Transcriptome Profiling of *Micromelalopha troglodyta* (Lepidoptera: Notodontidae) Larvae under Tannin Stress Using Solexa Sequencing Technology[1]

Kai Feng, Fang Tang[2], Yu Zhang, and Meiling Nong

Co-Innovation Center for the Sustainable Forestry in Southern China, College of Forestry, Nanjing Forestry University, Nanjing 210037, China

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Abstract Tannins are a large group of polyphenolic compounds and natural protective substances for plant survival. The differentially expressed genes (DEGs) of *Micromelalopha troglodyta* (Graeser) under tannin stress were studied by Solexa sequencing technology. A total of 51,797,038–54,991,822 and 51,674,478–52,307,172 clean reads were obtained from the tannin treatment (TT) library and the control (CK) library transcriptomes, respectively, and assembled into 21,236 nonredundant consensus sequences. The expression of 1,627 unigenes in the TT library was remarkably different from that of the CK library ; 885 genes were upregulated, and 742 genes were downregulated ($P \leq 0.001$). The expression of 18 DEGs was detected by real-time fluorescent quantitative PCR, and the trend of gene expression was consistent with that of transcriptome data. In the biological process category, the DEGs were primarily related to cellular processes, metabolic processes, and single-organism processes. In the molecular function category, the DEGs were mainly involved in binding and catalytic activity, and in the cellular component category, the DEGs were mainly related to the cell, cell part, and organelle. Pathway enrichment analysis indicated that drug metabolism-cytochrome P450 and glutathione metabolism may be associated with detoxification-related processes under tannin stress, and glutathione S-transferases and other detoxification enzyme genes play an important role in detoxifying tannins in *M. troglodyta* larvae. This study also provides important resources for further study of the genes related to pesticide targets and metabolic processes in *M. troglodyta*.

Key Words *Micromelalopha troglodyta* (Graeser), tannin, transcriptome, detoxification enzyme, cytochrome P450, glutathione metabolism

Transcriptome sequencing (RNA-Seq) technology has been developed in recent years using a new generation of high-throughput sequencing technology (Marguerat and Bähler 2009). Wilhelm et al. (2008) and Nagalakshmi et al. (2008) used RNA-Seq technology to study the fission yeast and *Saccharomyces cerevisiae* transcriptome, thus symbolizing the establishment of RNA-Seq technology. RNA-Seq yields abundant information about RNA transcripts with extremely high detection accuracies and enables the discovery of transcripts at lower abundance (Shen et al. 2011, Chen et al. 2018). The most unique application of RNA-Seq is the analysis of differentially expressed genes (DEGs) in different

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[2]Corresponding author (email: tangfang76@foxmail.com).

sample transcriptomes (Wall et al. 2009, Gu et al. 2019). The application of transcriptomics is very extensive (Qi et al. 2011, Liu et al. 2019) and includes structural studies of transcripts (border identification of introns, initiation codon identification, alternative splicing studies, and UTR identification), studies on the structural variations of transcripts (identification of fusion genes and study of the polymorphism of coding sequences), gene expression studies (Wang at el. 2010), functional studies of noncoding regions (e.g., studies of noncoding RNAs, microRNAs, and small interfering RNAs) (Clamp et al. 2007, Ponting et al. 2009), the discovery of new low-abundance recordings, and more.

Cao et al. (2013) used RNA-Seq to identify the DEGs of *Chironomus kiiensis* Tokunaga. In addition, pathway enrichment analysis also showed that these DEGs were annotated to the metabolic pathway. Pan et al. (2015) compared the transcriptomes of thiamethoxam-resistant strains and another susceptible strain of aphids. The results demonstrated that acetylcholine receptor gene changes, upregulated ribosomal protein, ecdysone uridine diphosphate glucose transferase, cytochrome C oxidase, esterase, and peroxidase were the main mechanisms of aphid resistance to thiamethoxam. Following the completion of the whole-genome sequencing of *Drosophila melanogaster* (Meigen) in 2000, many insect transcriptome sequences have been reported. With the emergence and development of high-throughput sequencing technology, this gene information is an important sequence resource in insect toxicology research.

However, there have been no studies on the transcriptome of *Micromelalopha troglodyta* (Graeser). Therefore, in this study, the deep transcriptome of *M. troglodyta* was sequenced by using Illumina HiSeq^{TM} 2000 sequencing technology. Then, the transcriptome was globally analyzed by assembly, annotation, and bioinformatics analysis, including classification, protein function prediction and classification, and metabolic pathway analysis. This study used data from RNA-Seq to obtain gene transcriptional expression levels. A comprehensive analysis of the DEGs was performed using transcriptome data. Furthermore, to understand the biological activity and biological processes of detoxification-related genes, the differentially expressed detoxification-related genes were further identified. This work establishes a foundation for the study of the *M. troglodyta* genome, which has great theoretical significance for studying genes related to pesticide targets and metabolic processes in *M. troglodyta*. At the same time, the study results also provide a theoretical basis for the study of the relationship between herbivores and host plants and the extensive interaction between them.

Materials and Methods

Insect rearing and sample preparation. The *M. troglodyta* population was collected from poplar (*Populus* × *euramericana* 'Nanlin 895') in Nanjing, Jiangsu Province, China. The larvae were reared in the incubator at 26 ± 1°C, relative humidity of 70–80%, and photoperiod of 16:8 (light:dark). The newly collected poplar leaves were used as food for larvae. Healthy third-instar larvae of a similar weight and appearance were used for the tannin treatments (TTs).

First, the tannin was dissolved with a small amount of ethanol and then was diluted with distilled water to the concentration of 0.1 mg/mL. Newly collected poplar

leaves were soaked in the tannin solution for 10 s. After the leaves dried, two treated leaves were placed into a triangular flask with five third-instar larvae. This procedure was repeated with 10 triangular flasks. The control (CK) group consisted of larvae feeding on leaves soaked in distilled water. After feeding on the treated leaves for 96 h, the larvae were collected in groups and dissected. The larvae of *M. troglodyte* were dissected on ice. After the peritrophic membrane containing midgut contents was removed, midguts were washed in 1.15% ice-cold KCl and collected. All experiments were independently conducted three times.

RNA isolation and sequencing. We used the RNprep Tissue Kit (TIANGEN) to separate the total RNA according to the manufacturer's guidelines. The concentration of RNA was measured with a spectrophotometer, and the integrity of RNA was detected by the agarose gel dissolution method with a concentration of 1%. The total RNA was purified by using poly-T oligo magnetic beads to obtain mRNA, and fragmentation buffer was to interrupt mRNA. First, we used random hexamers to synthesize the first cDNA strand, and then the second cDNA was synthesized with dNTPs, DNA polymer I, and RNaseH. Solexa sequencing using a Illumina HiSeq 2000 instrument was performed at the Shenzhen Huada Gene Research Institute.

Sequence assembly. Trinity were used to perform the *de novo* assembly and eliminate PCR duplication. Then, when the transcripts were aggregated into unigenes, Tgicl was usually used. Trinity is composed of three single-handed software modules, namely, Inchworm, Chrysalis, and Butterfly, which are successively used to process a large number of reads. Trinity divides the sequence data into a number of separate de Bruijn maps, with each map representing some point of view of the transcription complexity of a given gene or site. Each map is separately assessed to extract the full-length splicing subtype and separate the transcripts from homologous genes.

Multiple samples of the same species were used for sequencing. Sequence clustering software was used to splice and remove the single gene in each sample set so as to obtain the required nonredundant single gene. In the final step, we used Blast to compare unigenes with the five databases (NCBI nonredundant nucleic acid database [NT], NCBI nonredundant protein sequences [NR], Cluster of Orthologous Groups of proteins [COG], Kyoto Encyclopedia of Genes and Genomes [KEGG], and Swissprot) to obtain the result of annotation; Blast2GO with NR annotation was used to obtain the Gene Ontology (GO) annotation (Altschul et al. 1990, Conesa et al. 2005). We, thus, established five samples of *M. troglodyta*. The unigenes assembled with these six samples were further sequenced and deduplicated using sequence-based clustering software to obtain the longest possible nonredundant unigenes.

Sequence annotation. NR and Swissprot are protein databases, and COG is also a protein database based on complete genomes of bacteria, algae, and eukaryotes that provides a direct homologous classification based on system evolution. KEGG is a database containing gene function and cell metabolism and signaling pathway information. Unigenes were matched with four major protein databases (NR, Swissprot, KEGG, and COG) through BLASTx (evalue, <0.00001). Then, the unigenes were compared to the nucleic acid database NT (evalue, <0.00001). When the comparison between different databases was inconsistent, according to the priority of five databases (NR, Swissprot, KEGG, and COG), the

sequence direction of a Unigene was determined. Gene function and metabolic pathway classifications were analyzed by the sequence similarity method, and the unigenes were compared to the GO gene function classification database and KEGG metabolic pathway database to obtain the functional classification and the prediction of metabolic pathways.

Digital gene expression library preparation and analysis. The number of reads for RNA sequencing of different samples differed. If the number of matched reads is considered at the gene expression level only, there is confusion when comparing the expression levels of certain genes among different samples. To resolve this problem, the reads were standardized by matching the genes (Mortazavi et al. 2008) using the reads per kb per million reads (RPKM) value to represent the transcriptional expression level of the gene. The RPKM method successfully eliminates the effect of different gene lengths and sequence differences on the calculation of gene expression levels. Thus, the RPKM can be directly used to compare the differences in gene expression levels between the TTs and the CK. In order to identify the DEGs between TT and CK, the threshold of *P* value was ascertained by the false discovery rate (FDR) method. Generally, when the threshold FDR was $\geq$0.8 and | log2ratio | was $\geq$1, we think that there are significant differences in gene expression. Then, GO enrichment analysis and KEGG pathway enrichment analysis were used to further annotate genes expressed across different stress levels.

Real-time fluorescent quantitative PCR and data analysis. Real-time fluorescent quantitative PCR (qPCR) was conducted with a SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (Takara, Japan) kit in an ABI 7500 instrument (Applied Biological System). Software primer premier 5.0 was used to design gene-specific primers based on gene sequence template (Table 1). The amplification of cDNA by qPCR was performed in a 20-μL mixture that contained approximately 1μL of cDNA, 10 μL of SYBR Premix Ex Taq, 0.4 μL of Rox reference dye, 0.4 μL of both sense primer (10 μM) and antisense primer (10 μM), and 7.8 μL of double-distilled water. Actin was used as an internal standard (0.4 μL for each). The following qPCR procedure was used: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 34 s; and 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s for plate reading. At the end of each operation, a solution chain curve was produced for each sample to evaluate the purity of the amplified product. All experiments were independently conducted three times. The relative expression level of *M. troglodyta* mRNA was calculated by using the $2^{-\Delta\Delta CT}$ method (Giulietti et al. 2001).

Results

***M. troglodyta* transcriptome assembly.** The detoxification of *M. troglodyta* larvae under tannin stress was studied by RNA-Seq. Reads were assembled using Trinity. After data filtering, 51,797,038–54,991,822 reads for the TT library and 51,674,478–52,307,172 reads for the CK library were acquired. Trinity software was used to further assemble these clean reads into 52,246–55,203 contigs with a mean length of 363–421 bp in the TT, and 46,918–64,401 contigs with an average length of 349–400 bp in the CK were obtained. In our study, 33,008–36,353 unigenes were obtained with a mean length of 574–674 bp for the TT and 29,948–42,194 unigenes

Table 1. Primers used in real-time RT-PCR.

Gene Number	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
actin	GCGGCGCGACTCACCGACTAC	GGGAAGAGAGCCTCAGGGCAAC
CL2610.Contig1_All	CCTTGAACTGTTTAGTGCCGG	AACTATCGAACAACCGCAACC
CL3460.Contig3_All	TCGAGAAGCGAGTCATAATTAGG	GGAGATGTCGAAAGATCCTGCA
CL3584.Contig2_All	GACTTTTGGGCCAACATCAG	CAAATCTGGGCAAGAAGAACAC
CL6276.Contig2_All	GTTCACTACCCTGGAAGCATACC	CAGCCAACTTGCCACTCG
CL818.Contig2_All	CTGACCTGTCCCTTGGATGTAC	GAGGATTTCCACGGCGTTC
CL818.Contig3_All	GCATCTTCTTCATCAGGCTCA	AGGTACGCTTCCAGGGTAGTGA
CL818.Contig4_All	CAGTAGCTGACCTGTCCCTTGG	CCAGCCATTTTGTTGATCGTG
Unigene13733_All	CCGTTTTCGCCACATTTTACTC	CTCCTGGTCCGATTTGAAACA
CL1404.Contig3_All	ATTACTTCAGCGGCAGGGAG	GCGTGCAGTCTTGGATTAGG
CL1922.Contig1_All	ATCTACAGGATGACAACGCCAAG	TTCCTCAACGCTCCCCAA
CL1922.Contig2_All	GGAAGTGGTGCTAAGACGGA	ACTTGGCGTTGTCATCCTGT
CL1922.Contig3_All	ATCTACAGGATGACAACGCCA	TTCCTCAACGCTCCCCAA
CL3242.Contig1_All	TTGCTCGAAACCGCTATGG	TTGAACAGCATTTCGGGTCT
CL336.Contig1_All	TTACGATGGAAACTGATGCG	GGAAGGCTTTGTCCTAAACTCT
Unigene8938_All	TGGCTTCAAATGAGACACGA	GTTTGTTGCCGTCGATGTAGT
CL441.Contig5_All	GGATGGTTCTCCCCTTATTTG	CGAGCTTGGATTGATCTTATGC
CL4566.Contig1_All	CAACAAAACACTTTCCGACACC	GCCAACCTCTTCTTTTCTCCAA
CL5132.Contig1_All	GAGATTGTATCCACCAGTCCCG	GCCGCTGACTTTGGAATAGG

with a mean length of 526–638 bp for the CK. There were 46,518 unigenes with a mean length of about 906 bp. They were assembled from clean reads of N_{50} with a length of 1,568 bp (Tables 2 and 3). After assembly, 23,792 unigenes were annotated in 5 public databases (Table 4). All 21,236 unigenes were grouped with the NR database, 15,655 unigenes were matched with the Swissprot database, 14,032 unigenes were matched with the KEGG database, 7,678 unigenes were matched with the COG database, and 9,893 unigenes were matched with the GO database (Table 4).

Functional annotation of the *M. troglodyta* transcriptome. The distribution of the evalues of the identified *M. troglodyta* unigenes revealed that 30.4% of the unigenes shared the greatest homology with an evalue cut-off of $<$1e−100 (Fig. 1a). In addition, the semblance distribution of the distinguished unigenes indicated that the semblance of more than 63.0 % of unigenes to their closest homologous genes was greater than 60.0 % (Fig. 1b). Certainly, the greatest number of unigene matches were for the insect genome, and *Danaus plexippus* (L.) (62.7 %), *Bombyx mori* (L.) (9.3 %), *Papilio xuthus* (L.) (3.5 %), *Tribolium castaneum* (Herbst) (2.9 %), and *Helicoverpa armigera* (Hübner) (1.5 %) accounted for the top 5 unigenes based on the NR annotations. The rest (20.1 %) of the sequences showed good homology with those of other insects (Fig. 1c).

The GO database was commonly used for gene functional annotation (Ashburner et al. 2000). Blast2GO software was used for gene annotation and matched the transcriptome of *M. troglodyta* to 3 major functional processes, including 59 GO terms. In other words, according to the GO gene functional classification system, 9,893 unigenes were divided into the 3 main functional ontologies—biological process, molecular function, and cellular component (CC) (Fig. 2). In view of the GO analysis, approximately 54.42% of genes were in the biological processes category, and the rest of the genes were in cellular processes (29.06 %) and molecular processes (16.51 %). In biological processes, the main subcategories were cellular process (6,126) and metabolic process (5,005) and next was the single-organism process (4,619). For the cellular component category, cell parts (4,609), cells (4,610), and organelles (3,338) were the most frequently represented. In terms of molecular function, binding (4,954) and catalytic activity (4,928) were highest. Nevertheless, in these three main categories, few genes were allocated to virion, protein tag, and receptor regulator activity.

In total, 7,678 sequences were subjected to COG classifications; they were divided into 25 COG groups using WebMGA, with an evalue cut-off of $1e^{-5}$. In the 25 COG classifications, the greatest group was general function prediction (2,899), followed by replication, recombination and repair (1,314) and translation, and ribosomal structure and biogenesis (1,296). This may be related to the fact that there is still currently little data on *M. troglodyta* in the COG database. In all, 1,089 unigenes with unknown function were obtained by sequencing and are presumed to be new genes specific to *M. troglodyta* (Fig. 3).

DEGs of *M. troglodyta* in response to tannin stress. To explore the detoxification mechanism to tannin stress in *M. troglodyta*, detoxification response genes that were up- or downregulated in larvae under tannin stress were identified by using Illumina HiSeq 2000 sequencing. For the purpose of maximizing the accuracy of the measurement of expression levels, merged data from three replicates, including RPKM values, were computed and the results between the

Table 2. Statistical output of transcriptome sequencing in *M. troglodyta*.

Samples	Total Raw Reads	Total Clean Reads	Total Clean Nucleotides	Q20 Percentage	N Percentage	GC Percentage
CK1	55,468,690	52,307,172	4,707,645,480	97.93	0.0	47.34
CK2	56,179,062	51,674,478	4,650,703,020	97.57	0.00	47.44
CK3	55,275,274	52,274,752	4,704,727,680	97.98	0.00	46.55
TT1	56,003,020	52,131,146	4,691,803,140	97.77	0.00	46.73
TT2	55,530,018	51,797,038	4,661,733,420	97.82	0.00	47.72
TT3	59,682,090	54,991,822	4,949,263,980	97.59	0.00	48.16

Table 3. Assembly quality statistics for transcriptome sequencing in *M. troglodyta.*

Sample	Total Number	Total Length (nt)	Mean Length (nt)	N50 (bp)	Total Consensus Sequences	Distinct Clusters	Distinct Singletons
Contig							
CK1	48,496	18,236,888	376	670			
CK2	64,401	25,746,928	400	763			
CK3	46,918	16,367,413	349	578			
TT1	55,203	23,236,738	421	866			
TT2	53,653	20,097,612	375	680			
TT3	52,246	18,969,648	363	644			
Unigene							
CK1	31,536	18,453,011	585	997	31,536	3,189	28,347
CK2	42,194	26,939,120	638	1,156	42,194	4,735	37,459
CK3	29,948	15,741,797	526	823	29,948	2,994	26,954
TT1	36,353	24,495,447	674	1,270	36,353	4,513	31,840
TT2	34,294	20,290,864	592	1,025	34,294	3,592	30,702
TT3	33,008	18,943,896	574	981	33,008	3,471	29,537
All	46,518	42,137,143	906	1,568	46,518	15,221	31,297

nt, nucleotide.

Table 4. Annotation result statistics for transcriptome sequencing in *M. troglodyta*.

Sequence File	NR	NT	Swissprot	KEGG	COG	GO	ALL
All-Unigene.fa	21,236	15,107	15,655	14,032	7,678	9,893	23,792

replicates for the TT and CK groups were compared (Fig. 4). When FDR was $\geq$0.8 and |log2Ratio| was $\geq$1, the difference between TT and CK was considered significant. Among the 23,792 unigenes, a total of 1,627 DEGs were ascertained (Fig. 5). We found that 885 of these genes were upregulated and the other 742 were downregulated.

Real-time fluorescent quantitative PCR analysis. In order to further estimate the DEGs identified from the transcriptome library, some DEGs were selected and quantified by qPCR under tannin stress. The results illustrated that the expression

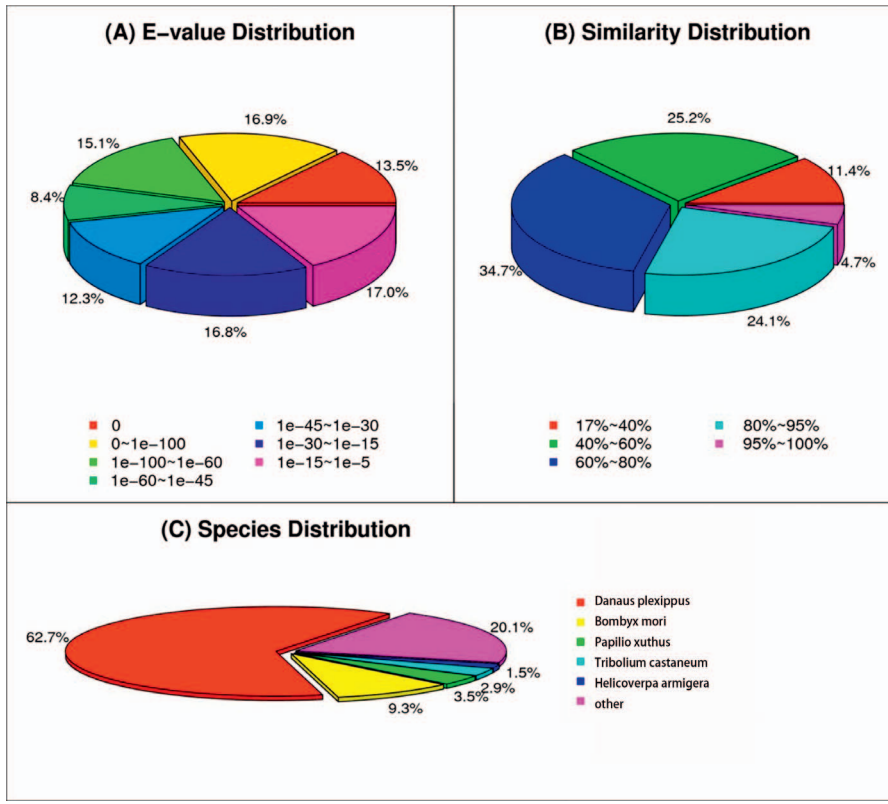

Fig. 1. NR classification of all *M. troglodyta* unigenes. (A) The evalue distribution from NR annotations; (B) NR annotation similarity distribution; (C) NR annotated species distribution.

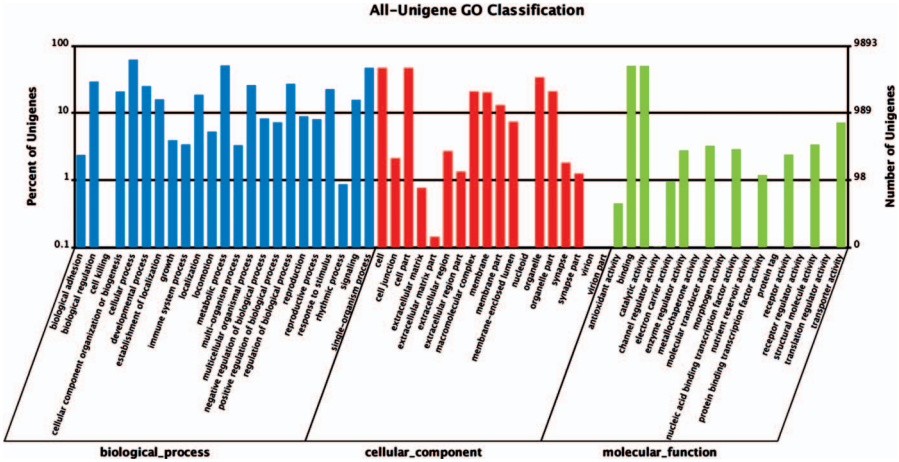

Fig. 2. GO function classification of all unigenes in *M. troglodyta*.

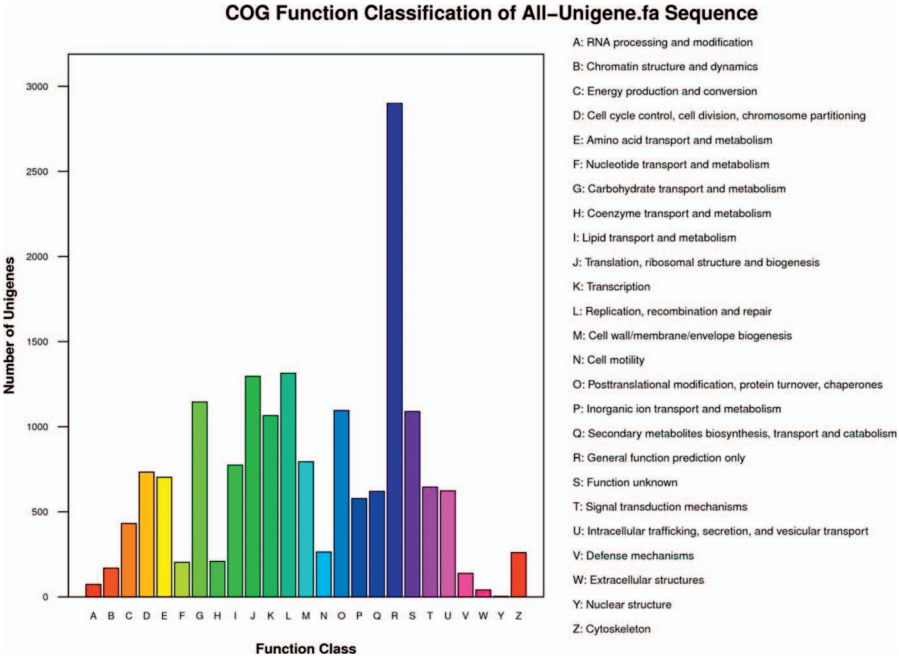

Fig. 3. COG annotations of putative proteins. All putative proteins were aligned to the COG database and can be classified into at least 25 molecular families.

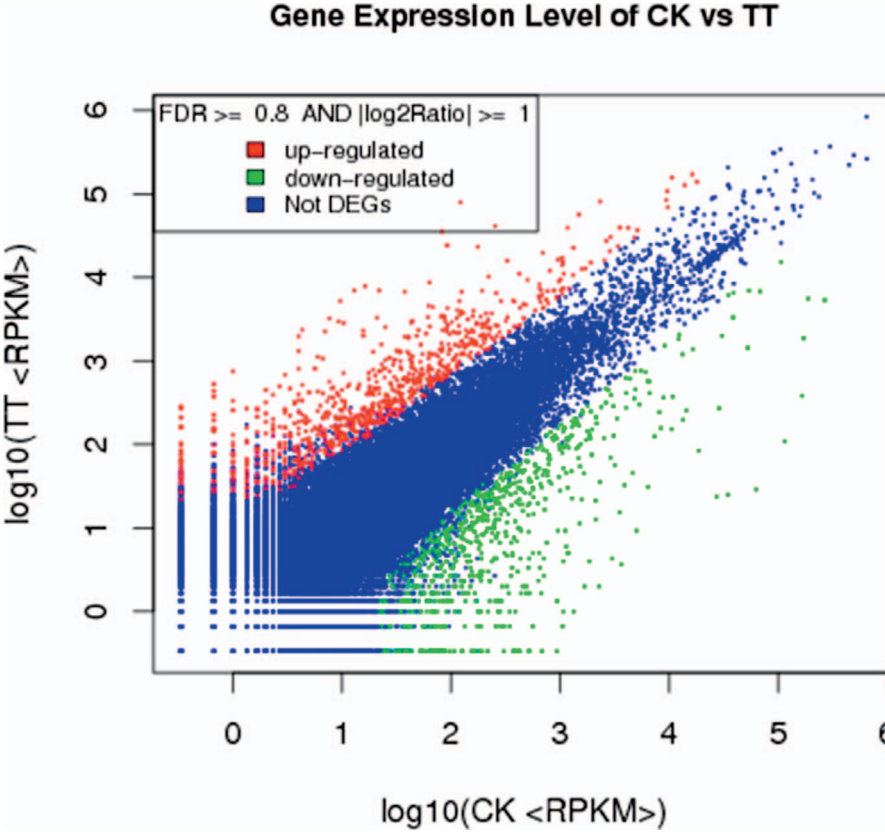

Fig. 4. Comparison of gene expression levels between the CK library and TT library. For comparing gene expression levels between the two libraries, each library was normalized to 1 million tags. The red dots represent transcripts that were more prevalent in the TT library. The green dots represent the transcripts present at a lower frequency in the infected tissue, and the blue dots indicate transcripts that did not change significantly. The parameters " FDR $\geq$ 0.8" and "log2 Ratio $\geq$ 1" were used as the thresholds with which to judge the significance of differences in gene expression.

level of the selected unigenes was the same as that obtained in *M. troglodyta* transcriptome data (Fig. 6). The expression of all 18 genes was consistent with the RNA sequence data. It has been reported that actin, which is stably expressed in insects, is an appropriate reference gene for data standardization. In general, the results of qPCR were consistent with the transcriptome data, and the changes detected in mRNA sequencing were confirmed to be true.

Differentially expressed detoxification-related genes. Detoxification-related DEGs were determined from the transcriptome library. In our study, the results revealed that tannin stress induced the expression of several different genes

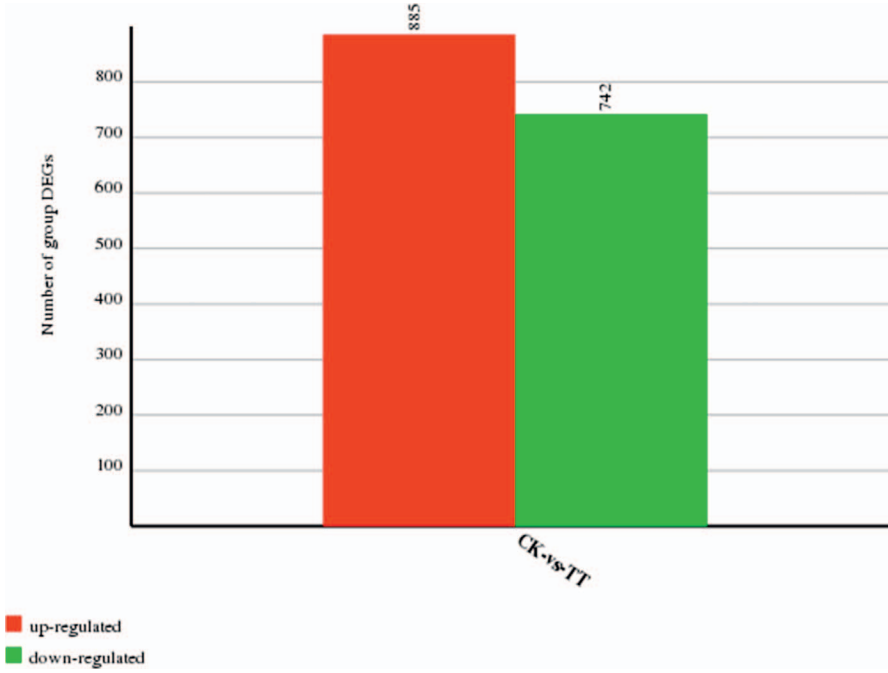

Fig. 5. Effect of TT on unigenes in *M. troglodyta*. The horizontal axis represents the CK, and the number of differentially expressed genes are shown on the vertical axis.

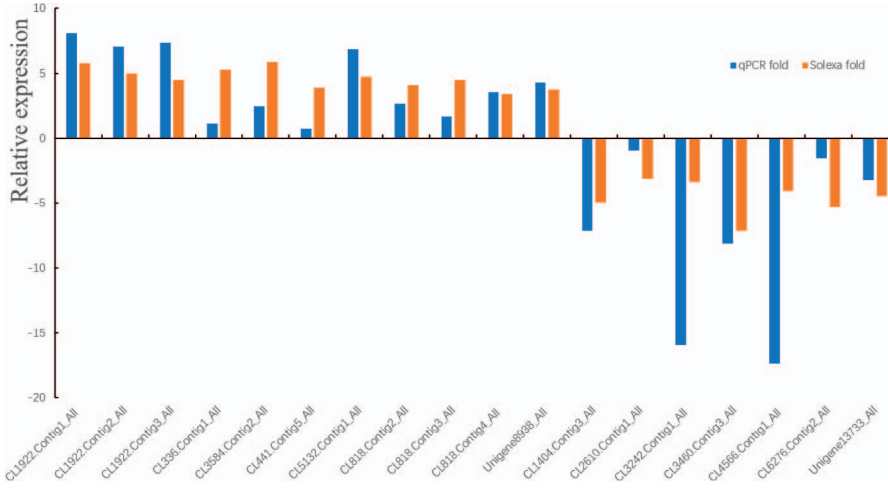

Fig. 6. qPCR validation of 18 selected DEGs.

involved in detoxification, such as glutathione S-transferase (GSTs), cytochrome P450s (CYP), and uridine diphosphate-glycosyl transferases (UGTs). UGTs are one of the most essential enzymes in phase II reactions, and CYPs are one of the main enzymes in phase I reactions. They also play very critical roles in the decomposition of endobiotics and xenobiotics (Feyereisen 2012, Sun et al. 2019). Our results indicated that TT resulted in the downregulation of 4 CYPs and the upregulation of 26 CYPs in the larvae of *M. troglodyta*. One UGT was downregulated by 3.83-fold in treated larvae, while three UGTs were upregulated by 3.14- to 4.5-fold under tannin stress. GSTs played a significant role in the insect detoxification process. Interestingly, DEG analysis indicated a series of GST genes that demonstrated different levels of induction or inhibition according to tannin stress. For example, the expression of five GSTs was downregulated in the TT library, whereas five of these genes were upregulated in the TT library (Table 5).

Discussion

Tannin, also known as gallic acid or tannic acid, widely exists in the roots, stems, leaves, fruits, and bark of plants. Tannin is a polyphenolic compound with a relative molecular weight of 500–3,000 u. According to the solubility of tannin, it can be divided into soluble tannin and insoluble tannin. Soluble tannin is considered the main substance causing astringency, and it combines with human oral mucosal proteins to produce strong astringency. Plant tannins are a type of compound that has been studied previously and frequently in natural products. Many studies have shown that phenolic hydroxyl groups in tannins can interact with enzymes in pathogens and, thus, produce toxicity to the gastrointestinal microorganisms of animals (Goel et al, 2005). In our study, we recognized many kinds of DEGs and signaling pathways involved in the *M. troglodyta* response to TT.

The theoretical basis of RNA-Seq is as follows: all RNA in a specific cell or tissue is isolated, a cDNA library is constructed, and the cDNA library sequences are randomly fragmented into small fragments. Alternatively, RNA fragmentation can be followed by reverse transcription. Sequencing is performed using a new generation of high-throughput sequencing. The reads are compared to the database (reference genome) or *de novo* assembled (no reference genome). Finally, a genome-wide transcriptome is formed, and the gene functional annotation, expression annotation, and participating metabolic pathways are analyzed (Morozova et al. 2009). Qin et al. (2011) used the HiSeq 2000 platform for deeply sequencing normal and regenerated tissue in *Dugesia japonica* Ichikawa & Kawakatsu and then established digital gene expression profiles and transcriptional maps, which provided a broad and deep molecular biology background for the development of this model organism, especially the exploration of genes involved in *D. japonica* regeneration (Qin et al. 2011). At present, the sequence data of *M. troglodyta* have not been reported domestically or internationally, but obtaining more information on the *M. troglodyta* sequence presents a better way to research gene functions in this species. This study used Illumina HiSeq 2000 sequencing technology and annotated the reference transcriptional database of *M. troglodyta*. Then, 28,365,876,720 bp of data were obtained, marking the first time that the RNA-Seq technology has been used to study and obtain the complete transcriptional

Table 5. Detoxification-related genes associated with tannin stress in *M. troglodyta*.

Gene	Gene ID	Gene Name	Fold Change (TT/CK)
Cytochrome P450	Unigene12568_All	Antennal cytochrome P450 CYP9 [*Mamestra brassicae*]	3.4136082
	CL1404.Contig3_All	Cytochrome P450 6AE32 [*Manduca sexta*]	−4.923214
	CL1922.Contig1_All	Cytochrome P450 6B45 [*Manduca sexta*]	5.7623847
	CL1922.Contig2_All	Cytochrome P450 6B45 [*Manduca sexta*]	4.9864118
	CL1922.Contig3_All	Cytochrome P450 6B45 [*Manduca sexta*]	4.4617819
	CL2145.Contig1_All	Cytochrome P450, partial [*Bombyx mori*]	3.6618083
	CL2145.Contig2_All	Cytochrome P450 CYP9A1v2 [*Heliothis virescens*]	3.2198201
	CL2189.Contig1_All	Cytochrome P450 [*Spodoptera litura*]	3.2231876
	CL2189.Contig2_All	Cytochrome P450 [*Spodoptera litura*]	2.8490278
	CL2189.Contig5_All	Cytochrome P450 [*Spodoptera litura*]	2.7652443
	CL2483.Contig1_All	Cytochrome P450 monooxygenase CYP6ab [*Mamestra brassicae*]	3.3981984
	CL289.Contig1_All	Cytochrome CYP333B3 [*Spodoptera littoralis*]	8.2400626
	CL3242.Contig1_All	Cytochrome CYP4G75 [*Spodoptera littoralis*]	−3.419367
	CL3242.Contig2_All	Cytochrome P450 4G49 [*Manduca sexta*]	3.3844023
	CL3273.Contig4_All	Cytochrome P450 [*Spodoptera litura*]	4.3025557

Table 5. Continued.

Gene	Gene ID	Gene Name	Fold Change (TT/CK)
	CL336.Contig1_All	Cytochrome p450 CYP337B1 [*Helicoverpa armigera*]	5.2573214
	CL441.Contig3_All	Antennal cytochrome P450 CYP9 [*Mamestra brassicae*]	3.4499757
	CL441.Contig4_All	Antennal cytochrome P450 CYP9 [*Mamestra brassicae*]	4.8108849
	CL441.Contig5_All	Antennal cytochrome P450 CYP9 [*Mamestra brassicae*]	3.8656539
	CL441.Contig6_All	Antennal cytochrome P450 CYP9 [*Mamestra brassicae*]	3.3702144
	CL4566.Contig1_All	Cytochrome P450 4G49 [*Manduca sexta*]	−4.064462
	CL5132.Contig1_All	Cytochrome P450 CYP4 L4 [*Mamestra brassicae*]	4.742774
	CL882.Contig1_All	Cytochrome CYP324A1 [*Spodoptera littoralis*]	4.9910473
	Unigene17603_All	Cytochrome 9A20 [*Bombyx mori*]	3.9547248
	Unigene18186_All	Cytochrome P450 [*Helicoverpa armigera*]	2.9317792
	Unigene27074_All	Cytochrome P-450 [*Heliothis virescens*]	3.7396736
	Unigene30060_All	Cytochrome P450 [*Bombyx mori*]	3.7074318
	Unigene3886_All	Putative cytochrome P450 [*Danaus plexippus*]	−3.661727
	Unigene6762_All	Cytochrome P450 332A4 [*Manduca sexta*]	3.0956518
	Unigene8938_All	Cytochrome P450 CYP332A1 [*Bombyx mori*]	3.7144864

Table 5. Continued.

Gene	Gene ID	Gene Name	Fold Change (TT/CK)
Glutathione	Unigene13733_All	Glutathione S-transferase [*Danaus plexippus*]	−4.467413
S-transferase	CL3584.Contig2_All	Glutathione S-transferase [*Choristoneura fumiferana*]	5.8764524
	CL818.Contig3_All	Glutathione S-transferase epsilon 2 [*Spodoptera litura*]	4.4431222
	CL6276.Contig3_All	Glutathione S-transferase epsilon 2 [*Spodoptera litura*]	4.3831039
	CL6276.Contig2_All	glutathione S-transferase epsilon 2 [*Spodoptera litura*]	−5.340891
	CL3460.Contig3_All	glutathione S-transferase omega 2 [*Bombyx mori*]	−7.149118
	CL2610.Contig1_All	glutathione S-transferase epsilon 2 [*Bombyx mori*]	−3.157138
	CL818.Contig2_All	glutathione S-transferase epsilon 2 [*Spodoptera litura*]	4.0839171
	Unigene1069_All	glutathione S-transferase sigma 1 [*Bombyx mori*],	−4.234424
	CL818.Contig4_All	glutathione S-transferase epsilon 2 [*Spodoptera litura*]	3.3943055
Uridine diphosphate	CL3392.Contig2_All	UDP-glycosyltransferase UGT33F2 [*Helicoverpa armigera*]	−3.832574
-glycosyltrans-ferases	Unigene9109_All	UDP-glycosyltransferase UGT40Q1 [*Helicoverpa armigera*]	3.4982524
	CL501.Contig2_All	UDP-glycosyltransferase UGT33F2 [*Helicoverpa armigera*]	3.1453869
	CL1560.Contig1_All	UDP-glycosyltransferase UGT40F2 [*Helicoverpa armigera*]	4.5468818

information of *M. troglodyta*. The results of this experiment provide extensive sequence resources for *M. troglodyta*. Thus, RNA-Seq was used to identify DEGs. In addition, RNA-Seq was used to lay the foundation for further in-depth studies and to systematically and comprehensively define mechanisms of action or resistance.

GST/glutathione metabolism associated with tannin stress. During evolution, plants produce secondary metabolites to protect themselves from phytophagous insects or interfere with their growth and development. Tannins are a large group of polyphenolic compounds and are a natural protective substance for plant survival. To adapt to their ecological environment, insects decompose toxic substances into nontoxic substances mainly by detoxifying enzymes (GSTs, CYP, and esterase), or the toxic substances are used by insects or excreted from their bodies.

Wang et al. (2004) used microarray and genomic techniques to study the Malpighian tubule of *D. melanogaster*. The researchers found that, in addition to osmotic regulation, the Malpighian tubule also functions in transferring tissue solutes (Wang et al. 2004). Therefore, the Malpighian tubule can excrete a wide range of tissue lysates and xenobiotic biological metabolites. The Malpighian tubule enhances its excretion mainly by expressing CYP enzymes, GSTs, and alcohol dehydrogenase in large amounts (Dow and Davies 2006). These three enzymes play a significant role in the metabolism and detoxification of endogenous lysates and xenobiotic organisms.

GSTs comprise an important metabolic enzyme system in organisms that participates in the primary and secondary metabolism of exogenous substances. GSTs catalyze the nucleophilic reaction of endogenous glutathione with a substrate by the conjugation of glutathione. GSTs mainly transfer a group of electrophilic substrates to the sulfur atom of endogenous reduced glutathione, which makes electrophilic substances hydrophilic and easy to excrete and detoxify (Enayati et al. 2005). The GST gene plays a significant role in the insect detoxification process. It also plays a major role in protecting insects from the reactive chemicals formed by the decomposition of endogenous compounds and the biotransformation of foreign compounds. (Maher 2005, Rinaldi et al. 2002). Furthermore, the GST gene plays an important role in the storage and transport of reduced sulfur, the synthesis of proteins and nucleic acids, the regulation of enzyme activity, the maintenance of the antioxidant properties of tissues, and the regulation of redox-sensitive signal transduction (Yan et al. 2014).

GSTs are widely distributed within living organisms. In mammals, GSTs are mainly distributed in liver microsomal cells, and GSTs are also active in serum. In insects, high levels of GSTs are found in the fat body, digestive tract, and Malpighian tubule. Studies have shown that GSTs are one of the most important enzymes for insects to metabolize insecticides, and they play an important role in resistance to some pesticides, especially organophosphate and carbamate pesticides. In addition, insect GSTs play an important role in insect resistance to plant secondary substances and other exogenous toxic substances (Chen and Gao 2005). Li et al. (2010) found that there were 3 specific upregulated expression patterns in 11 GST genes, of which 2 were upregulated in the genomic sigma family, and the expression folds (2.83- and 4.30-fold) were higher than those in the delta family (2.06) when alachlor was used to treat mosquitoes. These results suggest that sigma family GSTs may play a major role in the metabolism of alachlor,

which means that the degradation metabolism of alachlor in the mosquitoes mainly uses GSTs of the sigma family (Li et al. 2010). Chen et al. (2005) found that the activity of GSTs in *Plutella xylostella* (L.) treated with a low concentration of total alkaloids of *Tripterygium wilfordii* Hook. f. (LC_{10}) was significantly increased in 1st and 2nd instar larvae, reaching 2.83 times that of the CK. These results indicate that *T. wilfordii* alkaloids can significantly reduce the enzyme activity of GSTs at low concentrations (Chen et al. 2005). Gao et al. (1999) found that the activity of GSTs increased 4–8 times after feeding *H. armigera* with artificial diets containing 0.01% rutin, 2-tridecanone, and quercetin. After induction of the 2nd generation of *H. armigera* with 0.01% quercetin, it was found that the GST activity of the induced population was increased by nearly 15 times, and the activity of carboxylesterase was increased by 2 to 3 times. These results indicate that quercetin can induce overexpression of GSTs in *H. armigera*, suggesting that GSTs are one of the most important detoxification enzymes in *H. armigera* (Gao et al. 1999). These studies demonstrate that individual or multiple GST genes show significant changes under stress from insecticides or plant secondary metabolites.

We used transcriptomes to analyze the upregulation or downregulation of all GST genes in *M. troglodyta*. We selected 11 DEGs for GSTs from the transcriptome. For example, five GST genes were downregulated in the TT library, and five of these genes were upregulated in the TT library. We also found that 11 DEGs were involved in the glutathione metabolism pathway. GST is an important enzyme in glutathione metabolism. In addition to functioning in intracellular toxic substance binding, transport efflux, and glutathione reductase, GST also protects cells from oxidative damage. This implies that *M. troglodyta* may reply to tannin stress by increasing the expression of GSTs, which may illustrate the detected activation of GSTs under tannin stress.

Drug metabolism CYP associated with tannin stress. The CYP protein binds to CO catalyzed by Fe^{2+} and has a characteristic absorption peak at 450 nm, so it is named the CYP enzyme system. CYP enzymes are 46-to 60-kDa proteins with similar structures and different properties. CYP is an important oxidase system located in the smooth endoplasmic reticulum. This enzyme can synthesize and degrade insect pheromones and hormones and degrade host secondary metabolic toxins and insecticides in insects (Berenbaum 2002, Guo et al. 1991, Sandstrom et al. 2006, Scott et al. 1998). CYP also has oxidase, reductase, isomerase, and dehydrogenase activities (Mansuy 1998).

In recent years, studies have found that CYP enzymes play an important role in the metabolism of many endogenous substances in insects. CYP has functions related to the metabolism of juvenile hormones, juvenile hormone analogues, and antijuvenile hormone substances in some insects. Moreover, many studies have shown that insect CYP is closely related to ecdysterol, fatty acid metabolism, and the synthesis of hydrocarbons in insects. Because CYP has functions in detoxification in most organisms, the CYP enzyme system is often classified as a detoxifying enzyme system.

CYP was first discovered in mammalian liver microsomes. This enzyme has been found in many eukaryotes (such as animals, plants, and fungi) and many prokaryotes (such as bacteria). CYP not only exists in different organisms, but various CYPs have also been identified in different tissues of the same species (Omura 1999). For example, CYP is abundant in the insect midgut, fat body,

Malpighian tubule, and other organs or tissues, even including the head. Studies have shown that the sixth instar larvae of *H. armigera* have the highest CYP content in the midgut, followed by the body fat and the body wall (Qiu and Leng 1999). Insects rapidly metabolize ingested toxic substances (including plant secondary metabolites) through a detoxifying enzyme system concentrated in the midgut. The fat body is beneficial for the metabolism of toxic substances that enter the body through the epidermis or trachea (Yu et al. 2002). CYP LPR is encoded by CYP6A1 and CYP6D1 in houseflies and could be induced by phenobarbital and piperonyl butoxide but not by naphthalene, cyclopentadienes (such as dieldrin or aldrin), or beta-naphthoflavine, whereas ethanol could only induce CYP6A1 (Scott et al. 1996). Zhang et al. (2009) found that when gossypol and dimboa were added to food, the expression of CYP in *Pyrausta nubilalis* (Hübner) was positively correlated with the metabolism of these plant secondary metabolites (Zhang et al. 2009). Willoughby et al. (2006) used microarray technology to demonstrate that overexpression of the CYP gene plays an important role in insecticide-resistant *Drosophila* (Willoughby et al. 2006). Karunker et al. (2008) found that the resistance of B and Q biotypes of *Bemisia tabaci* (Gennadius) to imidacloprid was caused by overexpression of CYP6CM (Karunker et al. 2008). Bautista et al. (2009) found that the CYP gene CYP6BG1 was overexpressed in the fourth instar larvae of the permethrin-resistant strain of *P. xylostella*, and it was confirmed by gene silencing that overexpression of CYP6BG1 enhanced the metabolism of permethrin, resulting in resistance to this compound (Bautista et al. 2009). Because the role of CYP in the metabolism of endogenous compounds has been explained, the role of CYP in the metabolism of exogenous compounds has also been found. CYP drug metabolism mainly occurs in the liver and small intestine (Guengerich 2003). CYP 3A4 and CYP 2C9 had the highest expression in these tissues. Therefore, these enzymes were very common in drug metabolism (Guengerich 2003, Cao et al. 2013). It has been confirmed that CYP6AE14w was highly expressed in the midgut of *H. armigera* and was specifically induced by gossypol. The expression level of this gene was positively correlated with the growth of *H. armigera* in the presence of gossypol in food (Mao et al. 2007). CYP is involved in the metabolism of most pharmaceutical compounds (Cao et al. 2013).

In our study, 189 genes were identified in the drug metabolism-CYP pathway by transcriptome analysis, of which 29 were DEGs. Then, we found 30 DEGs for CYP from the transcriptome. The expression of these genes changed significantly under tannin stress, indicating that the CYP gene can be induced in *M. troglodyta*. Many studies have shown that insect resistance associated with CYP is usually characterized by overexpression of CYP, which is a common resistance mechanism (Feyereisen 1999). These results suggest that the drug metabolism pathway in *M. troglodyta* may mediate tannin-induced stress responses. Nevertheless, further studies are needed to determine the potential regulatory role of the drug metabolism pathway in *M. troglodyta* under tannin stress.

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