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Pyrethroid pesticide-induced differential gene expression in midguts of two silkworm (*Bombyx mori*) strains of different susceptibilities

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ABSTRACT: In many Asian countries, the silkworm (*Bombyx mori*) is economically important as a main silk-producing insect. Pesticide pollution often causes huge economic losses to the sericulture. Digital gene expression (DGE) was performed to investigate the gene expression profiles of Lan5 (sensitive) and Mysore (tolerant) silkworm strains at 48 h after exposure to fenvalerate. The results showed that a total of 1,575 differentially expressed genes (DEGs) were detected in the Lan5 DGE library, and 1,485 DEGs were detected in the Mysore DGE library. The possible functions of all DEGs in biological process, cellular component, and molecular function were determined using the gene ontology (GO) classification system. Pathway enrichment analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Furthermore, some selected DEGs were verified using quantitative real-time PCR (qRT-PCR). These findings provide abundant data on the effect of pesticide exposure on insects at the molecular level and a new clue to control strategy of Lepidoptera pests.

KEYWORDS: *Bombyx mori*, transcriptome, fenvalerate, differentially expressed genes, midgut

INTRODUCTION

Sericulture is a very prosperous industry in many Asian countries such as China and Thailand [1, 2]. As a kind of silk-producing insect, the silkworm, *Bombyx mori*, is not only an economically important insect, but also a good model insect for Lepidopteran research [3]. However, various diseases and pesticide poisoning caused huge economic losses to the sericulture every year [4–7]. Fenvalerate is an effective and low toxic pyrethroid pesticide, which is commonly used in the mulberry fields in autumn and winter in China [8]. In order to achieve effective result, the dosage and varieties of pesticides are continuously increased in agricultural production, which not only aggravates environmental pollution, but also pollutes mulberry leaves, leading to silkworm pesticide poisoning [9, 10].

The midgut of *B. mori* is the main organ for food digestion and nutrient absorption and a natural barrier against foreign substances [11]. As a part of the defense system of the midgut, the peritrophic membrane (PM) has numerous functions such as protecting epithelial cells, preventing the invasion of harmful substances, and stimulating digestion [12]. However, thus far, the effects of fenvalerate exposure on the transcriptome patterns in the midgut of 2 silkworm strains with different susceptibilities have not been researched and reported. In our previous research, the tolerance of 198 silkworm strains to fenvalerate was tested by bioassay, and the significant differences between different strains were found (data unpublished). Among the tested silkworm strains, the silkworm strain Lan5 from Zhejiang Province of China is sensitive to fenvalerate, while the strain Mysore from India is tolerant to fenvalerate.

As a kind of high-throughput sequencing technique, DGE profiling is known to be accurate, sensitive, and economic [13, 14]. DEGs and transcriptome from multiple samples can be well studied and analyzed by using this technique [15, 16]. In this research, the Illumina Genome Analyzer platform was employed to analyze the gene transcriptome profiles of the exposed and normal silkworms of the 2 strains. GO and KEGG pathway analyses were also performed. Some differentially expressed genes were identified and verified via qRT-PCR to determine the differences.

MATERIALS AND METHODS

Preparation of insects and fenvalerate solution

The silkworm strain Lan5 and Mysore were provided by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. They were reared at standard temperature under a photoperiod of 12 h of light and 12 h of dark. Fenvalerate solution (Sigma-Aldrich Trading Co., Ltd., USA) was diluted to a

0.02 mg/l concentration, which is less than LC_{50} for 24 h [17]. Mulberry leaves were immersed in the solutions for 5 s and allowed to dry naturally, before being fed to the fifth instar silkworms on the second day for 3 times a day.

Tissue dissection

At 48 h after exposure to fenvalerate solution coated leaves, the larvae and the normal control larvae were dissected. The midguts of 10 larvae were collected and rinsed 3 times in diethylpyocarbonate (DEPC)-treated double distilled water. All samples were stored at −80 °C for use. Each treatment was undertaken in triplicate.

RNA extraction

For Illumina sequencing, total RNA was isolated from the fenvalerate-exposed midgut as well as the control midgut by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. To remove any residual DNA, samples were pretreated with RNase-free DNase I (TaKaRa, Beijing, China) for 30 min at 37 °C.

Construction of DGE libraries

The RNA-seq libraries were prepared using TruSeq™ DNA Sample Preparation Kit-Set A (Illumina, San Diego, USA) following the manufacturer's protocol. Approximately 20 µg of total RNA from the fenvalerate-treated midgut or the wild midgut was used to isolate mRNA using Sera-mag Magnetic Oligo (dT) Beads (Illumina). To avoid cDNA synthesizing bias by priming, the purified mRNA was fragmented into small pieces (100–400 bp) using divalent cations at 94 °C for 5 min. The oligo (dT) method was used for reverse transcription to synthesize double-stranded complementary DNA (cDNA). The libraries were sequenced using Illumina Highseq 2000 platform (Illumina) according to the manufacturer's instructions.

GO and KEGG analysis

The possible functions of all DEGs were determined using the gene ontology (GO) classification system (http://www.geneontology.org). A corrected p value $\leqslant 0.05$ was selected as a threshold for significant enrichment of the gene sets. WEGO (Web Gene Ontology Annotation Plot) software was used for visualizing, comparing, and plotting GO annotation results [18]. Pathway enrichment analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg) [19]. Pathway with a corrected p value $\leqslant 0.05$ was designated as significantly enriched pathways in differentially expressed genes.

Quantitative real-time PCR

Total RNA was extracted from the midgut of both treated and control larvae after 48 h using Trizol reagent (Takara). A total of 20 µl volume of real-time PCR reaction solution contained 0.2 µg cDNA, 5 pmol of each primer, and 10 µl of SYBR Green Real-time PCR Master Mix (Takara). PCR reactions were run in triplicate with 2 biological replicates with thermal cycling parameters at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s, and 60 °C for 1 min according to the manufacturer's protocol. The specific primers of genes were designed based on the cDNA sequences (Table 1). The relative expression level of these genes was estimated according to the $2^{-\Delta\Delta Ct}$ method [20]. All the samples were measured independently 3 times.

RESULTS

Analysis of DGE libraries

In this project, 12 samples were sequenced on Illumina HiSeq Platform in total, and about 6.65 Gb was generated per sample. The average genome mapping rate is 79.14%, and the average gene mapping rate is 78.60%. Among all 13,625 genes identified, 12,525 of them are known genes, and 1,100 of them are novel genes. Of all 11,992 novel transcripts that were identified, 6,222 transcripts are previously unknown splicing events for known genes, 1,131 transcripts are novel coding transcripts without any known features, and the remaining 4,639 are long noncoding RNA.

Identification of DEGs after fenvalerate treated in two silkworm varieties

The differentially expressed genes in the infected midgut samples were identified by the standard of FDR $\leqslant 0.001$ and $|\log_2 \text{ratio}| \geqslant 1$. In the susceptible variety Lan5 DEG class, a total of 1,575 genes were found with 833 up-regulated and 742 down-regulated. In the tolerant variety Mysore DEG class, a total of 1,485 genes were found with 417 up-regulated and 1,068 down-regulated (Fig. 1).

GO analysis of DEGs

In order to understand the functions of these DEGs, all the DEGs were mapped to terms in the GO database and compared with the whole genome background. The DEGs were termed by GO ontology into 3 categories, namely cellular component, molecular function, and biological process (Fig. 2).

In the susceptible strain Lan5 DEG class, DEGs have been categorized into a total of 43 functional groups using the WEGO software. In each of the 3 main categories of the GO classification, "metabolic process", "cellular process", "cell", "cell part", "binding", and "catalytic activity" terms are dominant. In most terms, more up-regulated genes were observed than the down-regulated ones. Some genes related to the immune system process, biological adhesion, synapse, and antioxidant activity were all up-regulated.

Meanwhile, in the tolerant strain Mysore DEG class, DEGs have also been categorized into a total

Table 1 Primer pairs for real-time PCR.

Gene name	F primer sequence (5′–3′)	R primer sequence (5′–3′)
Cyp4g15	TGGCAACAGAACTCACAT	CAGAGCGAGGTTGACTAA
Cyp4l6	CAAAGCGGTAATGGGAAAC	AGGTGGAGACTACATCGCAAAT
Cyp4m9	TTACCACATAACACCGAAGG	GACGCAATACAGCAGACAGA
Cyp6ae2	ATCGAGATCCGGTCTGCTA	GACTATTTTCTCCGCCCTCT
Cyp6ae7	TGAAGTCATCACGCAGGGCATA	CCAGCAAATTCTCGAAAACACC
Cyp6ae9	AGAATTTTGGTGAGATGGCC	GGTTTTGATGACTTCAGGGTC
Cyp6ae21	TCGAGAACCGTGGCTACAA	CAATAAGGCGTCGTCAACC
Cyp6au1	GAGGCATTAAGTTCAGCAAGG	CTAAACATCGGCGTCAGGT
Cyp307a1	TCTGAAGCCGATACTGATGAAG	ACAGCGTAGCCCTGGTTGAT
GSTd1	CGGGCTATTCAAACCTATCTG	CTCAGGTACAAAGTGCCAAGA
GSTd3	ACTACCGCAACGCACCTC	TGCCACTAGCAGCACGAC
GSTe1	ATACGGCAAAGGCAGTTCA	TTTTCTCGCAAAGCAGGATA
CarE FE4	GGCGGTAAAGTTTCTGGC	CGGCGTAGTTGAAGTAATAGTC
JHE1	GTGACTCTGCTCCGTTGG	GGGATGCGGTGTTTCTAC
ae48	AACCTAAGACCGAGTGATT	CATTTCGTTGGGAGATAG
cce-7	TCCACTGGGTCAAGAACA	TAGAAAGCGGTGAAAGGT
ace1	GAAATCTAAATCGGCACG	GAATCTGGCAGGAAGTCG
CarE-11	GACTGCCTGCATCTCAAC	TACCAGCAAATCCTCTTC
Hsp20.1	GTAAGCACGAGGAGAGGCAGGA	GCCGTCAGAGGACAACCGAGAT
Hsp23.7	ATTTCATTCCGTTCTCCA	GCTTAGCCTCAACTACCAC
Hsp25.4	GGAGACAGCCCAGAAGAC	ATATGGAATCGGCAAGAA
Tret1-like	CTCCGTCGTATCTGGCTT	TGTGTCCCGTTTTCTTCT
SPZ1	GCCTCACAGTCACCCACA	CTCGCCCAACTTAGCAAC
GlcNAcase1	CATGACTTTCCCCGTTAC	ACCCTGATCCCTCTGTTC
Serpin9	ATTCTTGACTGTCGCCATTA	GACGCATTGTCGGTTTCT
Serpin12	CATTATTGGCTCTTCTTC	ACAGTTGATTCCGTTCTA
SP1	ATTGACTGGCGTAAGGGAG	GCTGGGAAGACGGATTTT
PCD P4	TCGCAAGAACAAGAAATACG	TCCAACGCTATCTCCACAA
UGT40B4	GGTCATCCAAATCGCACA	TACCGAAGCCACTAAAAA
PPO2	TACCGCTGGCACGCCTACA	TCCACCGACACGGACCTCAC
PGRP	GTCATCGTCCAGCACACA	ACACCTTGCCGTTACCTC

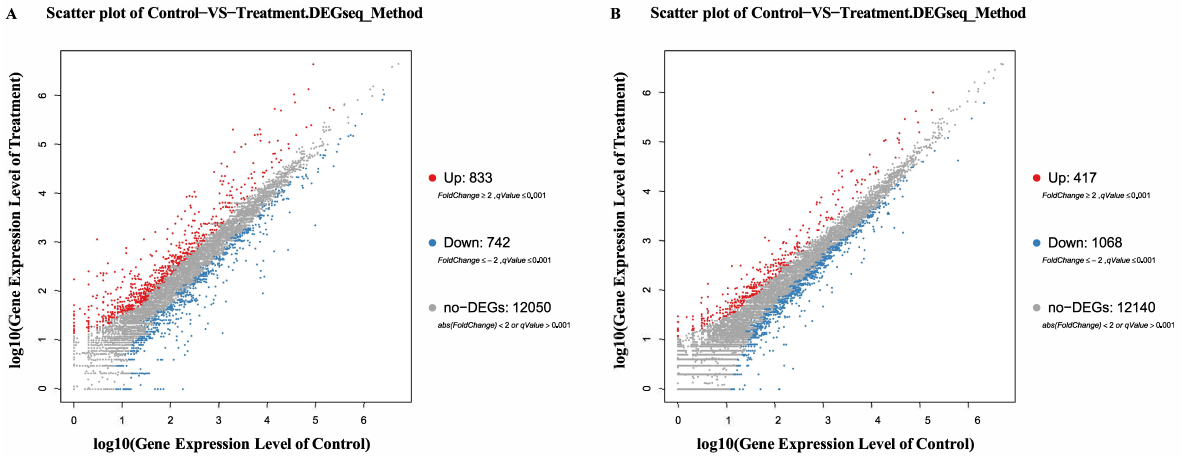

Fig. 1 Scatter plot of differentially expressed genes. (A) Lan5; (B) Mysore. X and Y axes represent $\log_{10}$ transformed gene expression level, red color represents the up-regulated genes, blue color represents the down-regulated genes, and gray color represents the non-DEGs.

of 39 functional groups using the WEGO software. In each of the 3 main categories of the GO classification, “metabolic process”, “cellular process”, “cell”, “cell part”, “binding”, and “catalytic activity” terms are dominant. Interestingly, some genes related to the biological adhesion, immune system process, growth, supramolecular complex, and electron carrier activity were down-regulated, whereas more down-regulated

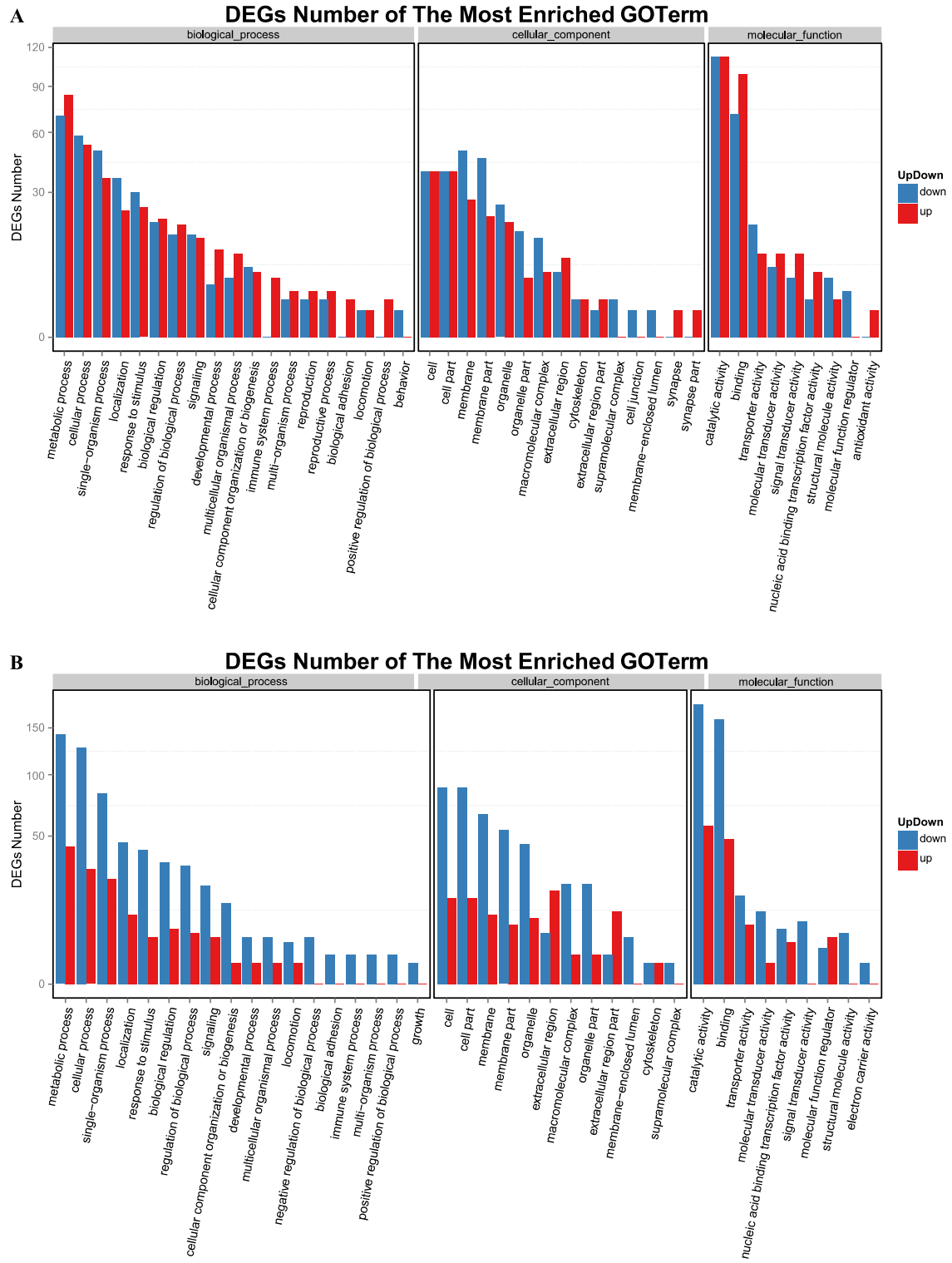

Fig. 2 GO classification of up-regulated and down-regulated genes. (A) Lan5; (B) Mysore. X axis represents GO term. Y axis represents the amount of up/down-regulated genes in a category.

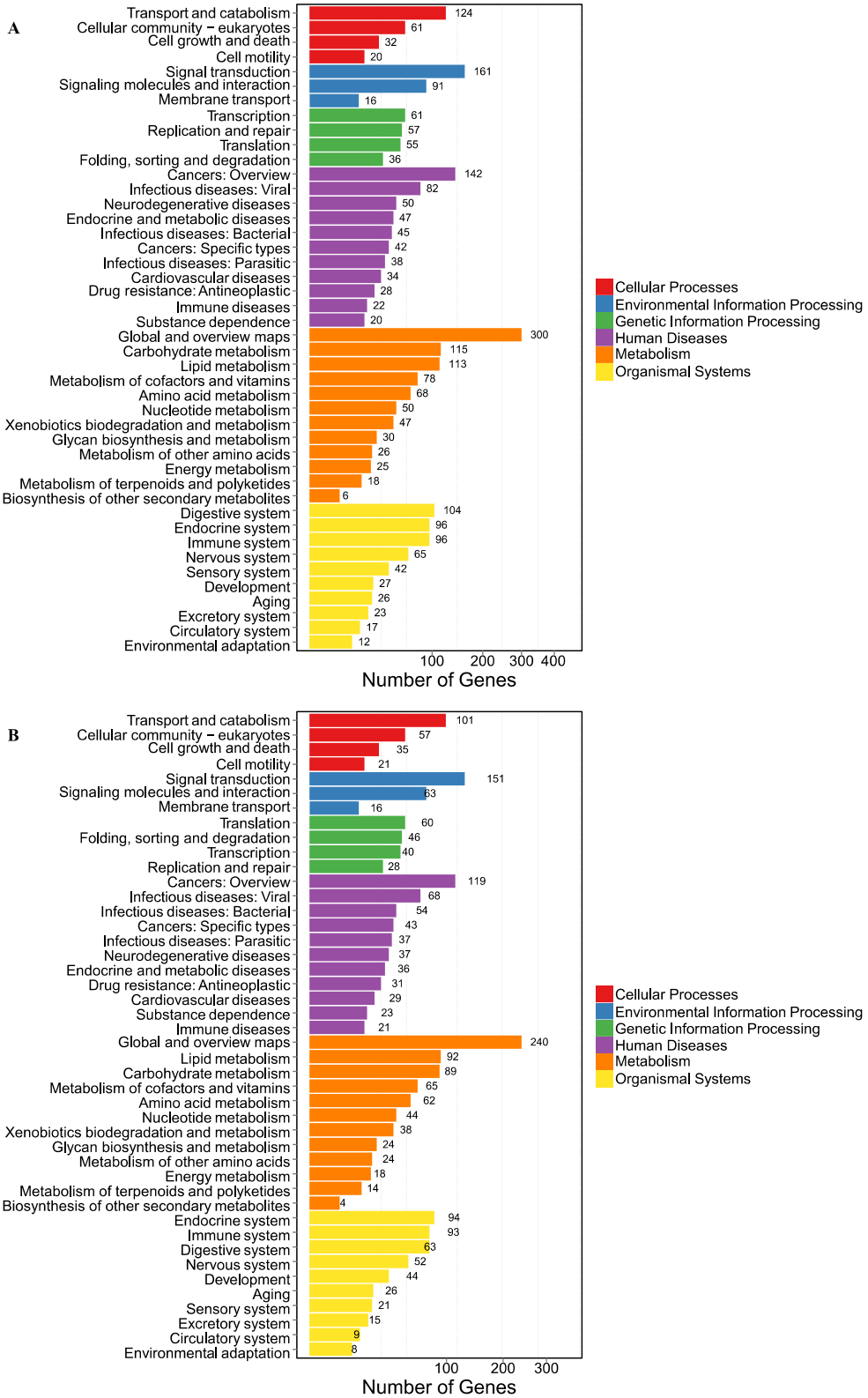

Fig. 3 Pathway classification of DEGs. (A) Lan5; (B) Mysore. X axis represents number of DEG. Y axis represents functional classification of KEGG. There are 6 branches for KEGG pathways: Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Disease (For animals only), Metabolism, and Organismal Systems.

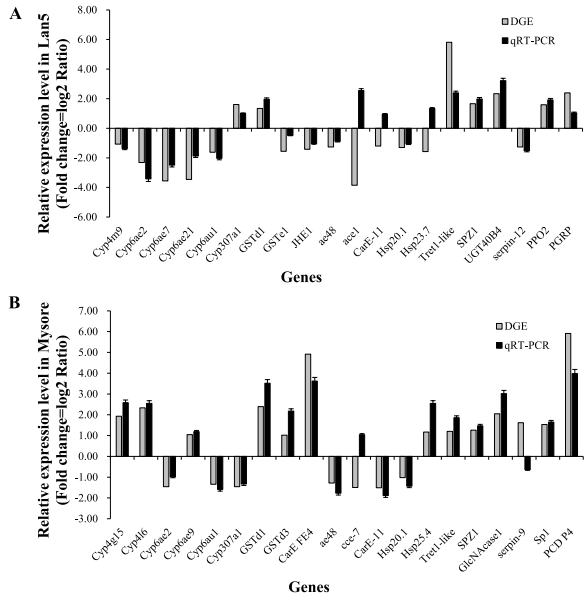

Fig. 4 Verification of transcriptomic results of DEGs by qRT-PCR. (A) Lan5; (B) Mysore. The Y axis indicates the relative expression level of gene mRNA transcripts (fold change = $\log_2$ ratio). The X axis indicates the selected genes. Vertical bars represent the mean ± SE ($n = 3$).

genes were observed than the up-regulated ones in other terms.

KEGG pathways of DEGs

In order to further understand the biological pathways in which the DEGs (Up and Down) are involved, KEGG (http://www.genome.jp/kegg) ontology assignments were used to classify functional annotations of the identified genes (Fig. 3). There are 6 branches for KEGG pathways: Cellular Processes, Environmental Information Processing, Genetic Information Processing, Human Disease, Metabolism, and Organismal Systems. There is a difference between up (down)-regulated pathways observed in Mysore compared with Lan5. In total, the pathways with the greatest representation in the DEGs were "Transport and catabolism", "Signal transduction", "Transcription", "Cancers: Overview", "Global and overview maps", "Digestive system", and "Endocrine system".

Determination of DEGs by qRT-PCR

Twenty DEGs were randomly selected respectively in Lan5 and Mysore and verified by using qRT-PCR. The results showed that most genes were concordant with the DGE libraries, but 3 genes from Lan5 (*ace1*, *CarE11*, and *Hsp23.7*) and 2 genes from Mysore (*cce7* and *serpin9*) were non-concordant (Fig. 4). Although some differences were observed in the results of qRT-PCR and DGE analysis, the overall regulate trend of most genes was consistent, which indicated that the results of the DGE data were accurate.

DISCUSSION

This study is the first report systematically analyzing the expression profile and regulation of genes in midguts of 2 silkworm strains with different susceptibilities after exposure to fenvalerate. RNA-Seq is a powerful and efficient technology for the rapid identification and analysis of majority part of whole transcriptome [21, 22]. In this study, the Illumina Genome Analyzer platform was employed to analyze the transcriptome changes. GO and KEGG pathway were analyzed to classify the functions of DEGs and identify the difference of pathways involved in the fenvalerate regulation, respectively.

Fenvalerate-exposed susceptible Lan5 and tolerant Mysore silkworms were subjected to DGE to perform a global analysis of their transcriptomes. The Lan5 silkworms yielded 1,575 DEGs, and the Mysore silkworms yielded 1,485 DEGs. These differences in the 2 silkworm strains might be involved in different susceptibilities or defense responses to fenvalerate, which is similar to the previous studies [23]. However, the possible mechanism of the susceptibility differences between 2 silkworm strains and molecular information on these DEGs still needs to be clarified in future research.

Many studies showed that the midgut is the major detoxification tissue in *B. mori*. There are some detoxification enzymes in midgut such as cytochrome P450s, glutathione-S-transferase, and carboxylesterase, which are able to hydrolyze insecticide and eliminate them from silkworm body through metabolism [24]. In this study, fenvalerate-induced up-regulation of many cytochrome P450 such as *Cyp4g15*, *Cyp4l6*, and *Cyp6ae9* were observed in the midgut of Mysore, which suggested their roles in detoxification of fenvalerate. At the same time, the detoxification gene with the highest up-regulation in the Mysore DGE was a glutathione-S-transferase gene (XM_012692362.1) with gene expression fold change of 5.7. And another up-regulated detoxification gene was CarE FE4 (XM_004921552.2) with gene expression fold change of 4.9.

As activators of the innate immune system in many organisms, heat shock proteins (HSPs) are a family of proteins that help protect organisms from environmental-induced cellular damage [25]. In this study, the up-regulations of transcription level of *Hsp25.4* was also found in the midgut of Mysore silkworm after exposure to fenvalerate. In *B. mori*, antimicrobial peptides (AMPs) such as moricins, gloverins, and attacins play an important role in eliminating invades [26]. Our study results showed that the transcription levels of *Mor*, *SPZ1*, and *PGRP-S1* were observed up-regulated after exposure to fenvalerate. Due to the up-regulation of these AMPs, the silkworm

larvae may become more resistant to infection of various microbes.

In conclusion, this is the first report that employed the DGE technique to examine the transcriptome differences between midguts of 2 silkworm strains after exposure to fenvalerate. These findings provide abundant data on the effect of pesticide exposure on insects at the molecular level and a new clue to control strategy of Lepidoptera pests.

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