EFFECTS OF CHLORBENZURON ON CUTICLE FORMATION OF *HYPHANTRIA CUNEA* (LEPIDOPTERA: EREBIDAE) AND ITS DETOXIFICATION METABOLISM

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Abstract. Despite the major role of benzoylureas (BPUs) in the control of pests in agricultural and forestry, their molecular mechanisms have in most cases remained elusive. BPUs cause molting impairments and were thought to interfere with chitin biosynthesis. Here, chlorbenzuron-treated *Hyphantria cunea* was subjected to extensive transcriptomic and proteomic analyses to investigate the toxicological mechanisms by which BPUs induce abortive molting and decrease chitin in insects. Following chlorbenzuron treatment, the transcriptomic analysis yielded 1973 differentially expressed genes (DEGs), while the proteomic analysis yielded 714 differentially expressed proteins (DEPs). The DEGs and DEPs were functionally annotated using Gene Ontology (GO) and analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Comprehensive transcript and protein analyses revealed that DEGs and DEPs were co-regulated in insect cuticle biosynthesis and detoxification metabolism. Of note, these genes and proteins, as well as their associated dysregulated pathways, could be promising targets for research into the precise action site of BPUs. This research provides large-scale omics data that can be used to better understand the overall mechanism of action of BPUs and the cause of death in insects.

Keywords: *benzoylphenyl urea, Hyphantria cunea, chlorbenzuron, insect cuticle, transcriptomic analysis, proteomic analysis*

Introduction

Given their strong growth-regulating effects in insects, benzoylphenyl ureas (BPUs) are considered to be promising insecticides (Hajjar and Casida, 1978; Sechser and Reber, 1998). Since the synthesis of the first benzoylurea pesticide diflubenzuron (DFB), several studies have investigated the mechanism of BPUs (Sun et al., 2015). As insect growth regulators (IGRs), BPUs primarily act on the egg stage and larval stage, to particularly control the development of young Lepidoptera larvae (Bouvier et al., 2002; Matsumura, 2010; Doucet and Retnakaran, 2012). Past studies have demonstrated that BPUs, as a type of Chitin synthesis inhibitor (CSI), block chitin biosynthesis in insects causing molting failure, and this may result in developmental malformation and death of larvae (Mommaerts et al., 2006; Merzendorfer, 2013; Liu et al., 2019). However, the exact action site of BPUs is currently not well understood.

The cuticle of insects performs the same function as skin and bone in vertebrates, and the main chemical components of the epicuticle and exocuticle are chitin and proteins (Merzendorfer, 2013; Wang et al., 2020). Insect cuticular proteins (ICP) and

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polysaccharide chitin are secreted in turns and interact with each other to form the orderly arrangement of the cuticular structure (Gillott, 2005; Moussian, 2010). The BPUs damaged the insect cuticle by inhibiting chitin synthesis, which affected the molting and metamorphosis of insects (Merzendorfer, 2013). BPUs can prevent the incorporation of N-acetylglucosamine into chitin by blocking the catalysis of chitin synthase (CHS), which plays an important role in the final step of chitin biosynthesis in insects (Fotakis et al., 2020; Xin et al., 2021). Sulfonylurea receptor (SUR), a member of the ABC-transporter family, is known to be a target of BPUs which can depolarize the cuticular vesicle in insect by inhibiting the K^+ channel and interfering with chitin biosynthesis (Abo-Elghar et al., 2004; Matsumura, 2010). It has been reported that BPUs not only affect the level of chitin, but also the expression of cuticular proteins in insects (Emam and Degheele, 1993; Tail et al., 2015). However, the effects of BPUs on insect cuticular proteins at the molecular level have not been sufficiently studied. Given the complicated regulation of insect cuticle formation, the mechanism of action of BPUs needs to be further explored.

In insects, detoxification metabolism is driven by multiple enzymes and transport carriers which regulate catalysis, hydrolysis and excrete toxins (Castañeda et al., 2009). Several genes associated with detoxification enzymes are expressed, and this is accompanied by enzymatic modifications and degradation to protect insects from pesticides and toxins (Castañeda et al., 2009). Although BPUs are utilized as specific pesticides to regulate insect growth, like many conventional pesticides, the use of BPUs may increase the activity of detoxifying enzymes in insects, which may contribute to metabolism-based resistance against insecticides (Homem and Davies, 2018; Chen et al., 2020). However, the genes and proteins involved the metabolism of BPUs in insects are poorly understood.

Further research is advocated to investigate BPUs' mode of action in insect growth and development. Studies have demonstrated that BPUs can cause abnormalities in multiple genes and metabolic pathways in insects, including insect hormone biosynthesis (Hamilton et al., 2021), energy substance metabolism (Olsvik et al., 2019), CHS/chitinase (CHT) genes (Tetreau and Wang, 2019), and some metabolic pathways associated with P450 genes (Homem and Davies, 2018). This suggests that in addition to interfering with the cuticle, BPUs have several indirect effects on insects. The combination of all these factors may lead to death of insects. The effects of BPUs on insects need to be investigated from multiple aspects.

To better understand the effect of BPUs on insect cuticle and its potential mechanism of action, we performed transcriptome and proteome analyses in *Hyphantria cunea* larvae, a destructive and highly polyphagous forest pest, following treatment with chlorbenzuron (2-chloro-*N*-[(4-chlorophenyl)carbamoyl]benzamide), a type of BPUs. In addition, the levels of chitin and protein in the cuticle as well as the activities of detoxification enzymes were explored. Further, qRT-PCR tests were conducted to investigate genes involved in cuticle formation, detoxification and those regulated by chlorbenzuron to cause multiple metabolic disorders in *H. cunea*. The findings of the study provide deeper insights into the overall mechanism of BPUs. Large-scale omics data were generated which can be used for further research into the structure-activity relationships (SARs) and development of BPUs.

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Materials and Methods

Insects and Chlorbenzuron Treatment

H. cunea eggs and the artificial diet were obtained from the Research Institute of Forest Ecology, Chinese Academy of Forestry (Beijing, China), and pure chlorbenzuron was obtained from the National Institute of Metrology (Beijing, China). In a beaker, 2 g AGAR and 27.5 g powder of artificial was added in100 ml water, then heated it to a boil. According to the volume of the artificial diet, the diet containing 100 ppm chlorbenzuron was configured. Until the artificial diet was coagulated into jelly at room temperature, the diet was cut into pieces. Larvae were reared in standard insect glass vials after eggs hatched, with a maximum of 50 animals per 20 g artificial diet. There were 500 and 500 newly molted 4^{th} instar larvae selected as two treatments (T, CK). The CK group was fed pure artificial diet and the T group was fed artificial diet with 100ppm chlorbenzuron for 48 h, respectively. Both treatments had been starved for 12 h. For each replicate experiment sample, only 10 surviving larvae with no noticeable signs of intoxication were randomly collected. Subsequently, the samples and the samples of cuticle were flash-frozen in liquid nitrogen and kept at −80 °C for paraffin sectioning, transcriptomic and proteomic analyses. Three biological replicates from 3 different *H. cunea* eggs were used in the study. The environmental chamber was set at 25±1°C, 60±10% relative humidity, and a photoperiod of 16 h:8 h light (L): dark (D).

Determination of Physiological Data

The samples and the samples of cuticle of *H. cunea* were selected as CK(0h), CK(48h) and T(48h). The samples of cuticle were prepared for determining the level of chitin and cuticular proteins in *H. cunea.* Then, the samples of *H. cunea* were used for determining the activity of mixed function oxidases (MFOs) and glutathione transferases (GSTs). Physiological experiments performed on Multiskan SkyHigh microplate reader (Thermo Fisher Scientific, MA, USA) were followed according to kit (Sangon, Shanghai, China) instructions for four substances (https://www.sangon.com). Data of the study was recorded and calculated by Excel 2017 and are presented as mean ± standard error (SEM). SPSS 19.0 was used for variance analysis and Dunnett's test ($P< 0.05$) compared the statistical significance in ANOVA.

Transcriptomic Analysis

cDNA Library Construction and Illumina HiSeq Sequencing

Total RNA was extracted from two groups (T, CK) using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) DNase (Promega, Madison, WI, USA) was added to remove contaminating DNA. The integrity of RNA was quantified and qualified by Agilent 2100 (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.), and 1% agarose gel electrophoresis. Total mRNA was enriched using magnetic beads with Oligo(dT) method and then fragmented into short fragments using fragmentation buffer. Next, a cDNA strand was synthesized by reverse transcription with random hexamers, followed by the addition of a buffer, dNTPs, and DNA polymerase I to synthesize a second cDNA strand. After that, AMPure XP beads were used to purify, repair, and select the segment size of double-stranded cDNA. The cDNA library was constructed by PCR amplification. The Agilent 2100 was used to determine the size of the insert fragment of the library. The effective concentration of the library

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was accurately quantified via the Q-PCR method. Illumina sequencing was performed at Allwegene, Beijing, China.

Transcriptomic Data Analysis

Raw reads (raw data) from each sample were processed by Trimmomatic (v0.33) software to generate high-quality clean data (Bolger et al., 2014). Q20, Q30, and GC content of the clean reads were calculated. All subsequent analyses relied on clean reads assembled into unigenes by Trinity (v2.6) software (Grabherr et al., 2011). The unigenes were then used as the reference sequence (ref), and the clean reads from each sample were mapped to the ref, using Expectation-Maximization (RSEM) software, which then counted the number of reads on each gene for each sample (Li and Dewey, 2011). The FPKM (fragments per kilobase of transcript per million mapped reads, FPKM) value of each gene was used to calculate gene expression levels (Trapnell et al., 2010).

Differentially Expressed Genes (DEGs) Analysis

Analysis of DEGs between the two groups (T, CK) was performed by the DESeq package (1.16.0). The Benjamini & Hochberg method was used to control the false discovery rate (FDR) by adjusting the resulting *P* values (McDermaid et al., 2019). After adjusting for FDR, genes with an adjusted *P*-value <0.05 and $|\log_2^{(\text{Fold change})}| \geq 1$ from DESeq were designated as DEGs. GO functional annotation and enrichment analysis were performed by GOseq (v1.22.0) (Young et al., 2010). KEGG pathway analysis was performed by KOBAS (v2.0) (Mao et al., 2005). The significance threshold for analysis results of the DEGs was set at *P*-value <0.05.

Proteomic analysis

Total Protein Extraction and Peptide Preparation

The tandem mass tag (TMT) technique was used to perform proteomic analysis on samples from two groups (T, CK). Total protein extraction was performed as the method described in Ross et al. (2004). The protein concentrations were determined by BCA Protein Assay Kit (Beyotime, Shanghai, China). Each sample protein was enzymolysis with the same amount, then the preparation of peptides was performed as the method described in Ross et al. (2004). To obtain the peptides, iodoacetamide (IAA) was added to achieve the final concentration of 11 mM and incubated at room temperature for 15 min in dark.

TMT Labeling of Peptides

The trypsin-treated peptides were desalted with Strata X C18 (Phenomenex) and vacuum-dried. Next, the peptides were dissolved in 0.5 M TEAB and labeled with a TMT kit (Thermo Fisher Scientific Inc.). The labeled reagent was defrosted and dissolved in acetonitrile, and then mixed with the peptide and incubated for 2 h, 25℃. The labeled peptide was vacuum-mixed, desalted, and freeze-dried.

HPLC Fractionation

The peptides were segmented by high pH reversed-phase HPLC on an Agilent 300Exextend C18 column (5 μm in size, 4.6 mm in diameter, 250 mm in length). The

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gradient of peptide fractionation was 8%-32% acetonitrile, pH 9.60, fractions were separated for 60 min, then the peptides were combined into 14 fractions and freeze-dried in the vacuum (Rauniyar and Yates III, 2014).

LC-MS/MS Analysis

The peptides were separated using an ultra-performance liquid phase system, then implanted into an NSI ion source for ionization and analyzed through Q Exactive ™ Plus mass spectrometry. The peptide parent ions and their secondary fragments were detected and analyzed using Orbitrap (Rauniyar and Yates III, 2014). The data acquisition mode uses a data-dependent scanning (DDA) program.

Data Analysis

In this experiment, the raw MS data was imported into MaxQuant (v1.6.15.0) to retrieve the secondary mass spectral data. The corresponding analysis parameters were set as follows: The database is Hyphantria cunea TX unique. fasta (71232 sequences), the anti-library is added to calculate the false positive rate (FDR) caused by random matching, and the database is updated with the common contamination library. To reduce the impact of contaminated protein on identification results, the digestion method was set to Trypsin/P; the number of missed cuts was set to 2; the minimum length of peptide segment was set to 7 amino acid residues. The number of peptide modifications was limited to 5. The mass error tolerance of primary parent ions was set to 20 PPM and 4.5 PPM for the First and Main searches, respectively, and the mass error tolerance of secondary fragment ions was set to 20 PPM. The Carbamidomethyl (C) was set to fixed modification, variable modification to methionine oxidation, N-terminal protein acetylation, and deamidation (NQ). The quantitative method was set to TMT-6plex, and the FDR for protein and PSM identification was set to 1%. The FDR accuracy in the three levels of the spectrum, peptide, and protein identification was set to 1%. A protein must contain at least one unique peptide in order to be identified.

Differentially Expressed Protein (DEP) Analysis

DEPs were screened using the criteria of a *P*-value <0.05 and a fold change less than 1 or higher than 1.3. GO annotation proteome was derived from the UniProt-GOA database (Young et al., 2010). KAAS was used to annotate protein in the KEGG database (Mao et al., 2005). The annotation result was then mapped on the KEGG pathway database using the KEGG mapper. In order to perform GO functional analysis and KEGG pathway analysis on the DEPs, a two-tailed Fisher's exact test was used to test the enrichment of the DEPs against all identified proteins. The results of the analysis with a corrected *P*-value < 0.05 were considered significant.

Integrated Analysis of the Transcriptome and Proteome

To conduct an integrated analysis of the transcriptome and proteome, the protein ID was converted to the corresponding transcript ID, and the crossover of the two omics quantification was counted based on the transcript ID. Next, DEGs and DEPs were subjected to KEGG and GO enrichment analyses to determine common pathway information and GO functions, respectively. BLASTx homology search against various protein databases was used to annotate DEGs and DEPs (Wang et al., 2018; Gharat et al., 2020).

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Gene Comparison and Validation Using qRT-PCR

To compare and validate the DEGs in the transcriptome data, 18 genes were selected to perform qRT-PCR experiment. Primer Premier (v6.25) software was used to design the gene-specific primers. qRT-PCR was performed on an iCycler RT detection system (Bio-Rad, CA, USA) with an iQ SYBR Green Supermix (Bio-Rad, CA, USA). The qPCR program comprised 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. the fold change in the gene expression levels was calculated with the $2^{-\Delta\Delta CT}$method.

Results

Chlorbenzuron Reduced Chitin Levels and Cuticular Proteins in the Cuticle of H. cunea

Transverse paraffin sections of the whole body were stained for chitin with CFW to investigate the effects of chlorbenzuron on chitin levels in the larval cuticle of *H. cunea* Significant reduction in CFW fluorescence after chlorbenzuron treatment implied decreased chitin levels in the cuticle of *H. cunea*. (*Fig. 1A, B, C*) There was a significant difference in the level of chitin in *H. cunea* between the T(48h) group and the CK(48h) group ($F=82.54$， $P<0.0001$) (*Fig. 2A*). Also, there was a significant difference in cuticular proteins of *H. cunea* after chlorbenzuron treated ($F=85.07$， $P<0.0001$) (*Fig. 2B*). CK(48h) and T(48h) groups have no significant difference in chitin levels and cuticular proteins (*Fig. 2A, B*).

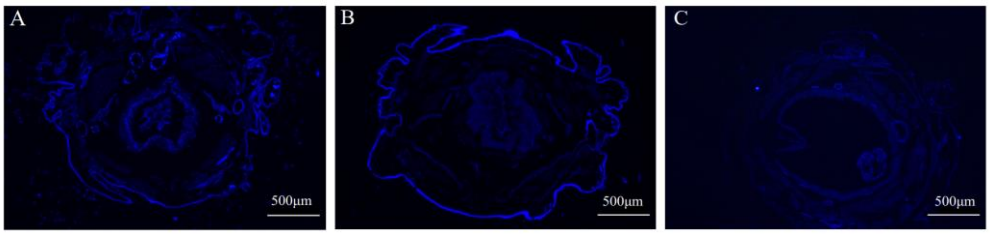

Figure 1. *Labeling of chitin in larval cuticles from transverse paraffin sections of H. cunea. (A) newly molted 4th instar larvae. (B) CK group was fed a control diet for 48 hours. (C) T group was fed a diet containing 100 ppm chlorbenzuron*

Chlorbenzuron Treated Increase the Activities of MFOs and GSTs in H. cunea

Measured as the activities of MFOs and GSTs, no significant difference between CK(0h) group and CK(48h) group (*Fig. 2C, D*). In the T(48h) group, the activities of MFOs was significantly higher than in the CK(48h) group ($F=59.72$, $P=0.0001$) (*Fig. 2C*). There was also a significant difference in the activities of GSTs between CK(48h) group and T(48h) group ($F=15.93$, $P=0.004$) (*Fig. 2D*).

H. cunea Transcriptome Assembly

Following the removal of N reads, low-quality reads, and adapter-related reads, the *H. cunea* transcriptome sequencing for CK and T groups was summarized as shown in *Table S1*. A total of 71869 unigenes were derived from 140251231 clean reads with an N50 length of 1814 bp (*Table S2*).

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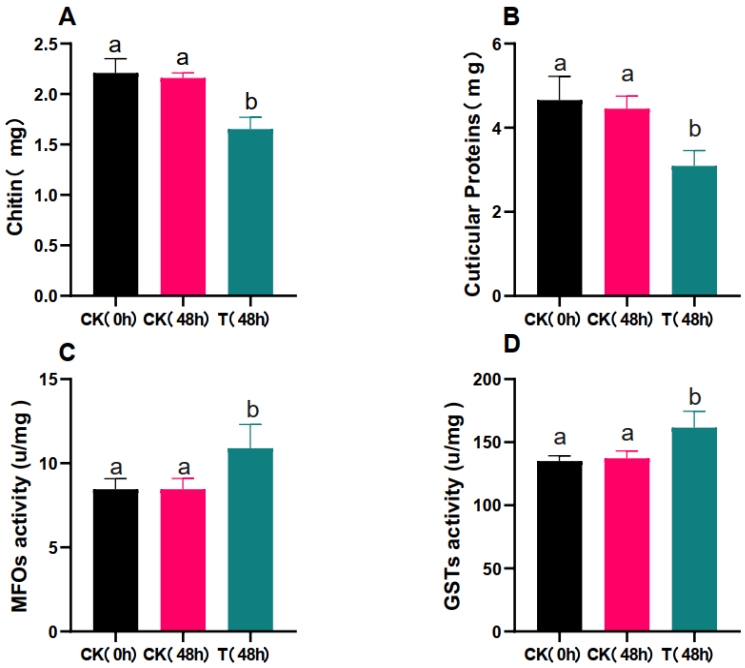

Figure 2. *Determination of physiological data of H. cunea. (A) the level of chitin in cuticle (B) cuticular proteins in cuticle. (C) the activities of MFOs. (D) the activities of GSTs. Data are presented as the Mean ± SE of three independent biological replications, and error bars indicated standard errors; One-way ANOVA was performed followed by Dunnett's test; Different letters following the data in a column show significant difference and statistical data was significant at P value<0.05*

Identification of DEGs by Transcriptomic Analysis of H. cunea Response to Chlorbenzuron Treatment

RNA-seq experiments were performed to elucidate the underlying mechanism of how chlorbenzuron treated changes the transcriptome of the *H. cunea*. The correlation analysis of gene expression levels was performed between each sample; the results revealed significant differences between the T and CK groups at the transcriptional level (*Fig. 3A*). A total of 1973 significant DEGs (1179 up-regulated genes and 758 down-regulated genes) were identified in the T groups (*Fig. 3B-D*). GO functional annotation of DEGs was divided into three categories: biological, molecular functions, and cellular components. The DEGs that were found to be differentially regulated in response to chlorbenzuron treatment that were mainly associated with catalytic activity, oxidoreductase activity, small molecule metabolic process, proteolysis, and obsolete electron transport (*Fig. 3E*). The KEGG pathway analysis was used to provide a detailed description of the specific biological pathways of the DEGs in response to chlorbenzuron treatment. The most enriched pathways included “Protein processing in endoplasmic reticulum” (bmor04141), “Glutathione metabolism” (bmor00480), and “Drug metabolism − cytochrome P450” (bmor00928), and the top 20 significantly enriched pathways were identified (*Fig. 3F*).

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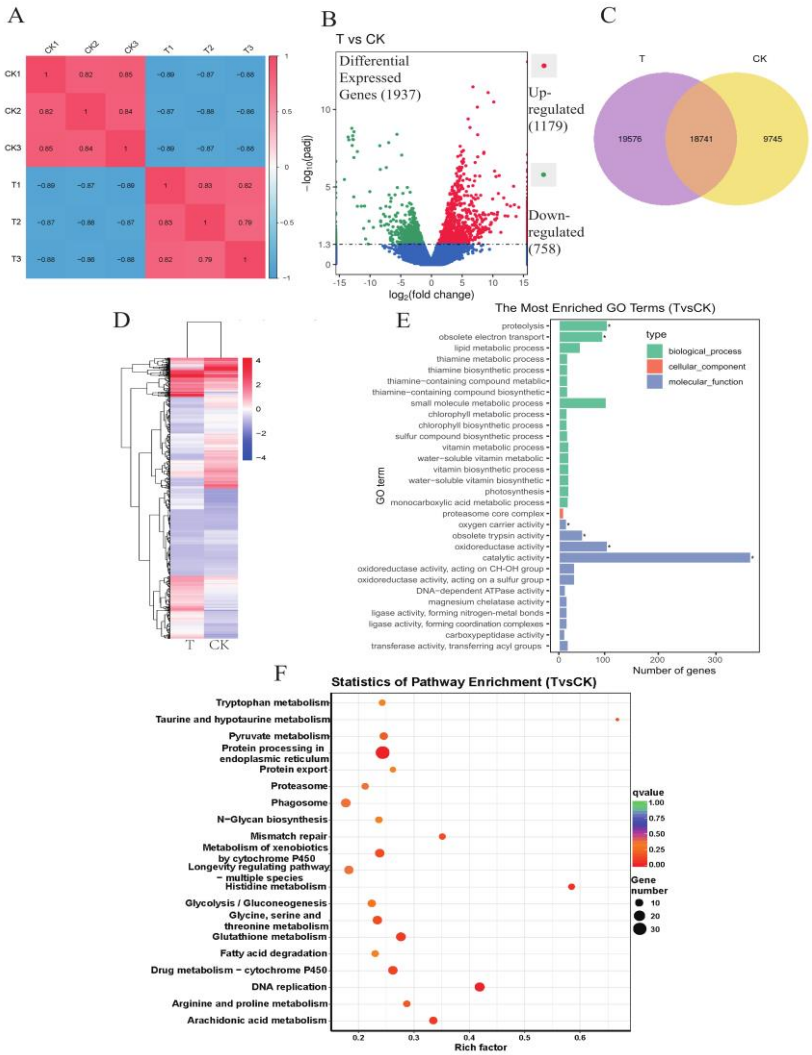

Figure 3. *Transcriptomic analysis of T and CK groups which were fed artificial diet and artificial diet with 100ppm chlorbenzuron for 48h respectively. (A) Correlation coefficients of the RNA-seq data. The abscissa and ordinate in the figure are the squares of the correlation coefficients of these samples. (B) Transcripts with a significant P value (<0.05) and a log2| FC| value>1 showing in the Volcano graphs were considered to be the DEGs between T and CK groups. The blue dots indicate the expression of these genes have no statistically significant difference. The green dots and the red dots represent significantly down-regulated genes and up-regulated genes respectively. (C) Venn diagram of the unique and shared DEGs in T and CK groups. (D) Heatmap representing gene expression levels of DEGs in T group compared to CK group. The column showing the genes were significantly up-regulated (red) or down-regulated (blue) in each group. (E) GO functional analysis and (F) KEGG pathway were performed to enrich the DEGs between T group and CK group*

Identification of DEPs by TMT-based Proteomic Analysis of H. cunea Response to Chlorbenzuron Treatment

We performed a TMT-based proteomic analysis to investigate differences in protein expression in chlorbenzuron-treated *H. cunea*. There were significant differences in protein between the T and CK groups (*Fig. 4A-B*). A total of 714 significant DEPs (283

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up-regulated proteins and 431 down-regulated proteins) were identified in the T groups (*Fig. 4C*). GO functional annotation was performed to gain an understanding of the response of chlorbenzuron-treated DEPs. The findings demonstrated that DEPs were primarily associated with the extracellular region, structural constituent of the chitin-based cuticle, structural constituent of the cuticle, chitin-based cuticle development, and peptidoglycan muralytic activity (*Fig. 4D*). Furthermore, the KEGG pathway analysis was performed to reveal the biological pathways of the DEPs. The most enriched pathways included "Necroptosis" (bmor04217), "Ribosome" (bmor03010), and "Apelin signaling pathway" (bmor04371) (*Fig. 4E*).

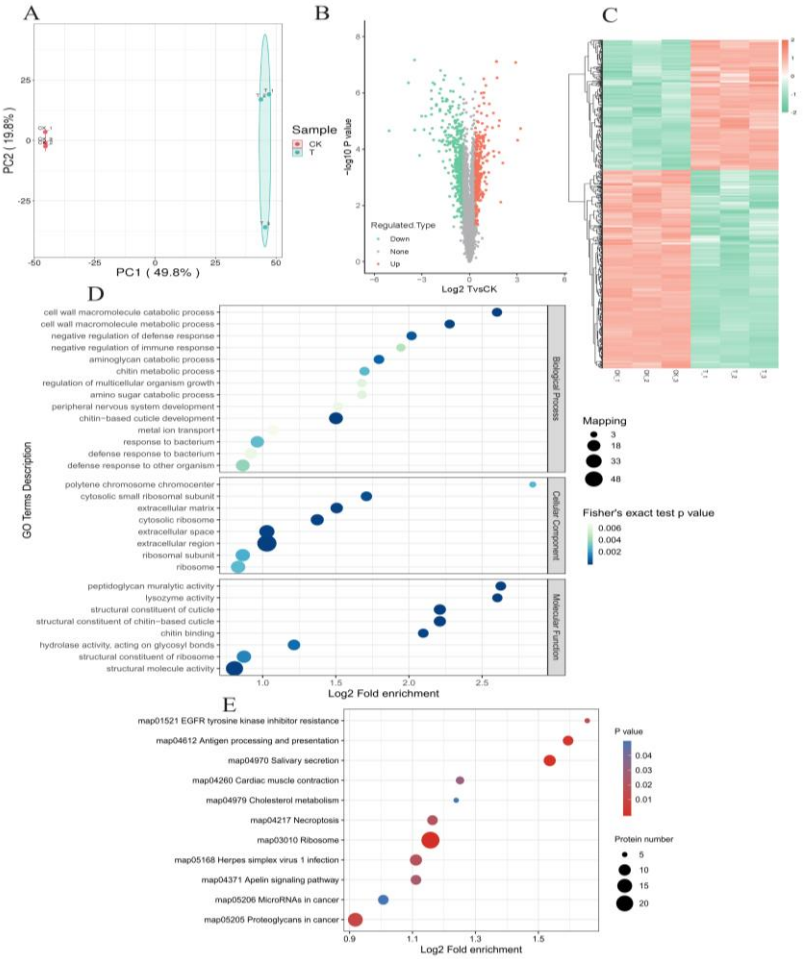

Figure 4. *Proteomic analysis of T and CK groups which were fed artificial diet and artificial diet with 100 ppm chlorbenzuron for 48h respectively. (A) Principal components analysis of the proteomic analysis showing the samples in T group and the samples in CK group. The samples were clustered into 2 different groups and the points represent biological replicates. (B) Proteins with a FC less than 1/1.3 or greater than 1.3 and a significant P value (<0.05) Volcano graph showing the relative abundances of proteins showing in the Volcano graphs were considered to be the DEPs between T and CK groups. The gray dots indicate the expression of these proteins have no statistically significant difference. The green dots and the orange dots represent significantly down-regulated proteins and up-regulated genes respectively. (C) Heatmap representing protein expression levels of DEPs in T group compared to CK group. The column showing the proteins were significantly up-regulated (orange) or down-regulated (green) in each group. (D) GO functional analysis and (E) KEGG pathway were performed to enrich the DEPs between T and CK groups*

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Integration of The Transcriptomic and Proteomic Data

Comparing the quantitative correlation between transcriptomics and proteomics can help researchers better understand the complex mechanisms of *H. cunea* response to chlorbenzuron treatment. Here, the protein expression level and mRNA expression level were combined using the above protein and transcript ID relationship, and both protein and transcriptome expression levels were converted to Log2 Ratio (Log2FC) (*Fig. 5A*). We list the proteins quantified in proteomics and transcriptomics as well as their corresponding expression levels. The significantly differentially expressed transcripts and proteins (DEGs and DEPs) in each group were obtained based on the different screening conditions of the transcriptome and proteome, and the crossover between them was demonstrated (*Fig. 5B*). Notably, each expression corresponds to a regulatory relationship.

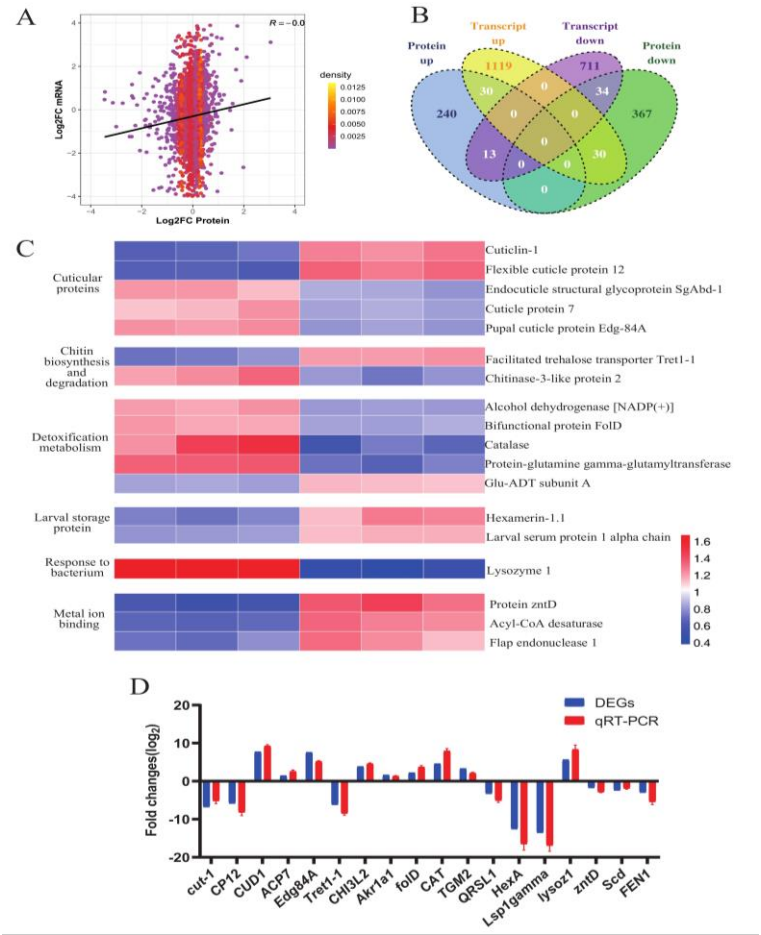

Figure 5. *Integration of the transcriptomic and proteomic analysis on T and CK groups which were fed artificial diet and artificial diet with 100ppm chlorbenzuron for 48h respectively. (A) Scatter plot of transcript and corresponding protein expression. The horizontal axis is protein expression and the vertical axis is transcript expression. The color of a point is the density of the point. (B) Venn diagrams were compared between differentially expressed proteins and differentially expressed transcripts in T and CK groups. (C) Comparison and validation between 18 DEGs and qRT-PCR of the genes. (D) Heatmap displaying the protein and gene were simultaneously up-regulated or down-regulated after chlorbenzuron treated. The column showing the proteins were significantly up-regulated (red) or down-regulated (blue) in each group*

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The regulatory relationships ("up-up" and "down-down") between transcripts and their corresponding proteins were selected to help researchers better understand the biological processes directly associated with proteins or transcripts with different regulatory relationships. The results of Nr annotation in the aforementioned DEGs and DEPs revealed that chlorbenzuron treatment primarily affected the insect cuticle metabolism and detoxification. Meanwhile, other genes and corresponding proteins related to physiological activities in *H. cunea* have been identified and investigated. Then, these 18 genes were selected to perform qRT-PCR experiment (*Fig. 5C*). The expression of 18 proteins corresponding to 18 genes was shown in (*Fig. 5D*).

Discussion

H. cunea is a destructive and highly polyphagous forest pest native to North America that has spread to other countries. Recent evidence shows that chemical control using BPUs to inhibit chitin biosynthesis in insects is highly effective and targeted (Hajjar and Casida, 1978; Edosa et al., 2019). However, the mechanism of action of BPUs remains poorly understood. The identification and characterization of genes and biological pathways associated with chitin biosynthesis are critical for understanding the mechanism of action of BPUs (Yao et al., 2021). The present investigation combined transcriptomic and proteomic analyses to determine the mechanism of action of chlorbenzuron on the 4th instar larvae of *H. cunea*. The regulatory relationships between genes and proteins and the metabolic pathways how *H. cunea* responds to chlorbenzuron treatment are shown in *Fig. 6*.

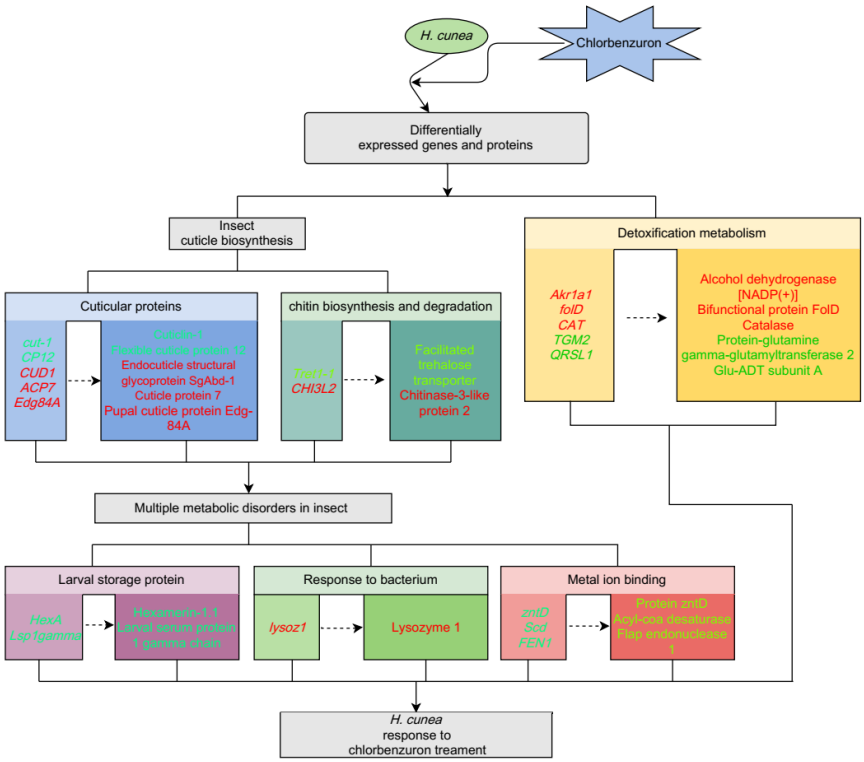

Figure 6. *The regulatory relationships and expressions between transcripts and their corresponding proteins. A proposed model of transcripts and corresponding proteins regulatory relationships in H. cunea after chlorbenzuron treated*

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Chlorbenzuron Affects the Formation of Cuticle

Proteins and chitin make up the insect cuticle. Chitin is covalently bound to the protein matrix, forming a chitin-protein complex. Cuticular proteins are crucial in the formation and development of insect cuticles (Rebers and Willis, 2001). The level of chitin and cuticular proteins in cuticle of *H. cunea* were significantly decreased after chlorbenzuron treated (*Fig. 2A, B*). The KEGG pathways of the DEGs and the GO functional analysis of the DEPs demonstrated that the effects of chlorbenzuron treatment were closely related to the metabolism of insect cuticles, including chitin-based cuticle development, structural constituent of the cuticle, structural constituent chitin-based cuticle, N-Glycan biosynthesis and chitin-binding. These biological processes point directly to the effect of chlorbenzuron on insect cuticles.

Herein, the expression of some genes and corresponding proteins was regulated simultaneously. Cuticle protein 7, endocuticle structural glycoprotein SgAbd-1, and pupal cuticle protein Edg-84A were up-regulated, while Cuticlin-1 and flexible cuticle protein 12 were down-regulated (*Fig. 5D*). Cuticlin-1 is a cuticle component that aids in the formation of extracellular envelopes that protects the organism from the environment. Cuticlin-1 was first reported in *Caenorhabditis elegans*, then was also found in Insecta such as *Bactrocera dorsalis* and *Ooceraea biroi* (Sebastiano et al., 1991; Geib et al., 2014; Oxley et al., 2014). The flexible cuticle protein 12 was discovered for the first time found in the flexible cuticles of all three metamorphic stages of *Hyalophora cecropia* (Binger and Willis, 1994). The protein contains the chitin-binding type R&R domain, which contributes to the flexibility and rigidity of the cuticle (Rebers and Riddiford, 1988; Willis, 2010). The insect cuticular protein family is divided into subfamilies based on their motifs, of which the CPR family contains R&R motif, including 3 subfamilies (RR-1, RR-2, and RR-3) is the largest (Zhou et al., 2016). The R&R motifs contain a Chitin-binding domain 4 (CBD4), which aids in chitin and protein matrix interactions (Qin et al., 2009). Studies have demonstrated that RR-1, RR-2, and other types of cuticular proteins may be involved in the formation of the exocuticle, while RR-1 and other cuticular proteins may participate in endocuticle formation (Shahin et al., 2016, 2018). Cuticle protein 7(ACP7), specifically expressed in the wings, is a member of the RR-2 subfamily and is required for the normal morphology and function of the wing cuticle (Zhao et al., 2017). SgAbd-1, an endocuticle structural glycoprotein, is a component of the abdominal endocuticle, belonging to the RR-1 subfamily (Andersen, 1998). The tetrapeptide (A-A-P-[AV]) repeats found throughout the protein are also found in many cuticle proteins (Marcu and Locke, 1998). Edg-84A, a protein found in the pupal cuticle of Drosophila and other insects, belongs to the RR-1 subfamily and has been found in both larval and pupal cuticles (Karouzou et al., 2007). Thus, abnormal responses to chlorbenzuron treatment in *H. cunea* larvae include the expression of cuticular proteins and corresponding genes. These changes in cuticular proteins directly influence the structure of the insect cuticle, interfering with the ecdysis and molting processes.

Researchers have demonstrated several roles of CHS, trehalase, and CHTs which are particularly important (Candy and Kilby, 1962; Jaworski et al., 1963; Tharanathan and Kittur, 2003). Although these enzymes have received the most attention, the evidence that BPUs directly act on CHTs or trehalase was few (Wang et al., 2020). Chlorbenzuron treatment regulated the expression of Facilitated trehalose transporter (Tret1-1) and Chitinase-3-like protein 2 as well as their corresponding genes. Tret1-1, the facilitated trehalose transporter was down-regulated (*Fig. 4C*). Trehalase catalyzes

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the conversion of trehalose to β-D-Fructofuranose, which is the first step in chitin biosynthesis. Tret1-1 is a trehalose low-capacity facilitative transporter, mediating the bidirectional transfer of trehalose. A study demonstrated that Tret1-1 facilitated the transport of trehalose synthesized in the fat body and the incorporation of trehalose into other tissues that needed a carbon source, thereby regulating trehalose levels in the hemolymph (Kanamori et al., 2010). Furthermore, Tret1-1 downregulation directly decreased trehalose levels in *H. cunea* hemolymph, indicating that chitin synthesis was inhibited and the demand for substrate (trehalose) was reduced. Also, Chitinase-3-like protein 2 was up-regulated (*Fig. 4C*). This protein lacks a functional description in insects, but it has been shown in humans to bind chitooligosaccharides and other glycans with high affinity (Schimpl et al., 2012). Recent studies have demonstrated that insects have 11 CHTs and CHT-like proteins that are primarily responsible for chitin degradation (Tetreau et al., 2015). Chitinase-3-like protein 2 up-regulation may hasten chitin degradation in *H. cunea* larval cuticles.

Chlorbenzuron Causes Detoxification Metabolism

MFOs, catalases, and GSTs are the three major enzyme groups involved in the etoxification of xenobiotics in insects (Li et al., 2007; Ketterman et al., 2011; Liu and Nannan, 2015). The activities of MFOs and GSTs in *H. cunea* were significantly increased after chlorbenzuron treated (*Fig. 2C, D*). Also, the KEGG pathways of the DEGs and the GO functional analysis revealed chlorbenzuron treatment influenced cytochrome P450-mediated metabolism of xenobiotics, glutathione metabolism, drug metabolism-cytochrome P450, and oxidoreductase activity. These pathways and processes were linked to detoxification metabolism. Chlorbenzuron treatment simultaneously regulated the expression of some genes and corresponding proteins in *H. cunea* associated with the three detoxifying enzyme groups.

The alcohol dehydrogenase [NADP (+)] and bifunctional protein FolD were both up-regulated (*Fig. 5D*). Studies show that the catalytic activity of these two proteins increases the supply of NADPH (D'Ari and Rabinowitz, 1991; Takahashi et al., 2012). In the catalytic process of MFOs, NADPH is required for electron transfer, whereby NADP is generated, followed by xenobiotic detoxification (Guengerich, 2001). The catalase was up-regulated in this study (*Fig. 5D*). Catalase is a detoxifying enzyme of insects, found in almost all aerobically respiring organisms that protect cells from the toxic effects of hydrogen peroxide (Missirlis et al., 2001; Zeng et al., 2020). Protein-glutamine gamma-glutamyltransferase 2 was up-regulated, while Glu-ADT subunit A was down-regulated in this study (*Fig. 5D*). The catalytic reaction of these enzymes is related to the glutathione (GSH) detoxification pathway and GSH biosynthesis (Shan et al., 2015; Lai et al., 2016). GSTs typically catalyze the conjugation of reduced GSH with electrophilic substrates, converting reactive molecules into less toxic conjugates that are easily excreted from the body (Konanz and Nauen, 2004).

Chlorbenzuron Decreases the Overall Biosynthetic Capacity

Chlorbenzuron affects chitin biosynthesis, the most important polysaccharide metabolism process in insects. The KEGG pathways of the DEGs and GO functional analysis of the DEPs revealed that chlorbenzuron treatment influenced glycolysis/ gluconeogenesis, amino sugar catabolic process, and metabolism of several amino acids. This suggested that chlorbenzuron plays a role in decreasing overall biosynthetic capacity and energy metabolism efficiency in *H. cunea*. The GO functional analysis of

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the DEPs revealed chlorbenzuron treatment influenced the defense response to bacterium and lysozyme activity. This indicated that the damage in the formation of cuticle increased the likelihood of xenobiotics infecting *H. cunea*.

The expression of Hexamerin-1.1 and Larval serum protein 1 alpha chain, as well as their corresponding genes, was decreased (*Fig. 5C, D*). These are larval storage proteins (LSP), which are expressed in the arthropod fat body and 23 other tissues and may serve as a store of amino acids for adult proteins synthesis (Burmester et al., 2010; Colomb et al., 2017). These results suggested that LSP released amino acids as a secondary source of input into the TCA (tricarboxylic acid, TCA) cycle. Of note, the process is linked to the mobilization of energy resources in insects to combat stress and is involved gluconeogenesis (Thompson, 1995). Chlorbenzuron treatment decreased LSP levels, as evidenced by some of the significant DEGs in KEGG pathways related to amino acids metabolism.

The expression ofLysozyme 1, as well as the corresponding gene were increased (*Fig. 5C, D*). Research evidence shows that Lysozyme 1 have antibacterial activity and are associated with insect innate immunity (Lemos and Terra, 1991; Souhail et al., 2016), demonstrating bacterial overgrowth in the chlorbenzuron-treated *H. cunea*. Furthermore, chlorbenzuron disrupted the formation of the insect cuticle, making xenobiotics, such as bacteria, more likely to infect *H. cunea*. Chlorbenzuron treatment may cause a skewing of the levels of normal and pathogenic/opportunistic bacteria in *H. cunea.* (Whitten et al., 2014).

Previous research has linked the effects of BPUs on insects to metal ion channels such as potassium and calcium channels (Nakagawa and Matsumura, 1993, 1994). In the present study, Protein zntD, Acyl-CoA desaturase, and Flap endonuclease 1 were found to be down-regulated, as were their corresponding genes (*Fig. 5C, D*). UniProtKB (https://www.uniprot.org/) electronic annotation revealed that these proteins can bind Zn^{2+}, Fe^{2+}, and Mg^{2+}, respectively. Zinc, which was discovered in mandibles of insect larvae, improved the ability of larva to penetrate the food. Zinc concentration in the mandible, however, depends on when they begin feeding (Morgan et al., 2003). Chlorbenzuron was revealed to decrease the overall biosynthetic capacity and cause antifeedant in *H. cunea*, then reducing the need for zinc. Also, a decrease in lipid biosynthesis and respiration related to Acyl-CoA desaturase in *H. cunea* reduced the need for Fe^{2+} and heme-binding (Bai et al., 2015). Flap endonuclease 1 binds two magnesium ions per subunit and is essential for DNA replication and repair (Pascale et al., 2011). As such, down-regulation of Flap endonuclease 1 suggested that chlorbenzuron treatment caused DNA damage in *H. cunea*.

Conclusions

This study demonstrates that chlorbenzuron induces regulation and expression of particular genes and proteins in *H. cunea*. Chlorbenzuron has the potential to disrupt insect cuticle biosynthesis and disrupt detoxification metabolism. Furthermore, chlorbenzuron disrupts the formation of the insect cuticle, and reduces the biosynthetic capacity, influencing the stability of insect energy metabolism and increasing the likelihood of xenobiotics infecting insects. Also, some metal ions that bind in these metabolic pathways are impacted.

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APPENDIX

Table S1. *Summary of sequences analysis*

Sample	Raw Reads	Clean Reads	Clean Bases	Q20	Q30	GC Content
CK1	26587711	26192313	3.93G	98.47%	95.43%	47.37%
CK2	21418479	21022998	3.15G	97.94%	94.07%	48.63%
CK3	25613474	25196165	3.78G	98.02%	94.11%	46.11%
T1	23558569	23139075	3.47G	98.05%	94.23%	47.01%
T2	24626530	24290894	3.64G	98.04%	94.16%	45.62%
T3	20682607	20409786	3.06G	98.01%	94.12%	46.50%

Table S2. *Length distribution of non-redundant (Nr) database consensus sequences*

Length of Nr unigenes	Total number
<300bp	28313
300-500bp	16963
500-1000bp	11446
1000-2000bp	7468
>2000bp	7679
All unigenes(bp)	71869
N50*(bp)	1814

Note: N50 = median length of all unigenes

Table S3. *Abbreviation list*

Abbreviation	Definition
BPU	benzoylphenyl urea
DEG	differentially expressed gene
DEP	differentially expressed protein
GO	Gene Ontology
KEGG	Encyclopedia of Genes and Genomes
IGRs	insect growth regulators
CSI	chitin synthesis inhibitor
ICP	insect cuticular proteins
CHS	chitin synthase
CHT	chitinase
SUR	sulfonylurea receptor
MFO	mixed function oxidases
GST	glutathione transferase
FPKM	fragments per kilobase of transcript per million mapped reads
TMT	tandem mass tag
LCP	larval storage proteins

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