Eurasia Journal of Science and Technology
ISSN: 2663-1016
DOI: https://doi.org/10.61784/ejst3033

TRANSCRIPTOME ANALYSIS OF THIACLOPRID-RESISTANT MYZUS PERSICAE REVEALS THE OVEREXPRESSION OF METABOLIC DETOXIFICATION GENES

JinFeng Hu*, XianZhi Zhou, Lei Lin
Fujian Engineering Research Center for Green Pest Management, Key Laboratory for Monitoring and Integrated Management of Crop Pests, Institute of Plant Protection, Fujian Academy of Agricultural Sciences, Fuzhou, China.
Corresponding author: JinFeng Hu, Email: hujinfeng007@sina.com

Abstract: The green peach aphid *Myzus persicae* (Sulzer, 1776) (GPA) is an economic pest that damages many agricultural crops and has evolved resistance to various classes of insecticides, including neonicotinoids. Thiacloprid is a broad-spectrum neonicotinoid with low toxicity to bees. After fifty selection cycles from a susceptible strain (FAA-S), a strain (THG-R) with 1,201.2-fold resistance to thiacloprid was obtained under laboratory conditions. The THG-R strain exhibited a high level of cross-resistance to imidacloprid (894.3-fold), moderate levels of cross-resistance to acetamiprid (48.7-fold), dinotefuran (44.7-fold), flupyradifurone (24.9-fold), and sulfoxaflor (14.6-fold), and low levels of cross-resistance to thiamethoxam (9.4-fold) and clothianidin (6.4-fold). Synergism and activity tests indicated the possible involvement of cytochrome P450 in the detoxification resistance to thiacloprid. Transcriptome profiling of the THG-R and FAA-S strains identified 72 differentially expressed genes (DEGs) related to insecticide detoxification, including 15 upregulated and 57 downregulated genes. Resistance was not conferred by known detoxification mechanisms of resistance to neonicotinoid insecticides and sulfoxaflor, but rather by overexpression of the P450 genes *CYP6CY3* and *CYP380c40* and UDP-glucuronosyltransferase (UGT) gene UGT344P2. The adenosine triphosphate (ATP)-binding cassette transporters (ABCs) *Mplethal(2)03659.1* and thioesterase *MpThem6* were highly overexpressed (30.3-fold and 7-fold, respectively) in the THG-R strain. These findings provided another possible enhanced metabolic route responsible for neonicotinoid resistance in GPA.
Keywords: *Myzus persicae*; Thiacloprid; Resistance development; Cross-resistance; Detoxification mechanism

1 INTRODUCTION

The green peach aphid *Myzus persicae* (Sulzer, 1776) (GPA) is one of the most economically important agricultural pests. It can feed on more than 400 plant species belonging to 50 families, including potato, tobacco, and eggplant. The GPA is notorious because it can act as a vector to transmit 115 different plant viruses, accounting for 67.7% of the aphid-vector viruses [1]. Insecticides are the foundation of the management of the GPA in China and other countries. More than 70 active ingredients across a range of modes of action (MOA) groups have been registered for use to control GPA globally [2]. However, heavy repeated applications of insecticides have led to field populations of GPA developing resistance to most used insecticides, such as organophosphates, carbamates, pyrethroids, and pyridine azomethine derivatives [3-5].

Neonicotinoids are systemic insecticides that act on the nicotinic acetylcholine receptors (nAChR) of insects and have become the most widely used pesticides in the world [6]. The major neonicotinoids in commercial pesticides are acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, and thiamethoxam [2]. Pest resistance to neonicotinoids remains a growing concern, with GPA resistance to imidacloprid in fields in Japan reported in 1996 [7]. Based on the Arthropod Pesticide Resistance Database (APRD) [8], 132 cases of neonicotinoid resistance involve the GPA, with only *Bemisia tabaci* and *Nilaparvata lugens* exhibiting more cases of resistance. Field monitoring data have shown that GPA has developed resistance to imidacloprid [9, 10], thiamethoxam [9], and acetamiprid [10]. The first chloronicotinyl insecticide, thiacloprid [(Z)-3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylidenecyanamide] was discovered by Bayer and commercialized in 2000 with a much broader spectrum of pests targeted and lower acute toxicity to bees than other neonicotinoids [11,12]. Thiacloprid entered the Chinese market in 2014 and has been registered to control GPA in cabbage, potato, and peach. To date, neonicotinoids have remained an effective control measure against GPA in China [4], although recent resistance monitoring work has indicated a lack of efficacy of imidacloprid in some districts [5]. Previous studies have mainly focused on the mechanism of imidacloprid resistance development in GPA [9,13,14], but the existing knowledge of thiacloprid is limited to its cross-resistance with other neonicotinoids.

Metabolic resistance is much more common in insects resistant to neonicotinoids, highlighting the role of cytochrome P450 as the principal agent. The overexpression of P450 genes has been widely reported to be responsible for neonicotinoid resistance in insects, such as *Drosophila melanogaster* (*CYP6G1*) [15], *B. tabaci* (*CYP6CM1*, *CYP4C64*) [16], *Aphis gossypii* (*CYP6CY14, CYP6CY22*, and *CYP6UN1*) [17], and *N. lugens* (*CYP6AY1*, *CYP6ER1*, *CYP4CE1*, and *CYP6CW1*) [18]. Biochemical surveys have also indicated an increase in the activities of detoxification enzymes, such as cytochrome P450 monooxygenases (P450), glutathione S-transferases (GSTs), and carboxylesterases (CarEs) in

imidacloprid-resistant aphids [4]. In GPA, the enhanced expression of a P450 gene, *CYP6CY3*, associated with imidacloprid resistance is much more common in individuals in the field than the R81T mutation of nAChR, and the molecular mechanisms of *CYP6CY3*-mediated resistance to some neonicotinoids have been well documented [19]. In recent years, new techniques and advances in genomic research revealed other metabolic enzymes that were also attributed to neonicotinoid resistance. For example, the overexpression of uridine diphosphate (UDP)-glycosyltransferases (UGTs) is associated with sulfoxaflor resistance in GPA [20] and thiamethoxam resistance in *A. gossypii* [21].

Under laboratory conditions, we obtained a high thiacloprid-resistant strain of GPA by successively screening a susceptible strain with thiacloprid. In the present study, we investigated the cross-resistance spectrum between thiacloprid and other insecticides. The present study has also identified genome-wide transcriptional changes between the thiacloprid-susceptible and resistant GPA strains. Genes involved in insecticide metabolic and detoxification, including P450, UGTs, adenosine triphosphate (ATP)-binding cassette transporters (ABCs), GSTs. and esterase, were identified, which provided insights into the metabolic mechanisms of GPS to thiacloprid. This information could be used to slow-down and eventually overcome pest resistance to thiacloprid.

2 MATERIALS AND METHODS

2.1 Insects

Two GPA strains, (FAA-S and THG-R), were used in the study. The THG-R strain was reared in the laboratory without exposure to insecticides after being collected from *Arabidopsis thaliana* in the Jianxin District of Fuzhou, China, in 2008 and was susceptible to neonicotinoids. The THG-R strain was established from the FAA-S strain by successive screening with thiacloprid for more than 50 generations in the laboratory. More than 50,000 adults were selected with direct spraying in each generation. Both GPA strains were reared on pakchoi cabbage seedlings, *Brassica chinensis*, under controlled conditions of 19−22°C, 60% relative humidity, and a photoperiod of 16:8 h (light:dark).

2.2 Pesticides, SYNERGISTS, and Other Chemicals

The insecticides used for bioassays included thiacloprid (97.5% purity; Bayer AG, Germany), imidacloprid (97% purity; Bayer AG, Germany), flupyradifurone (96% purity; Bayer AG, Germany), acetamiprid (99% purity; Ningbo Sanjiang Yinong Chemical Co., Ltd., China), thiamethoxam (98%purity; Syngenta Group, Switzerland), clothianidin (98% purity; Hailir Chemicals Co., Ltd., China), dinotefuran (96% purity; Hebei Veyong Bio-chemical Co., Ltd., China), sulfoxaflor (95.9% purity; Corteva Agriscience Chile Ltda. of China, China) and triflumezopyrim (95.9% purity; Corteva Agriscience Chile Ltda. of China, China). Synergists piperonyl butoxide (PBO; reagent grade), S,S,S-tributyl phosphorotrithioate (DEF; reagent grade), and diethyl maleate (DEM; reagent grade) and Chemicals, were purchased from Sigma-Aldrich Shanghai Trading Co., Ltd., United States.

Ethylenediaminetetraacetic acid (EDTA), albumin bovine (BSA), p-nitrophenol, reduced glutathione (GSH), coenzyme NADPH and sodium dodecyl sulfate (SDS) were from Shanghai Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). Eserine, p-nitroanisole, fast blue B salt, α-naphthyl acetate (α-NA), n-phenylthiourea (PTU), coomassie brilliant blue G250, 1-chloro-2,4-dinitrochlorobenzene (CDNB), phenylmethylsulfonyl fluoride (PMSF), DL-dithiothreitol (DTT), Triton X-100 and other chemicals were also purchased from Sigma-Aldrich Shanghai Trading Co., Ltd., United States

2.3 Susceptible Test Methods

The leaf-dip method was used to measure the toxicity of insecticides, as recommended by the Insecticide Resistance Action Committee (IRAC) [2]. Five to seven concentrations of insecticides were prepared using 0.1% Triton-100 and four replicates were conducted for each treatment. Clean leaf-discs of *Brassica oleracea* L. were cut using metal tubes and dipped in the test liquid for 10 s. The air-dried leaf-discs were placed onto 1% agar plate (20 mm depth) in a petri dish (30 mm diameter and 40 mm depth). Twenty apterous adults were then transferred onto each of the leaf discs using a paint brush and each unit was covered with a close-fitting, ventilated lid. Mortality was assessed after 3 d. Aphids that could not right themselves within 10 s once turned on their back were considered to be dead.

The effects of three synergists (piperonyl butoxide (PBO), S,S,S-tributyl phosphorotrithioate (DEF), and diethyl maleate (DEM)) in combination with thiacloprid against GPA were evaluated using the bioassay method mentioned above. The highest doses of PBO, DEF, and DEM for the susceptible strain that led to zero mortality were 0.8, 0.5, and 1 g L^{-1}, respectively, using the bioassay method. Apterous adult aphids were exposed to leaf discs that were treated with PBO, DEF, or DEM and the thiacloprid mixtures.

2.4 Determination of P450s, GST, and Carboxylesterase Activity

The monooxygenase enzyme (MFO) activity was measured according to Shang's method [22]. Sixty adult aphids were homogenized in 2.0 mL ice-cold phosphate-buffered saline (PBS, 0.04 mol L^{-1}, pH 7.8). The supernatant obtained by centrifuging 10,000 × *g* for 10 min at 4°C was added to a reaction unit containing NADPH and nitroanisole (0.05 mol L^{-1} in acetone) as a substrate. Hydrochloric acid (HCl, 1 mol L^{-1}) was added to terminate the reaction after incubation

for 30 min at 37°C. Then, the reaction unit was extracted by a sodium hydroxide (NaOH) and chloroform solution. Finally, the optical density (OD) of the enzyme source was recorded at 400 nm using a microplate reader. The specific activity was obtained according to a nitrophenol standard curve and the protein concentration of the enzyme source.

Carboxylesterase activity was determined using α-naphthyl acetate (α-NA) as the substrate according to the method reported by Van Asperen [23], with some modifications. Sixty adult aphids were homogenized in 2.0 mL of ice-cold PBS (0.04 mol L^{-1}, pH 7.0) and centrifuged at 4°C at 10,000 × *g* for 10 min. The supernatant fluid was used as the enzyme source. The supernatant fluid was kept on ice before the analysis. After the substrates a-NA (3×10^{-4} mol L^{-1}) and physostigmine (10^{-4} mol L^{-1}) were incubated with the enzyme source for 10 min at 37°C, the color developing agent [mass fraction 5%, SDS; mass fraction, 1% fast blue B salt = 5:2 (v/v)] was added. Absorbance at 600 nm (450 nm) was recorded at the same time in a microplate reader (SPECTRA max PLUS384, Molecular Devices, San Jose, CA, USA). The specific activity of CarEs was calculated based on an a-naphthol standard curve and the protein concentration of the enzyme source.

The activity of GSTs was determined according to a slightly modified published method involving 1-chloro-2, 4-dinitrobenzene (CDNB) [24]. Sixty adult aphids were homogenized in 2.0 mL ice-cold PBS (0.04 mol L^{-1}, pH 7.5), and the supernatant solution was used after being centrifuged at 10,000 × *g* for 10 min at 4°C. Briefly, a 300 μL reaction mixture containing 100 μL diluted enzyme solution, 100 μL CDNB (1.2 mM) substrate solution and 100 μL glutathione (GSH) (6 mM) was prepared, after which the absorbance was measured at 340 nm using the kinetic model for 10 min. The results were determined based on the protein concentration of an enzyme source and the specific activity was converted from an OD value.

The protein contents of the enzyme solutions were determined by the Bradford method [25]. Serial dilutions of a bovine serum albumin (BSA) solution and samples were measured together and the protein content of the samples was calculated by a standard curve based on the BSA solutions. The diluted enzyme solutions (50 μL) were mixed with Coomassie Blue (200 μL). After incubating at 25°C for 10 min, the absorbance at 595 nm was measured. All experiments were repeated three times, and the average values were obtained from the triple-replicated data.

2.5 Sample Collection and RNA Isolation

About 1800 aphids (300 mg, all instars, winged and wingless nymphs, and adults) from susceptible and thiacloprid-resistant strains were collected and the total RNA was extracted with TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the product protocol. The RNA integrity was assessed using the RNA Nano 6000 assay kit from the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

2.6 Library Preparation for Transcriptome Sequencing

The sequencing library was prepared by Novogene Co., Ltd. (Beijing, China) and sequenced on an Illumina Novaseq platform. Briefly, mRNA isolated from the FAA-S and THG-R strains, with three replicates per strain, was purified from the total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. After the adenylation of 3′ ends of DNA fragments, an adaptor with a hairpin loop structure was ligated to prepare for hybridization. Finally, the polymerase chain reaction (PCR) products were purified (AMPure XP system, Beckman Coulter, Brea, CA, USA) and the library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.7 Read Mapping and Expression Quantification

The clean reads were mapped to the GDP clone O genome using Hisat2 v2.0.5, and the mapped reads were counted using Htseq v0.11.2 [26] based on the MyzpeCyc clone O database. The expected number of Fragments Per Kilobase of Transcript Sequence per Million base pairs sequenced (FPKM) was used to estimate gene expression levels [27].

2.8 Differential Expression Analysis and Annotation

The DEG-Seq 2R package (1.20.0) was used to identify significant differentially expressed genes (DEGs). Genes with an adjusted P-value < 0.05 and a fold-change > 2 found by DEG-Seq 2R were assigned as differentially expressed. The functional annotation and classification of the DEGs were performed using the gene ontology (GO) database, and biological pathway annotations were obtained using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database by the clusterProfiler R package.

2.9 Quantitative Real-Time PCR (qRT-PCR) Analysis

A qRT-PCR analysis was performed to validate the expression profiles of selected metabolic detoxification genes, including DEGs and other detoxification genes that were reported to be associated with insecticide resistance in GPA, such as the cytochrome P450 genes, g7429, g25170, CYP6CY3 (KF218356.1) and CYP380C40 (OM677847)), GSTs, E4 (X74554.1), ABCs (g10538, g20740, and g10536), and the UDP-glucuronosyltransferase (UGT) genes ((UGT344P2 (OM677846), g20497, g20587, g20497, and g3205), and β-actin, EF1α as the internal control. The primers used in the

qRT-PCR are summarized in Table 1. The qRT-PCR reactions were conducted on a qTOWER 2.0/2.2 qRT-PCR thermal cycler (Analytik Jena, Jena, Germany) using 2 × SYBR® Green Master Mix (DBI). Three replicates were set using independent samples for each qRT-PCR experiment. The relative gene expression was calculated automatically using the qPCRsoft 3.2 software. The relative gene expression $2^{\wedge-\triangle\triangle Ct}$ method was used to calculate the relative fold gene expression of samples [28].

Table 1 Primers Used for the qRT-PCR Validation of DEGs

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
β-actin	GGTGTCTCACACACAGTGCC	CGGCGGTGGTGGTGAAGCTG
EF1α	CCGATGTCTATGTCTGCTAAGG	CATGATTTGAGCCTCGCCAA
GAPDH	GCGGTTTCGACGTGTCAGTTTG	CCGGAGCCCACAATGCACAC
CYP6CY3	CGGGGTGACGATCATCTATT	GGGTGGTCTTTTGACAAAGC Ss
E4	AAACTTTCCTTTTACACCGTT	TCTAAGCCAAGAAATGTTGAAA
GST	ACCCATGAGATTCCTTCTGTCC	TTCCAACACAGGCACTTTGC
CYP380C40	GCCAGTGATACTCAGAGAACT	AGCGACATTTTCTGGGCTGAA
UGT344P2	AATGGTTTCCTCAGCGTGAAA	GGCTGATCATAGAACATGGG
g10536	TGGACGCTGGTACAATAGTT	TTGAGAATCTGTTGACATCTGC
g20740	CGTGTTTGACCATCCATT	GCCACATAATACCGCTTAA
g10538	CTTGGCTAGAGCAATTATACGA	TACCGTACCAGCGTCAAT
g24259	GATCGTGCTTCTGTACTG	CGAGTTGTTCATGTGGAA
g25170	ACTCAGACAACTCCAAGATG	GGATTCGGATACAACTCAGG
g7429	GAAGCTACGCAGGATTACCA	TCCATCACCAAACGGCATAT
g20587	TGGAACTCATTAACGACGAA	CATTGTGACGATGTATGTACTC
g20497	ACGCCTCAAGAGTCTGTT	AGTGCTTCGGATTTCAAGTG
g3205	ATAAGGCAATGATCCAGAGTCG	GCTCCATCGTTCCGAATGA

2.10 Data Analysis

A probit analysis was conducted using the Data Processing System (DPS) software [29] to calculate the slope, LC_{50}, 95% confidence interval (CI) value, and the χ^2 value of each insecticide. Resistance ratios (RRs) were calculated using the results for a Lab-HN strain as the factor divisor. The insecticide resistance levels were classified according to Zhang et al. [30] i.e., susceptible (RR ≤ 5.0), low level of resistance (5.0 < RR ≤ 10.0), moderate level of resistance (10.0 < RR ≤ 100.0), and high level of resistance (RR > 100.0). Statistics analysis was also performed using he Data Processing System (DPS) software [29].

3 RESULTS

3.1 Thiacloprid Resistance Selection and Cross-Resistance Spectrum

The THG-R strain was obtained from the susceptible FAA-S strain after more than 50 generations of continuous selection with thiacloprid. A toxicity test indicated that the THG-R strain developed a very high level of resistance to thiacloprid (1201.2-fold, LC_{50} = 2270 mg·L^{-1}) compared to the FAA-S strain (LC_{50} = 1.89 mg·L^{-1}) (Table 1). The results of a cross-resistance test between thiacloprid and other insecticides for the THG-R strain of GPA are also shown in Table 2. Compared to the sensitive strain (SS), the cross-resistance ratios of the resistant strain (RS) for imidacloprid, acetamiprid, thiamethoxam, clothianidin, dinotefuran, sulfoxaflor, flupyradifurone, and Triflumezopyrim were 894.3-, 48.7-, 9.4-, 6.4-, 44.7-, 14.6-, 24.9-, and 2.36-fold, respectively, which indicated a high cross-resistance between thiacloprid with imidacloprid, a moderate cross-resistance with acetamiprid, dinotefuran, sulfoxaflor, and flupyradifurone, and a low cross-resistance with thiamethoxam and clothianidin. There was no cross-resistance between thiacloprid and triflumezopyrim in the THG-R strain.

Table 2 Resistance and Cross-Resistance of the FAA-S and THG-R Strains of GPA to Seven Insecticides

Insecticides	Strains	No.	Slope (SE)	LC_{50} (95% CI) Mg·L^{-1}	χ^2	RR[a]
Thiacloprid	FAA-S	480	4 (0.33)	1.89 (1.74-2.06)	3.32 (df=3)	
	THG-R	560	1.43 (0.13)	2270 (1861-2802)	2.52 (df=4)	1201.2
Imidacloprid	FAA-S	560	2.68 (0.21)	1.09 (0.97-1.22)	2.12 (df=4)	
	THG-R	560	1.44 (0.13)	974.74 (654.22-978.07)	4.19 (df=4)	894.3
Acetamiprid	FAA-S	480	2.02 (0.19)	2.63 (2.23-3.08)	3.85 (df=3)	
	THG-R	560	1.8 (0.15)	128.11 (108.5-152.03)	3.52 (df=4)	48.7
Thiamethoxam	FAA-S	560	4.71 (0.36)	2.57 (2.4-3.74)	5.5 (df=4)	
	THG-R	480	1.82 (0.18)	24.06 (20.18-28.67)	2.75 (df=3)	9.4
Clothianidin	FAA-S	560	3.5 (0.3)	3.21 (2.93-3.51)	4.19 (df=4)	

Dinotefuran	THG-R	480	1.93 (0.19)	20.41 (17.2-24.2)	3.91 (df=3)	6.4
	FAA-S	480	3.31 (0.26)	4.13 (3.76-4.53)	4.62 (df=3)	
Sulfoxaflor	THG-R	560	1.86 (0.15)	184.76 (157.04-217.85)	5.69 (df=4)	44.7
	FAA-S	480	2.68 (0.24)	1.34 (1.17-1.51)	2.61 (df=3)	
Flupyradifurone	THG-R	480	1.96 (0.18)	19.55 (16.6-23.28)	1.55 (df=3)	14.6
	FAA-S	480	3.26 (0.31)	1.98 (1.77-2.20)	2.76 (df=3)	
Triflumezopyrim	THG-R	480	2.03 (0.19)	49.38 (42.07-58.02)	5.06 (df=3)	24.9
	FAA-S	480	2.05 (0.19)	27.25 (23.28-32.05)	4.51 (df=3)	
	THG-R	480	2.02 (0.19)	64.24 (54.76-76.14)	2.18 (df=3)	2.5

[a]RR = the resistance ratio, obtained by the LC_{50} of each insecticide for the THG-R strain divided by the LC_{50} for the FAA-S strain.

3.2 Synergism of the Enzyme Inhibitors

The synergism results (Table 3) of the enzyme inhibitors showed that PBO exhibited significant synergistic action on the THG-R strain, with synergistic ratios of 2-fold, but no significant synergistic action on the FAA-S strain, with synergistic ratios of 1.03-fold. There was no significant synergistic action of DEF on the two strains, with low synergistic ratios of 1.02- and 1.26-fold, respectively. There was a significant synergistic action of DEM on the THG-R strain with a synergistic ratio of 1.86-fold, but no significant synergistic action on the FAA-S strain, with a synergistic ratio of 1.06-fold.

Table 3 Synergism of Three Enzyme Inhibitors on Thiacloprid Toxicity to the THG-R Strain of GPA

Insecticides	Treatment	No.	Slope±SE	LC_{50} (95% CI)	χ^2	Synergistic ratios (SR)
FAA-S	THC	480	4(0.33)	1.89(1.74-2.06)	3.32(df=3)	
	THC+PBO	480	3.82(0.32)	1.83(1.67-1.99)	3.61(df=3)	1.03
	THC+DEF	480	3.71(0.32)	1.85(1.69-2.02)	3.36(df=3)	1.02
	THC+DEM	480	4.01(0.33)	1.78(1.63-1.94)	3.39(df=3)	1.06
THG-R	THC	560	1.43(0.13)	2270.26(1861.38-2802.38)	2.52(df=4)	
	THC+PBO	560	1.56(0.14)	1142.75(948.56-1381.1)	4.59(df=4)	2
	THC+DEF	560	1.45(0.13)	1799.16(1482.75-2205.3)	0.86(df=4)	1.26
	THC+DEM	560	1.78(0.15)	1220.61(1032.17-1450.8)	5.88(df=4)	1.86

3.3 Detoxification of Enzyme Activities

The specific activities of detoxification enzymes (CarEs, MFO, and GSTs) in the FAA-S and THG-R strains were determined to analyze the biochemical mechanism of resistance in GPA to thiacloprid (Table 4). Compared to the FAA-S strain, the P450 activity in the THG-R strain increased significantly by 1.91-fold ($p < 0.01$) and the GST activity in the THG-R strain also significantly increased by 1.79-fold ($p < 0.05$). However, there was no significant difference in CarEs activity between the FAA-S and THG-R strains. According to the enzyme activity and synergism results, P450 may contribute to the metabolic resistance of GPA to thiacloprid.

Table 4 Detoxifying Enzyme Activities in the FAA-S and THG-R Strains of GPA

Metabolic enzyme	Strains	Activity	Ratio[b]
Specific activity of CarE[a] (μ mol μg^{-1} 10 min^{-1})	FAA-S	6.7±1.02	
	THG-R	8.17±2.01	1.22
Specific activity of GSTs[a] (n mol 0μg^{-1} min^{-1})	FAA-S	0.0047±0.00055	
	THG-R	0.0084±0.0026*	1.79
Specific activity of MFO[a] (n mol μg^{-1} 30 min^{-1})	FAA-S	0.34±0.052	
	THG-R	0.66±0.14**[a]	1.91

[a]Values within a column followed by different letters are significantly different (*significant at the 0.05 level and **significant at the 0.01 level using Tukey's test. [b]Ratio: activity of an enzyme in the THG-R strain / activity of an enzyme in the FAA-S strain.

3.4 Summary of the RNA-seq Data

The GPA gene expression in the THG-R strain and FAA-S strain (control) was quantified by RNA-seq and a total of 38.27 GB clean sequence data were obtained. The guanine-cytosine (GC) content was 40.07–43.41%, and the Q20 and Q30 values were ≥96.82% and ≥91.37%, respectively. The average total mapping ratio and average unique mapping ratio were 86.28 and 82.82%, respectively (Table 5).

Table 5 Summary of the RNA-seq Data

Sample	Raw reads	Clean bases (Gb)	Q20 (%)	Q20 (%)	GC (%)	Total reads	Total map	Unique map
FAA-S1	44,928,742	6.74G	96.95	91.76	40.73	43,616,450	39,529,693(90.63%)	37,511,121(86.0%)
FAA-S2	43,566,522	6.53G	97.02	91.83	38.49	42,309,780	37,711,668(89.13%)	36,182,851(85.52%)
FAA-S3	44,473,064	6.67G	96.82	91.37	36.81	43,284,852	36,691,863(84.77%)	35,268,102(81.48%)
THG-R1	42,067,498	6.31G	98.11	94.37	36.4	41,613,804	33,803,075(81.23%)	32,639,599(78.43%)

THG-R2	40,871,230	6.13G	98.15	94.46	40.02	40,035,794	36,427,134(90.99%)	35,126,460(87.74%)
THG-R3	39,251,802	5.89G	97.98	93.94	36.06	38,643,186	31,272,750(80.93%)	30,034,784(77.72%)

FAA-S1, S2 and S2: three repeats from a susceptible GPA strain raised under control conditions with no pesticide contact; THG-R1, R2 and R3: three repeats from the FAA-S strain screened continuously by thiaclopid for more than 50 generation; Q20: the percentage of bases with a Phred value > 20; Q30: the percentage of bases with a Phred value > 30. Total reads: the number of clean reads after quality control; Total map: the number and percentage of clean reads mapped with the reference genome; Unique map: the number and percentage of clean reads mapped with the unique locus of the reference genome.

3.5 Differentially Expressed Genes between the FAA-S and THG-R Strains

A total of 18,581 genes were merged and assembled based on all the mapped reads from the four strains using Stringtie (1.3.3b) [31]. The distributions of the expression levels of all genes were similar between the FAA-S and THG-R strains. Using the DEG-Seq 2R package (1.20.0), a total of 1,670 DEGs were identified between the FAA-S and THG-R GPA transcriptomes. More than 62.1% of the genes (1,037) were overexpressed (log_2foldchange > 1) and approximately 37.9% of the genes (633) were downregulated in the THG-R strain (Figure 1).

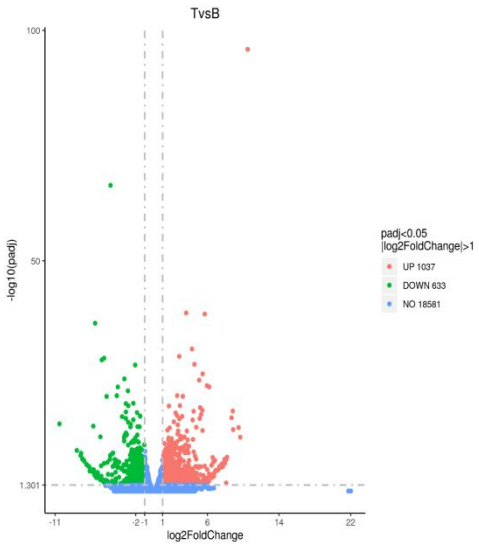
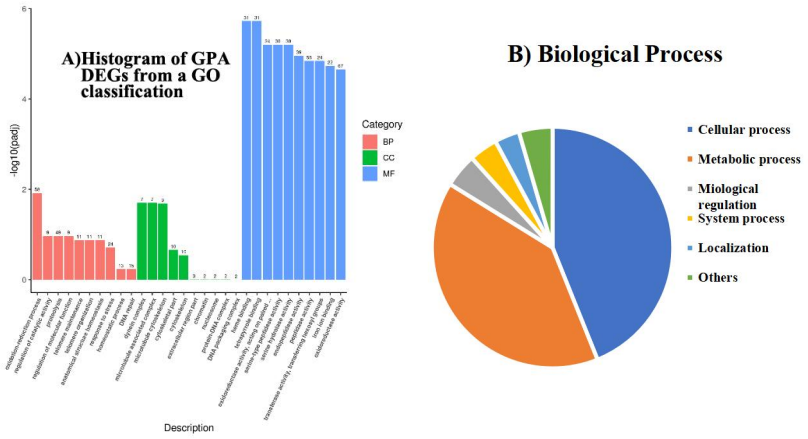


Figure 1 The DEGs between the GPA FAA-S and THG-R Strains

All DEGs were classified into three main GO categories: biological processes, cellular components, and molecular functions (Figure 2). For biological processes, the categories most represented were cellular processes (GO: 0008151, 643 out of 1,464 DEGs; 43.9%) and metabolic processes (GO: 0008152, 584 out of 1,464 DEGs; 25.3%) (Figure 2B). For cellular components, the genes involved in organelle parts (GO: 00043226, 90 out of 229 DEGs; 39.3%) and intracellular anatomical structure (GO: 0005622, 77 out of 229 DEGs; 33.6%) were the most abundant (Figure 2C). For molecular functions, catalytic activity (GO: 0003824, 796 out of 1,746 DEGs, 45.6%), transporter activity (GO:0005215, 383 out of 1,746 DEGs, 21.9%), and binding (GO: 0005488, 326 out of 1,746 DEGs, 18.7%) were the most highly represented categories (Figure 2D).



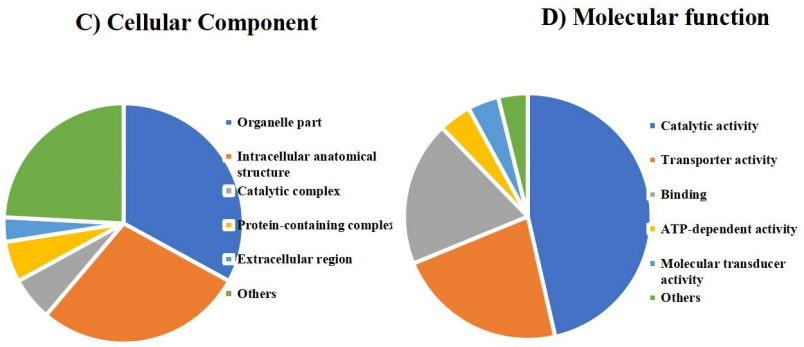


Figure 2 A GO Analysis of the DEGs of the GPA Transcriptome Annotated to UniGene cDNA Reference Data. The Analysis was Performed with Blast2GO-PRO. A) Histogram of GPA Differentially Expressed Sequences from a GO Classification. Three Main Ontologies (Biological Processes, Cellular Components, and Molecular Functions) are Shown on the X-axis. The Y-axis Indicates the Percentage of Total Genes, and the Right y-axis is the Number of Genes in each Category. B) The Distribution of Transcripts in the Biological Processes Category. C) The Distribution of Transcripts in the Cellular Components Category. D) The Distribution of Transcripts in the Molecular Functions Category.

A total of 547 DEGs were mapped onto 103 KEGG pathways (Figure 3). The largest category was lysosome, which contained 26 annotated DEGs (10.1%), followed by drug metabolism-cytochrome P450, pentose, and glucuronate interconversions, The metabolism of xenobiotics by cytochrome P450, fatty acid metabolism, and drug metabolism-other enzymes had 22 DEGs (8.1%), respectively.

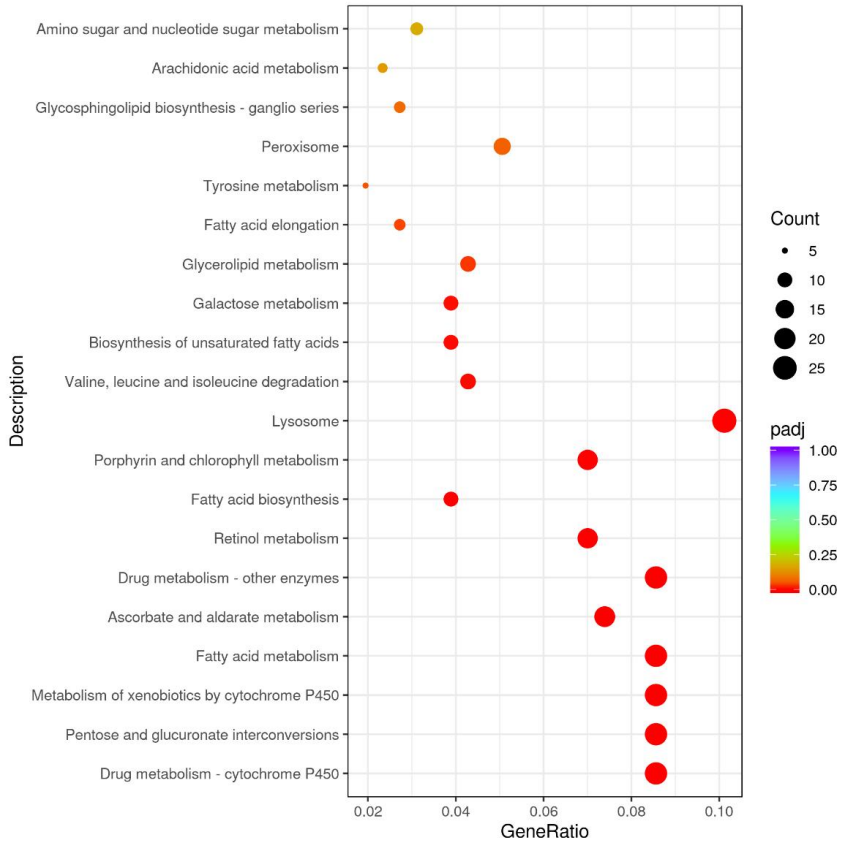


Figure 3 The Results of a KEGG Pathway Enrichment Analysis

3.6 Analysis of the Putative Genes Involved in the Regulatory Mechanism of Insecticide Detoxification

Among all the DEGs, 72 genes were identified that were involved in insecticide detoxification (Figure 4), including 15 upregulated detoxification genes and 57 downregulated detoxification genes. These overexpression genes are listed in Table 5, including five genes related to UGTs (*MpUgt2c1.3* (g20587), *MpUgt2c1.1* (g3205), *MpUgt2c1.2* (g10743),

MpUgt2b9 (g21618), and *MpUgt344j3* (g20497)); two genes related to P450s (cytochrome-P450) (*MpCyp4c1* (g25170) and *MpCyp6a14* (g7429)); two genes related to esterases (*MpPde11a* (g18568) and *MpThem6* (g24259)); and six genes related to ABCs (ATP-binding cassette transporters) (*Mplethal(2)03659.1* (g10536), *MpAbcg23.3* (g20740), *MpAbcg23.1* (g4805), *MpAbcg23.2* (g26047), *MpAbcb1b* (g21645), and *Mplethal(2)03659.2* (g10538)). The distribution of these upgraded detoxification genes on GPA chromosomes is shown in Figure 5.

Table 5 Selected Genes of the Detoxification Enzymes Identified by a Microarray as Significantly Differentially Transcribed between the THG-R and FAA-S Strains

Family	protein ID	blast result	name	Rename abbr.
ABCs	g10536.p1	XP_022166670.1	probable multidrug resistance-associated protein lethal(2)03659 isoform X2	*Mplethal(2)03659.1*
ABCs	g20740.p1	XP_022180695.1	ABC transporter G family member 23-like	*MpAbcg23.3*
ABCs	g4805.p1	XP_022180629.1	ABC transporter G family member 23-like isoform X1	*MpAbcg23.1*
ABCs	g26047.p1	XP_022175210.1	ABC transporter G family member 23-like	*MpAbcg23.2*
ABCs	g21645.p1	XP_022176197.1	multidrug resistance protein 1B-like isoform X1	*MpAbcb1b*
ABCs	g10538.p1	XP_022166683.1	probable multidrug resistance-associated protein lethal(2)03659	*Mplethal(2)03659.2*
Esterases	g18568.p1	XP_022168710.1	dual 3',5'-cyclic-AMP and -GMP phosphodiesterase 11A-like	*MpPde11a*
Esterases	g24259.p1	XP_022182351.1	protein THEM6-like	*MpThem6*
P450s	g25170.p1	XP_022175934.1	cytochrome P450 4C1-like	*MpCyp4c1*
P450s	g7429.p1	XP_022171832.1	probable cytochrome P450 6a14	*MpCyp6a14*
UDPGT	g20587.p1	XP_022182837.1	UDP-glucuronosyltransferase 2C1-like	*MpUgt2c1.3*
UDPGT	g3205.p1	XP_001949897.2	UDP-glucuronosyltransferase 2C1	*MpUgt2c1.1*
UDPGT	g10743.p1	XP_022166626.1	UDP-glucuronosyltransferase 2C1-like	*MpUgt2c1.2*
UDPGT	g21618.p1	XP_022162085.1	UDP-glucuronosyltransferase 2B9-like isoform X9	*MpUgt2b9*
UDPGT	g20497.p1	ATN96079.1	UDP-glucuronosyl transferase 344J3	*MpUgt344j3*

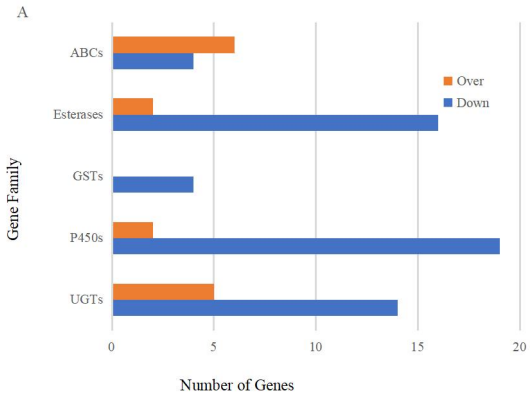


Figure 4 The DEGs between the THG-R and FAA-S Strains (Control) of *Myzus persicae*. A) The Number of Genes Encoding ABC Transporters, Esterases, GSTs, P450s, and UGTs that were Commonly Differentially Expressed between Thiacloprid Resistant and Susceptible Strains

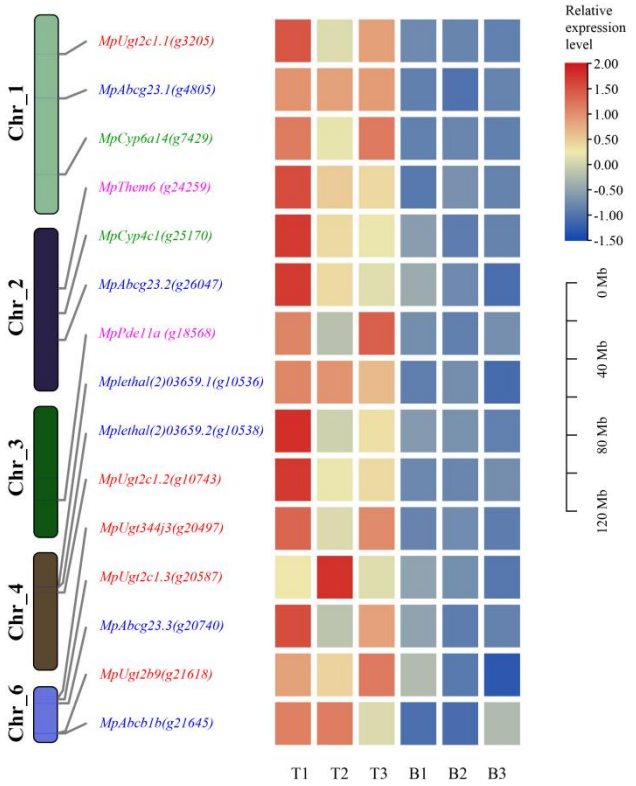


Figure 5 Distribution of the Detoxification Genes on *Myzus persicae* Chromosomes and Expression Heat Map (T: THG-R, B: FAA-S)

UDPGT Family Genes Shown in Red, ABC Transporter Family Shown in blue, P450 Family Shown in Green, Esterase Family Shown in Purple. These Genes were Distributed in each Chromosome, Except Chr_5. Gene Expression Levels are Indicated with a Rainbow Color Scale from Blue (Low Expression) to Red (High Expression)

3.7 Expression Levels of Insecticide Detoxification Genes in the FAA-S and THG-R Strains

Among the upregulated detoxification genes, *Mplethal(2)03659.1* was the most highly overexpressed (fold-change = 33.8), followed by *MpUgt2c1.3* (fold-change = 22.2) (Figure 6), *MpThem6* (fold-change = 4.1), *Mplethal(2)03659.2* (fold-change = 3.5), *MpUgt344j3* (fold-change = 3.4), *MpAbcg23.3* (fold-change = 3.3) and *MpUgt2c1.1* (3-fold), while the P450 genes *MpCyp4c1* and *MpCyp6a14* exhibited 2.9- and 2.7-fold-changes. For the other six upgraded detoxification genes, the fold-changes were below 2.7 (Fig. 6). The downregulated genes included all four sequences encoding GSTs with a negative fold-change of -1 to -2, and 19 sequences encoding cytochrome P450s, including *MpCyp380c40* with a fold-change of -1.5 to 2.5.

To further clarify the association between the overexpression of detoxification genes and the thiacloprid resistance mechanism of THG-R (Figure 7), we determined and compared the differences in the expression levels of nine upgraded detoxification enzyme genes (fold-change ≥ 2.7) in a transcript analysis and five other genes (*CYP6CY3*, *CYP380C40*, *FE4*, *GST*, *UGT344P2*) that were previously reported to be involved in resistance to Group 4 insecticides between the THG-R and FAA-S strain. The high expression levels of *MpUgt2c1.3*, MpThem6, and *Mplethal(2)03659.1* were 2.4-, 7-, and 30.3-fold, respectively. In contrast, the expression levels of *CYP380C40*, *MpCyp6a14*, and *FE4* were downregulated in the THG-R compared with the FAA-S.

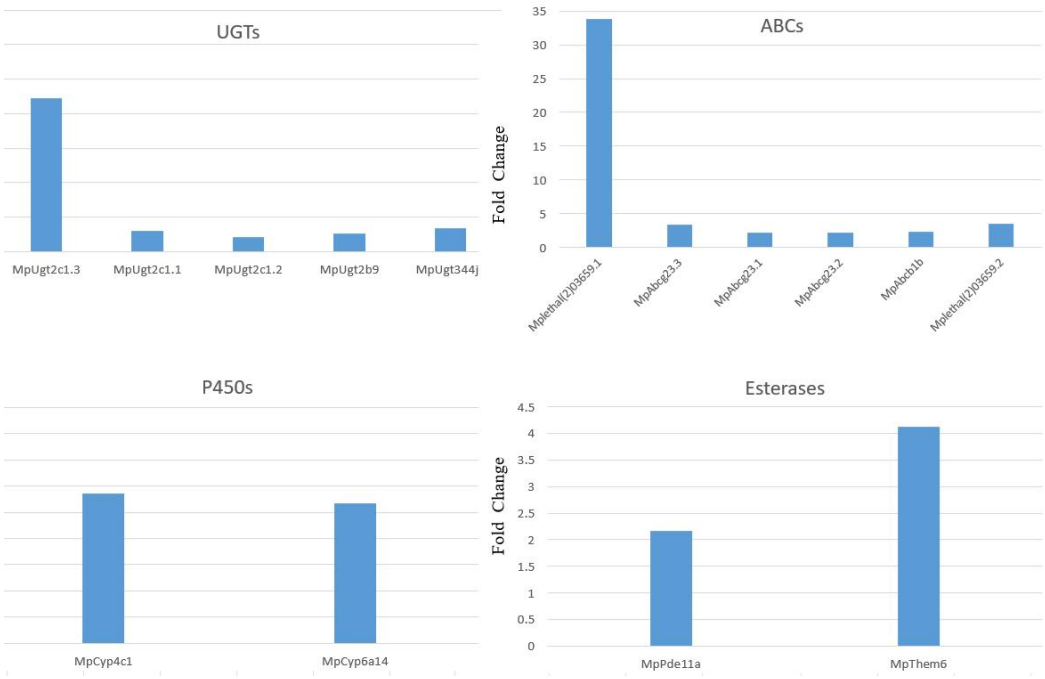
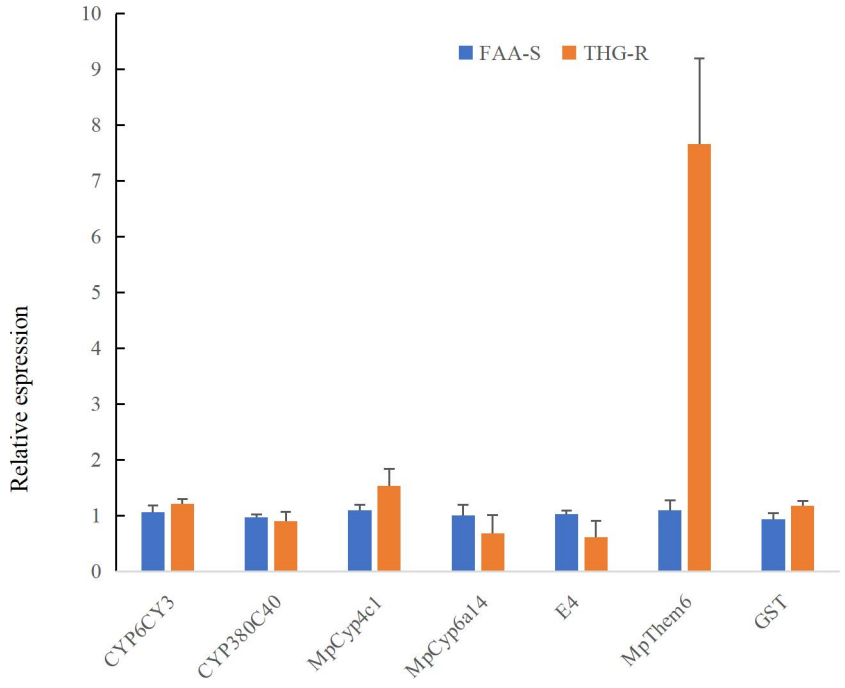


Figure 6 The Fold-Changes of Selected Genes Identified by a Microarray as Significantly Differentially Transcribed between the THG-R and FAA-S Strains



A) cytochrome P450, Carboxylesterase & Glutathione S-transferases

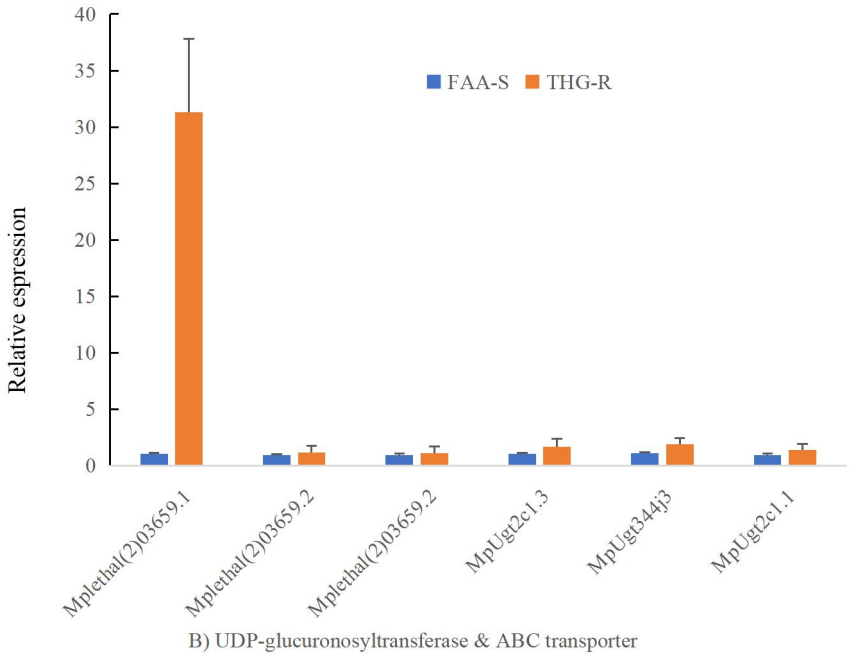


Figure 7 Quantitative Real-Time PCR (qRT-PCR) Validation of the Expression of DEGs Identified using RNA-sequencing. The Expression Levels were Normalized to the GAPDH, EF1α, and β-actin Genes. A) Expression of the Genes for P450, Esterases, GST, B) Expression of the ABCs and UGTs in each Pairwise Comparison of the Thiacloprid Resistant (THG-R) Strain and three Susceptible (FAA-S) Strains

4 DISCUSSION

Neonicotinoid resistance in GPA has become a serious worldwide problem. In this study, the susceptible GPA developed a very high-level resistance to thiacloprid (RR = 1201.2) after 50 generations of selection, which indicated a high risk of evolving thiacloprid resistance. Several studies have also documented that selection with neonicotinoids in the laboratory may result in highly resistant GPA strains. The GPA developed a 124.5-fold resistance to imidacloprid after a 20-generation selection [32], while a 75.6-fold resistance to thiamethoxam was generated after a 15-generation selection [33] and 57.5-fold acetamiprid resistance was achieved after a five-generation selection [34]. The intensity of selection in the field by neonicotinoids also causes GPA field populations to evolve a high resistance to neonicotinoids. For example, GPA in the FRC strain from southern France exhibited a 1679- and 225-fold resistance to imidacloprid and thiamethoxam, respectively [9], and strain 99H1 collected from Italy was found to have a more than 600-fold resistance to imidacloprid [35]). Risk management practices should be implemented to combat and slow thiacloprid resistance in GPA, such as resistance monitoring and rotations with different insecticides.

Laboratory bioassays were conducted to evaluate the cross-resistance in the THG-R strain between thiacloprid and other insecticides. The eight other insecticides were allocated to the IRAC Group 4 of compounds that act as nicotinic acetylcholine receptor (nAChR) competitive modulators. We found that the selection of GPA with thiacloprid after more than 50 generations in the laboratory induced a very high level of cross-resistance to imidacloprid (894.3-fold), moderate levels of resistance to acetamiprid (48.7-fold), dinotefuran (44.7-fold), flupyradifurone (24.9-fold), and sulfoxaflor (14.6-fold), and very low levels of resistance to thiamethoxam (9.4-fold) and clothianidin (6.4-fold). Although the five insecticides, imidacloprid, acetamiprid, dinotefuran, thiamethoxam, and clothianidin are neonicotinoids (Group 4A), they showed great differences in cross-resistance with thiacloprid (Group 4A). Previous studies indicated the presence of a high cross-resistance between thiacloprid and imidacloprid in GPA. Five highly imidacloprid-resistant field populations ($156 \leq RR \leq 412$) exhibited more than a 110-fold cross-resistance to thiacloprid [36]. Similarly, the FRC strain (RR > 2500 for imidacloprid) [14] exhibited > 2500-fold resistance to thiacloprid, > 80-fold resistance to acetamiprid, and 54-fold resistance to dinotefuran. However, the FRC strain also had a high (> 2,500-fold) resistance to clothianidin and > 100-fold resistance to thiamethoxam, but the THG-R strain had a low cross-resistance to the two neonicotinoids. The THG-R strain also developed a moderate cross-resistance to flupyradifurone (Group 4C) and sulfoxaflor (Group 4A), and the FRC strain also conferred a 43-fold resistance to sulfoxaflor [14]. However, the absence of cross-resistance between flupyradifurone and imidacloprid was observed in a low imidacloprid-resistant GPA strain [37]. Although cross-resistance did not occur between triflumezopyrim (Group 4D) and thiacloprid in the THG-R strain, the relatively low toxicity of triflumezopyrim against GPA limited its application. Based on our results, Group 4 insecticides cannot be used as an alteration tool for GPA thiacloprid resistance management strategies.

The toxicity of thiacloprid to THG-R GPA was enhanced by PBO and DEM, indicating that P450 and GST may be related to the decreased susceptibility to thiacloprid in GPA. The obviously increased activities of P450 and GST in the THG-R strain further supported the hypothesis of this study. Previous studies have demonstrated that increased

detoxification through metabolic enzymes significantly contributed to resistance to Group 4 insecticides in GPA, but this did not always occur. A significant synergism was detected in which the pretreatment of the FRC strain of GPA with PBO reduced the RRs from 1,679-fold to 234-fold for imidacloprid and from 225-fold to 26-fold for thiamethoxam [9], and for the clone 5191A it led to a 14.5-fold synergism of imidacloprid [38], with a much higher synergism than that observed in this study (2-fold). However, PBO synergism was not detected in acetamiprid-resistant GPA [34]. Another synergist, DEM, was also found to increase the toxicity of imidacloprid to *Rhopalosiphum padi* by 8.32-fold [39]. The activities of P450 monooxygenases and GST were reported to be 2.03- and 1.57-fold higher in thiamethoxam-resistant GPA than in a susceptible strain [33], which was similar to our result (1.91-fold). These results indicate that cytochrome P450 and GST may be involved in thiacloprid resistance in GPA. The thiamethoxam-resistant GPA also exhibited a 6.12-fold higher CarEs activity than the susceptible strain [33]. Our study yielded contrasting results in which no increase in activity was found in the THG-R strain, and DEF also had no obvious synergistic effect on the THG-R strain, which demonstrated the CarEs was not associated with thiacloprid resistance in GPA.

The molecular mechanisms governing high levels of neonicotinoid in GPA have been widely studied, but these resistant strains were collected in the field. Here, we attempted to determine the molecular mechanisms of thiacloprid resistance in GPA using an RNA-seq library approach. We discovered approximately 1,670 DEGs (1,037 up-regulated and 633 down-regulated) for susceptible and artificially selected high thiacloprid-resistant populations of GPA (THG-R). The number of DEGs was much higher than other neonicotinoid-resistant GPA strains. In the Cascade209, EastMunglinup209, and EastNaernup209 clones of GPA that are resistant to sulfoxaflor, a total of 1,033, 1,046, and 860 genes were consistently differentially expressed compared to the three susceptible clones, respectively [20]. Only 273 DEGs were identified between the insecticide resistant clone 5191A (27.5-fold to imidacloprid and 41.31-fold to thiacloprid) and the susceptible clone 4106A [40]. In general, the GO enrichment of DEGs in the THG-R strain indicated that the metabolic process was significantly enriched (Figure 2A, 2B), while the “metabolic pathway” (including drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450, fatty acid metabolism, and drug metabolism-other enzymes) was also enriched in a KEGG analysis. These metabolic pathways are known to be involved in insecticide metabolism [41,42].

The most widely studied genes belonging to the three gene families that typically participate in the detoxification of metabolic resistance to insecticides are *cytochrome P450* (P450s proteins, encoded by *CYP* genes), *glutathione S-transferases* (GST), and *carboxylesterases* (CarEs). The P450s are a superfamily of enzymes that can catalyze the oxidation of steroids, fatty acids, and xenobiotics [19]. The cytochrome P450 enzymes are important in the adaptation to the detoxification of insecticides in phytophagous insects and cytochrome P450-mediated enhanced metabolism has been proven to be responsible for insecticide resistance, including neonicotinoid resistance. In this study, we showed that the enhanced transcription of two P450 genes *MpCyp4c1* and *MpCyp6a14* in the THG-R strain was due, at least in part, to an approximately 3-fold amplification, while *MpCyp4c1* was found to exhibit a 2.13-fold overexpression by qPCR verification. However, the overexpression and duplication of *CYP6CY3* contributed to the differences in neonicotinoid-resistant GPA, such as a 22-fold overexpression in the 5191A clone [40], 9-36-fold overexpression in field collected populations in Greece [10], and 2.8−6.7-fold overrepresentation with copy numbers in field collected populations in Australia [43]. Nakao et al. [44] confirmed that *CYP6CY3* showed metabolic activity against imidacloprid, as well as acetamiprid, clothianidin, and thiacloprid. Furthermore, 21−76-fold highly overexpressed *CYP380C40*, which is another P450 gene, was detected in the sulfoxaflor resistant GPA clones [20]. However, no significant overexpression of the two P450 genes was found in the THG-R strain. From the results of the P450 inhibitor experiment, it was concluded that the P450 might not be the main enzyme involved in laboratory selected thiacloprid resistant GPA. Similarly to our results, *CYP6CY3* was also found to not be upregulated in a high acetamiprid-resistant (57.5-fold) GPA strain [34]. This difference revealed the existence of another enhanced metabolic route for neonicotinoids in addition to the P450 routes in GPA. Carboxylesterase was significantly down-regulated in the THG-R strain, which was consistent with the results of a previous study that showed that the FE4 gene did not involve the detoxification of thiacloprid in the pest [45]. The GSTs exhibited no significant different expression between the THG-R and FAA-S strains, which was consistent with other studies. From our study, we found that the three detoxification enzymes did not play the key role in the thiacloprid resistance of GDP [20]. However, the esterase *MpThem6* gene exhibited a 7-fold overexpression, which indicated that it probably had an important role in thiacloprid resistance.

The UGTs are a superfamily of enzymes that catalyze the transfer of glycosyl residues from activated nucleotide sugars to hydrophobic molecules (aglycones) and are also known as biotransformation enzymes that participate in the detoxification process [46]. The UGT gene family that was found to be significantly overexpressed in the THG-R strain included *MpUgt2c1.3* (2.43-fold), *MpUgt344j3* (2.28-fold), *MpUgt2c1.1* (2.04-fold), and *UGT344P2* (1.44-fold) (Figure 2). The gene *UGT344P2* was overexpressed by 6-33-fold in the sulfoxaflor resistant GPA clones (Pym et al., 2022), which may be correlated with cross-resistance to sulfoxaflor in the THG-R strain. Another two UGTs, namely *UGT352A4* and *UGT352A5,* were found to be 2.66-fold and 1.9-fold overexpressed, respectively, compared to the susceptible strain, and the knockdown of *UGT352A5* resulted in a decrease in the thiamethoxam resistance in the thiamethoxam-resistant (THQR) *B. tabaci* [47]. In a thiamethoxam-resistant strain of *A. gossypii*, the transcripts of 13 UGTs (*UGT344J2*, *UGT348A2*, *UGT344D4*, *UGT341A4*, *UGT343B2*, *UGT342B2*, *UGT350C3*, *UGT344N2*, *UGT344A14*, *UGT344B4*, *UGT351A4*, *UGT344A11*, and *UGT349A2*) were increased by approximately 2.0-fold and the suppression of selected UGTs significantly increased the insensitivity of resistant aphids to thiamethoxam [48]. We considered that the UGTs may play an important role in the cross-resistance to Group 4 insecticides in the THG-R

strain.

The ABC transporters are responsible for the translocation of a variety of substrates (e.g., metabolites, lipids, inorganic ions, and xenobiotics) by pumping them out of the cells through the cell membrane. The ABCs involved in insecticides have received much attention. Three ABC genes, *Mplethal(2)03659.1*, *MpAbcg23.3*, and *Mplethal(2)03659.2*, were found to be overexpressed in the THG-R strain, with *Mplethal(2)03659.1* overexpressed by 30.32-fold. The suppression of five overexpressed ABCs (*ABCA2*, *ABCD1*, *ABCD2*, *ABCE1*, and *ABCG15*) significantly increased the thiamethoxam sensitivity of resistant *A. gossypii* (Pan et al., 2020), while 10 ABCs (*ABCA1*, *ABCA2*, *ABCB1*, *ABCB5*, *ABCD1*, *ABCG7*, *ABCG16*, *ABCG26*, *ABCG27*, and *MRP7*) were up-regulated and the knockdown of *ABCA1* and *ABCD1* significantly increased the sulfoxaflor sensitivity in sulfoxaflor-resistant *A. gossypii* [49]. Furthermore, the knockdown of *AgABCG7* and *AgABCG26* increased thiamethoxam and imidacloprid mortality in a field multi-resistant population of *A. gossypii* (SDR) [50]. These data also confirmed that the diverse metabolic processes of *Mplethal(2)03659.1* were involved in thiacloprid resistance.

Mplethal(2)03659.1 and *MpThem6* attracted our attention because of their strong expression in the THG-R strain, which suggested that the two genes played a vital role in thiacloprid metabolism and transport. However, the involvement of the two genes in the metabolism related to insecticides is poorly understood. *Mplethal(2)03659.1* is an ATP-binding cassette transporter C (ABCC) family protein that has been widely detected in the genomes of other aphids, such as *A. gossypii* (XM_050204743.1), *Rhopalosiphum maidis* (XM_026951384.1), and *Diuraphis noxia* (XM_015510588.1). It is a probable multidrug resistance-associated protein that is similar to the human multidrug resistance-associated protein 1 that mediates the export of organic anions and drugs from the cytoplasm (probable multidrug resistance-associated protein lethal). *Mplethal(2)03659.1* has a possible involvement in cantharidin detoxification in two blister beetle species, *Lydus trimaculatus* (Fabricius, 1775) (tribe Lyttini) and *Mylabris variabilis* (Pallas, 1781) (tribe Mylabrini) [51]. Further studies should be conducted to investigate if overexpression of the two genes is widespread in the field resistant MPA population and to determine the effects of knockdown of the two genes on the susceptibility of neonicotinoids to their resistant populations.

5 CONCLUSIONS

Our results suggested that the GPA has the potential to rapidly evolve resistance to thiacloprid under continuous selection in the laboratory. Our experiment also revealed evidence for cross-resistance to Group 4 insecticides, except for triflumezopyrim, in the THG-R strain. Group 4 insecticides were therefore not recommended for use in rotation with thiacloprid when resistance occurred in the field. Enzyme activities and synergism tests indicated that the evolution of resistance to thiacloprid in the THG-R was associated with cytochrome P450. We constructed RNA-seq libraries to investigate the DEGs between THG-R and FAA-S. A comparative analysis of the DEGs between THG-R and FAA-S revealed that genes for detoxification enzymes, including cytochrome P450, UGTs, ABCs and thioesterase, were significantly overexpressed. The *MpUgt2c1.3*, *MpThem6*, *Mplethal(2)03659.1, MpCyp4c1*, and *MpCyp6a14* genes were identified as being overexpressed in the THG-R strain by qPCR. Conversely, the genes previously reported to be responsible for neonicotinoid resistance in GPA, including *CYP6CY3*, *CYP380C40*, and *UGT344P2*, were not significantly upregulated in the THG-R strain. We considered that the ABC gene *Mplethal(2)03659.1* and the thioesterase gene *MpThem6* may play a vital role in the metabolic mechanism of thiacloprid resistance in GPA. A complete identification of the function of these genes is necessary to fully understand their role in thiacloprid resistance in GPA.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

FUNDING

This work was supported by Fujian Natural Science Foundation (2022J01461), the program of Fujian Provincial Department of Science & Technology (2020R10240011), program of Science and Technology Innovation Foundation of FAAS Supported by Financial Department of Fujian Government (CXTD2021002-1, XTCXGC2021017).

REFERENCES

[1] Yi XU, Gray S M. Aphids and their transmitted potato viruses: A continuous challenges in potato crops. Integr. Agric. 2020, 19: 367-375.
[2] IRAC. The IRAC Mode of Action Classification, 2023.
[3] Hlaoui A, Chiesa O, Figueroa C C, et al. Target site mutations underlying insecticide resistance in tunisian populations of Myzus persicae (sulzer) on peach orchards and potato crops. Pest Manag. Sci. 2022, 78: 1594-1604.
[4] Li Y, Xu Z, Li S, et al. Insecticide resistance monitoring and metabolic mechanism study of the green peach aphid, Myzus persicae (sulzer) (Hemiptera: Aphididae), in Chongqing, China. Pestic. Biochem. Physiol. 2016, 132: 21-28.

[5] Tang Q L, Ma K S, Hou Y M, et al. Monitoring insecticide resistance and diagnostics of resistance mechanisms in the green peach aphid, Myzus persicae (sulzer) (hemiptera: aphididae) in China. Pestic. Biochem. Physiol. 2017, 143: 39-47.
[6] Casida J E. Neonicotinoids and other insect nicotinic receptor competitive modulators: Progress and prospects. Annu. Rev. Entomol. 2018, 63: 125-44.
[7] Nauen R, Strobel J, Tietjen K, et al. Aphicidal activity of imidacloprid against a tobacco feeding strain of Myzus persicae (Homoptera: Aphididae) from Japan closely related to Myzus nicotianae and highly resistant to carbamates and organophosphates. Bull. Entomol. Res. 1996, 86: 165-171.
[8] Mota-Sanchez D, Wise JC. The Arthropod Pesticide Resistance Database. Michigan State University, 2023.
[9] Bass C, Puinean AM, Andrews M, et al. Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. BMC Neurosci, 2011, 12(1): 51.
[10] Ch Voudouris C, Kati AN, Sadikoglou E, et al. Insecticide resistance status of Myzus persicae in Greece: long-term surveys and new diagnostics for resistance mechanisms. Pest Manag. Sci, 2016, 72: 671-83.
[11] Alptekin S, Bass C, Nicholls C, et al. Induced thiacloprid insensitivity in honeybees (Apis mellifera L.) is associated with up-regulation of detoxification genes. Insect Mol. Biol, 2016, 25:171-80.
[12] Jeschke P, Moriya K, Lantzsch R, et al. Thiacloprid (Bay YRC 2894) - a new member of the chloronicotinyl insecticide (CNI) family. Pflanzenschutz-Nachr. Bayer, 2001, 54: 147–160.
[13] Foster SP, Cox D, Oliphant L, et al. Correlated responses to neonicotinoid insecticides in clones of the peach-potato aphid, Myzus persicae (Hemiptera: Aphididae). Pest Manag. Sci, 2008, 64: 1111-1114.
[14] Cutler P, Slater R, Edmunds AJ, et al. Investigating the mode of action of sulfoxaflor: A fourth-generation neonicotinoid. Pest Manag. Sci, 2013, 69: 607-19.
[15] Le Goff G, Hilliou F. Resistance evolution in Drosophila: the case of CYP6G1. Pest Manag. Sci. 2017, 73: 493-499.
[16] Yang X, Xie W, Wang SL, et al. Two cytochrome P450 genes are involved in imidacloprid resistance in field populations of the whitefly, Bemisia tabaci, in China, Pestic. Biochem. Physiol, 2013, 107: 343-350.
[17] Chen A, Zhang H, Shan T, et al. The overexpression of three cytochrome P450 genes CYP6CY14, CYP6CY22 and CYP6UN1 contributed to metabolic resistance to dinotefuran in melon/cotton aphid, Aphis gossypii Glover. Pestic. Biochem. Physiol. 2020,167,104601.
[18] Zhang Y, Yang Y, Sun H, et al. Metabolic imidacloprid resistance in the brown planthopper, Nilaparvata lugens, relies on multiple P450 enzymes. Insect Biochem. Mol. Biol. 2016, 79: 50-56.
[19] Nauen R, Bass C, Feyereisen R, et al. The role of cytochrome P450s in insect toxicology and resistance. Annu. Rev. Entomol. 2022, 67: 105-124.
[20] Pym A, Umina PA, Reidy-Crofts J, et al. Overexpression of UDP-glucuronosyltransferase and cytochrome P450 enzymes confers resistance to sulfoxaflor in field populations of the aphid, Myzus persicae. Insect Biochem. Mol. Biol. 2022, 143: 103743.
[21] Pan Y, Tian F, Wei X, et al. Thiamethoxam resistance in Aphis gossypii glover relies on multiple UDP-glucuronosyltransferases. Front Physiol. 2018, 9, 322.
[22] Shang CC, Soderlund DM. Monooxygenase activity of tobacco budworm (Heliothis virescens) larvae: Tissue distribution and optimal assay conditions for the gut activity, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 1984, 79: 407–411.
[23] Van Asperen K. A study of housefly esterase by means of a sensitive colorimetric method. Insect Physiol. 1962, 8: 401–416.
[24] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. Biol. Chem. 1974, 249: 7130–7139.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72: 248–254.
[26] Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015, 31:166-9.
[27] Trapnell C, Williams BA, Pertea G, et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 2010, 28: 511-515.
[28] Kenneth JL, Thomas DS. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25: 402-408.
[29] Tang QY, Zhang CX. Data Processing System (DPS) software with experimental design, statistical analysis and data mining developed for use in entomological research. Insect Sci, 2013, 20(2): 254-260.
[30] Zhang XL, Liao X, Mao KK, et al. Neonicotinoid insecticide resistance in the field populations of Sogatella furcifera (Horváth) in Central China from 2011 to 2015. Asia Pac. Entomol. 2017, 20: 955–958.
[31] Pertea M, Kim D, Pertea GM, et al. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown, Nature Protocols. 2016, 11: 1650-1667
[32] Choi BR, Lee SW, Yoo JK. Resistance development and cross-resistance of green peach aphid, Myzus persicae (homoptera: aphididae), to imidacloprid. Pestic. Sci. 2002, 6: 264-270.
[33] Zhang P, Zhou X. Metabolic mechanism of resistance to thiamethoxam in Myzus persicae. Plant Protection. 2015, 41: 39-44.

[34] Berber G, Demîrcî B, Toprak U, et al. Acetamiprid resistance in the green peach aphid Myzus persicae (Sulzer) (Hemiptera: Aphididae): Selection, cross-resistance, biochemical and molecular resistance mechanisms. JAFAG 2022, 39: 136-142.
[35] Panini M, Dradi D, Marani G, et al. Detecting the presence of target-site resistance to neonicotinoids and pyrethroids in Italian populations of Myzus persicae. Pest Manag. Sci, 2014, 70: 931-938.
[36] Mottet C, Fontaine S, Caddoux L, et al. Assessment of the dominance level of the R81T target resistance to two neonicotinoid insecticides in Myzus persicae (Hemiptera: Aphididae). Econ. Entom. 2016, 109(5): 2182-2189.
[37] Nauen R, Jeschke P, Velten R, et al. Flupyradifurone: A brief profile of a new butenolide insecticide. Pest Manag. Sci, 2015, 71: 850-862.
[38] Philippou D, Field L, Moores, G. Metabolic enzyme(s) confer imidacloprid resistance in a clone of Myzus persicae (Sulzer) (Hemiptera: Aphididae) from Greece. Pest Manag. Sci. 2010, 66: 390-395.
[39] Gong P, Chen D, Wang C, et al. Susceptibility of four species of aphids in wheat to seven insecticides and its relationship to detoxifying enzymes. Front. Physiol. 2021, 11:623612.
[40] Puinean AM, Foster SP, Oliphant L, et al. Amplification of a cytochrome P450 gene is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. PLoS Genet. 2010, 6: e1000999.
[41] Bonizzoni M, Ochomo E, Dunn W A, et al. RNA-seq analyses of changes in the Anopheles gambiae transcriptome associated with resistance to pyrethroids in Kenya: identification of candidate-resistance genes and candidate-resistance SNPs. Parasit. Vectors. 2015, 8: 474.
[42] Safi NHZ, Ahmadi AA, Nahzat S, et al. Evidence of metabolic mechanisms playing a role in multiple insecticides resistance in Anopheles stephensi populations from Afghanistan, 2017, 16: 100.
[43] de Little SC, Edwards O, van Rooyen AR, et al. Discovery of metabolic resistance to neonicotinoids in green peach aphids (Myzus persicae) in Australia. Pest Manag. Sci, 2017, 73: 1611-1617.
[44] Nakao T, Kawashima M, Banba S. Differential metabolism of neonicotinoids by Myzus persicae CYP6CY3 stably expressed in Drosophila S2 cells. Pestic. Sci, 2019, 44: 177-180.
[45] Foster SP, Denholm I, Thompson R. Variation in response to neonicotinoid insecticides in peach-potato aphids, Myzus persicae (Hemiptera: Aphididae). Pest Manag. Sci, 2003, 59: 166-173.
[46] Brierley CH, Burchell B. Human UDP-glucuronosyl transferases: chemical defence, jaundice and gene therapy. Bioessays, 1993, 15: 749-754.
[47] Du T, Fu B, Wei X, et al. Knockdown of UGT352A5 decreases the thiamethoxam resistance in Bemisia tabaci (Hemiptera: Gennadius). Int. Biol. Macromol, 2021, 186: 100-108.
[48] Pan Y, Zeng X, Wen S, et al. Multiple ATP-binding cassette transporters genes are involved in thiamethoxam resistance in Aphis gossypii glover. Pestic Biochem Physiol, 2020, 167: 104558.
[49] Wang L, Zhu J, Cui L, et al. Overexpression of multiple UDP-glycosyltransferase genes involved in sulfoxaflor resistance in Aphis gossypii glover. Agric. Food. Chem. 2021, 69: 5198-5205.
[50] Lv Y, Li J, Yan K, et al. Functional characterization of ABC transporters mediates multiple neonicotinoid resistance in a field population of Aphis gossypii Glover. Pestic. Biochem. Physiol, 2022, 188: 105264.
[51] Fratini E, Salvemini M, Lombardo F, et al. Unraveling the role of male reproductive tract and haemolymph in cantharidin-exuding Lydus trimaculatus and Mylabris variabilis (Coleoptera: Meloidae): a comparative transcriptomics approach. BMC Genomics, 2021, 22: 808.