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Molecular mechanisms of cytochrome P450-mediated detoxification of tetraniliprole, spinetoram, and emamectin benzoate in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

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Research Paper

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Abstract

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) is a highly damaging invasive omnivorous pest that has developed varying degrees of resistance to commonly used insecticides. To investigate the molecular mechanisms of tolerance to tetraniliprole, spinetoram, and emamectin benzoate, the enzyme activity, synergistic effect, and RNA interference were implemented in *S. frugiperda*. The functions of cytochrome P450 monooxygenase (P450) in the tolerance to tetraniliprole, spinetoram, and emamectin benzoate in *S. frugiperda* was determined by analysing changes in detoxification metabolic enzyme activity and the effects of enzyme inhibitors on susceptibility to the three insecticides. 102 P450 genes were screened via transcriptome and genome, of which 67 P450 genes were differentially expressed in response to tetraniliprole, spinetoram, and emamectin benzoate and validated by quantitative real-time PCR. The expression patterns of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* were analysed in different tissues and at different developmental stages in *S. frugiperda*. Silencing *CYP340L16* significantly increased the susceptibility of *S. frugiperda* to tetraniliprole, spinetoram, and emamectin benzoate. Furthermore, knockdown of *CYP340AX8v2*, *CYP9A75,* and *CYP341B17v2* significantly increased the sensitivity of *S. frugiperda* to tetraniliprole. Knockdown of *CYP340AX8v2* and *CYP340AA4* significantly increased mortality of *S. frugiperda* to spinetoram. Knockdown of *CYP9A75* and *CYP341B15v2* significantly increased the susceptibility of *S. frugiperda* to emamectin benzoate. These results may help to elucidate the mechanisms of tolerance to tetraniliprole, spinetoram and emamectin benzoate in *S. frugiperda*.

Introduction

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) is a moth of the genus *Spodoptera* Guenée of the Noctuidae family, and is native to tropical and subtropical regions of the Americas and is highly migratory (Johnson, 1987; Wang *et al*., 2020; Wu *et al*., 2021; He *et al*., 2021a, 2021b). Since 2016, *S. frugiperda* has spread to several countries in Africa and Asia, and appeared in 18 provinces in mainland China in 2019, and by 2020, the outbreak had spread rapidly to 27 provinces and 1338 districts (Jing *et al*., 2020). The *S. frugiperda* feeds on a wide range of crops, including corn, soybeans, cotton, and rice, causing huge agricultural losses (Huang *et al*., 2021; Yang *et al*., 2021). The frequent and continuous use of insecticides is currently the main strategy for the management of *S. frugiperda*, which inevitably leads to the development of resistance. The *S. frugiperda* is currently resistant to a wide range of chemical insecticides, mainly organophosphates, carbamates, and pyrethroids, as well as some new chemical insecticides such as diamides, spinosyns, and benzoylureas (Yu and McCord, 2007; Carvalho *et al*., 2013; do Nascimento *et al*., 2016; Bolzan *et al*., 2019; Lira *et al*., 2020). More importantly, when *S. frugiperda* migrates, the alleles that produce resistance persist in resistant populations (Arias *et al*., 2019).

Insects, as an extremely abundant group of organisms in nature, have evolved a variety of resistance strategies under the pressure of insecticide selection. Increased expression or activity of insect detoxification metabolic enzymes is an important mechanism by which insects respond to insecticides. Genes encoding cytochrome P450 monooxygenase (P450), glutathione S-transferase (GST), and carboxylesterase (CarE) enhance insecticide resistance through sequence amplification, enhanced transcription, and mutation (Feyereisen 1999; Scott, 1999; Hemingway

et al., 2004). The P450s have been the focus of insecticide resistance research and have a wide range of substrates that can mediate resistance to almost all insecticides by metabolising a wide range of endogenous and exogenous compounds (Niwa et al., 2004; Shi et al., 2018). The P450s metabolise organophosphates, pyrethroids, neonicotinoids, and other insecticides through hydroxylation and epoxidation, evolving resistance to different insecticides in insects (Li et al., 2007).

In our previous study, we found that tetraniliprole, spinetoram, and emamectin benzoate had high insecticidal activity against *S. frugiperda* (Yang et al., 2022a; Zhang et al., 2022a). Tetraniliprole is a new diamide insecticide developed by Bayer CropScience, which is effective against the *S. frugiperda* (Li et al., 2019; Xu et al., 2020). Spinetoram is a semi-synthetic product of spinosyn, derived from a soil inhibiting microorganism (*Saccharopolyspora spinosa*), effective in the management of lepidopteran pests (Zheng et al., 2019; Sparks et al., 2021). Emamectin benzoate is a new type of highly effective semi-synthetic antibiotic insecticide derived, synthesised, optimised, and modified from the fermentation product avermectin B1 as a lead compound (Yen and Lin, 2004; Ioriatti et al., 2009). Spinetoram and emamectin benzoate are both broad-spectrum, high effective, low toxicity, low residue, and easily degradable green insecticides. However, in actual production, some pests have developed different degrees of resistance to spinetoram, tetraniliprole, and emamectin benzoate (Che et al., 2015; Liu et al., 2016; Gutiérrez-Moreno et al., 2019; Huang et al., 2021).

In this study, the enzymatic activities of P450, CarE, and GST were determined after tetraniliprole, spinetoram, and emamectin benzoate treatments, and the synergistic effects of piperonyl butoxide (PBO) and diethyl maleate (DEM) on insecticides in *S. frugiperda* were validated. Moreover, a total of 102 P450 genes were identified in the transcriptome and genome of *S. frugiperda*, of which 67 P450 genes showed up-regulated in expression in response to tetraniliprole, spinetoram, and emamectin benzoate. The expression patterns of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* were analysed in different tissues and at different developmental stages of *S. frugiperda*. The functions of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* in the susceptibility of *S. frugiperda* to insecticides were verified via RNA interference (RNAi). The results of this study provide a basis for further exploration of the mechanisms of tolerance to tetraniliprole, spinetoram, and emamectin benzoate in *S. frugiperda*.

Materials and methods

Insect rearing

The *S. frugiperda* population without exposure to insecticides was collected from Tancheng (Shandong, China) in 2019. It was reared in an illumination incubator at 26 ± 1 °C with 60–70% relative humidity and 16 h light: 8 h dark photoperiod. The *S. frugiperda* larvae was reared with an artificial diet (Bowling, 1967), and the adults were fed with 10% honey solution referring to our previous research.

Insecticide and chemical

Tetraniliprole technical (99.00%) was purchased from Bayer Crop Science Co., Ltd (Beijing, China). Spinetoram technical (85.80%) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Emamectin benzoate technical (95.00%) was provided by Shandong Lukang Biopesticides Co., Ltd (Dezhou, China). Triton X-100 was purchased from Sigma-Aldrich (St. Louis, MO, United States). Dimethyl sulfoxide (DMSO) was purchased from Solaibao Technology Co., LTD (Beijing, China).

Toxicity assay and sample collection

Tetraniliprole, spinetoram, and emamectin benzoate were firstly dissolved with a small amount of DMSO to 10,000 mg L^{-1}, then further diluted in distilled water with 0.05% Triton X-100 to their LC_{50} concentration of 0.092 mg L^{-1}, 0.161 mg L^{-1}, and 0.194 mg L^{-1}, and equal amounts of DMSO and Triton X-100 were added to the distilled water as control. The maize leaves were cut into rectangle of 2 cm × 0.5 cm, soaked in the medicaments for 15 s, and then took out to dry. The 3rd-instar larvae were fed on the treated maize leaves, and reared at 25 ± 1 °C, photoperiod 16 h: 8 h and relative humidity 50%–70%. Each treatment was repeated for three times with thirty larvae in each replication. The survival active insects were collected and frozen in liquid nitrogen and then stored at −80 °C 48 h after treatment. For tissue dissection, the *S. frugiperda* were rinsed three times with 75% ethanol and washed three times with sterilised deionised water. With a stereomicroscope, the tissues of the head, cuticula, malpighian tubule, fat body, and midgut of *S. frugiperda* were dissected in chilled 1 × phosphate-buffered saline (pH 7.4) with sterile forceps. Tissues dissected from fifteen 3rd-instar larvae and each tissue sample included three independent biological replications. In addition, the egg, the first-instar to sixth-instar larvae, prepupa, pupa, male adult, and female adult of *S. frugiperda* were collected. The tissue and instar samples of *S. frugiperda* were stored at −80 °C for the following experiments.

Detoxification enzyme activity assay

The protein concentration was determined by BCA protein quantification/concentration assay kit from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). The activities of glutathione S-transferase (GST), Carboxylesterase (CarE), and cytochrome P450 monooxygenase (P450) were determined by GST Kit, CarE enzyme activity assay kit and MFO kit from Suzhou Comin Biotechnology Co., LTD (Suzhou, China).

About 0.1 g of the sample was added into the extract in the 1 ml kit and homogenised in ice bath to obtain crude enzyme solution. Determination of P450 enzyme activity: The crude enzyme solution was centrifuged at 4 °C for 20 min at 12,000 g, and the absorption value was determined at 400 nm with a microplate reader. The production of 1 nmol p-nitrophenol per milligram of protein per minute is defined as a unit of enzyme activity. GST enzyme activity determination: The crude enzyme solution was centrifuged at 4 °C for 10 min at 8000 g, and the absorption value was determined at 340 nm wavelength. At 37°C, 1 μmol CDNB was catalysed to bind to GST per mg of protein per minute as a unit of enzyme activity. CarE enzyme activity determination: The crude enzyme solution was centrifuged at 4°C for 30 min at 15,000 rpm and the absorption value was determined at 450 nm. At 37°C, an increase of 1 per milligram hiprotein per minute in catalytic absorbance was defined as one unit of enzyme activity.

Synergism bioassay

In the synergism experiment, 50 μg of piperonyl butoxide (PBO) and diethyl maleate (DEM) were dissolved in 0.5 μl dimethyl

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sulfoxide, and then injected into the antesternum of 3rd-instar larvae of *S. frugiperda*. Two hours later, the injected *S. frugiperda* was treated with tetraniliprole at the concentration of 0.092 mg L^{-1}, spinetoram at the concentration of 0.161 mg L^{-1} and emamectin benzoate at the concentration of 0.194 mg L^{-1}. The mortality was checked 48 hours later.

RNA isolation, library construction and sequencing

The total RNA was extracted by TRIzol reagent (Invitrogen, United States) following the manufacturer's procedure. The total RNA quantity and purity were analysed using a |Bioanalyzer 2100 (Agilent, United States) with RIN number >7.0. The twelve RNA samples from *S. frugiperda* of the three insecticides with LC_{50} treatment groups and the control were then used for mRNA preparation and cDNA library construction, and followed by NovaSeq 600 sequencing using a 150 bp paired-end sequencing strategy. After the final transcriptome was generated, StringTie was used to estimate the expression levels of all transcripts. StringTie was used to perform expression level for mRNAs by calculating FPKM (Fragments Per Kilobase of transcript sequence per Million base pairs). The differentially expressed mRNAs were selected with fold change >2 or fold change <0.5 and with parametric F-test comparing nested linear models (p value < 0.05) by R package edgeR. The sequencing data had been deposited in the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) with the accession number GSE226546.

Identification of P450 genes and phylogenetic analysis

The sequences of P450 genes were searched in the transcriptome and genome of *S. frugiperda* according to the annotation information. The short (<500 bp) and repetitive sequences were removed, and the amino acid sequences of the remaining P450 genes were blasted against the NCBI non-redundant (Nr) protein database to remove the redundant sequences and faulty annotated sequences. A phylogenetic tree was generated by the ClustalW alignment of the amino acid sequences with P450 genes from other insect species using the neighbour-joining method in MEGA 5.05 with 1000 bootstrap replications.

Quantitative real-time PCR

Reverse transcription to cDNA from 1 μg of total RNA was performed using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). The relative mRNA expression was analysed by RT-qPCR. It was carried out by an IQ™ 5 multicolour real-time PCR detection system (BIO-RAD, USA) with the SYBR PrimeScript™ RT-PCR Kit (Takara, Japan). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as an endogenous control and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression (Livak and Schmittgen, 2001). The RT-qPCR reaction included three independent technical and biological replications. The primers were listed in Supplementary Table S1.

RNA interference

The double-stranded RNA (dsRNA) was synthesised using a T7 high yield transcription kit (Invitrogen, USA) according to the manufacturer's instructions. The primers for dsRNA synthesis were listed in Supplementary Table S1. The 3rd-instar larvae of *S. frugiperda* was injected with 0.5 μl (4 μg/μl) dsRNA of target gene. The 3rd-instar larvae of *S. frugiperda* was injected with the ds*EGFP* (enhanced green fluorescent protein) as a control. The injected 3rd-instar larvae were transferred onto the artificial diet for rearing. The experiments included three biological replications, and each replication included fifteen insects. The knockdown efficiency of the dsRNA was analysed through RT-qPCR in 24 h and 48 h, respectively. To assess the sensitivity of the injected *S. frugiperda* to insecticides, the 3rd-instar larvae were treated with tetraniliprole, spinetoram, and emamectin benzoate at LC_{50} concentration. The mortality of *S. frugiperda* was checked after 48 h.

Statistical analysis

The statistical analysis was performed with SPSS 20.0 software (IBM Corporation). The Student's t-test was used to compare the differences between treatments. Data were shown as mean ± standard error (SE). The $P < 0.05$ and $P < 0.01$ were considered statistically significant and very significant differences, respectively.

Results

Effects of tetraniliprole, spinetoram, and emamectin benzoate on the detoxification enzyme activity

The relative level of MFO activity in *S. frugiperda* was significantly increased by 34.32-fold, 29.35-fold, and 3.77-fold under tetraniliprole, spinetoram, and emamectin benzoate treatment at 48 h compared to the control (fig. 1a, d, g). The relative level of GST activity was significantly increased by 1.46-fold, 1.21-fold, and 2.25-fold under tetraniliprole, spinetoram, and emamectin benzoate treatment at 48 h compared to the control (fig. 1c, f, i). The relative level of CarE activity was no significant difference under tetraniliprole and emamectin benzoate treatment at 48 h, respectively (fig. 1b, h). After spinetoram treatment, the activity of CarE decreased significantly compared to the control (fig. 1e).

Synergistic effects of PBO and DEM on the mortality of *S. frugiperda*

After PBO injection, the mortality of *S. frugiperda* was significantly increased by 1.78-fold, 1.72-fold, and 1.41-fold under tetraniliprole, spinetoram, and emamectin benzoate treatment compared to the control (fig. 2a–c). After DEM injection, there was no significant synergistic effect on the mortality of *S. frugiperda* treated with tetraniliprole, spinetoram, and emamectin benzoate compared to the control group (fig. 2d–f).

Overall analysis of sequencing data

Twelve independent cDNA libraries constructed from control, tetraniliprole, spinetoram, and emamectin benzoate treatments were sequenced on the HiSeq 2500 platform, yielding an average of 45,658,671 clean reads (Table S2). Data quality evaluation showed good data quality with Q20 and Q30 ratios of >99% and >98% for all samples and GC counts ranging from 49% to 51%. The total clean reads of the twelve libraries were merged and de novo assembly using Trinity software, yielding a total of 45,707 unigenes (Fig. S1). The distribution of unigenes was between 1000–2000 bp, accounting for 15.4%, with 4487 (9.8%) unigenes with nucleotide lengths greater than 2000 bp.

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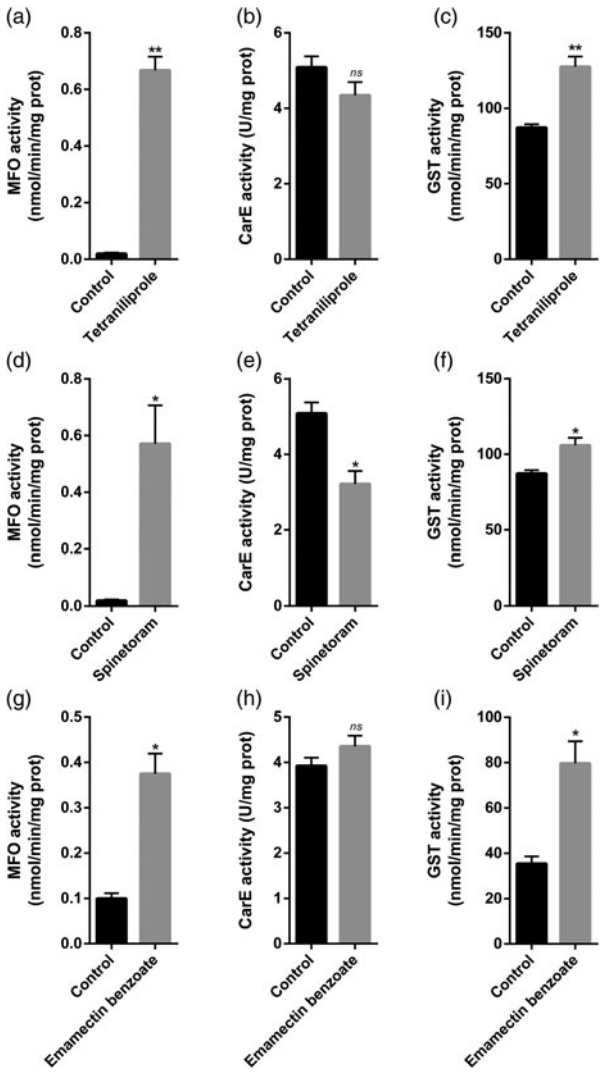

Figure 1. Detoxification enzyme activity after insecticides treatment. (**a, d, g**) MFO activity after treatment of tetraniliprole, spinetoram, and emamectin benzoate. (**b, e, h**) CarE activity after treatment of tetraniliprole, spinetoram, and emamectin benzoate. (**c, f, i**) GST activity after treatment of tetraniliprole, spinetoram, and emamectin benzoate. The asterisk (*) and asterisks (**) indicate $P < 0.05$ and $P < 0.01$. *ns* represents no significant difference.

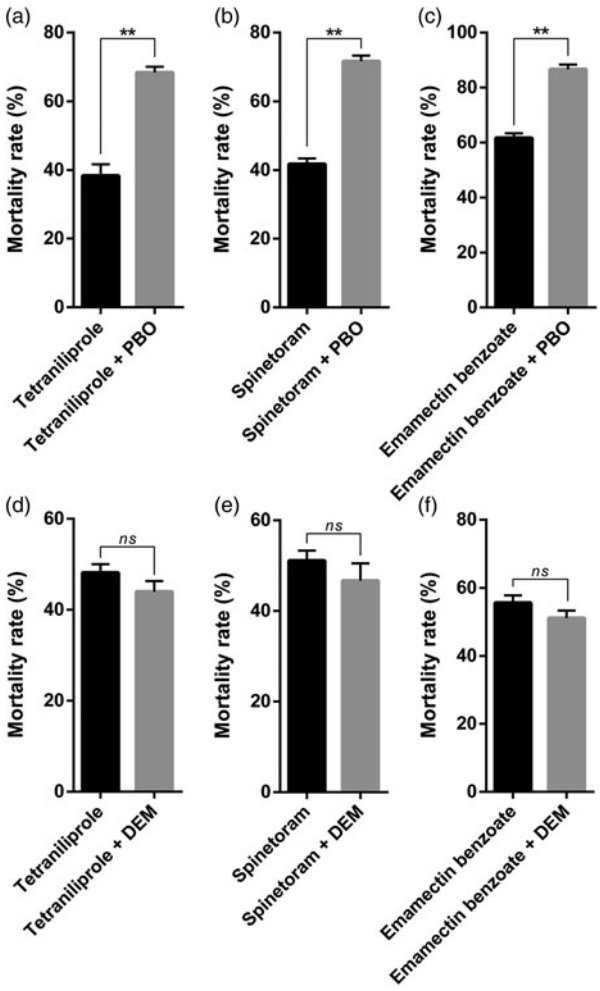

Figure 2. Effects of synergists on mortality of *S. frugiperda*. (**a-c**) The synergism of PBO on *S. frugiperda* mortality after treatment of tetraniliprole, spinetoram, and emamectin benzoate. (**d-f**) The synergism of DEM on *S. frugiperda* mortality after treatment of tetraniliprole, spinetoram, and emamectin benzoate. The asterisks (**) indicate $P < 0.01$. *ns* represents no significant difference.

Functional annotation and analysis of differentially expressed genes (DEGs)

By Blastx homology search, 21,374 of 45,707 unigenes matched entries in the NCBI non-redundant (Nr) protein database with a cut-off *E*-value of 10^{-5}. Most of the unigenes had homologies with *Spodoptera litura* (59.03%) (fig. 3a). To further elucidate the biological functions of these unigenes, Kyoto Encyclopedia of Genes and Genomes (KEGG) assignments were used to classify 45,707 unigenes into different functional groups according to KEGG pathways. Based on sequence homology, 12,731 unigenes (27.85%) were annotated and classified into six biological processes (fig. 3b). Ultimately, a total of 3477 unigenes were aligned to metabolism, including carbohydrate (18.98%), lipid (15.90%), amino acid (14.98%), and cofactors and vitamins (10.07%). Among 12,731 annotated unigenes, most were aligned to genetic information processing (2374), cellular process (1850), and organismal systems (1800).

To identify DEGs associated with the three insecticides (tetraniliprole, spinetoram, and emamectin benzoate versus control samples) at LC_{50} concentration, the DESeq method was used. Of the 3891 DEGs identified in the control and tetraniliprole samples, 2043 were up-regulated and 1848 were down-regulated (fig. 4a). A total of 979 DEGs were detected in the control and spinetoram samples, of which 632 were up-regulated and 347 were down-regulated (fig. 4b). Of the 1634 DEGs identified in the control and emamectin benzoate samples, 721 were up-regulated and 913 were down-regulated (fig. 4c). Cluster heat map showing visual comparison of DEGs between insecticide treatment and control (fig. 4d-f).

Identification and screening of differentially expressed P450 genes

The sequences of P450 genes were identified by a BLAST search against the database of transcriptome and genome in *S. frugiperda*. The complete coding region was confirmed by ORF finder and protein BLAST results (Table S3). These identified P450 genes were classified into different subfamilies via alignment between *S. frugiperda* and other insect species. A total of 102

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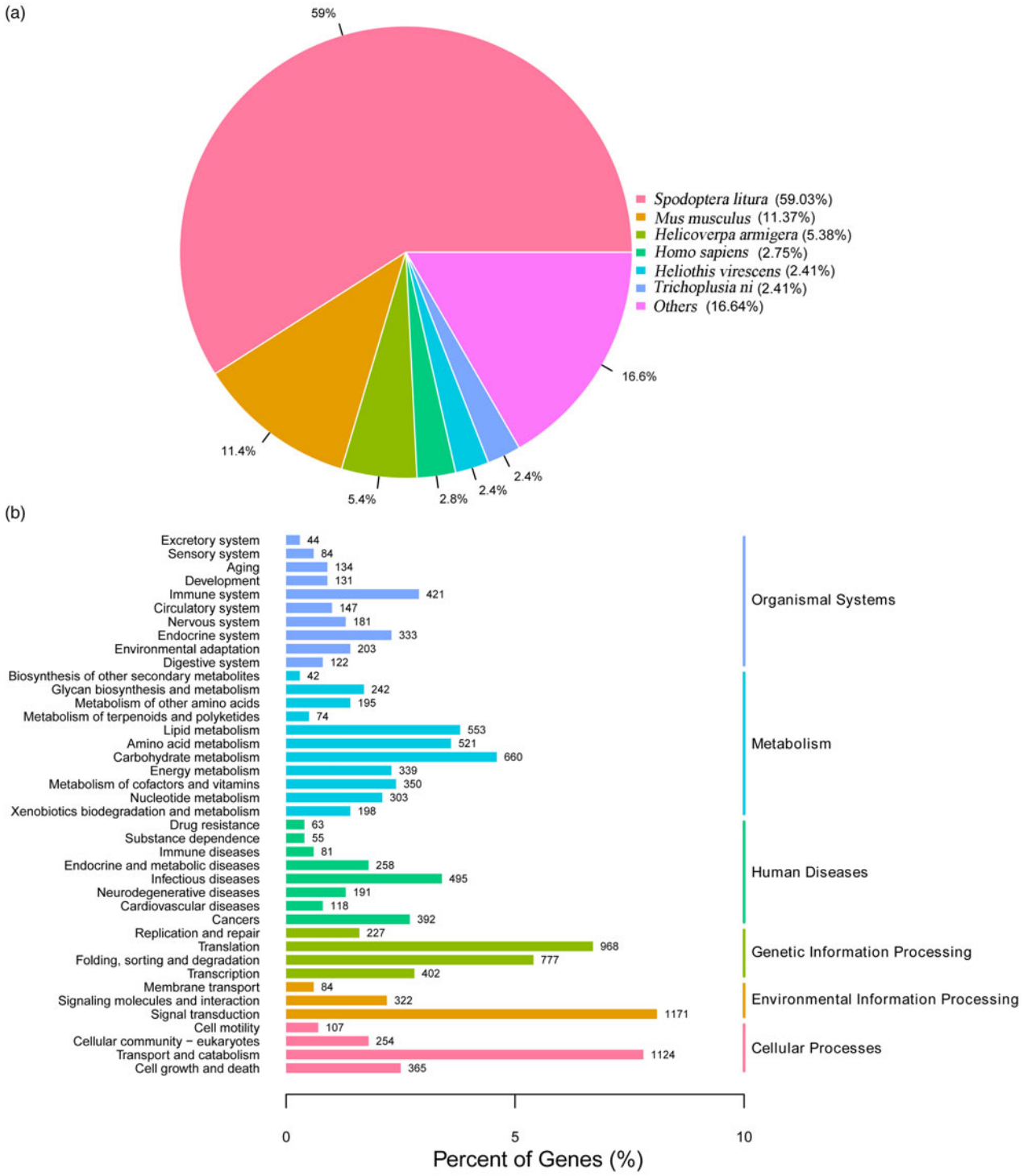

Figure 3. Annotation of *S. frugiperda* transcriptome. (**a**) Species distributions of top Blastx matches to Nr database. (**b**) The unigenes involve in different KEGG classifications.

P450 genes were identified in *S. frugiperda*, including seven in CYP2, forty in CYP3, forty-seven in CYP4, and eight in mitochondrial clade (Table S3, fig. 5). To obtain potential P450 genes that might be involved in the tolerance of tetraniliprole, spinetoram, and emamectin benzoate, the expression induction of sixty-seven differentially expressed P450 genes identified in the transcriptome of *S. frugiperda* was detected after insecticide treatment with LC_{50} concentration by RT-qPCR.

Thirty-one P450 genes were significantly up-regulated after tetraniliprole treatment, including sixteen in CYP3 and fifteen in CYP4 (fig. 6). Twenty-seven P450 genes were significantly up-regulated after spinetoram treatment, including nine in CYP3 and eighteen in CYP4 (fig. 6). Seven P450 genes were significantly up-regulated after emamectin benzoate treatment, including four in CYP3 and three in CYP4 (fig. 6). Among the P450 genes, the expression of ten genes was changed by more

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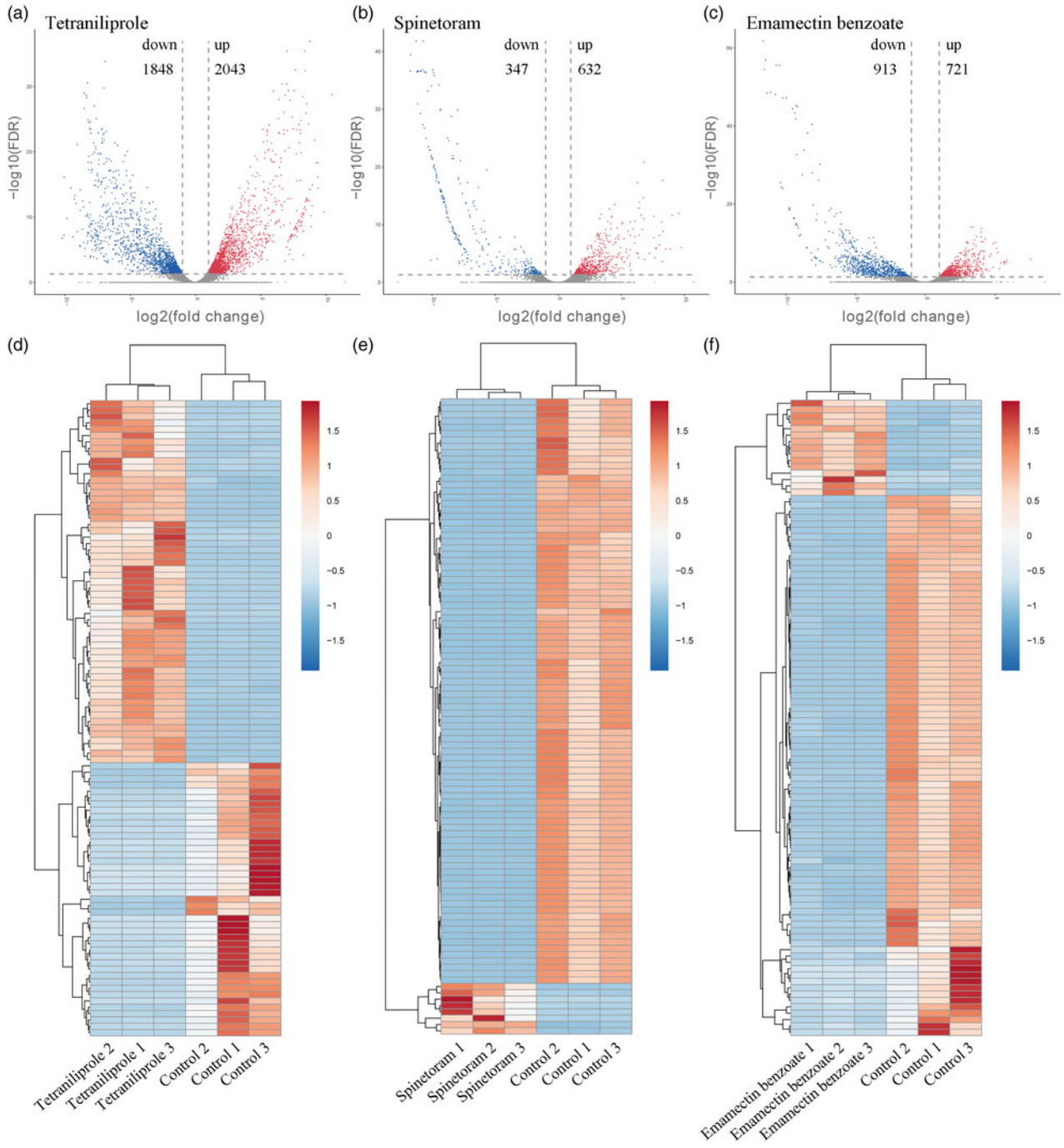

Figure 4. Effect of tetraniliprole, spinetoram, and emamectin benzoate treatments on differential gene expression in *S. frugiperda*. (**a**) Number of up- and down-regulated DEGs and Volcano plot of DEGs in tetraniliprole exposure. (**b**) Number of up- and down-regulated DEGs and Volcano plot of DEGs in spinetoram exposure. (**c**) Number of up- and down-regulated DEGs and Volcano plot of DEGs in emamectin benzoate exposure. Red data points indicated up-regulated unigenes and green data points indicated down-regulated unigenes. (**d**) Heatmap of DEGs between tetraniliprole and control. (**e**) Heatmap of DEGs between spinetoram and control. (**f**) Heatmap of DEGs between emamectin benzoate and control.

than 2.0-fold with tetraniliprole treatment, among which *CYP6AE43v2*, *CYP340AX8v2*, and *CYP341B17v2* were up-regulated more than 4.0-fold. After spinetoram treatment, the expression of twenty P450 genes was changed by more than 2.0-fold, among which *CYP9G17*, *CYP340AA3v2*, *CYP340AA4*, *CYP340L1v2*, *CYP340L7v1*, *CYP340L8v2*, *CYP341B15v2*, and *CYP367A1v1* were up-regulated more than 4.0-fold. Only two P450 genes (*CYP9A75* and *CYP341B15v2*) were up-regulated by more than 2.0-fold after emamectin benzoate treatment. *CYP340L16* was significantly up-regulated after tetraniliprole, spinetoram, and emamectin benzoate treatments (fig. 7). Thirteen genes, including *CYP6AB58v1*, *CYP6AE75*, *CYP9G17*, *CYP321A10v2*, *CYP321A8v2*, *CYP4L13*, *CYP4S9*, *CYP340AX8v2*, *CYP340G2*, *CYP340L19v1*, *CYP340L1v2*, *CYP340L7v1*, and *CYP340L16*, were significantly up-regulated in both tetraniliprole and spinetoram treatments (fig. 7). Five genes, including

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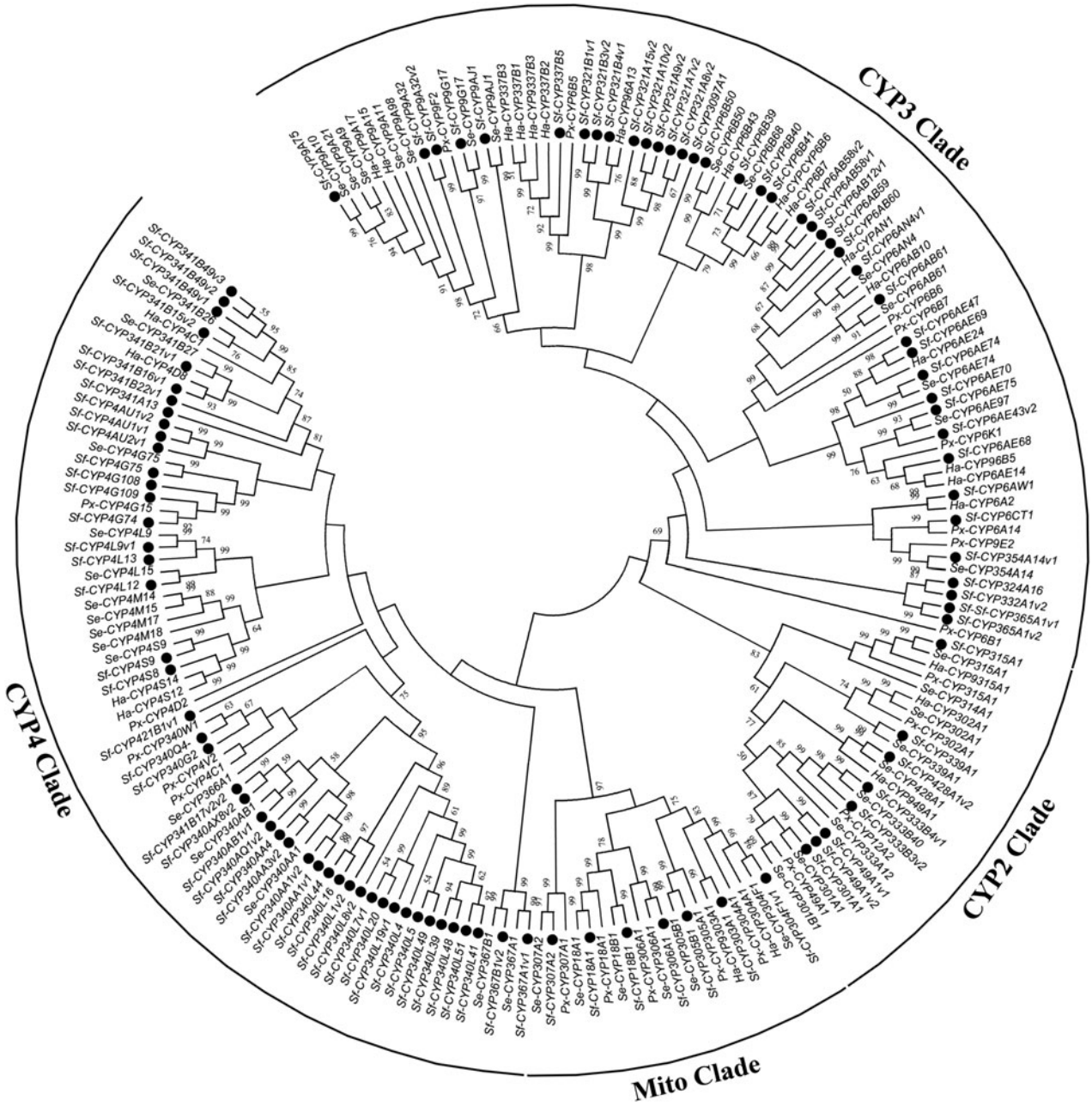

Figure 5. Phylogenetic analysis of P450 genes of *S. frugiperda*. The numbers above the branches indicate the support for the phylogenies, and only values above 50% are shown. Blackened circles indicate the *S. frugiperda* position. Sf, *S. frugiperda*; Se, *Spodoptera exigua*; Px, *Plutella xylostella*; Ha, *Helicoverpa armigera*.

CYP6AE69, *CYP6AE74*, *CYP9A75*, *CYP4G75,* and *CYP340L16*, were significantly up-regulated in both tetraniliprole and emamectin benzoate treatments (fig. 7). Three genes, including *CYP6AB60*, *CYP341B15v2,* and *CYP340L16*, were significantly up-regulated in both spinetoram and emamectin benzoate treatments (fig. 7).

Expression profiles analysis of CYP9A75, CYP340AA4, CYP340AX8v2, CYP340L16, CYP341B15v2, and CYP341B17v2 in S. frugiperda

CYP9A75, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* were selected for further functional study and the expression profiles of them were examined in the tissues of head, cuticula, malpighian tubule, fat body, and midgut and at different developmental stages of *S. frugiperda* (Fig. S2). Tissue-specific expression analysis showed that *CYP9A75* was highly expressed in the midgut, with relative lower expression in cuticula, head, fat body, and malpighian tubule (fig. 8A). The expression of *CYP340AA4* was more abundant in the cuticula (fig. 8B). *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* were exclusively expressed in the head, with considerable low expression levels in the remaining four tissues of cuticula, malpighian tubule, fat body, and midgut (fig. 8c-f).

The results of RT-qPCR analysis showed that the expression of these six P450 genes at different developmental stages had some specificity. *CYP9A75* expression was high in the male pupal

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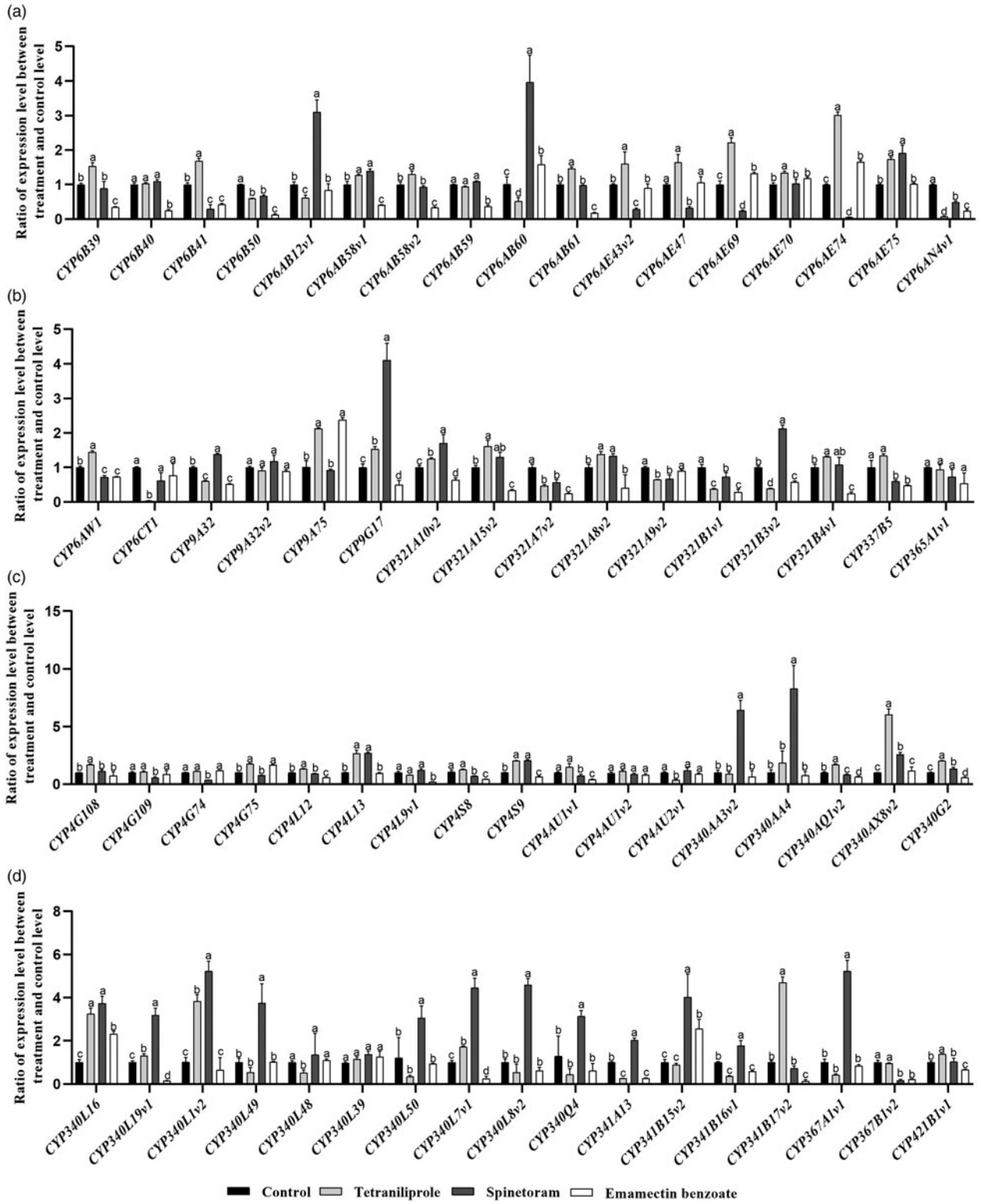

Figure 6. Verification of expression level of P450 genes in *S. frugiperda*. (**a-d**) Relative expression of 67 P450 genes in *S. frugiperda* susceptible strain after exposure to tetraniliprole, spinetoram and emamectin benzoate at the LC_{50} dose. Data and error bar represent the mean and standard error (SE) of three biological replications. Different letters indicate significant differences ($P < 0.05$).

stage at 1 d, relatively low in eggs (fig. 8g). *CYP340AA4* was mainly expressed in 2nd-instar and 4th-instar larvae, with relatively low expression in male pupal stages at 7 d and in eggs (fig. 8h). *CYP340AX8v2* was expressed at all developmental stages, with higher expression in 1st-instar larvae, followed by 2nd-instar larvae and male pupal stages at 1 d (fig. 8i). *CYP340L16* was most abundantly expressed in 4th-instar larvae, followed by 2nd- and 3rd-instar larvae (fig. 8j). *CYP341B15v2* was mainly expressed in adults, followed by

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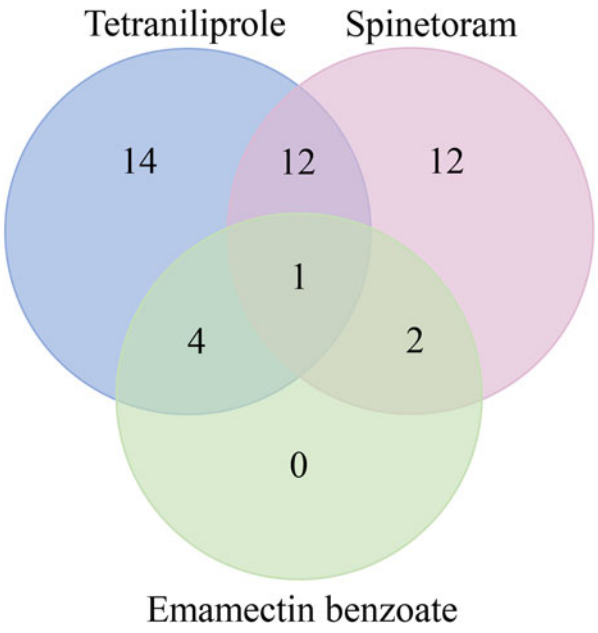

Figure 7. Venn diagram analysis of the numbers of up-regulated P450 genes by tetraniliprole, spinetoram, and emamectin benzoate.

4th-instar and 5th-instar larvae (fig. 8k). *CYP341B17v2* was more highly expressed in 1st-instar larvae (fig. 8l).

CYP340L16, CYP340AX8v2, CYP9A75, CYP341B15v2, CYP341B17v2, and CYP340AA4 impact on the susceptibility of S. frugiperda *to insecticides*

To further investigate the relationship between P450 genes and insecticides tolerance, RNAi was used to knockdown the expression of *CYP340L16*, *CYP340AX8v2*, *CYP9A75*, *CYP341B15v2*, *CYP341B17v2,* and *CYP340AA4* based on the results of insecticide induction. The expression of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* significantly decreased by 46.42%, 60.52%, 52.08%, 68.61%, 46.85%, and 57.33% after injection of dsRNAs compared to the control larvae injected with ds*EGFP* in 12 h (fig. 9a), while the 24 h expression significantly decreased by 59.34%, 74.59%, 62.45%, 54.99%, 37.22%, and 38.26% in comparison to the control (fig. 9h).

The subsequent bioassays demonstrated that knockdown of *CYP340L16* dramatically increased the mortalities of larvae treated with tetraniliprole, spinetoram, and emamectin benzoate by 36.67%, 22.22%, and 23.33% in 12 h, and increased the mortalities by 45.56%, 17.78%, and 43.33% in 24 h (fig. 9b, i). Injection of ds*CYP340AX8v2* significantly increased the mortalities of larvae treated with tetraniliprole and spinetoram by 31.11% and 25.56% in 12 h, and increased the mortalities by 62.22% and 54.44% in 24 h (fig. 9c, j). Injection of ds*CYP9A75* significantly increased the mortalities of larvae treated with tetraniliprole and emamectin benzoate by 16.67% and 34.44% in 12 h, and increased the mortalities by 46.67% and 30.00% in 24 h (fig. 9d, k). Injection of ds*CYP341B15v2* significantly increased the mortalities of larvae treated with emamectin benzoate by 30.00% and 34.44% in 12 h and 24 h (fig. 9e, l). However, no significant difference was observed in mortality between ds*CYP341B15v2*-treated and ds*EGFP*-treated larvae following exposure to spinetoram (fig. 9e, l). Knockdown of *CYP341B17v2*

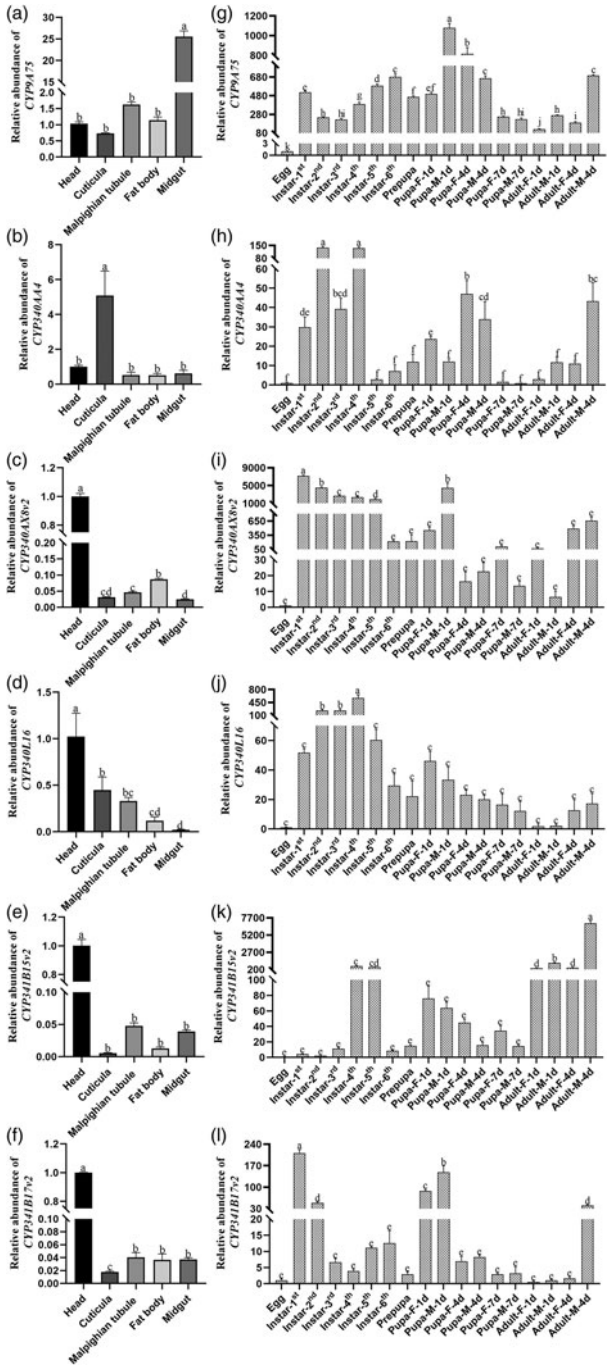

Figure 8. Expression patterns of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* in *S. frugiperda*. (**a-f**) Expression of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* in different tissues of *S. frugiperda*. (**g-l**) Expression of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* at different developmental stages of *S. frugiperda*. Pupa-F-1 d, the female pupal stage at 1 d; Pupa-M-1 d, the male pupal stage at 1 d; Pupa-F-4 d, the female pupal stage at 4 d; Pupa-M-4 d, the male pupal stage at 4 d; Pupa-F-7 d, the female pupal stage at 7 d; Pupa-M-7 d, the male pupal stage at 7 d; Adult-F-4 d, the female adult stage at 4 d; Adult -M-4 d, the male adult stage at 4 d. Data and error bar represent the mean and standard error (SE) of three biological replicates. Different letters indicate significant differences ($P < 0.05$).

dramatically increased the mortalities of larvae treated with tetraniliprole by 20.00% in 12 h and 18.89% in 24 h (fig. 9f, m). Knockdown of *CYP340AA4* dramatically increased the mortalities

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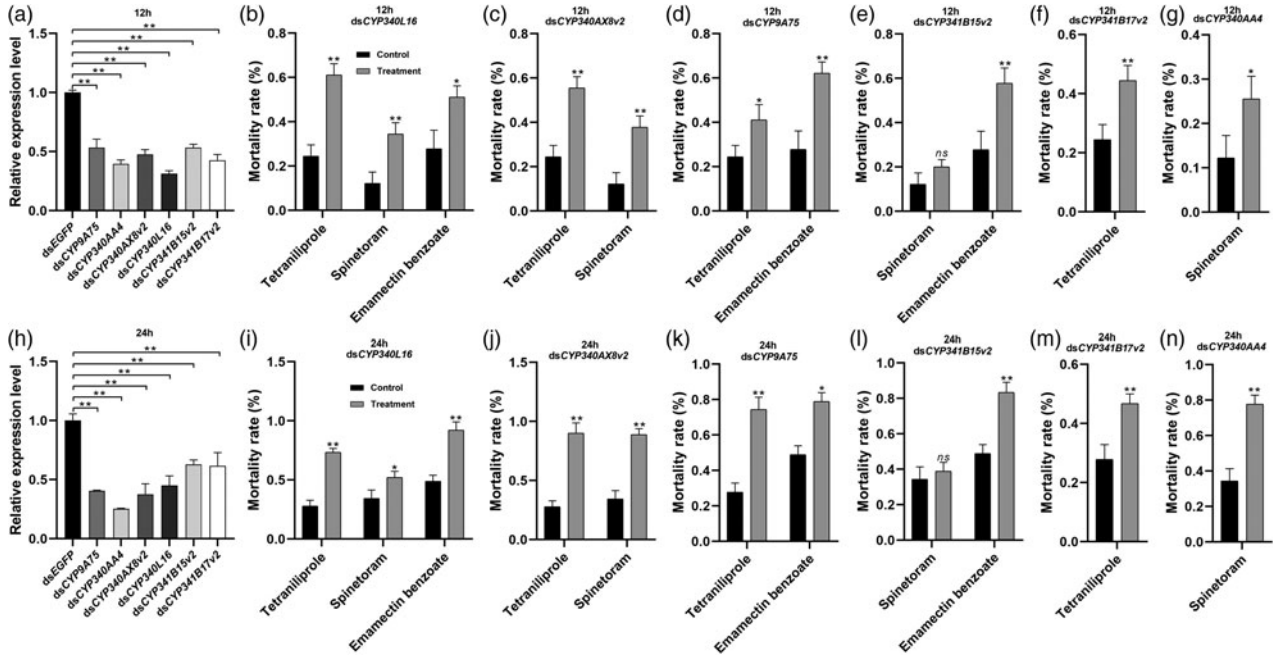

Figure 9. Functional analysis of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B18v2* by RNAi. (**a, h**) Expression of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B18v2* in *S. frugiperda* at 12 h and 24 h after dsRNA injection. (**b, i**) Mortality rates of ds*CYP340L16* injected larvae treated with LC_{50} concentration of tetraniliprole, spinetoram, and emamectin benzoate at 12 h and 24 h. (**c, j**) Mortality rates of ds*CYP340AX8v2* injected larvae treated with LC_{50} concentration of tetraniliprole and spinetoram at 12 h and 24 h. (**d, k**) Mortality rates of ds*CYP9A75* injected larvae treated with LC_{50} concentration of tetraniliprole and emamectin benzoate at 12 h and 24 h. (**e, l**) Mortality rates of ds*CYP341B15v2* injected larvae treated with LC_{50} concentration of spinetoram and emamectin benzoate at 12 h and 24 h. (**f, m**) Mortality rates of ds*CYP341B18v2* injected larvae treated with LC_{50} concentration of tetraniliprole at 12 h and 24 h. (**g, n**) Mortality rates of ds*CYP340AA4* injected larvae treated with LC_{50} concentration of spinetoram at 12 h and 24 h. The asterisk (*) and asterisks (**) indicate $P < 0.05$ and $P < 0.01$. *ns* represents no significant difference.

of larvae treated with spinetoram by 13.33% in 12 h and 43.33% in 24 h (fig. 9g, n).

Discussion

Tetraniliprole, spinetoram, and emamectin benzoate have high insecticidal activity against lepidopteran pests and are widely used in the control of *S. frugiperda*. The active ingredient of spinetoram is a natural product from the fermentation of the soil-borne *Saccharopolyspora spinosa*. Emamectin benzoate is derived, synthesised, optimised and modified from the fermentation product avermectin B1 as a lead compound. Spinetoram and emamectin benzoate are both broad-spectrum, high effective, low toxicity, low residue, and easily degradable green insecticides. Tetraniliprole is a new diamide insecticide. In addition, mechanisms of resistance to biogenic and chemical insecticides may differ in insects. Therefore, these three insecticides were used to treat *S. frugiperda* and analyse the resistance mechanism of them.

Metabolic resistance is one of the main mechanisms of insect resistance to insecticides. P450, GST, and CarE are three major detoxifying enzymes of insects, which can metabolise exogenous compounds such as insecticides (Wei *et al.*, 2001; Li *et al.*, 2007; Cui *et al.*, 2011). In this study, enzymatic activities of P450 and GST were significantly increased after treatment with tetraniliprole, spinetoram, and emamectin benzoate compared to the control group. PBO and DEM are multifunctional oxidase and esterase inhibitors that act synergistically by inhibiting metabolic enzymes in insects and reducing the degradation of insecticides in their tissues. PBO injection significantly increased the susceptibility of *S. frugiperda* to insecticides, while DEM injection had no significant effect on the susceptibility. Enhanced P450 detoxification enzyme activity was identified as the major driver of tolerance to insecticides (Lu *et al.*, 2019a, 2019b, 2020). It has been demonstrated that the cytochrome P450 is involved in the detoxification metabolism of tannin in *S. litura* and could increase the tolerance of the *Tribolium castaneum* to plant toxicants (Zhang *et al.*, 2021; Zhao *et al.*, 2022). Therefore, the cytochrome P450 might be involved in the detoxification metabolism of tetraniliprole, spinetoram, and emamectin benzoate in *S. frugiperda*.

Transcriptome sequencing has given us the opportunity to gain a comprehensive understanding of gene expression following insecticide treatment and to detect genes that may be involved in insecticide metabolism. In this study, 102 P450 genes were identified in *S. frugiperda*. Previous studies showed that there were 180 P450 genes in *Culex quinquefasciatus* (Yang and Liu, 2011), 90 P450 genes in *Drosophila melanogaster* (Tijet *et al.*, 2001), 85 P450 genes in *Plutella xylostella* (Yu *et al.*, 2015), 84 P450 genes in *Bombyx mori* (Ai *et al.*, 2011) and 36 P450 genes in *Pediculus humans* (Lee *et al.*, 2010). Differences in the number of insect P450 genes may be related to the depth of sequencing or the insect's adaptation to its environment. In insects, CYP3 and CYP4 clades are highly correlated with the development of insecticide resistance (Yang *et al.*, 2013; Li *et al.*, 2018; Wang *et al.*, 2019). In this study, forty and forty-seven P450 genes were identified in CYP3 and CYP4 clades, respectively. In *C. quinquefasciatus*, 72 P450 genes in CYP3 clade and 83 P450 genes in CYP4 clade were identified, and there were 36 P450 genes in CYP3 clade and 32 P450 genes in CYP4 clade, 26 P450 genes

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in CYP3 clade and 36 P450 genes in CYP4 clade, 36 P450 genes in CYP3 clade and 33 P450 genes in CYP4 clade, 11 P450 genes in CYP3 clade and 9 P450 genes in CYP4 clade, in *D. melanogaster*, *P. xylostella*, *B. mori* and *P. humans*, respectively (Tijet *et al.*, 2001; Lee *et al.*, 2010; Ai *et al.*, 2011; Yang and Liu, 2011; Yu *et al.*, 2015). These results suggested that P450 genes in CYP3 and CYP4 clades might play important roles in the metabolism of tetraniliprole, spinetoram, and emamectin benzoate in *S. frugiperda*.

It has been shown that chlorantraniliprole, emamectin benzoate, and *Bacillus thuringiensis* affected several P450 genes in *S. frugiperda* resulting in up-regulation of *CYP4G75*, *CYP6AB12*, *CYP321A7*, *CYP321A8*, *CYP321A9*, *CYP321A10*, *CYP321B1*, *CYP321B5*, *CYP6AE44,* and *CYP6AE43* (Zhang *et al.*, 2020a). In order to obtain potential P450 genes that may be associated with detoxification of tetraniliprole, spinetoram, and emamectin benzoate, sixty-seven differentially expressed P450 genes were selected for RT-qPCR validation. Thirteen P450 genes of *CYP6AB58v1*, *CYP6AE75*, *CYP9G17*, *CYP321A10v2*, *CYP321A8v2*, *CYP4L13*, *CYP4S9*, *CYP340AX8v2*, *CYP340G2*, *CYP340L19v1*, *CYP340L1v2*, *CYP340L7v1,* and *CYP340L16* were significantly up-regulated in tetraniliprole and spinetoram exposure. Five P450 genes of *CYP6AE69*, *CYP6AE74*, *CYP9A75*, *CYP4G75,* and *CYP340L16* were significantly up-regulated in tetraniliprole and emamectin benzoate exposure. Three P450 genes of *CYP6AB60*, *CYP341B15v2,* and *CYP340L16* were significantly up-regulated in spinetoram and emamectin benzoate exposure. The gene *CYP340L16* was significantly up-regulated in tetraniliprole, spinetoram, and emamectin benzoate exposure. These results suggested that different types of insecticides could induce up-regulation of some of the same P450 genes in the same insect species, thereby leading to the development of cross-resistance. In *Bemisia tabaci*, *CYP6CM1* conferred the cross-resistance between neonicotinoid and pymetrozine (Nauen *et al.*, 2013). The P450, *CYP6Z1* conferred cross-resistance between carbamate and pyrethroid in a major African malaria vector, *Anopheles funestus* (Ibrahim *et al.*, 2016). In *Tetranychus cinnabarinus*, *CYP389C16* contributed to the cross-resistance between cyflumetofen and pyridaben (Feng *et al.*, 2020). In addition to the up-regulated genes, four P450 genes were significantly down-regulated after induction of tetraniliprole, spinetoram, and emamectin benzoate, namely *CYP6B50*, *CYP6AN4v1*, *CYP321A7v2*, and *CYP321B3v2*. They may be involved in pest fitness cost or other biological processes.

Cytochrome P450 genes were expressed in one or some specific developmental stages or tissues of insects and might be associated with specific functions (Chung *et al.*, 2009). We selected six genes that were highly up-regulated following induction by at least one of the three insecticides and analysed their tissue and stage-specific expression patterns. Tissue-specific expression analysis showed that *CYP9A75* was highly expressed in the midgut, which is the primary digestion tissue and important site for storage of various metabolic enzymes (Terra *et al.*, 2018). CYP340 has been reported to be a lepidoptera-specific family with midgut-specific expression and abundant transposable elements for each gene in *P. xylostella*, and family members are organised in clusters (Yu *et al.*, 2015). But in our research, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* were exclusively expressed in the head. It has been reported that lepidopteral specific CYP367s expressed mainly in the head tissue in *P. xylostella* suggested a role in olfaction or detoxification (Yu *et al.*, 2015). And in sects, the head tissue was the control centre for many behaviours (Jia *et al.*, 2010; Liu *et al.*, 2019). Therefore, we have reason to think that these genes might also be involved in the behaviour regulation of *S. frugiperda* in addition to the detoxification of insecticides. The expression of resistance-related genes was closely linked to the developmental period of the insects (Mao *et al.*, 2009). In our study, most of the genes were highly expressed during the feeding stage, especially during the 2th-instar to 6th-instar larval stage, when the larva ingests and digests more food, and thus are more likely to be exposed to foreign substances such as pesticides.

To further investigate the role of P450 genes in the development of insecticide resistance, functional validation was performed using RNAi technology. As a powerful tool to study the function of specific genes *in vivo* through function loss experiments, RNAi has been widely used in many insects (Zhang *et al.*, 2020b, 2022b; Xiao and Lu, 2022; Yang *et al.*, 2022b). In this study, silencing *CYP340L16* dramatically increased the mortality of *S. frugiperda* larvae treated with tetraniliprole, spinetoram, and emamectin benzoate at 12 h and 24 h, indicating that *CYP340L16* might be a broad-spectrum P450 gene responding to multiple selection pressures from insecticides. *CYP340AX8v2* and *CYP9A75* played an important role in the sensitivity of two insecticides in *S. frugiperda*. *CYP341B15v2*, *CYP341B17v2,* and *CYP340AA4* only played an important role in individual insecticide sensitivity in *S. frugiperda*. Different P450 gene members have different substrates, but they may overlap to adapt to changes in the environment. Expression of multiple P450 genes induced in response to exogenous compounds such as pesticides showed evolutionary plasticity (Qiu, 2014).

In summary, our study described the effects of tetraniliprole, spinetoram, and emamectin benzoate on P450 enzyme activity and P450 gene expression in *S. frugiperda*. The expression patterns of *CYP9A75*, *CYP340AA4*, *CYP340AX8v2*, *CYP340L16*, *CYP341B15v2,* and *CYP341B17v2* in *S. frugiperda* were analysed in different tissues and at different developmental stages, and the functions in insecticides detoxification of these six P450 genes were further validated by RNAi. The results provide a basis for further exploration of the detoxification mechanism to tetraniliprole, spinetoram, and emamectin benzoate in *S. frugiperda*.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S000748532300038X.

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Competing interests. None.

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