fennel was significantly increased after different concentrations of mucor spore suspension in the frozen soil, and the number of deaths of 1.0×107 spores/mL in the treatment group was the largest, which was significantly higher than that of other groups. The suspension of 1.0 × 107 spores/mL was the optimal concentration for infection.

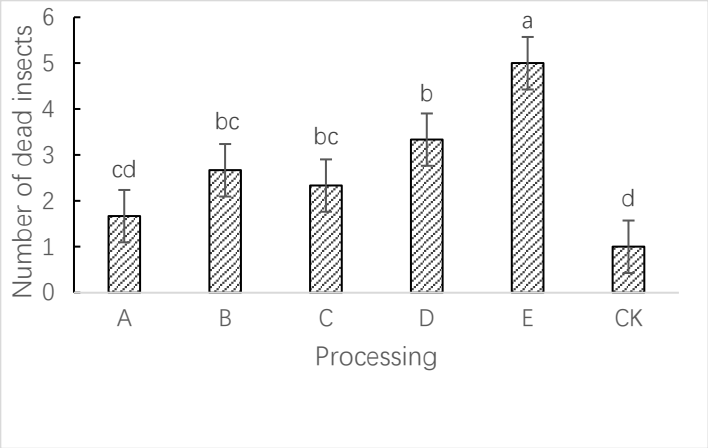

FIG.1 INFECTION AND MORTALITY OF SPORE SUSPENSIONS WITH DIFFERENT CONCENTRATIONS

Note: CK Indicates Control, A Means 1.0 × 103 Spores /Ml, B Means 1.0 × 104 Spores /Ml, C Means 1.0 × 105 Spores /Ml, D Means 1.0 × 106 Spores /Ml, E Means 1.0 × 107 Spores /Ml

3.2 TRANSCRIPTOME RESULT ANALYSIS

3.2.1 QUALITY ANALYSIS OF TRANSCRIPTOME SEQUENCING DATA

Relying on high-throughput sequencing technology, double-ended sequencing data of a large number of samples have been successfully obtained. To ensure the high quality and reliability of this data, the data was filtered using fastp software before the information was analyzed. Through this operation, invalid data such as low-quality reading segments and joint sequences are eliminated, so as to reduce the possible interference of invalid data in subsequent analysis.

Transcriptome sequencing of 15 samples yielded a total of 110.03 G of CleanData (high-quality data). The effective data volume of each sample is in the range of 6.33~7.05 G. Generally speaking, a higher percentage of Q30 means better data quality. The distribution ratio of Q30 bases ranged from 97.4% to 97.74%, and the average GC content reached 45.04%. In addition, 42119 Unigenes were obtained by splicing, with a total length of 54076937 bp and an average length of 1283.91 bp. The quality of the data obtained by this sequencing is in line with the standard, and there is no obvious bias problem, which can be used for subsequent in-depth analysis.

3.2.2 TRANSCRIPTOME SPLICING AND ANNOTATION

The data was stitched using Trinity software, and a total of 42,119 Unigenes were obtained. Among them, there are 29,001 Unigenes ⩾ 500 bp in length and 16,999 Unigenes ≥ 1,000 bp in length. Annotate Unigenes using multiple databases.

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TABLE 2 ANNOTATIONS OF UNIGENES IN THE DATABASE

data base	Number of annotated genes	Percentage（%）
NR	22473	53.36%
SwissProt	13087	31.07%
KEGG	1470	3.49%
KOG	11584	27.50%
eggNOG	18751	44.52%
GO	11718	27.82%
Pfam	12053	28.62%

3.2.3 GENE EXPRESSION LEVEL ANALYSIS

The expression levels of Unigenes were calculated by Bowtie2 and eXpress software, and it was found that there were significant differences in gene expression levels between different samples. The differential expression analysis of all genes in the treatment group and the control group was carried out by DESeq2 software, and the screening criteria were: log2fold change|>1, and the q-value <0.05. A total of 357, 444, 198, 890 differentially expressed Unigenes.

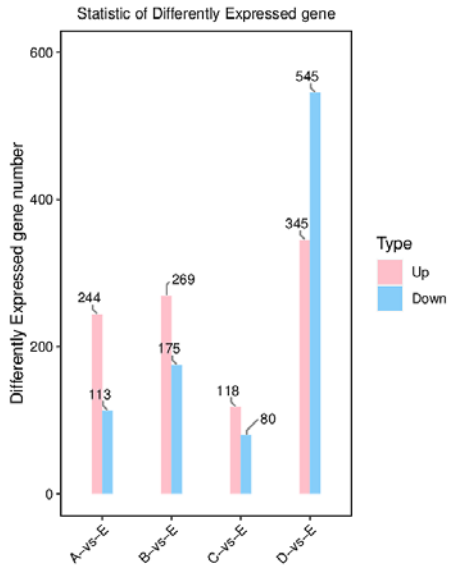

FIG 2 STATISTICAL CHART OF DIFFERENTIALLY EXPRESSE UGENES

3.2.4 FUNCTIONAL ENRICHMENT ANALYSIS

In order to further understand the biological functions of differentially expressed genes, GO and KEGG enrichment analysis was performed on differentially expressed genes, and it was found that multiple genes were related to the growth and development, metabolic pathways and environmental adaptability of insects. GO enrichment analysis showed that differentially expressed genes were mainly enriched in cell components, molecular functions, and biological processes. KEGG enrichment analysis revealed multiple pathways related to insect metabolism and signaling, such as "oxidative phosphorylation", "glycolysis/gluconeogenesis", and "MAPK signaling pathway".

(1) GO enrichment analysis

After the differentially expressed Unigene was obtained, the differential genes in the treatment group and the control group were GO enriched.

The results of differential gene enrichment analysis in A (24 h treatment group) and E (control group) showed that significant differential genes were enriched into three major taxa, namely Biological Process (BP) and Cellular Component (CC).) and Molecular Function (MF). In the biological process part, polysaccharide catabolic process, metabolic process, and regulation of antimicrobial humoral reaction has high statistical significance. In terms of cell components, cells, organelle membranes, and retrotransposon nucleocapsids had high statistical significance. suggests that these cellular structures may have changed under A-vs-E conditions. In the molecular function part, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, monooxygenase activity and iron ion binding.

The results of differential gene enrichment between B (48 h treatment group) and E (control group) showed that in the biological process category, the defense response to bacterium and innate immune response were indicated) has the highest statistical significance. In addition, fatty acid oxidation and glycolytic processes also showed high significance, suggesting changes in energy metabolism. Among the cell component categories, the extracellular region and organellar membrane had high statistical significance, indicating that these cellular structures may have changed under B-vs-E conditions. Among the molecular functional categories, the structural constituent of cuticles and iron ion binding had the highest statistical significance.

The results of differential gene enrichment between C (72 h treatment group) and E (control group) showed that the most significant biological process categories included DNA-mediated transposition and gluconeogenesis) and cell surface receptor signaling pathway had the highest statistical significance. Among the cell component categories, the highest significance included mitochondrion, extracellular region, and membrane. These correlate with mitochondria, extracellular regions, and membrane structure, suggesting the importance of these cellular components in the C-vs-E comparison. Among the molecular function categories, the highest significance included RNA-directed DNA polymerase activity and G-protein-coupled

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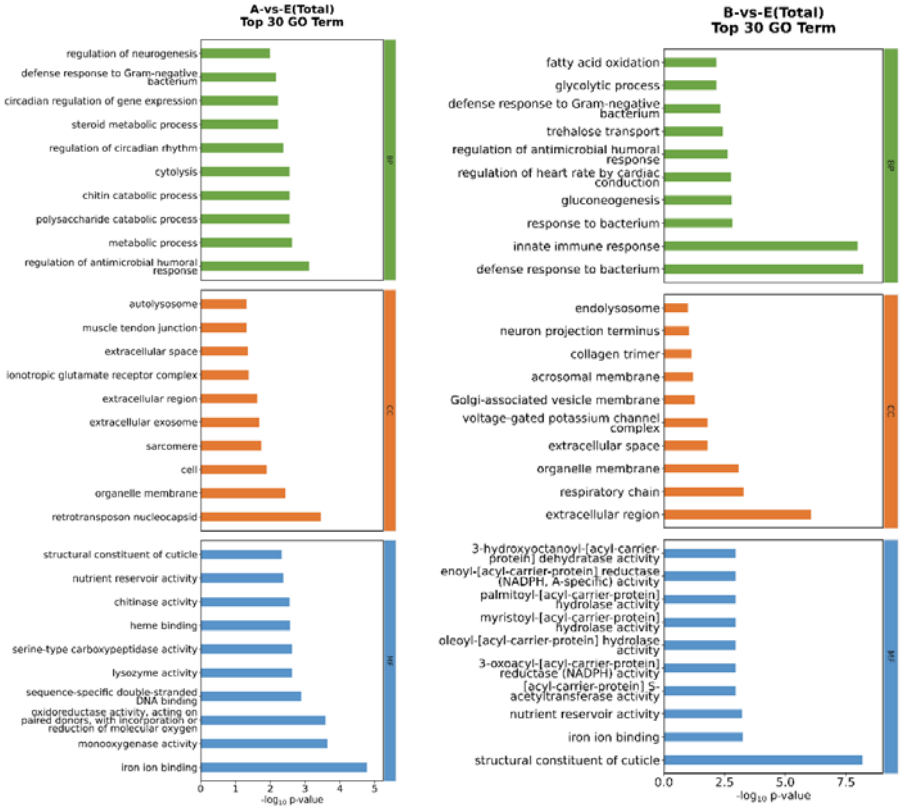
receptor activity (G). protein-coupled receptor activity) and 3-oxoacyl-[acylcarrier protein] reductase (NADPH, B-specific) activity. These involve RNA-directed DNA polymerase activity, G-protein-coupled receptor activity, and specific enzyme activity, indicating that these molecular functions differ significantly in the C-vs-E comparison.

The results of differential gene enrichment between D (96 h treatment group) and E (control group) showed that the significant differences in biological processes included antifungal humoral response and negative regulation of coagulation coagulation), xanthine catabolic process, etc. These processes may be related to differences in metabolism, immune response, and cellular interactions between samples D and E. In terms of cell composition, significant differences include peroxisome, extracellular space, and extracellular regionetc. Differences in these components may reflect differences in the cellular structure and extracellular environment of samples D and E. In terms of molecular function, significant differences include calcium channel inhibitor activity, iron ion binding, and structural constituent of stratum corneum cuticle) and so on. These differences in function may be related to the different activity and binding properties of samples D and E at the molecular level.



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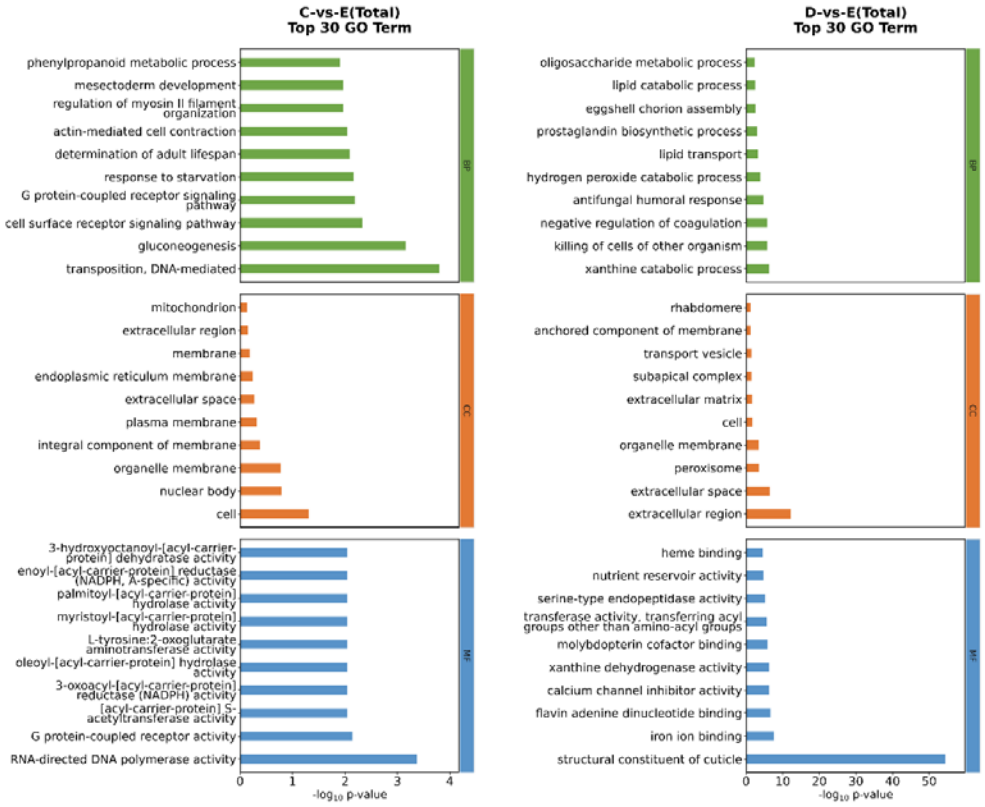

FIG 3 GO ENRICHMENT ANALYSIS SECONDARY CLASSIFICATION ITEMS

KEGG ENRICHMENT ANALYSIS

The KEGG database integrates data from genomic information, biochemical pathways, disease information, and chemical substances and chemical reactions. Through KEGG analysis, genes can be associated with known metabolic and signaling pathways, thereby revealing the systemic role of genes in organisms.

According to a set threshold, i.e. | log2fold change | If it is greater than 1 and the FDR is less than 0.05, the genes are screened to find the genes that are different. These screened differential genes are then mapped to the KEGG PATHWAY pathway for further analysis of the role and associated mechanisms of these differential genes in biological pathways. The results showed that a total of 1470 (3.49%) Unigenes were annotated to the KEGG database in the treatment and control groups, involving multiple metabolic pathways and signaling pathways, and the first 20 significantly enriched pathways were analyzed in this study. These differential genes are mainly enriched in environmental information processing-signal transduction, human diseases-infectious diseases: viruses , Human Diseases-Cancer: overview, Cellular Processes-Cellular Processes-Transport and catabolism, Organismal Systems-Endocrine system, Metabolism - Metabolism-Lipid metabolism, Metabolism-Carbohydrate metabolism), Human Diseases-Neurodegenerative disease Genetic Information Processing-Translation , Organismal Systems-Immune System, Organismal System-Nervous System , Human Diseases-Cardiovascular disease, Metabolism-Metabolism-Xenobiotics biodegradation and metabolism, Metabolism-Nucleotide metabolism), Cellular Processes-Cellular community - eukaryotes, Human Diseases - Endocrine and Metabolic Diseases , Human Diseases-Cancer: specific types, Organism Systems-Digestive system , Metabolism-Metabolism of other amino acids, metabolism - Metabolism of cofactors and vitamins. Among them, Environmental Information Processing-Signal transduction) enriched the most DEGs, with 262 DEGs. Signal transduction genes, immune system genes and endocrine system genes were enriched multiple times in these 20 enrichment pathways, indicating that the fennel thin-winged borer induced and activated related immune responses after being stressed by Mucorderium permafrost, which played an important role in resisting the infection of Mucor permafrost.

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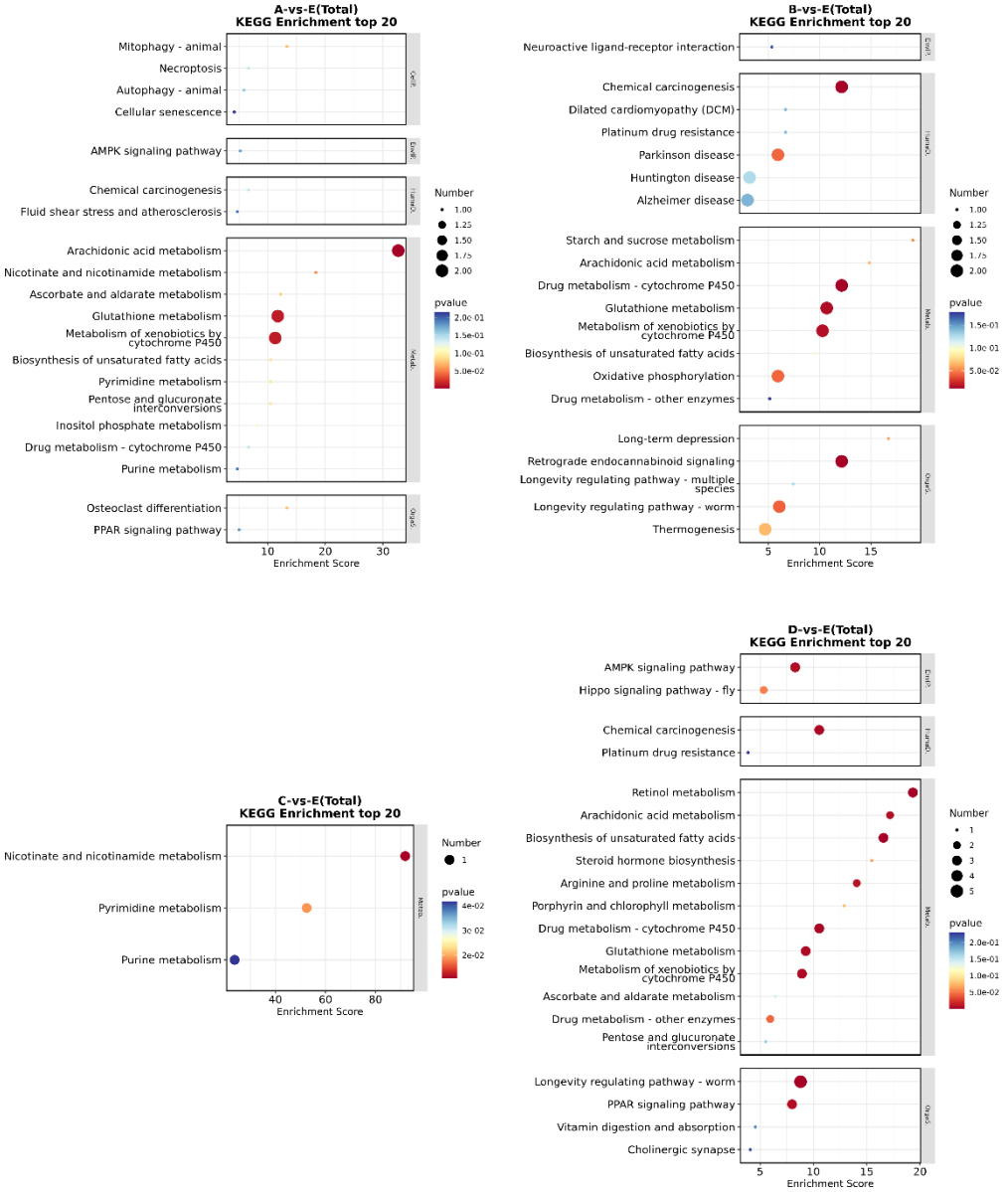

FIG 4. KEGG ENRICHMENT ANALYSIS BUBBLE CHART

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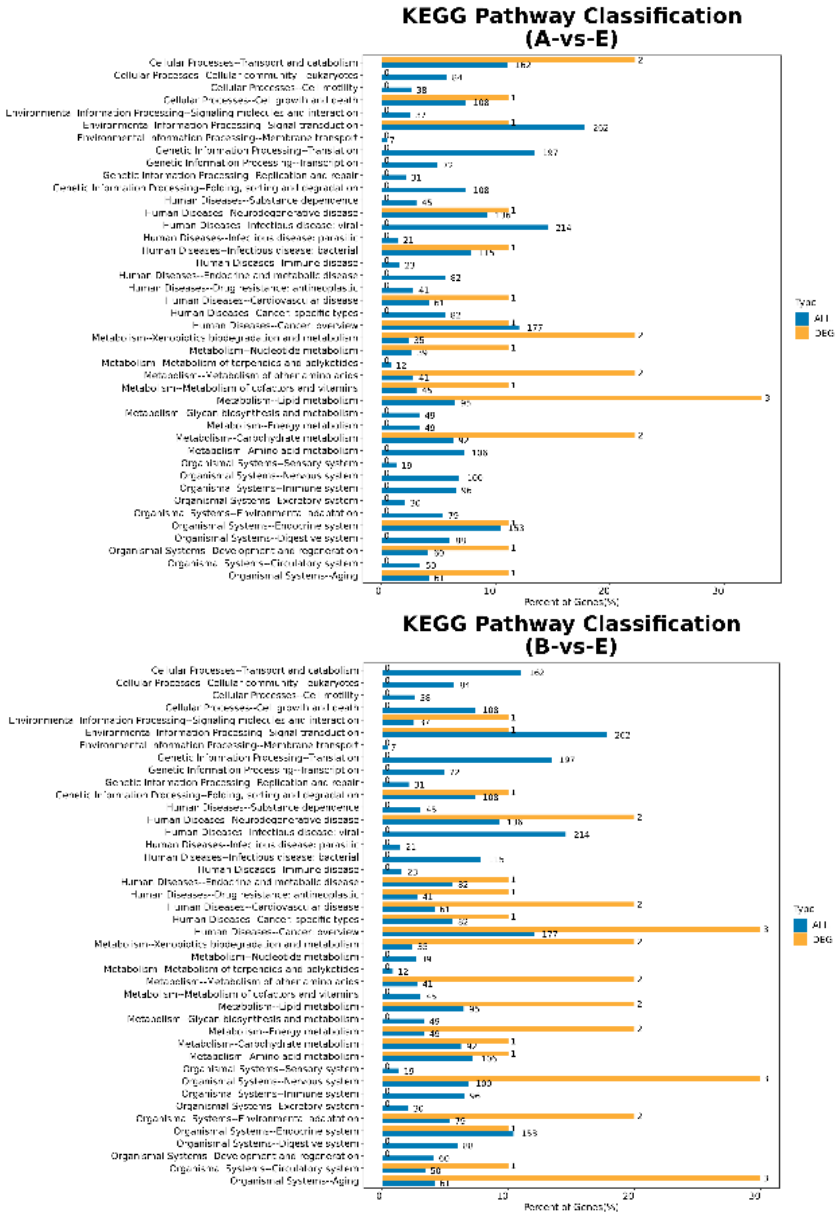

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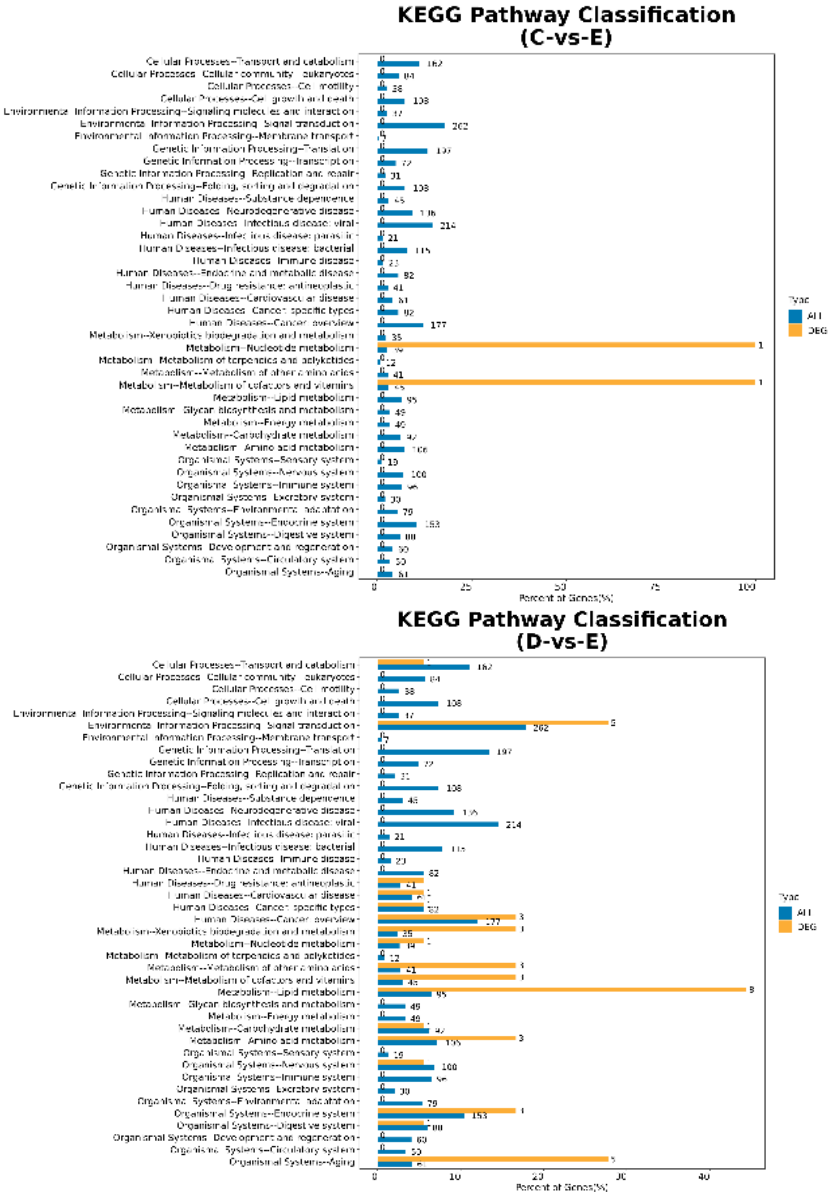

FIG 5 COMPARISON OF LEVEL 2 DISTRIBUTION OF KEGG ENRICHMENT ANALYSIS

Note: The blue bar represents the percentage of all genes (ALL), and the orange bar represents the percentage of differentially expressed genes (DEG)

3.2.5 *TRANSCRIPTION FACTOR ANNOTATION*

Transcription factors (TFs) play a key role in the regulation of gene expression and are proteins that can regulate gene transcription. Transcription factors By annotating transcription factors, we can gain insight into the gene regulatory network of F. fennel thin-winged borer. By annotating Unigenes with transcription factors, 1471 Unigenes were found to be annotated to the transcription factor database. Among the differentially expressed genes, multiple transcription factor families showed significant expression differences, indicating that these transcription factors may play an important role in the environmental adaptability of F. fennel.

TABLE 3 ANNOTATION RESULTS OF TRANSCRIPTION FACTORS

Transcription factor family	Number of annotated genes	percentage
bHLH	120	8.16%
C2H2	110	7.48%
ERF	95	6.46%
MYB	80	5.44%

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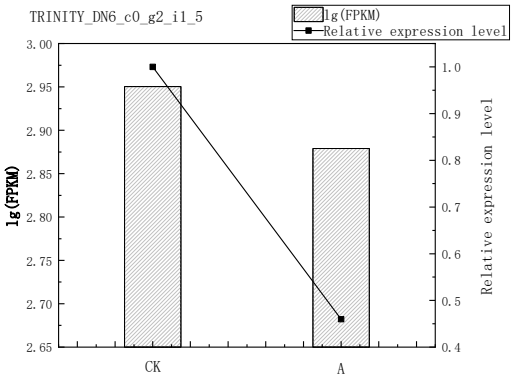
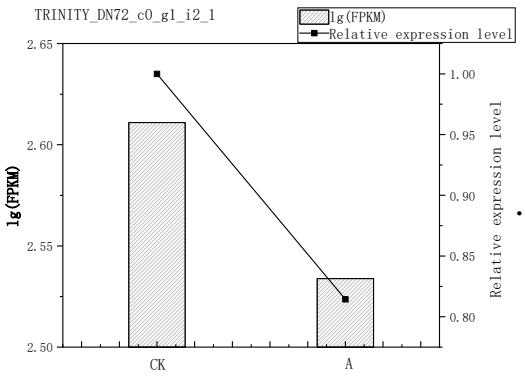
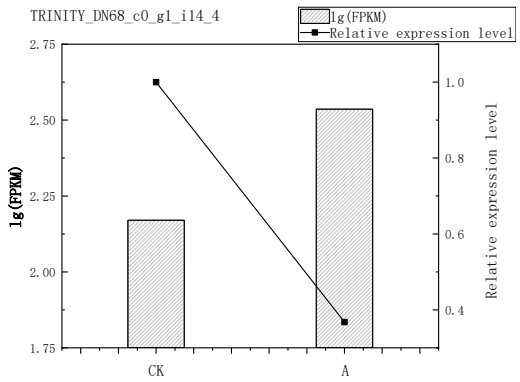
bZIP	70	4.76%
AP2/ERF	60	4.08%
WRKY	50	3.40%
NAC	45	3.06%
HSF	40	2.72%
ARF	35	2.38%
other	806	54.80%

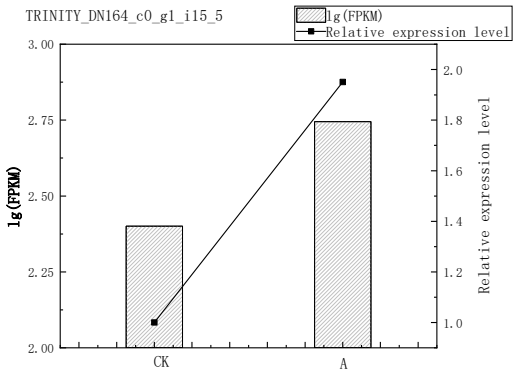
3.2.6 TRANSCRIPTOME VALIDATION

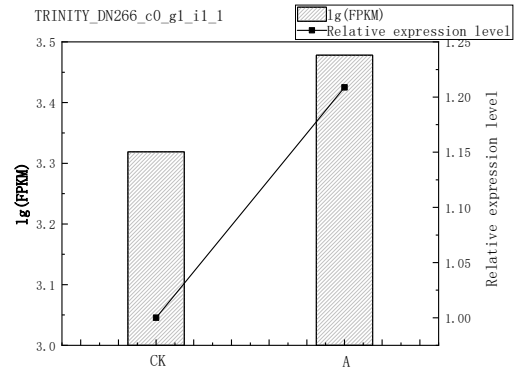
In order to verify the accuracy of the transcriptome data, 9 genes with large differences were screened out from the sequencing results for real-time PCR verification, and the results showed that the validation results of these 9 genes with large differences were basically consistent with the expression trend of transcriptome sequencing, indicating that the transcriptome data had high accuracy and reliability.

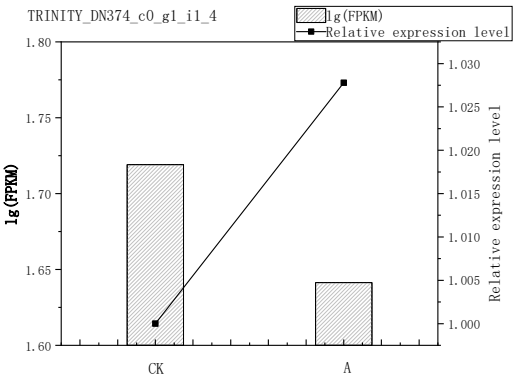












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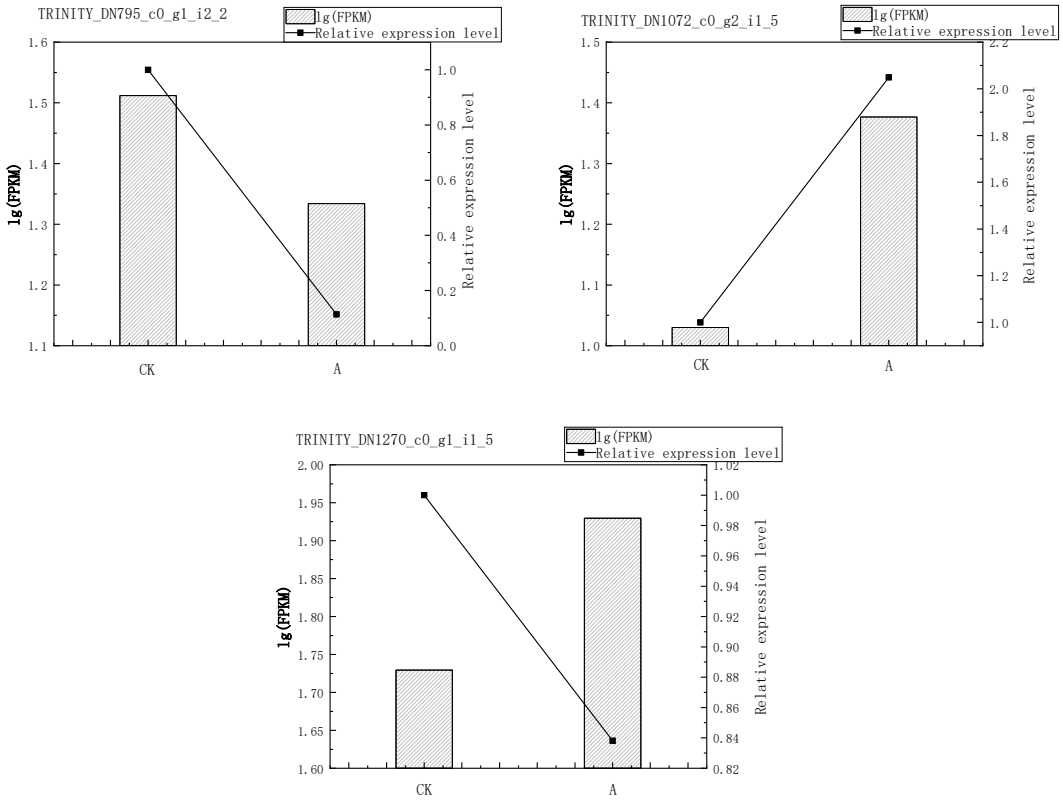

FIG 6 DIFFERENTIAL GENE RT QPCR ANALYSIS

4 DISCUSSION

Larvae are a critical period for pest control. Previous studies have shown that the use of fungi plays an important role in biological control of pests and diseases. Chen Dasong et al. [18] measured the virulence of B. thuringiensis and showed that Bacillus thuringiensis had a good killing effect on A. s. s. and had a high safety profile. Zhang Xu [19] et al. showed that the strain had a certain lethality rate against S. frugi, and after 8 days of infection of the 3rd instar larvae of S. lai, only 1×108 spores per mL had a cumulative mortality rate of 100%, while the cumulative mortality rate of the 5th instar larvae did not reach 100%. After 36 h, 48 h, 60 h and 72 h of 8/mL spru suspension of Chlorella laeferi 1×10 8/mL spore suspension, the enzyme activities of CAT, SOD and CarE were the highest at 36 h and then gradually decreased, while the enzyme activities of POD and GST were the highest at 48 h and then decreased.

With the development of high-throughput sequencing technology, transcriptome technology has been widely used to study the mechanism of insect resistance to environmental stress, and the mechanism of action of F. fennel at the molecular level after stress is further understood through transcriptome technology. A large number of relevant research literatures have been reported on diamondback moth, grassland moth, cotton bollworm, etc. Han Lijuan et al. [20] performed transcriptome sequencing of brown planthoppers infected with C. aeruginosa, combined with bioinformatics analysis of differentially expressed genes and signaling pathways, and further speculated genes related to the immune response of brown planthoppers. Yang Yuanxue et al. [21] analyzed the molecular mechanism of detoxification and metabolism of S. frugia by transcriptome technology, and experiments showed that CYP3 and CYP4 family cluster genes may be related to the molecular mechanism of detoxification and metabolism of S. frugalis.

Fungi-based biological control technologies have received widespread attention in recent years, and have been widely used due to their environmental friendliness, resistance to pests, and continuous control effects [22]. In this study, the transcriptome data were analyzed by using permafrost mucormycetes to infect A. fennel, and the stress resistance mechanism of A. fennel was further understood at the molecular level, which provided a theoretical basis for the efficient and safe control of A. fennel in agricultural production.

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