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Multiple Insecticide Resistance and Associated Metabolic-Based Mechanisms in a *Myzus Persicae* (Sulzer) Population

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Abstract: The green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is an economically devastating crop pest worldwide. The *M. persicae* (SEF-R) population of a cabbage field in China was tested for susceptibilities to 13 insecticides. Compared with the susceptible population (FFJ-S), extremely high and high resistance to beta-cypermethrin (324-fold) and imidacloprid (106.9-fold) was detected in SEF-R. More importantly, this is the first report of resistance in the field *M. persicae* population to sulfoxaflor (32.4-fold), flupyradifurone (9.5-fold), pymetrozine (34.8-fold), spirotetramat (8.1-fold), flonicamid (5.8-fold), and broflanilide (15.8-fold) in China when compared with FFJ-S. The resistance factor decayed to a low level to sulfoxaflor and pymetrozine after 15 generations without any selection pressure. The resistance-related mutations (R81T and kdr) detected in SEF indicated target-site resistance to neonicotinoids and pyrethroids, respectively. Biochemical assays revealed the involvement of monooxygenase, carboxylesterase, superoxide dismutase, and peroxidase in a multi-insecticide resistance mechanism. The overexpression of P450s, esterases, and a UDP-glycosyltransferase might be responsible for the multi-insecticide resistance in SEF-R. The knockdown of CYP6CY3 in SEF-R increased its susceptibility to imidacloprid, thiacloprid, and thiamethoxam, which verified that P450s play vital roles in neonicotinoid metabolism. Our findings provide guidance for the rational use of insecticides to delay resistance development in GPA.

Keywords: *Myzus persicae*; insecticides; resistance monitoring; detoxification enzymes; protective enzymes; metabolic mechanism

1. Introduction

The green peach aphid (GPA) *Myzus persicae* (Sulzer, 1776) is an enormously polyphagous pest worldwide. Reported to feed on more than 400 kinds of plants belonging to 50 families, including varieties of crop, vegetable, fruit, and tobacco, infestations of GPA reduce plant growth rates and cause reductions in crop quantity and quality [1]. In addition, the aphid vectors over 100 plant viruses, accounting for 67.7% of the aphid vector viruses, which can lead to large yield reductions [2]. As a highly variable species, aphids evolve different strains and biotypes. These are distinguished by biology, host-plant preference, and insecticide resistance [3], favoring spread and rapid colonization. The cabbage *Brassica oleracea* var. capitata Linnaeus is the primary GPA vegetable target, having the largest vegetable planting area in China of 0.4 million hectares, which accounts for 25–30% of Chinese vegetables. GPA is a destructive pest of *B. oleracea* throughout the country. Effective control is crucial to protect *B. oleracea* from aphid infestation and to prevent viral transmission.

Although some other integrated pest management measures, such as natural enemies, cultural control, and mechanical control, have been used to control GPA, chemical control is still the primary means of suppressing the pest in practice. Based on IRAC, there are 16 groups of effective insecticides that kill the GPA by acting on nerves and muscles

(10 groups), growth and development (four groups), and respiration targets (two groups) [4]. In China, the systematic use of insecticides has played a vital role in controlling this pest since the introduction of organophosphates and carbamates insecticides in the 1950s [5]. To date, more than 600 pesticide products have been registered to manage the cabbage GPA in China. Among these products, 336, accounting for 55.17%, contain neonicotinoids, and 158 products, accounting for 25.94%, contain pyrethroids [6], which indicates that these two insecticides remain the major insecticides used to eliminate the GPA of cabbage, even though they have been applied for several decades. Overreliance on insecticides has led to the occurrence and expansion of resistance in the GPA and threatens their continued efficacy. Insecticide resistance monitoring data in China indicates that GPA field populations have high resistance to organophosphate insecticides, such as dimethoate and malathion, carbamate insecticides, such as pirimicarb and methomyl, pyrethroids, such as fenvalerate and cypermethrin, and neonicotinoids, such as imidacloprid [5,7,8]. If we are to regain the ability to use these insecticides effectively against GPA, the assessment of resistance stability is necessary. As is widely known, unstable resistance means that the effectiveness of an insecticide to some resistant insects may be restored. Resistance stability has been widely studied in some insects that are resistant to different insecticides after the selection pressure is removed [9,10]. For example, resistance to imidacloprid in the resistant strain of *Musca domestica* L. was unstable, while resistance to cypermethrin was stable [11]. Conversely, a highly resistant field strain of Aedes aegypti displayed a significant reversion towards susceptibility to permethrin after 10 generations in the absence of insecticide [12]. However, there are limited data on the stability of insecticide resistance in GPA. The evolution and spread of resistance in GPA represent major threats to its sustainable control [13]. Understanding resistance mechanisms is critical for the development of rational strategies to prolong the lives of insecticides currently in use. To date, seven independent mechanisms of resistance have been found in this species, including alterations in target site, metabolic mechanisms, and physical resistance mechanisms [13]. In terms of target site resistance, the R81T substitution onto the β1 subunit of nicotinic receptors of acetylcholine (nAChR) and the two amino acid substitutions (L1014F and M918T) in the voltage-gated sodium channel confer target site resistance to neonicotinoids and pyrethroid insecticides, respectively [13]. The genetic modification of the target site will automatically confer cross-resistance to all ingredients in the same sub-group, which has been used to monitor resistance. The enhanced expression of insecticide-detoxifying enzymes is the most common mechanism in pests and often presents the greatest challenge, being responsible for multi-resistance. The overproduction of carboxylesterases (E4 and EF4) causes the resistance of *M. persicae* to organophosphates and carbamates [14,15]. The amplification of the cytochrome P450 gene CYP6CY3 is associated with low-level resistance to neonicotinoids [16–18], whereas the upregulation of glutathione S transferase (GST) and UDP-glycosyltransferases (UGTs) has been shown to affect insecticide resistance [19,20]. In addition, the protective antioxidant enzymes, i.e., superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), which play important roles in immunity, help to maintain a state of equilibrium in insects after insecticide exposure [21–24]. Field populations are exposed to different insecticides under variable selection pressures, favoring the evolution of multiple resistance through resistance mechanisms. Understanding these mechanisms will help us to estimate the prevalence of resistant phenotypes when bioassays cannot be used to detect resistance [25,26].

Brassica species are cultivated year-round in Fujian Province, southeast China, and are vulnerable to GPA and other notorious pests, such as *Plutella xylostella*, *Phyllotreta striolata* (Fabricius), *Spodoptera litura* Fabricius, and *Pieris rapae*. Various insecticides are frequently applied by farmers to control the damage from these pests. Presently, some active ingredients, such as flonicamid, cyantraniliprole, spirotetramat, afidopyropen, sulfoxaflor, and flupyradifurone, which have excellent aphicidal activities, have been registered for GPA management in China [27]. Furthermore, other insecticides, such as broflanilide, have also entered the Chinese market and showed high potential in aphid control, although they have not been registered for aphid management [28]. These compounds belong to

different groups of insecticides and may be alternated using pyrethroids or neonicotinoids in the field. Resistance to some insecticides, such as spirotetramat [29], flupyradifurone [30], and sulfoxaflor [20], has been assessed in other countries. However, the resistance status of GPA is unavailable in China, including Fujian Province. In order to warn against, delay, or prevent the development of resistance in GPA to these compounds and better guide management practices, we conducted resistance monitoring and resistance stability assessments in a GPA field population collected from Fujian Province. To further investigate the resistance mechanisms at play and monitor insecticide resistance, biochemical assays and molecular methods were used to determine the activities and expression levels of detoxifying and protective enzymes, as well as those of related candidate genes.

2. Materials and Methods

2.1. Insects

The susceptible laboratory strain of the GPA (FFJ-S) was continuously reared on *Brassica campestris* ssp. Chinensis (L.) without insecticide exposure in the Fujian Institute of Plant Protection, Fujian Academy of Agricultural Science, Fuzhou, China, after it was collected from *Arabidopsis thaliana* in Jianxin District of Fuzhou, China, in 2008. The field population (SEF-R) (more than 2000 individuals) was collected from cabbage in the Jin'an District of Fuzhou (26°12′84″ N, 119°33′59″ E) on 3 March 2022, where high-frequency insecticide application was carried out to control aphids and other pests. Both GPA strains were reared on seedlings of *Brassica campestris* ssp. Chinensis (L.) at $21 \pm 2\,^{\circ}\mathrm{C}$ and $65\% \pm 5\%$ relative humidity under a photoperiod of 16:8 h (light: dark). The SEF-R was reared one generation before being subjected to a bioassay.

2.2. Pesticides, Synergists, and Other Chemicals

The insecticides used for bioassays included flupyradifurone (96% purity; Bayer AG, Leverkusen, Germany), thiacloprid (97.5% purity; Bayer AG), imidacloprid (97% purity; Bayer AG), esfenvalerate (90% purity; Shandong Huayang Technology Co., Ltd., Tai'an, China), beta-cypermethrin (95% purity; Nanjing Red Sun Co., Ltd., Nanjing, China), flonicamid (96% purity; Ishihara Sangyo Kaisha, Ltd., Nishi-ku, Osaka, Japan), cyantraniliprole (FMC Corporation, Philadelphia, PA, USA), spirotetramat (96% purity; Bayer AG), afidopyropen (92.5% purity; BASF SE, Ludwigshafen, Germany), broflanilide (98% purity; Mitsui Chemical lnc., Tokyo, Japan), thiamethoxam (98% purity; Syngenta Group, Basel, Switzerland), pymetrozine (95% purity; Syngenta Group, Switzerland), and sulfoxaflor (95.9% purity; Corteva Agriscience, Indianapolis, IN, USA).

Synergists piperonyl butoxide (PBO; reagent grade), S,S,S-tributyl phosphorotrithioate (DEF; reagent grade), and diethyl maleate (DEM; reagent grade) were purchased from Sinopharm Group (Holding) Co., Ltd., Shanghai, China. Ethylenediaminetetraacetic acid, albumin bovine (BSA), and sodium dodecyl sulfate were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). Eserine, α-naphthyl acetate, fast blue B salt, p-nitroanisole, n-phenylthiourea, Coomassie Brilliant Blue G250, DL-dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.3. Bioassay Methods

In order to assess the susceptibility of GPA to selected insecticides, the leaf-dip method recommended by IRAC [4] was used under laboratory conditions. The nymphs born to the field-collected aphids (F0 generation) were considered the F1 generation, and the F1 apterous females were used to generate the F2 generation. More than 20,000 (F2 generation) apterous aphids were used in susceptibility tests, synergism experiments, biochemical assays, and molecular analyses. Stock solutions were prepared by dissolving technical grad insecticide in acetone. Then, the stock insecticide solutions were dissolved in 0.1% Triton X-100 to prepare five to six concentrations, and the 0.1% Triton X-100 alone was used as the control. Clean leaf discs obtained from healthy cabbage balls of *B. oleracea* L. were

dipped in insecticide solutions for 10 s. After being air-dried under room conditions, the leaf discs were placed onto 1% agar plate (20 mm depth) in a Petri dish (30 mm diameter and 40 mm depth). Spirotetramat and pymetrozine act primarily on immature life stages of aphids, but the other 11 insecticides are effective against female adults. Then, 20 apterous aphids (2-d-old) (for spirotetramat and pymetrozine test) or apterous adults (for other insecticides) were each transferred onto a treated leaf disc using a paint brush, and each unit was covered with a close-fitting, ventilated lid. Mortalities were observed 4 d later in order to determine the impact of spirotetramat and pymetrozine and 3 d later to determine other insecticide effects. Aphids that could not right themselves within 10 s once turned on their backs were considered dead. Four replicates were conducted for each treatment.

2.4. Resistance Stability

In order to test the stability of resistance, the SEF-R strain was reared for 15 generations (equivalent of approximately 1 year in the field) without insecticide exposure in order to investigate whether resistance to imidacloprid, beta-cypermethrin, sulfoxaflor, and pymetrozine was stable. GPA of the 1st (G1), 4th (G4), 7th (G7), 10th (G10), 12th (G12), and 15th (G15) generations were subjected to a bioassay. The average rate of change in response per generation (R) was estimated in accordance with Tabashnik [31], as follows:

$$R = \log_{10}[\text{final LC}_{50}] - \log_{10}[\text{initial LC}_{50}]/n,$$

where n represents the number of generations. An increase or decrease in resistance is indicated by positive and negative R values, respectively.

2.5. Synergism Experiment

The effects of three synergists (PBO, DEF, and DEM) against GPD, each used in combination with a thiacloprid or cypermethrin mixture, were evaluated using the bioassay method. The highest doses of PBO, DEF, and DEM on the susceptible strain that led to zero mortality were found to be 0.08, 0.05, and 0.06 g L^{-1}, respectively, using the bioassay method. Apterous adult aphids were exposed to leaf discs that were treated with PBO, DEF, or DEM combined with the thiacloprid and cypermethrin mixture. The concentrations used in FFJ-S were 4 mg L^{-1}, 3 mg L^{-1}, 2 mg L^{-1}, 1 mg L^{-1}, 0.6 mg L^{-1} and 0.4 mg L^{-1} for imidacloprid or imidacloprid and 40 mg L^{-1}, 20 mg L^{-1}, 10 mg L^{-1}, 5 mg L^{-1}, 2.5 mg L^{-1}, 1.25 mg L^{-1}, and 0.625 mg L^{-1} for beta-cypermethrin, while the concentrations used in the SEF-R strain ranged from 2 mg L^{-1} to 450 mg L^{-1} for imidacloprid and ranged from 20 mg L^{-1} to 8000 mg L^{-1} for beta-cypermethrin. Four replicates were performed for each concentration in the bioassay.

2.6. Detoxification Enzyme Activity Assays

Aphids from the FJJ-S and SEF-R strains were used directly for biochemical assay. The esterase activity was measured using a microplate reader in accordance with the work of Byrne and Devonshire [32]. Individual aphids were homogenized in 60 µL of ice-cold phosphate-buffered saline (PBS) (0.02 M, pH 6.5) and centrifuged at 4 °C and 10,000× *g* for 10 min. The supernatant was used as the esterase source. After incubating for 5 min at room, 100 µL substrate α-naphthyl acetate (107 mM) and 100 µL of eserine (107 mM) were mixed with the color development reagent (6 mg fast blue RR salt dissolved in 10 mL of 0.02 M PBS, pH 6.5) in the dark. Then, 150 µL of this reaction liquid was added to a 96-well microplate (Corning Life Sciences, Tewksbury, MA, USA), followed by the addition of 50 µL of supernatant fluid and 50 µL of PBS per well. The absorbance at 450 nm was measured for 5 min continuously using the kinetic model in a microplate reader (SPECTRA max PLUS384, Molecular Devices, San Jose, CA, USA). The esterase activity was reported as mOD450/min/aphid. For each population, approximately 100 individuals were measured.

The methodology used for the determination of the GST activity is quite a well-known routine procedure involving 1-chloro-2,4-dinitrobenzene, with slight modifications [33]. Briefly, 60 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 $10,000\times g$ for 10 min at 4 °C. Briefly, a 300 μL reaction mixture containing 100 μL diluted enzyme solution, 100 μL 1-chloro-2,4-dinitrobenzene (1.2 mM) substrate solution, and 100 μL glutathione (6 mM) was prepared, after which the absorbance was measured at 340 nm for 10 min using the kinetic model. The results were determined based on the protein concentration of an enzyme source, and the specific activity was converted from an OD value.

The monooxygenase enzyme (MFO) activity was measured in accordance with Shang's method [34]. Briefly, 60 adult aphids were homogenized in 2.0 mL ice-cold PBS (0.04 mol L^{-1}, pH 7.8). The supernatant obtained via centrifuging $10,000\times g$ 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 (1 mol L^{-1}) was added to terminate the reaction after incubation for 30 min at 37 °C. Then, the reaction unit was extracted using a solution of sodium hydroxide (NaOH) and chloroform. Finally, the optical density (OD) of the enzyme source was recorded at 400 nm using a microplate reader. The specific activity was obtained using a nitrophenol standard curve and the protein concentration of the enzyme source.

2.7. Determination of Protective Enzyme Activities in M. persicae

In total, 30 adult aphids from the FJJ-S and SEF-R strains were homogenized in a phosphate buffer (0.1 M, pH 7.0) at 0 °C using an electric mechanical homogenizer. After centrifuging the homogenate at 8000 g for 10 min at 4 °C, the supernatant was used to measure the activity of CAT (catalog no. A007-2), POD (catalog no. A084-1), and SOD (catalog no. A001-3) using commercial assay kits in accordance with the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A spectrophotometric method, operating based on the ultraviolet absorption of peroxide released from the activity of CAT on hydrogen peroxide, was used to determine the CAT level at 240 nm, and the nitroblue tetrazolium reduction method and the guaiacol method were used to determine the SOD activity at 560 nm and POD activity at 470 nm, respectively.

The protein contents of the enzyme solutions were determined using the Bradford method [35]. Serial dilutions were made of a BSA solution, and the samples were measured together. The protein contents of the samples were calculated using a standard curve based on the BSA solution. The diluted enzyme solutions (50 μL) were mixed with Coomassie Brilliant Blue (200 μL). After incubating the solutions at 25 °C for 10 min, the absorbance at 595 nm was measured. All the experiments were repeated three times, and the average values were obtained from the triple-replicated data.

2.8. Detection of R81T, L1014F and M918T Mutations

Genetic analyses of GPA from SEF-R were conducted to determine target site resistance. This included the nicotinic acetylcholine receptor mutation R81T and the voltage-gated sodium channel knockdown resistance mutations L1014F (kdr) and M918L (Super-kdr), responsible for neonicotinoid and pyrethroid resistance in *M. persicae*, respectively. DNA from a single individual GPA was extracted using DNAVzol reagent kit (ThermoFisher SCIENTIFIC, Waltham, MA, USA) according to the manufacture's recommendations. The real-time TaqMan assays were used for the mutation identification of genome DNA in a MasterCycler ep RealPlex4 machine (Eppendorf, Hamburg, GER) in accordance with the methods described in other studies [36,37].

2.9. Gene Expression Analysis

The mRNA levels of different enzyme-encoding genes in the FFJ-S and SEF-R strains were measured using RT-qPCR with SYBR® Green Supermix (ThermoFisher, USA) in a qTOWER 2.2 real-time quantitative PCR system (Analytikjena, Jena, Germany). Total RNA (30 apterous adult aphids per sample) was extracted using TRIzol according to the manufacture's instructions (Invitrogen, Waltham, MA, USA) and quantified using a ScanDrop 100 spectrophotometer (Analytikjena) in accordance with the manufacturer's protocols. The RNA concentration was diluted to 0.8 μg $μL^{-1}$ using diethyl pyrocarbonate-treated H_2O, and 0.8

µg of RNA was then reverse-transcribed in a 20 µL reaction volume using a TUREscript 1st Stand cDNA Synthesis Kit (Aidlab, Beijing, China), with the beta actin gene as an internal control (NCBI gene ID: XM_022321094.1). In total, 15 genes belonging to the detoxification of protective enzymes were quantified, namely, CYP6CY3 (Gene ID: KF218356), CYP6K1 (Gene ID: XM_022323152), CYP6CY23 (Gene ID: MF155662), CYP380C40 (Gene ID: OM677847), CYP6CY9 (Gene ID: XM_022316140), CYP6CY56 (Gene ID: MT472683), CYP4G15 (Gene ID: XM_022323310), CYP3CY06 (Gene ID: XM_022312311), E4 (Gene ID: X74554), THEM (Gene ID: XM_022326659), GST (Gene ID: 111036096), UGT344P2 (Gene ID: OM677846), POD (Gene ID: 112683716), SOD (Gene ID: 111035379), and CAT (Gene ID: 111041019). The primers for these genes were designed using the Primer Express 3.0 software based on the sequences of target genes in NCBI or other research, and they are listed in Supplementary Materials.

Each RT-qPCR was conducted in a 20 µL mixture containing 1 µL of sample cDNA, 1 µL of each primer (200 nM), 6 µL of diethyl pyrocarbonate-treated H_2O, and 10 µL of $2\times$ SYBR® Green Supermix. The qPCR cycling parameters were as follows: 95 °C for 3 min, followed by 39 cycles of 95 °C for 10 s and 58 °C for 30 s. A plate reader was used for the analysis. Melting curve generation was performed from 60 to 95 °C (+1 °C (+1 ti, holding time 4 s). After adding all the components, the samples were centrifuged at $6000\times g$ for 1 min to keep all the components in the tube bottom. To check the reproducibility of the assay results, a was performed qPCR for each sample using three technical replicates and three biological replicates. The relative gene expression was calculated automatically using qPCRsoft3.2 software. The comparative $2^{-\Delta\Delta CT}$ method [38] was used for the relative quantification calculation.

2.10. In Vivo RNAi of CYP6CY3 and Bioassays

A knockdown of CYP6CY3 was performed using RNAi in SEF-R to evaluate the effect of CY6CY3 on the resistance of Group 4 insecticides. Fragments of CYP6CY3 were cloned into pGEM-T (Promega Corporation, Madison, WI, USA) and used as templates for dsRNA-CYP6CY3 synthesis using a T7 RiboMAX™ Express RNAi System (Promega Corporation, Madison, WI, USA) in accordance with the work of Peng et al. [39]. The dsRNA-CYP6CY3 (100 ng mL^{-1} in diet) was added to the artificial diet [40], and DEPC water and dsGFP (dsRNA of GFP) were also added to the artificial diet as the control. The dsRNA-CYP6CY3-treated artificial diet, containing imidacloprid (100 mg L^{-1}), thiacloprid (40 mg L^{-1}), thiamethoxam (30 mg L^{-1}), sulfoxaflor (50 mg L^{-1}), or flupyradifurone (10 mg L^{-1}), was used in the toxicity assessment. Additionally, the diet supplemented with DEPC water or dsGFP, albeit without insecticides, was used as the control. The experiments were performed in triplicate. To determine the efficiency of the dsRNA knockdowns of CYP6CY3, the aphids were collected after they fed on the artificial diet for 48 h, and the samples were used for qRT-PCR analysis. The mortality of the aphids was also calculated for 48 h. Each treatment was replicated in triplicate.

2.11. Data Analysis

The enzymatic activities and the gene expression data between SEF-R and FJJ-S were compared using Student's *t* tests, and the mortalities of the GPA that were RNAi- plus insecticide-treated were compared versus the mortalities of the control via a one-way analysis of variance in combination with Fisher's least significant difference multiple comparison tests. These experiments were analyzed using a DPS Data Processing System (Hangzhou Ruifeng Technology LTD., Hangzhou, China) [41]. All the data are presented as the means $\pm$ standard errors (SEs). Differences were considered significant at *p* value < 0.05 or 0.01. The median lethal concentrations (LC$_{50}$) of each insecticide for adult apterous aphids were obtained using the DPS Data Processing System. LC$_{50}$ values without overlap among 95% confidence intervals (CIs) were considered significantly different. The resistance factor (RF) was calculated as the ratio of the LC$_{50}$ value of the field population to the LC$_{50}$ value of the FFJ-S strain. Resistance levels were classified in accordance with the standard reported by World Health Organization [42]: susceptibility (RF < 3-fold), decreased susceptibility (

RF = 3- to 5-fold), low resistance (RF = 5- to 10-fold), moderate resistance (RF = 10- to 40-fold), high resistance (RF = 40- to 160-fold), and extremely high resistance (RR > 160-fold).

3. Results

3.1. Insecticide Resistance

The bioassays of the field and susceptible populations indicated that the field population had a reduced susceptibility to the 13 tested insecticides according to the WHO standard (Table 1). The LC_{50} of the SEF-R strain showed a reduced susceptibility to five insecticides of Group 4 in IRAC: imidacloprid (116.48 mg L^{-1}), thiacloprid (54.86 mg L^{-1}), thiamethoxam (33.2 mg L^{-1}), sulfoxaflor (51.51 mg L^{-1}), and flupyradifurone (12.67 mg L^{-1}). When compared with the FFJ-S strain, the SEF-R exhibited a 106.9-fold resistance to imidacloprid, 29-fold resistance to thiacloprid, 12.9-fold resistance to thiamethoxam, 32.4-fold resistance to sulfoxaflor, and 9.5-fold resistance to flupyradifurone.

Table 1. Resistance of FFJ-S and SEF-R strains of GPA to 13 insecticides.

Insecticides.	Group [a]	Strains	No.	Slope (SE)	LC_{50} (95% CI) mg·L^{-1}	χ^2	RF [b]
Thiacloprid	Group 4	FFJ-S	480	4 (0.33)	1.89 (1.74–2.06)	3.32 (df = 3)	
		SEF-R	560	1.57 (0.14)	54.86 (45.64–66.52)	3.04 (df = 4)	29
Imidacloprid	Group 4	FFJ-S	560	2.68 (0.21)	1.09 (0.97–1.22)	2.12 (df = 4)	
		SEF-R	480	1.83 (0.18)	116.48 (97.95–138.88)	2.45 (df = 3)	106.9
Thiamethoxam	Group 4	FFJ-S	560	4.71 (0.36)	2.57 (2.4–3.74)	5.5 (df = 4)	
		SEF-R	480	1.64 (0.18)	33.2(27.37–40.49)	2.61 (df = 3)	12.9
Sulfoxaflor	Group 4	FFJ-S	480	2.79 (0.25)	1.59 (1.41–1.79)	4.25 (df = 3)	
		SEF-R	480	2.31 (0.26)	51.51 (42.81–61.19)	1.6 (df = 3)	32.4
Flupyradifurone	Group 4	FFJ-S	480	2.68 (0.24)	1.34 (1.17–1.51)	2.61 (df = 3)	
		SEF-R	480	1.99 (0.19)	12.67 (10.78–14.92)	2.94 (df = 3)	9.5
Esfenvalerate	Group 3	FFJ-S	480	2.04 (0.19)	9.66 (8.24–11.33)	3.76 (df = 3)	
		SEF-R	480	1.77 (0.17)	764.4 (639.34–915.07)	3.09 (df = 3)	79.1
beta-Cypermethrin	Group 3	FFJ-S	560	1.8 (0.15)	7.32 (6.17–8.69)	3.15 (df = 4)	
		SEF-R	480	1.9 (0.18)	2371.54 (2006.7–2823.87)	0.64 (df = 3)	324
Flonicamid	Group 29	FFJ-S	560	2.02 (0.16)	1.35 (1.15–1.57)	2.47 (df = 4)	
		SEF-R	480	1.79 (0.18)	7.85 (6.55–9.42)	2.53 (df = 3)	5.8
Pymetrozine	Group 9B	FFJ-S	560	1.84 (0.15)	0.86 (0.73–1.01)	3.54 (df = 4)	
		SEF-R	480	1.99 (0.19)	29.89 (25.45–35.4)	3.88 (df = 3)	34.8
Afidopyropen	Group 9D	FFJ-S	480	2.03 (0.19)	0.74 (0.62–0.87)	2.38 (df = 3)	
		SEF-R	480	2.1 (0.2)	2.92 (2.49–3.44)	2.81 (df = 3)	4
Spirotetramat	Group 23	FFJ-S	480	2.09 (0.19)	1.05 (0.9–1.23)	2.51 (df = 3)	
		SEF-R	480	2.05 (0.19)	8.45 (7.22–9.95)	4.92 (df = 3)	8.1
Cyantraniliprole	Group 28	FFJ-S	480	1.95 (0.19)	8.38 (7.07–9.87)	3.55 (df = 3)	
		SEF-R	480	1.81 (0.18)	28.81 (24.21–34.55)	0.94 (df = 3)	3.4
Broflanilide	Group 30	FFJ-S	560	1.78 (0.15)	0.98 (0.83–1.17)	4.88 (df = 4)	
		SEF-R	480	1.82 (0.18)	15.23 (12.81–18.27)	0.43 (df = 3)	15.5

[a], IRAC MoA Classification; [b], RF (resistance factor) = LC_{50} value of a field population/LC_{50} value of the Lab-SS strain.

The SEF-R strain showed a high resistance to esfenvalerate (RF = 79.1-fold; LC_{50} = 764.4 mg L^{-1}) and an extremely high resistance to beta-cypermethrin (RF = 324-fold; LC_{50} = 2371.54 mg L^{-1}). The SEF-R strain also developed high resistance to pymetrozine (RF = 34.8-fold; LC50 = 29.89 mg L^{-1}) and broflanilide (RF = 15.5-fold; LC_{50} = 15.23 mg L^{-1}). For the other three insecticides, low resistance to spirotetramat (RF = 8.1-fold;

LC_{50} = 8.45 mg L^{-1}) and flonicamid (RF = 5.8-fold; LC_{50} = 7.85 mg L^{-1}) was detected in SEF-R. The LC_{50} values for afidopyropen and cyantraniliprole in the SEF-R were 4.0 and 3.4 times higher than those in FFJ-S, respectively (Table 1), which indicated a decreased susceptibility in the SEF-R to the two insecticides.

3.2. Stability of Resistance to Selected Insecticides

The field population of GPA, SEF-R, was raised under laboratory conditions for 15 generations without insecticide exposure. Susceptibility to imidacloprid, beta-cypermethrin, sulfoxaflor, and pymetrozine in GPA were evaluated in G1, G4, G7, G10, G12, and G15, respectively. The GPA became much more susceptible to the selected four insecticides in the absence of insecticide selection. The RF of GPA against imidacloprid decreased from 106.9-fold in G1 to 18.7-fold in G15 (Figure 1A) relative to the susceptible strain FFJ-S. The resistance level of GPA to imidacloprid dropped slowly from G1 (106.9-fold) to G4 (101.7-fold), reduced sharply from G4 to G12, and then maintained a stable level from G12 to G15.
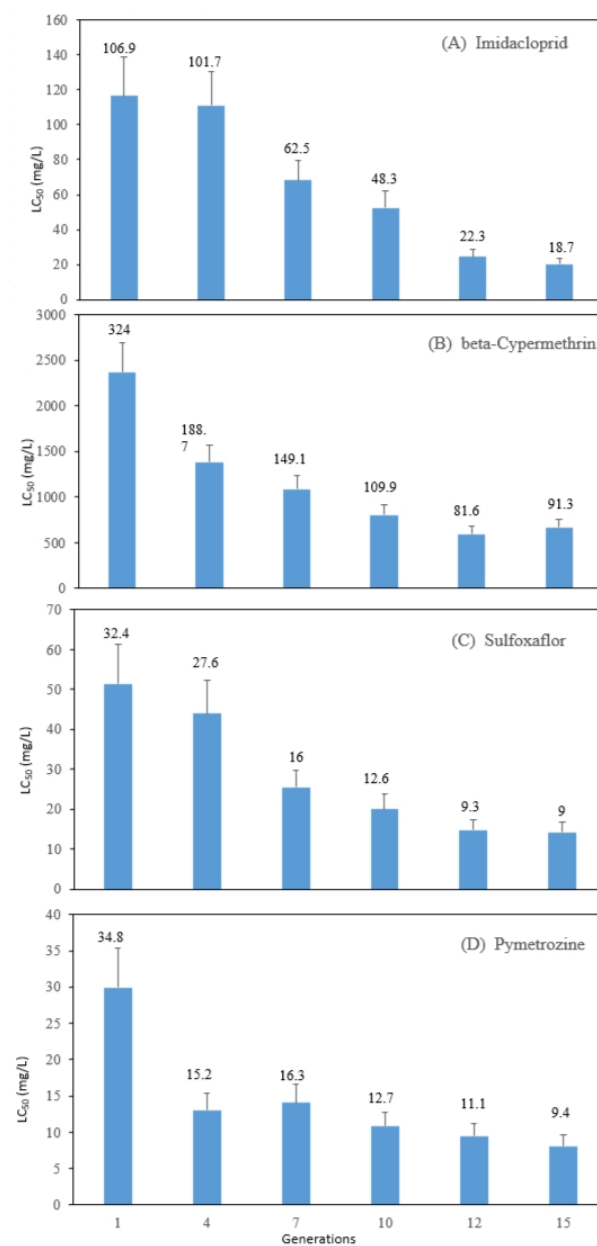

Figure 1. Change in LC_{50} values (95% CI) of imidacloprid (**A**), beta-cypermethrin (**B**), sulfoxaflor (**C**), and pymetrozine (**D**) to *M. persicae* collected from the field and reared in the laboratory without

exposure to any insecticides. Numbers on the tops of the graph bars represent resistance ratios compared with those of susceptible strain Pila.

The resistance level of GPA to beta-cypermethrin quickly declined from 324-fold in the G1 to 109.9-fold G10 and then remained steady from G10 to G15 (Figure 1B). The resistance level of GPA to sulfoxaflor decreased slowly from G1 to G4, and then it quickly dropped from G4 to G10 (Figure 1C). Meanwhile, the RF of the GPA to pymetrozine sharply dropped from 34.8-fold (G1) to 15.2-fold (G4) and trended towards being stable from G4 to G15 (Figure 1D). The declines in rates of resistance against imidacloprid, beta-cypermethrin, sulfoxaflor, and pymetrozine were −0.05, −0.04, −0.04, and −0.04 in G15, respectively.

3.3. Synergist Assessments and Enzyme Activity Levels

The synergistic effects of PBO, DEF, and DEM on the toxicity of imidacloprid and beta-cypermethrin to the resistance field strain SEF-R were evaluated. The synergism ratios of PBO, DEF, and DEM on imidacloprid to SEF-R were 4.16-, 2.15-, and 3.2-fold (Table 2), respectively, and those on beta-cypermethrin to SEF-R were 6.43-, 3.84-, and 1.91-fold (Table 3), respectively. These three synergists had little synergistic effects on the applicability of imidacloprid or beta-cypermethrin to susceptive strain FFJ-S (Tables 2 and 3).

Table 2. Synergism of three enzyme inhibitors on imidacloprid toxicity to GPA.

	Treatment	No.	Slope ± SE	LC$_{50}$ (95% CI)	χ^2	SR [b]
FFJ-S	IMDP [a]	560	2.68 (0.21)	1.09 (0.97–1.22)	2.12 (df = 4)	
	IMDP + PBO	480	3.18 (0.28)	1.1 (0.99–1.22)	4.07 (df = 3)	0.99
	IMDP + DEF	480	3.3 (0.28)	1.13 (1.02–1.25)	5.08 (df = 3)	0.96
	IMDP + DEM	480	2.7 (0.26)	1.01 (0.9–1.14)	5.31 (df = 3)	1.08
SEF-R	IMDP	480	1.83 (0.18)	116.48 (97.95–138.88)	2.45 (df = 3)	
	IMDP + PBO	560	1.56 (0.14)	27.99 (23.23–33.81)	6.37 (df = 4)	4.16
	IMDP + DEF	560	1.63 (0.16)	54.13 (45.19–64.94)	6.42 (df = 4)	2.15
	IMDP + DEM	560	1.55 (0.14)	36.36 (30.14–43.91)	5.43 (df = 4)	3.2

[a], IMDP: imidacloprid; [b], SR (synergism ratio) = LC$_{50}$ of imidacloprid/LC$_{50}$ of imidacloprid with synergist.

Table 3. Synergism of three enzyme inhibitors on beta-cypermethrin toxicity to GPA.

Insecticides	Treatment	No.	Slope ± SE	LC$_{50}$ (95% CI)	χ^2	SR [b]
FFJ-S	CYPE [a]	560	1.8 (0.15)	7.32 (6.17–8.69)	3.15 (df = 4)	
	CYPE + PBO	560	1.75 (0.14)	7.09 (5.98–8.42)	3.26 (df = 4)	1.03
	CYPE + DEF	560	1.62 (0.14)	7.1 (5.93–8.52)	2.35 (df = 4)	1.03
	CYPE + DEM	560	1.82 (0.15)	7.61 (6.43–9.03)	3.74 (df = 4)	0.96
SEF-R	CYPE	480	1.9 (0.18)	2371.54 (2006.7–2823.87)	0.64 (df = 3)	
	CYPE + PBO	480	1.81 (0.18)	368.74(309.07–439.99)	3.6 (df = 3)	6.43
	CYPE + DEF	480	1.98 (0.18)	617.18 (524.91–732.39)	2.95 (df = 3)	3.84
	CYPE + DEM	480	2.1 (0.21)	1241.92 (1054.77–1468.27)	1.2 (df = 3)	1.91

[a], CYPE: beta-cypermethrin; [b], SR (synergism ratio) = LC$_{50}$ of beta-cypermethrin/LC$_{50}$ of beta-cypermethrin with synergist.

The SEF-R strain possessed significantly higher P450 (3.06-fold), carboxylesterase (3.04-fold), GST (1.5-fold), SOD (1.2-fold), and POD (3.4-fold) activities compared with the FFJ-S strain (Figure 2). The CAT activity was reduced significantly in the SEF-R.
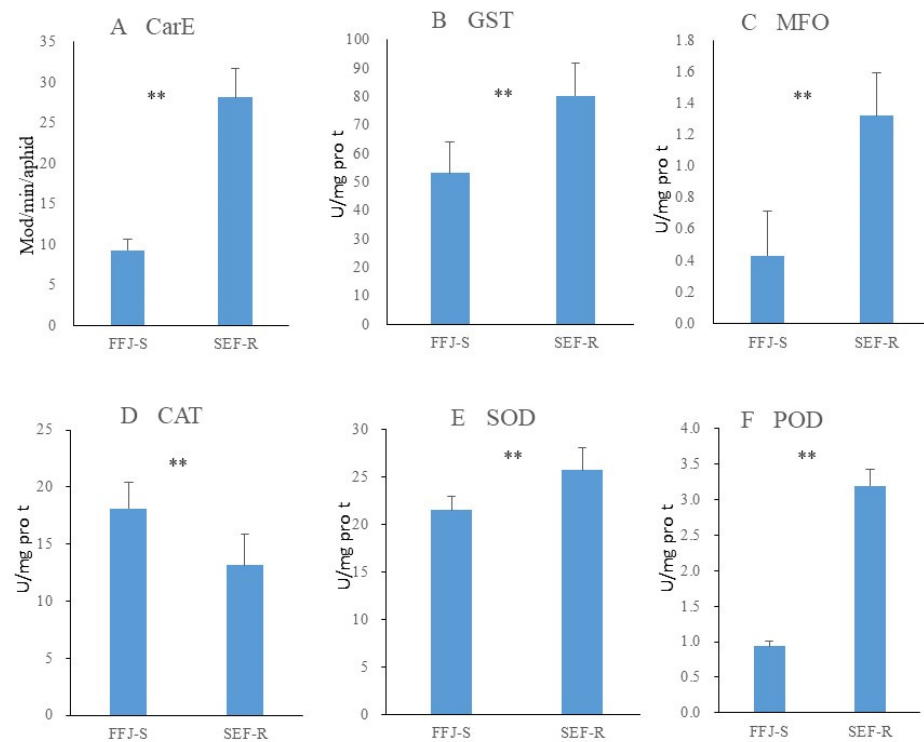
Figure 2. Activities of detoxifying enzymes and protective enzymes in FFJ-S and SEF-R. The asterisk ** in the Figure indicates significant differences as determined by Student's *t* test ($p < 0.01$).

3.4. R81T, kdr and Super-kdr Resistance

The R81T and kdr mutations were detected in the SEF-R, but a super-kdr mutation was not found. In the SEF-R, the predominant genotype was the heterozygote for the R81T and kdr mutations. Its percentage of heterozygote was 64.29% for R81T and 79.35% for kdr, while the percentage of RR was 10.2% for R81T, respectively (Figure 3).

Figure 3. Frequency of insecticide resistance genotypes (R81T, L1014F and M918T mutations) using the Taqman PCR assay. One hundred individual aphids were tested per genotype test. SS: wild type; RS: heterozygous resistant; RR: homozygous resistant.

3.5. Enzyme Genes Expression

The expression differences in 15 metabolic and protective enzyme-encoding genes between SEF-R and FFJ-S were assessed (Figure 4) via qPCR analysis. Among the eight

selected P450 genes, six, namely CYP6CY3 (3.63-fold), CYP6CY23 (3.13-fold), CYP380C40 (2.51-fold), CYP6CY9 (2.46-fold), CYP4G15 (5.24-fold), and CYP3CY06 (1.71-fold), were significantly upgraded. However, the CYP6K1 and CYP6CY56 genes were downgraded in the SEF-R population (Figure 4). A significant overexpression of all three esterase genes, E4 (7.11-fold), and THEM (11.47-fold), was observed in the SEF-R population. The expression of UGT344P2 was estimated to have increased 18-fold in SEF-R, which made it the most highly overexpressed gene tested. The GST showed no significant difference in expression level between the SEF-R and FFJ-F. For the three protective enzyme genes, POD (2.04-fold) was significantly upregulated and SOD showed no significant difference, but the CAT level (0.57-fold) was significantly downregulated in the SEF-R compared with its level in the FFJ-S.
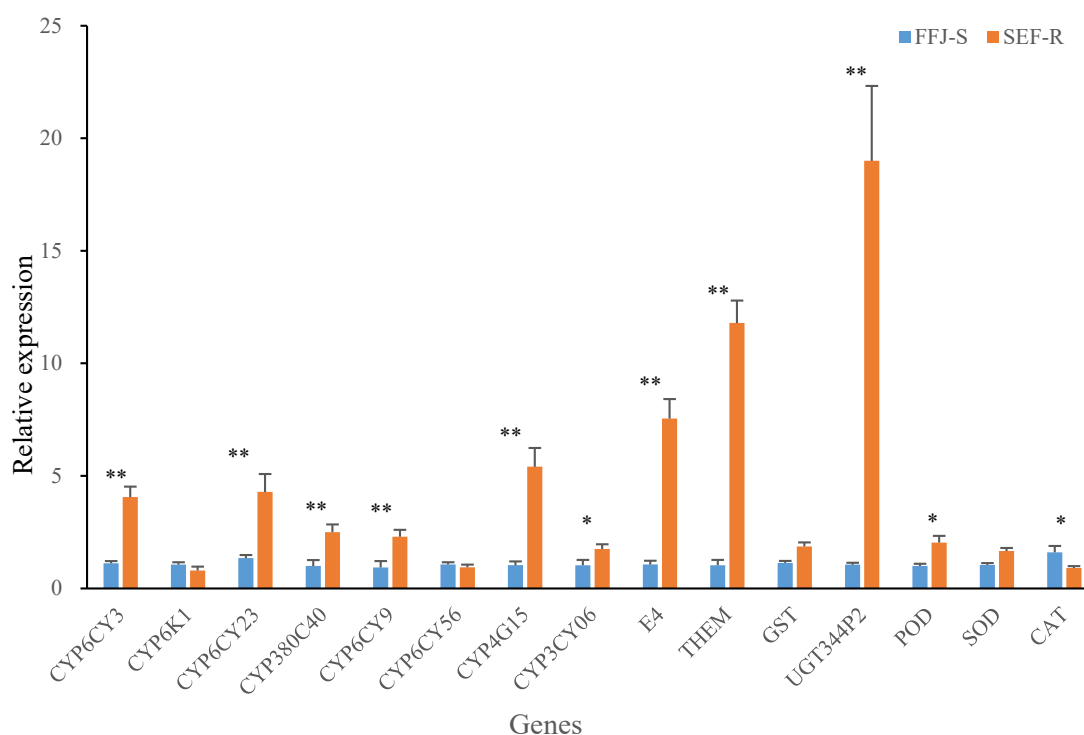

Figure 4. Quantitative real-time PCR validation of the expression levels of differentially expressed P450 genes identified using RNA-sequencing. The expression levels were normalized to GAPDH and β-actin genes. The asterisk * in the Figure indicates significant differences as determined by Student's *t* test ($p < 0.05$). The asterisk ** in the Figure indicates significant differences as determined by Student's *t* test ($p < 0.01$).

3.6. Knockdown of CYP6CY3 Increases the Sensitivity of M. persicae to Group 4 Insecticides

To evaluate the functional role of CYP6CY3 in resistance to Group 4 insecticides in GPA, the expression levels of CYP6CY3 were decreased via RNA interference in the SEF-R population, and the toxicity levels of imidacloprid, thiacloprid, thiamethoxam, sulfoxaflor, and flupyradifurone were tested after RNAi exposure. After GPA were fed a dsRNA-incorporated diet for 48 h, the transcript levels of CYP6CY3 were reduced 0.62-fold compared with that of the control, which contained dsGFP (Figure 5A). The mortality levels after dsCYP6CY3 treatment, imidacloprid exposure, thiacloprid exposure and thiamethoxam exposure were 78.33%, 66.67%, and 68.33%, respectively, which were significantly higher than those of the control at the diagnostic dose of sulfoxaflor (Figure 5B). However, sulfoxaflor and flupyradifurone exposure did not affect the mortality rate.
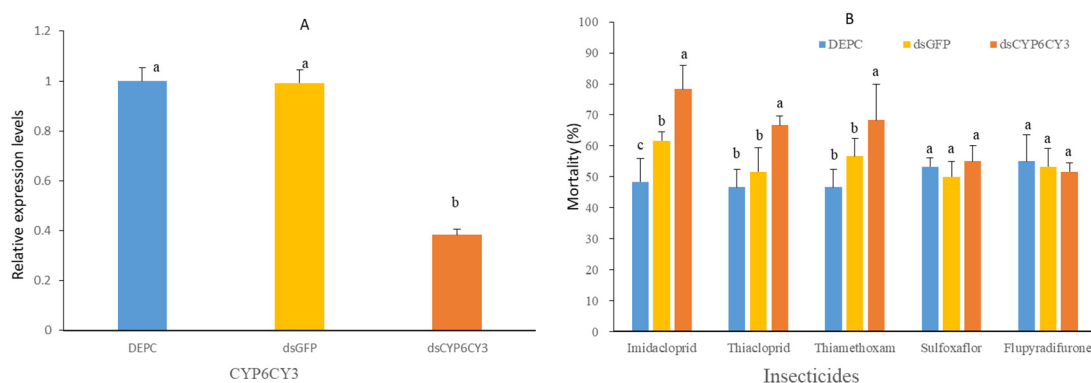
Figure 5. Knockdown of *CYP6CY3* increased the sensitivity of resistant aphids to imidacloprid, thiacloprid, thiamethoxam, sulfoxaflor, and flupyradifurone. (**A**) Relative expression levels of CYP6CY3, (**B**) (%) mortality at 48 h of dsRNA-fed adult GPA after treatment with imidacloprid (100 mg L^{-1}), thiacloprid (40 mg L^{-1}), thiamethoxam (30 mg L^{-1}), sulfoxaflor (50 mg L^{-1}), and flupyradifurone (10 mg L^{-1}). The bars with lowercase letters (a,b) on top are significantly different according to the one-way analysis of variance followed by Tukey's multiple comparison test ($p < 0.05$).

4. Discussion

GPA evolution and the spread of insecticide resistance in its population are considered major challenges in the management and elimination of the pest and its transmitted virus. To date, the GPA has developed resistance to almost all insecticides used worldwide. An aphid-monitoring survey suggested that the GPA in a cabbage field was not susceptible to the 13 tested insecticides categorized into six MoA groups [4]. Multi-insecticide resistance might drastically reduce the number of insecticides that can be used to control the insect in this district. In order to delay the development of resistance and prolong the product's effectiveness, implementing insecticide resistance management strategies, including rational applications of insecticides, is urgently needed according to monitoring data.

As in most other countries, neonicotinoids (Group 4A) are among the most important chemical classes of insecticides used against the GPA in China [43]. More than 55% of all registered products in China contain neonicotinoids, which play key roles in the tactics of GPA management [6]. However, according to the Arthropod Pesticide Resistance Database (APRD), GPA have the second-greatest number of cases of neonicotinoid resistance, behind only those of *Bemisia tabaci* [44]. This represents a serious threat to the effective control of GPA with neonicotinoids [40]. The high level of imidacloprid resistance detected in SEF-R reflected its status in cabbage fields, and the results were consistent with other monitoring data. Since susceptibility in the GPA was shown to decrease in 2005 [45], elevated levels of resistance to imidacloprid have been observed in most of the provinces of China in which GPA have been tested, such as Beijing [46], Hunan [47], Jiangsu, and Shanghai [48]. According to published documents, confirmed resistance to imidacloprid is much higher in vegetable aphid populations (RF = 32–350.9-fold) than in tobacco aphid populations (RF = 6.03–22-fold) in China [8]. Additionally, GPA collected from a cabbage field in Longyao, Heibei Province, exhibited the highest resistance (2853-fold) [48]. We speculated that selection pressures rather than host plants shaped the evolution of imidacloprid resistance because GPA collected from tobacco fields in Europe evolved a moderate-to-high resistance to imidacloprid (RF > 15 detected in 86.7% populations in 2015) [49]. Less insecticide needed to be used against GPA because of the short growing season of tobacco in China, which also indicated that the limited application of imidacloprid delayed resistance development. The present study revealed a moderate-level resistance in the SEF-R clone to the other two neonicotinoids, thiacloprid and thiamethoxam, that were registered in 2015 to kill aphids on cabbage. Zhang [50] found that GPA collected from cabbage fields in Guangdong and Hunan Provinces, in southern and southeastern China, respectively, were still susceptible to thiamethoxam. There are few products of thiamethoxam (eight products) and thiacloprid (four products) allowed in cabbage aphid management, which may reduce

their spray applications in fields [6]. We inferred that the moderate resistance to the two nicotinoids in SEF-R was caused by cross-resistance of thiacloprid or thiamethoxam with imidacloprid, as verified in other studies [51,52].

As nAChR-competitive modulators, sulfoxaflor (Subgroup 4C) and flupyradifurone (Subgroup 4D) belong to the novel classes sulfoximines and butenolides, respectively. Previous studies showed that the two compounds are effective against some GPA populations exhibiting neonicotinoid resistance [53,54], but subsequent research confirmed the cross-resistance of sulfoxaflor or flupyradifurone with imidacloprid in some field populations [52,55]. SEF-R exhibited resistance to the two compounds. Today, sulfoxaflor (Tefuli®) and flupyradifurone (Jinxian®) are only registered by Corteva Agriscience and Bayer AG, respectively, which easily allows us to establish their application history. Growers did not apply the two compounds in the field in recent years. However, field populations of GPA in other countries have developed high resistance to the two compounds. In total, 25% of the field-collected GPA in Greece had an RF > 119.2 for flupyradifurone [30], and field populations collected in Western Australia and Spain exhibited 4–23-fold and 0.6–61-fold resistance to sulfoxaflor, respectively [20]. Our study first reported the resistance of GPA to sulfoxaflor and flupyradifurone in China, which might be caused by high resistance to imidacloprid. The loss of sensitivity of GPA to sulfoxaflor and flupyradifurone threatened the applications of the two insecticides as alternative tools for integrated pest and resistance management. The resistance of SEF-R to the five Group 4 insecticides makes it difficult to select related products. Stability experiments showed that the RFs of imidacloprid and sulfoxaflor dropped to 18.7- and 9-fold, respectively, after 15 generations without selection (Figure 1A). The study highlighted that the cessation of selective pressure could help to restore the susceptibility of GPA to imidacloprid and sulfoxaflor, but that their use should be prohibited for at least one year. For Group 4 compounds, thiamethoxam and flupyradifurone should be recommended as alternative insecticides to manage GPA in the field. However, practitioners should avoid repeated applications.

Synthetic pyrethroids (Group 4A) form another common insecticide group that have been used for several decades to control GPA in China. While GPA collected from Beijing and Hebei exhibited extremely high resistance levels to fenvalerate (RF = 675) and deltamethrin (RF = 1468) in 1990 [56], pyrethroid resistance became widespread across China by the late 1990s. Surveys from 2011 to 2014 revealed that more than 85.5% of the 62 collected field populations had developed a high resistance to beta-cypermethrin [48] in China. The SEF-R population exhibited extremely high resistance to beta-cypermethrin (RF = 324-fold) and high resistance to esfenvalerate (RF = 79.1-fold). The recommended doses for GPA control ranged from 25 to 90 mg L^{-1}, far below the LC50 values of esfenvalerate (764.4 mg L^{-1}) and beta-cypermethrin (2371.54 mg L^{-1}) (Table 1), which led to control failures of GPA after the application of the two pyrethroids at recommended concentrations. After the SEF-R was reared for 15 generations without exposure to any insecticides, the GPA maintained a 91.3-fold resistance to beta-cypermethrin (LC$_{50}$ = 667.97 mg L^{-1}) (Figure 1B). The two pyrethroids were not advised for use to control GPA in this field.

The IRAC classified pymetrozine [57] and flonicamid [58] into Group 9, "Selective homopteran feeding blockers", with pymetrozine in Subgroup 9B and flonicamid in Subgroup 9C. The two compounds target the chordotonal organ, resulting in an inhibitory effect on aphid feeding, and they offer attractive alternatives to conventional insecticides due to a lack of cross-resistance. Pymetrozine has been present the Chinese market for about two decades, and flonicamid has also been used for 10 years. According to the Arthropod Pesticide Resistance Database [44], there have been no reports of field resistance to flonicamid in GPA worldwide. In Greece, RFs to pymetrozine ranged from 1.1 to 2.2) in 2018 [59], even lower than those found in 2010 (1.0–3.7 and 1.0–6.0 in clones from peach and tobacco, respectively) [60] although ~10 years had passed. Astonishingly, the SEF-R evolved 5.8- and 29.89-fold resistance to flonicamid and pymetrozine, respectively. However, 205.9-fold resistance to pymetrozine has been documented in A. gossypii collected from Cheongju, South Korea [61], and 1100-fold resistance to pymetrozine has also been

detected in *B. tabaci* [62]. These reports suggest possible resistance development in GPA to the two compounds. Furthermore, resistance to pymetrozine decreased quickly during the first four generations and remained stable in the subsequent nine generations. Owing to the quick increase in susceptibility due to the suspended application of pymetrozine and the relative susceptibility of the SEF-R to flonicamid, they could be used as an alternative to other insecticides. The insecticides spirotetramat (Group 23), afidopyropen (Group 9D), and cyantraniliprole (Group 28) were recently used as aphicides against GPA in China. Spirotetramat has a new mode of action and is drawn from a class of chemicals called tetramic acids that act as lipid biosynthesis inhibitors. Afidopyropen is a derivative of pyripyropene A that has a new mode of action as a chordotonal organ transient receptor potential vanilloid-type channel modulator, and cyantraniliprole is a member of the bisamides class of insecticides with an excellent aphicidal activity [27]. Except for the detection of high levels of resistance to spirotetramat in two populations of GPA collected from Queensland (170.7-fold resistance in Alloway171 and 105.6-fold resistance in Osborne171) [29], there has been little evidence of variation in susceptibility to afidopyropen or cyantraniliprole. No obvious resistance has been observed to either afidopyropen or cyantraniliprole, and only low resistance to spirotetramat (RF = 8.1) was identified in SEF-R. The three insecticides should be applied as alternatives to insecticides that provoke high resistance levels.

Broflanilide, a new meta-diamide pesticide that acts on the γ-aminobutyric acid receptor in insect pests through a novel mechanism of action, is highly active against a broad range of pests, including lepidoptera, coleoptera, and homoptera [63]. Resistance to broflanilide in the GPA has not been documented in previous studies. To date, broflanilide is not permitted for the control of GPA, but it has been registered for the management of *Plutella xylostella* and *Phyllotreta striolata* (Fabricius) on cruciferous vegetables in China. We confirmed that growers tend to use broflanilide spray more than five times per year. Selection pressures from the extensive use of broflanilide to control *P. xylostella* and *P. striolata* might lead to the evolution of resistance in the GPA in the field. In the future, risk assessment of resistance in GPA is necessary prior to the registration of broflanilide use on cabbage.

Resistance monitoring data indicated that SEF-R developed multifold resistance to different insecticides through multiple mechanisms. The study of resistance in GPA has shown the important role target site mechanisms play in developing high levels of resistance by affecting insecticide binding to receptors [13]. Since it was first detected in France in GPA [64], the R81T mutation in the nAChR gene has been proven responsible for high resistance to neonicotinoids and also been found to confer cross-resistance to other insecticide classes that function as agonists on the nAChR, such as sulfoxaflor [42,53,55] and butenolides [52]. This resistance mutation has been detected in SEF-R and has also been observed in Chinese populations of GPA [65]. We speculated that the R81T mutation conferred resistance in SEF-R to all five tested Group 4 compounds, and sulfoxaflor and butenolides were not used as alternatives that could be rotated with neonicotinoids. IRAC also recommended using a maximum of one Group 4 insecticide application per crop cycle against GPA. Otherwise, the SEF-R possessed a kdr mutation (L1014F) in the sodium channel with 79.35% genotypic frequencies of RS heterozygotes, but evidence of super-kdr mutation was not found. Similarly, Tang [48] detected the L1014F kdr mutation in 35 of 45 field populations with 2.7–100% genotypic frequencies of RS heterozygotes in China. The kdr mutation also made it difficult to select other pyrethroid compounds, such as deltamethrin, bifenthrin, and fenpropathrin, to control aphids in the field. Enhanced detoxification using pesticide detoxification enzymes is an important mechanism for reducing susceptibility to insecticides. PBO and DEF had useful synergistic effects with imidacloprid and beta-cypermethrin, but DEM only had useful effects with cypermethrin. Synergism of PBO with neonicotinoids or permethrins has been comprehensively documented in field-collected resistant GPA and shows usefulness with imidacloprid [8,66] and cyhalothrin [66], which is consistent with our results. Increases associated with MFO, CarE, and GST have also been observed. The results suggested the possible involvement of the metabolic resistance mechanisms

of the three detoxification enzymes in SEF-R. This agreed with reports on field resistant populations collected from Hunan and Chongqing Provinces [50,63] and Korea [67].

To further investigate the functions of the MFOs that were related to multi-resistance in SEF-R, we chose eight P450 genes to undergo expression analysis. Compared with in FFJ-S, seven P450 genes were more than 2-fold upregulated in the SEF-R, with CYP6CY3 (3.63-fold), CYP6CY23 (3.13-fold), CYP380C40 (2.51-fold), CYP6CY9 (2.46-fold), and CYP4G15 (5.24-fold) being significantly overexpressed. The overexpression of CYP6CY3 (10–75-fold) is responsible for neonicotinoid resistance in the GPA, which confers a moderate level of resistance to neonicotinoids [13,16]. However, SEF-R was found to have only 3.13-fold more upregulated overexpression of CYP6CY3 than the susceptible strain. The knockdown of CYP6CY3 in SEF-R increased the susceptibility of SEF-R to imidacloprid, thiacloprid, and thiamethoxam, but it did not affect the susceptibility to sulfoxaflor and flupyradifurone, which is consistent with the results of Nakao et al. [17]. CYP6CY23 also ungraduated greatly in SEF-R, but it showed no capacity to metabolize nicotine [68]. Indeed, there is a need to investigate its function in GPA. Additionally, two GPA clones with different copy numbers of CYP6CY3 displayed low levels (3–20-fold) of resistance to imidacloprid and thiamethoxam, but the two clones survived and reproduced well on Brassica napus seedlings grown from seed treated with commercial levels of neonicotinoids [18] in a large-scale semi-field trial.

The reduced penetration of insecticide is an additional mechanism of resistance in the imidacloprid-resistant 5191A clone of GPA [13,16]. Additionally, CYP4G15 was significantly overexpressed in SEF-R. The CYP4 clan in insects is involved in the last step of cuticular hydrocarbon biosynthesis [5,69,70]. Although there was no evidence of the CYP4 clan's involvement in insecticide resistance in the GPA before our study, the overexpression levels of CYP4EM10 [71] and CYP4PR1 [72] in *Triatoma infectants* and CYP4G19 in *Blattella germanica* [73] are associated with pyrethroid resistance, and the increased expression levels of CYP4C68 and CYP4G70 are associated with imidacloprid resistance in *Diaphorina citri* Kuwayama [74]. Further studies are needed to confirm the function of CYP4G15 in the GPA. The overexpression of CYP380C40 (21–76-fold) and UDP-glucuronosyltransferase UGT344P2 (6–33-fold) has been identified in field populations of the GPA in Australia, and this proved partially responsible for sulfoxaflor resistance in the aphid [20]. The expression levels of CYP380C40 and UGT344P2 were estimated to be 2.51- and 18-fold, respectively, and the overexpression could be responsible for the 32.4-fold increase in resistance to sulfoxaflor in SEF-R compared with in FFJ-S. Although the function of some P450s in GPA has been clearly identified, co-upregulation of several P450 genes is often observed and the ways in which these genes work together is unclear.

Additionally, we evaluated the differences in the expression levels of two esterase genes, E4 and THEM, between SEF-R and FFJ-S and found that they all had increased expression levels in SEF-R. The carboxylesterase, E4, mainly confers resistance to carbamates and organophosphates [15]. Resistance to these two kinds of insecticides in the GPA are widely distributed throughout China, although resistance levels have not been investigated in SEF-R. THEM is a type of thioesterase that catalyzes the cleavage of thioester bonds present in a wide range of glutathione, acyl-carrier proteins, fatty acyl-coenzyme A substrates, and other cellular molecules [75]. Thioester-containing proteins may result from different environments and selective pressures that trigger the innate immune response [76]. The GST gene was not upregulated in SEF-R, which indicated that it was not associated with insecticide resistance. Antioxidant mechanisms were also investigated. A biochemical analysis revealed that the activities of SOD and POD were higher, and the CAT activity was lower, in SEF-R than in FFJ-S. Higher SOD and POD activities have also been observed in another type of field resistance, which is consistent with our results. However, CAT activity also increased in that resistant strain, which is contrary to our results [67]. Quantitative PCR showed increased expressions of SOD and POD and a decreased expression of CAT in SEF-R. These results are consistent with their activity levels. Protective enzymes often work as part of a basic immune response.

Previous resistance monitoring surveys in China put emphasis on conventional insecticides, such as neonicotinoids and pyrethroids. New ingredients with aphicidal activities are often not included in monitoring programs. SEF-R exhibited resistance to some newly introduced or unregistered insecticides, such as broflanilide, which meant the careful monitoring of susceptibility to these compounds was necessary. The risk of insecticide resistance, including the establishment of a susceptibility baseline, needs to be assessed when a new ingredient is registered for new target pest insects in China. However, other target pest insects are not considered in the risk assessment. Based on our results, neonicotinoids and pyrethroids are not suitable to control SEF-R, but afidopyropen and cyantraniliprole are good alternative to the two kinds of insecticides that effectively control SEF-R. Flonicamid and spirotetramat are also recommended in SEF-R management in order to maintain a diversity of modes of action and implement resistance management in practice, which are critical steps to preventing or delaying resistance development. Other control tactics, such as biological control, should be integrated with insecticides for enhanced protection from aphid damage and reduced insecticide application.

5. Conclusions

Our study demonstrated that a field population of *M. persicae* (SEF-R) exhibited an extremely high level of resistance to beta-cypermethrin, high levels of resistance to imidacloprid and esfenvalerate, moderate levels of resistance to thiacloprid, thiamethoxam, sulfoxaflor, pymetrozine, and broflanilide, and low levels of resistance to flupyradifurone, flonicamid, and spirotetramat. Afidopyropen and cyantraniliprole were recommended as the primary insecticides and flonicamid and spirotetramat were used as alternatives with which to control SEF-R. Molecular analysis showed a high frequency of R81T mutation and kdr mutation, conferring target site resistance to neonicotinoids and pyrethroids in SEF-R. Exposure to the PBO synergist restored susceptibility to imidacloprid and beta-cypermethrin in SEF-R, suggesting the role of MFO in neonicotinoids and pyrethroids. The activities of MFO, CarE, and GST in SEF-R were significantly higher than those in FFJ-S, confirming that a metabolic detoxification mechanism is involved in multi-resistance to selected insecticides. RNA-seq results revealed that the overexpression of P450s (CYP6CY3, CYP6CY23, CYP380C40, CYP6CY9, and CYP4G15), esterases (E4, and THEM), and a UGT (UGT344P2) might be responsible for the multi-resistance of SEF-R. A knockdown of CYP6CY3 in SEF-R increased the susceptibility of SEF-R to imidacloprid, thiacloprid, and thiamethoxam, which verified that the P450 plays a vital role in neonicotinoid metabolism. The increased activities of POD and SOD enzymes, and the upregulation of the two encoding genes, showed that the two protective enzymes play some role in the resistance of SEF-R to various insecticides. However, more work is required in order to determine the exact detoxification mechanism conferring multi-resistance in SEF-R.

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