Research Article

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Characterisation of imidacloprid resistance in the bird cherry-oat aphid, *Rhopalosiphum padi*, a serious pest on wheat crops

Kang Wang,[a,†] Meng Zhang,[b,†] Yanna Huang,[a] Zhuolin Yang,[a] Sha Su[a] and Maohua Chen[a*]

Abstract

BACKGROUND: *Rhopalosiphum padi* is a destructive insect pest of wheat worldwide. Studies have shown that *R. padi* has developed resistance to different insecticides, including imidacloprid. We studied the mechanisms conferring resistance to imidacloprid at the biochemical and molecular levels.

RESULTS: An *R. padi* imidacloprid-resistant (IM-R) strain and a susceptible (SS) strain were established. Fitness analysis using life-tables showed that the IM-R strain had obvious disadvantages in several parameters, indicating reduced fitness. Profiles of cross-resistance of IM-R and SS to seven insecticides were detected. Both synergistic and enzyme activity data suggested that P450 plays a role in resistance. Furthermore, the mRNA expression levels of cytochrome P450 (CYP) genes *CYP6CY3-1* and *CYP6CY3-2* were significantly increased in the IM-R strain. No target-site mutation within the nicotinic acetylcholine receptor (nAChR) subunits was detected in the IM-R strain. Interestingly, the expression levels of the nAChR α1, α2, α3, α7-2, and β1 subunit genes were significantly decreased, suggesting that down-regulation of these subunits may be involved in resistance.

CONCLUSION: Multiple mechanisms confer imidacloprid resistance in *R. padi*.

Keywords: *Rhopalosiphum padi*; fitness; cross-resistance; P450; nicotinic acetylcholine receptor (nAChR)

1 INTRODUCTION

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), is an important pest of wheat worldwide.[1] Aside from direct feeding damage, *R. padi* also transmits the barley yellow dwarf virus (BYDV), which causes an economically important disease in small grains, leading to reduced quality and yield.[2] In recent years, outbreaks of this pest have often occurred in China,[3,4] and it has shown an increasing degree of resistance to some insecticides, including organophosphates and carbamates.[5–7] Since its introduction in the early 1990s, imidacloprid has rapidly become one of the most commonly used neonicotinoid insecticides against aphids, whiteflies, and planthoppers.[8] Regional susceptibility analyses of 12 *R. padi* field populations showed that some populations had different resistance levels to imidacloprid, with the highest resistance ratio being 13.6; however, the resistance levels of *Myzus persicae* and *Aphis gossypii* field populations to imidacloprid were much higher.[9–11]

Studies have revealed that both metabolic and target-site mechanisms are associated with neonicotinoid resistance.[10,12] Imidacloprid acts as an antagonist on insect nicotinic acetylcholine receptors (nAChRs), which mediate fast cholinergic synaptic transmission in the insect central nervous system.[13,14] Target-site resistance was first characterised in laboratory-selected imidacloprid-resistant (IM-R) *Nilaparvata lugens*, in which a single point mutation (Y151S) within the extracellular domain of two nAChR subunits (Nlα1 and Nlα3) led to reduced imidacloprid affinity on receptors.[15,16] A single point mutation (R81T), an arginine to threonine substitution, was discovered in the loop D region of the nAChR β1 subunit in *M. persicae*, which was the first example of field-evolved target-site resistance to imidacloprid.[12] Three target-site mutations (R81T, V62I, and K264E) within the nAChR β1 subunit were detected in *A. gossypii* IM-R and field populations.[17] The down-regulation of single or several insect nAChR subunits is also involved in the resistance mechanism for imidacloprid. Both point mutations and decreased mRNA expression of nAChR β1 were found to be involved in imidacloprid resistance in *A. gossypii*.[17] Toxicity bioassays indicated that α1 RNAi treatment greatly decreased the sensitivity to imidacloprid and thiamethoxam in *Leptinotarsa decemlineata*.[18]

The metabolic mechanisms in insects against synthetic and natural xenobiotics include cytochrome P450 monooxygenases

* *Correspondence to: M Chen, Northwest A&F University, Yangling 712100, China. E-mail: maohua.chen@nwsuaf.edu.cn*

† *These two authors contributed equally to this work.*

a *Northwest A&F University, State Key Laboratory of Crop Stress Biology for Arid Areas, Key Laboratory of Crop Pest Integrated Pest Management on the Loess Plateau of Ministry of Agriculture, Yangling, Shaanxi Province, China*

b *Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou, Henan Province, China*

(cytochrome P450s), esterases [especially carboxylesterases (CarEs)], and glutathione S-transferases (GSTs).[19] Cytochrome P450-mediated imidacloprid resistance was reported in several insect species, including *Aedes aegypti*,[20] *Musca domestica*,[21,22] *Bemisia tabaci*,[23–25] *N. lugens*,[26–29] *Laodelphax striatellus*,[30] *M. persicae*,[12,31] and *A. gossypii*.[32] Single or multiple P450 genes were over-expressed in imidacloprid-resistant strains and/or populations of these insect species. Synergistic and metabolic enzyme assays in an IM-R strain of *A. gossypii* (RF75) suggested that cytochrome P450 may play important roles in imidacloprid resistance. *In vitro* biochemical assays and *in vivo* differential synergism studies using differential effects of enzyme inhibitors indicated that the enhanced oxidase activity increased imidacloprid resistance in *M. persicae*.[33] Microarray analysis revealed constitutive over-expression (22-fold) of a single P450 (*CYP6CY3*) in an imidacloprid-resistant *M. persicae* clone (5191A).[31]

In the current study, we collected *R. padi* from a wheat field in 2013 and used it as the progenitor population to derive an IM-R strain and a susceptible (SS) strain. Bioassays, life tables, a synergistic assay, enzyme measurement, gene expression, and mutation detection were used to investigate the development and mechanisms of insecticide resistance. The profiles of cross-resistance of these two strains to seven other insecticides were also characterised.

2 EXPERIMENTAL METHODS

2.1 Insects

An *R. padi* population was collected from a wheat field in Gansu Province, China, in 2013 and used to establish an imidacloprid-resistant strain (IM-R) and a susceptible (SS) strain on seedlings of wheat (cultivar 'Xiaoyan 22') in mesh cages (41×41×41 cm) in the laboratory. The wheat seedlings were planted in 10-cm-diameter plastic pots. Twenty wheat seeds were planted in nutrient soil per pot to obtain three-leaf-stage seedlings. Five pots with seedlings were placed in each mesh cage. All insects were reared in the laboratory at 23 ± 1°C and a photoperiod of 16:8 h light:dark. *Rhopalosiphum padi* took 6 to 10 days to complete a generation under the laboratory conditions. To normalise the bioassay and insecticide selection time, we considered 7 days as the generation time in this study. The IM-R strain was selected by successively exposing adults to imidacloprid for > 52 generations, and the SS was reared in parallel without any insecticide contact. In the process of insecticide selection, the toxicity of imidacloprid was evaluated every four generations to confirm the median lethal concentration (LC_{50}) value, which was used as the selected concentration for the following four generations. Every 14 days, five pots with three-leaf seedlings 24 hours post-spraying with the respective concentration of imidacloprid were placed inside the cage. The previous pots were removed once the seedlings had died. Aphids from the 52nd generation were used for the life-table analysis, synergism bioassays, enzyme activity analysis, mutation survey of the nAChR subunit genes, and reverse transcription–polymerase chain reaction (RT-PCR) analysis.

2.2 Insecticides, chemicals, and synergists

The insecticides used for bioassays included the neonicotinoids imidacloprid (95% purity; Jiangsu Changlong Chemical Co., Ltd, Nanjing, China), thiamethoxam (96% purity; Shandong Sino-Agri United Biotechnology Co., Ltd, Jinan, China) and acetamiprid (96% purity; Shandong Sheda Crop Science Co., Ltd, Jinan, China), as well as chlorpyrifos (96% purity; Shangdong Moderne Chemical Co., Ltd, Jinan, China), malathion (95% purity; Tianjin Aigefu Co., Ltd, Tianjin, China), beta-cypermethrin (96% purity; Yancheng Nongbo Bio-technology Co., Ltd, Yancheng, China), isoprocarb (95% purity; Anhui Huaxing Chemical Industry Co., Ltd, Hefei, China), and sulfoxaflor (98% purity; Dow AgroSciences Inc., Indianapolis, IN, USA).

Additional materials used were piperonyl butoxide (PBO; reagent grade) (Sigma-Aldrich, St Louis, MO, USA), triphenyl phosphate (TPP; reagent grade) and diethyl maleate (DEM; reagent grade) (Shanghai Chemical Reagent Co, Ltd, Shanghai, China), α-naphthol and fast blue B salt (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), α-naphthyl acetate (α-NA) (Solarbio, Beijing, China), eserine, reduced glutathione (GSH), and Coomassie brilliant blue G250 (Sigma-Aldrich) and 1-chloro-2,4-dinitrochlorobenzene (CDNB) (Roche, Mannheim, Germany).

2.3 Bioassays

It was reported that a bioassay method that combined direct contact with an insecticide and subsequent exposure to residues on leaves gave a measurable resistance factor in the determination of resistance of *M. persicae* to neonicotinoids, while the resistance level could not be measured because of insufficient mortality at the highest concentration when using a bioassay method that applied the insecticide directly to the dorsal surface of individual aphids.[9] We adopted a leaf-dipping bioassay method that combined direct contact and residue exposure for the insects in this study.[5] Stocks of insecticides were prepared in acetone (10 g L^{-1}) and diluted in a series of concentrations containing 0.01% (v/v) Triton X-100 for bioassays. Wheat leaves with apterous adult aphids were dipped into insecticide solutions for 10 s, and the residual droplets of the solution on the leaves were absorbed by dry filter paper. Treated leaves were then placed in a Petri dish with moisten filter paper. Control aphids were treated with distilled water containing 0.01% (v/v) Triton X-100 and 0.01% acetone. At each concentration, 50–60 apterous adults were treated. Each concentration was replicated at least three times, and aphid mortality was assessed after 24 h. The LC_{50} values, 95% confidence intervals (CIs), and slopes were calculated by probit analysis using SPSS software (SPSS Inc., Chicago, IL, USA). The resistance ratios (RRs) were estimated at the LC_{50} level as RR = LC_{50} of IM-R/LC_{50} of SS.

2.4 Life-table analysis of IM-R and SS strains

To confirm the fitness costs of resistance to imidacloprid in *R. padi*, 50 apterous adults from the SS and the IM-R strains were collected randomly, and each aphid was transferred to a 10-cm-diameter plastic dish (one per dish) supplied with wheat leaves (the first leaves cut from the three-leaf-stage wheat seedlings). All aphids were reared at 23 ± 1°C with a photoperiod of 16:8 h light:dark. After 12 h of larviposition, one newborn larva from each dish was transferred to a new plastic dish with new leaves for the life-table analysis. New leaves were added and the old leaves were removed every 2 days. The molting and mortality of the aphids in the dishes were recorded every 12 h. After maturity, the adult mortality and fecundity (offspring/individual) were also recorded every 12 h. The newborn nymphs were counted and removed at each time-point until the death of the adult. Life table analysis was calculated using the TWOSEX-MSChart program.[34] The net reproductive rate was calculated as $R_0 = \sum l_x m_x$ where x is the age in days, l_x is the proportion of individuals surviving to age x, and m_x is the number of offspring produced per aphid at age x. The intrinsic rate of increase (r) was estimated using the Euler-Lotka

equation $\sum_{i=1}^{N} e^{-r(x+1)} l_x m_x = 1$ with age indexed from 0. The finite rate of increase was calculated as $\lambda = e^r$. The mean generation time was then calculated as $T = \ln R_0 / r$. The gross reproduction rate was calculated as $GRR = \sum m_x$.

2.5 Synergistic bioassays

Insecticide toxicity in the presence and absence of the synergists PBO, TPP, and DEM was evaluated using the bioassay method described above. The maximum sublethal doses for PBO, TPP, and DEM were determined for SS with the bioassay method described above. At least five concentrations of each synergist and a control (acetone only) were used. The maximum dose that led to zero mortality in SS was adopted as the maximum sublethal concentration in our study. Individuals of *R. padi* were treated with a PBO, TPP, or DEM (which was applied at the maximum sublethal dose; final concentration 100 mg L^{-1}) and imidacloprid mixture. The synergistic ratio was calculated using the conventional approach of dividing the LC_{50} without the synergist by the LC_{50} with the synergist. Probit analysis was conducted using SPSS software.

2.6 Enzyme activity analysis

Protein content was determined using the Bradford method with bovine serum albumin as the standard.[35]

CarE activity was determined by a modified version of the method of Chen *et al.*.[36] The enzyme source was the supernatant of 10 apterous adults homogenised in 1 mL of pre-chilled phosphate-buffered saline (PBS, 0.1 mol L^{-1}, pH 7.0, containing 1.0 $\times 10^{-3}$ mol L^{-1} EDTA) on ice and centrifuged at 4°C and 12 000 ***g*** for 10 min. The supernatant was kept on ice before analysis. After the substrate α-NA (3.0×10^{-3} mol L^{-1}) and physostigmine (10^{-4} mol L^{-1}) had been mixed for 10 min on a microplate reader at 30°C, fast blue B salt (3.0×10^{-3} mol L^{-1}) was added to each microplate well. The optical density (OD) at 600 nm was recorded at intervals of 25 s for 10 min at 30°C with an iMark Microplate Reader (Bio-Rad, Hercules, CA, USA).

A GST activity test was performed following a slightly modified version of a previously reported method.[36,37] The enzyme source was prepared with 10 apterous adults in 1 mL of pre-chilled PBS (0.1 mol L^{-1}, pH 7.6, containing 1.0 $\times$ 10^{-3} mol L^{-1} EDTA) on ice and centrifuged at 4°C and 12 000 ***g*** for 10 min. The supernatant was kept on ice before analysis; 1.2 mM CDNB and 6 mM GSH were incubated in a microplate reader for 20 min at 27°C. Three replicates were performed for each enzyme. The OD at 340 nm was recorded at intervals of 30 s for 5 min at 27°C with an iMark Microplate Reader.

Cytochrome P450 activity was estimated by measuring the haem peroxidase activity. Quantification of haem activity can be expressed as cytochrome P450 activity. The enzyme activity was quantified by this method.[38–41] The enzyme source was prepared with 10 apterous adults in 1 mL of pre-chilled PBS (0.1 mol L^{-1}, pH 7.5, containing 1.0 $\times$ 10^{-3} mol L^{-1} EDTA) on ice and centrifuged at 4°C and 15 000 ***g*** for 10 min. Haem peroxidase activity was measured using the substrate 3,3′5,5′-tetra-methylbenzidine (TMBZ) (Sigma Aldrich). Four aliquots of 20 μL of enzyme solution, 80 μL of 0.625 M potassium phosphate buffer (pH 7.2), 200 μL of TMBZ solution, and 25 μL of hydrogen peroxide (3%) were pipetted into separate wells of a 96-well microplate. Plates were incubated at room temperature for 2 h before reading at 450 nm at 25 °C. Control wells consisted of 20 μL of distilled water, 80 μL of 0.625 M potassium phosphate buffer, 200 μL of TMBZ solution, and 25 μL of hydrogen peroxide (3%). Three replicates were performed for each enzyme. A standard curve for haem peroxidase activity was prepared using different concentrations of cytochrome C from horse heart (Sigma Aldrich). Monooxygenase levels were expressed as equivalent units (EUs) of cytochrome P450 mg^{-1} protein using the standard curve of cytochrome C.

The enzyme activity assays were replicated using three independent biological sample preparations, and the enzyme solution from each replicate was tested three times. Data represent the mean values of three replicates with the standard error.

2.7 Cloning of the P450 *CYP6CY3* gene from *R. padi*

Molecular studies of imidacloprid resistance have demonstrated that a single cytochrome P450, CYP6CY3, confers resistance to neonicotinoid insecticides in aphids.[12,31] The full-length cDNA of *CYP6CY3* was cloned by RT-PCR and rapid amplification of cDNA ends (RACE). Degenerate primers designed according to the *CYP6CY3* nucleotide sequences of *Acyrthosiphon pisum*, *M. persicae*, and *A. gossypii* were used in putative fragment amplification. Based on the two partial putative fragments, 5′-RACE and 3′-RACE were performed using the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) following the manufacturer's protocols. To confirm the full-length assembly of the *CYP6CY3* gene, specific primer pairs were used to amplify the full length of the gene in different strains. All primers used are shown in Table 1. All PCR products were purified with a Wizard PCR Preps kit (Promega, Madison, WI, USA). The purified PCR products were cloned into the pGEM-T easy vector (Promega) and transformed into *Escherichia coli* DH5α competent cells. Five positive clones from each sample were randomly chosen for bidirectional sequencing on an Applied Biosystems 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

2.8 Mutation survey in nAChR subunit genes

Total RNA was isolated from apterous adult aphids using TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesised from the total RNA (1.0 μg) using the M-MLV reverse transcriptase cDNA Synthesis Kit (Promega). The gene-specific primers for amplifying the *R. padi* nAChR subunit gene from IM-R and SS followed Zhang *et al.*[42] PCR was performed with an initial denaturation at 94°C for 3 min followed by 35 cycles of 30 s at 94°C, 1 min at annealing temperature of 55°C Tm, 2 min at 72°C, and a final extension at 72°C for 10 min. Then, the PCR products were analysed on 1% agarose gels. PCR sequencing was performed at Sangon Biotech, Shanghai, China. The sequencing was repeated with at least 10 individuals for the IM-R and SS strains.

The nucleotide sequences from SS were taken as the standard models. All nucleotide sequences were aligned using CLUSTALX, and single nucleotide polymorphisms (SNPs) were ascertained in DNASP v5.

2.9 Quantitative real-time PCR (qRT-PCR) and data analysis

qRT-PCR was performed on a Bio-Rad IQ5 Real-Time PCR system (Bio-Rad) with Fast Start SYBR Green I Master Mix (Roche) in accordance with the manufacturer's instructions. The cDNA template was prepared using the PrimeScript RT Reagent Kit with the M-MLV reverse transcriptase cDNA Synthesis Kit (Promega, Madison, WI, USA) as described above. In a final 20-μL volume reaction, 1 μL of cDNA template, 10 μL of 2 $\times$ FastStart Essential DNA Green Master, 0.8 μL of forward and reverse primer (Table 1), and 7.4 μL of

Table 1. Primers used for sequence cloning, RACE and qRT-PCR

Primer application	Primer name	Sequence (5′–3′)
Sequence cloning	CYP6CY3F1	AACCATGACATACAGACTGRARA
	CYP6CY3R1	GGTGACGGTCCAAGAATGTGYAT
	CYP6CY3F2	GACTTCTTCCACTCCGCCTTYAARG
	CYP6CY3R2	GCCTTGGTGGAAATGCTGACNAART
5′-RACE	CY3-1R1	CCCAGCAGCAAACATTACAAAAGCAT
	CY3-1R2	GTGGATACTTACGGAGTGATTCTGCT
	CY3-2R2	GACAGTTTTAGTTGAATCTCTTGACG
	CY3-2R2	TTTCTGAACAAAGCGACCAGTGCGG
3′-RACE	CY3-1F1	ACCTTCACTTAGAACGCTTTTCAGAG
	CY3-1F2	GTTTTGAATGAAAATTTGCCCAAACGT
	CY3-2F2	CACTCCGTAAGTATCCCGCACTGGTCG
	CY3-2F2	TGCTTTTGTAATGTTTGCTGCTGG
Cloning full length	CYP6CY3-1F	TTTCGTCGTCCACCACCG
	CYP6CY3-1R	CCATCGCCAAACGGAAGA
	CYP6CY3-2F	AGTGTGGGACTATGTATT
	CYP6CY3-2R	ATTTCATTTCCATCTCAG
qRT-PCR	qRpα1F	TATGGCTGGAACACGAATG
	qRpα1R	TTCGCCGTCAGCATTATTG
	qRpα2F	AACAGACTCATCAGACCCGT
	qRpα2R	TCCATTCTTGCTCAACCCAG
	qRpα3F	GCCTTCCTGCGAAATAGATG
	qRpα3R	CAACCCGTGAACCGCTTACT
	qRpα4F	TGACAACGAGTGTGTGGCTG
	qRpα4R	CCGCTTTTGTCATTAGGGT
	qRpα5F	TTGCGACGAACCGTATTTAG
	qRpα5R	CGTCACTTTTTCACCGCTGT
	qRpα7-1F	AATGGTGAATGGGACCTGCT
	qRpα7-1R	AACACGCACGGGACTATCAG
	qRpα7-2F	CTCAATCCAAACCGACTGTG
	qRpα7-2R	CGCCATCTGATTTGAGCACT
	qRpβ1F	ACAGTTTGGCTTAGCATTCGT
	qRpβ1R	GTAGTCTGCCTCGTCCCATT
	qCYP6CY3-1F	CAATCGTGGTGTTTACTCGGA
	qCYP6CY3-1R	TCTTGAGTTTTGCGGATGTG
	qCYP6CY3-2F	TCTTGAGTTTTGCGGATGTG
	qCYP6CY3-2R	CTGAAGTAGTAGGCGACGGTA
	qβ-actinF	TGAGACATTCAACACCCCTG
	qβ-actinR	CCTTCATAGATTGGGACAGTG
	qEF-1αF	GCTCTATTGGCTTTCACCTT
	qEF-1αF	GATGTAACTGCTGACTTCTTTC

RNase-free water were included. Thermal cycling conditions were 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15s, and extension at 72°C for 20 s. The melting curve was obtained by raising the temperature from 65°C to 95°C in 0.5°C increments. The experiment was conducted using three independent biological sample preparations, and each replicate was performed three times. The β-actin and elongation factor 1α (*EF-1*α) genes of *R. padi* were used as the internal reference genes to normalise the target gene expression levels, and the expression was normalised for each of the target genes to the SS strain. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.10 Statistical analysis

For qRT-PCR and the enzyme activity results, normality and homoscedasticity of the data were tested first. The data were arcsine-square-root-transformed to meet the assumptions of normality and homoscedasticity. The significance of the differences was determined by Student's *t*-test with the level of significance at $P < 0.05$. Data analyses were performed using SPSS software.

For life-table analysis, the data were log-transformed if needed to meet the assumptions of normality and homoscedasticity required for these analyses. The significance of the differences in the life-table parameters between the IM-R and SS strains was determined using one-way analysis of variance (ANOVA). The mean values were compared using the least significant difference (LSD) test ($P < 0.05$) when a significant difference ($P < 0.05$) was detected for parameters. All analyses were conducted using SPSS software.

3 RESULTS

3.1 Establishment of IM-R and SS strains

The intergenerational dynamics of imidacloprid toxicity during the selection process are shown in Figure 1. The LC_{50} of IM-R to imidacloprid gradually increased from 0.646 to 26.069 mg L^{-1} over 52 generations of selection, and the resistance ratio increased 40.35-fold.

3.2 Resistance cost

With the susceptible strain used as the control, the relative fitness of the IM-R strain was analysed by constructing a life table. The results are presented in Table 2. There was a significantly prolonged nymph period and shortened life span for the IM-R strain, and the fecundity of the strain was significantly reduced. The net reproductive rate (R_0), the finite rate of increase (λ), and the intrinsic rate of increase (r) were significantly reduced in the IM-R strain compared with SS.

3.3 Cross-resistance pattern

The profiles of cross-resistance of IM-R and SS to seven insecticides were determined (Table 3). Compared with SS, the IM-R strain showed high levels of cross-resistance to thiamethoxam (10.72-fold), acetamiprid (6.21-fold), and isoprocarb (6.30-fold) and low levels of cross-resistance to chlorpyrifos (2.48-fold) and malathion (3.42-fold). No significant cross-resistance to beta-cyhalothrin and sulfoxaflor was observed.

3.4 Synergistic effect to toxicity in susceptible and resistant strains

The synergistic effects of PBO, TPP, and DEM on imidacloprid susceptibility of IM-R and SS are shown in Table 4. The level of resistance of the IM-R strain was suppressed by the oxidase inhibitor PBO (3.59-fold) and esterase inhibitor TPP (2.45-fold), whereas the synergistic effect of DEM (1.13-fold), a glutathione depletor, was low. The synergistic effects of TPP, DEM, and PBO were not remarkable in SS. These results suggest that P450s are involved in imidacloprid resistance in the IM-R strain. They also suggest that P450s and CarEs are possibly involved in imidacloprid resistance in the IM-R strain, whereas GSTs are not significantly involved.

3.5 Detoxification of enzyme activities

CarE, GST, and cytochrome P450 activities were detected in the IM-R and SS strains (Table 5). Cytochrome P450 activity was significantly higher in the IM-R strain than in SS, with an enzyme

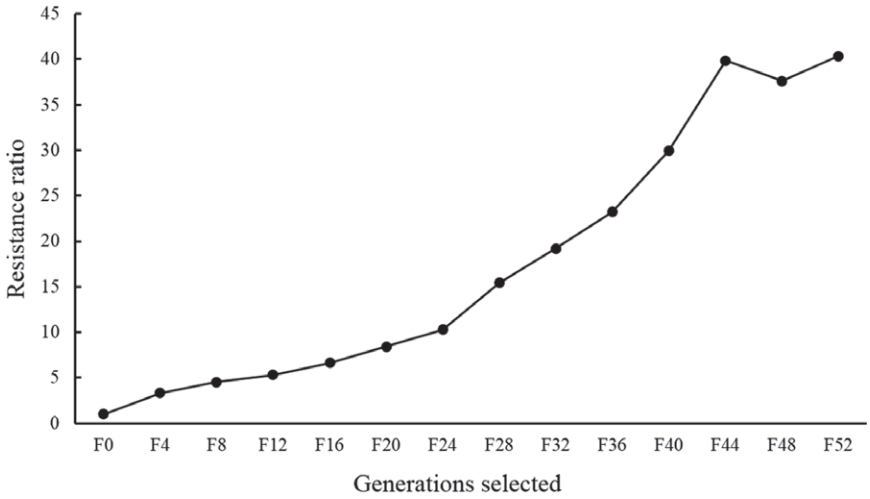

Figure 1. The dynamics of imidacloprid resistance in *R. padi* after 52 generations of continuous selection with imidacloprid. Resistance ratios were calculated by dividing the LC_{50} values of the current generation by the LC_{50} of the susceptible (SS) strain. A 'generation' refers to the normal time for *R. padi* to complete one generation in the laboratory (i.e. 7 days).

Table 2. Comparison of development time, longevity and population parameters between *R. padi* imidacloprid-resistant (IM-R) and susceptible (SS) strains on cut leaves

Stage/parameters	Strain	
	SS	IM-R
1st instar (days)	1.16 ± 0.037 a	1.35 ± 0.036 b
2nd instar (days)	1.05 ± 0.045 a	1.20 ± 0.033 b
3rd instar (days)	1.14 ±0.038 a	1.25 ± 0.074 a
4th instar (days)	1.08 ± 0.030 a	1.53 ± 0.061 b
Nymph period (days)	4.43 ± 0.058 a	5.33 ± 0.066 b
Lifespan of adult (days)	19.41 ± 0.453 a	10.45 ± 0.971 b
Fecundity	64.23 ± 2.43 a	41.61 ± 2.54 b
R_0	73.904 ± 1.772 a	46.989 ± 3.746 b
λ	1.694 ± 0.0100 a	1.595 ± 0.016 b
r	0.527 ± 0.006 a	0.467 ± 0.010 b
GRR	74.260 ± 1.700 a	68.420 ± 2.690 a
T	8.161 ± 0.087 a	8.237 ± 0.158 a

The standard error of the mean was calculated by using 10 000 bootstrap replications, and data followed by different letters in the same row indicate significant differences based on the LSD test ($P < 0.05$).
R_0, net reproductive rate; λ, finite rate of increase (day^{-1}); r, intrinsic rate of increase (day^{-1}); GRR, gross reproduction rate; T, mean generation time (days).

ratio of 1.88. CarE activity was 2.674 nmol μg^{-1} min^{-1} in the IM-R strain, significantly higher than the 1.943 nmol μg^{-1} min^{-1} in SS. No significant difference ($P < 0.05$) in GST activity was observed between the two strains.

3.6 Identification of *R. padi* CYP6CY3

Two *CYP6CY3* genes (*CYP6CY3-1* and *CYP6CY3-2*) were amplified and cloned from *R. padi*, and sequence analysis showed high levels of similarity in nucleotides (84%) and amino acids (78%). The deduced amino acid identity between *R. padi CYP6CY3-1* and other *CYP6CY3* genes (*A. pisum*, XP_001948581; *M. persicae*, AHB52749; and *A. gossypii*, AEZ51832) ranged from 76% to 79%. The amino acid sequence similarity between *CYP6CY3-2* and the other *CYP6CY3* genes ranged from 76% to 84%.

3.7 Detection of site mutations in the nAChR subunits of *R. padi*

cDNA fragments encoding eight *R. padi* nAChR subunits were amplified separately. Only three single nucleotide mutations (G150A in *Rpα1*, G1305C in *Rpα2*, and A1254T in *Rpα4*) were observed (data not shown), and none caused any amino acid point mutations.

3.8 Expression profiles of nAChR subunits and *CYP6CY3* in *R. padi*

The expression profiles of eight *nAChR* and two *CYP6CY3* genes from *R. padi* were determined in IM-R and SS. The mRNA transcriptional levels of the nAChR α1, α2, α3, α7-2, and β1 subunit genes decreased significantly in the IM-R strain compared with SS (Fig. 2). The relative expression levels of *CYP6CY3-1* and *CY6CY3-2* were 2.81- and 2.67-fold higher, respectively, in the IM-R strain than in SS.

4 DISCUSSION

Imidacloprid acts as an antagonist of insect nAChRs, and is highly efficient against many insect pests. It is extensively used worldwide and is the primary insecticide for controlling phloem-feeding pest insects.[43] Soon after its introduction in China, it became the most commonly used insecticide. Imidacloprid has been used to control aphids in China for > 20 years and is used widely in nearly all areas harbouring populations of *R. padi*. To investigate possible mechanisms of the development of imidacloprid resistance in *R. padi*, an insecticide-resistant strain was selected in the laboratory, and the development and mechanisms of resistance and cross-resistance to other insecticides were analysed in this study.

The high level of resistance to imidacloprid in *R. padi* field populations documented here has not previously been reported;[7,44] however, two other aphid species, *A. gossypii* and *M. persicae*, have been reported to have developed extremely high levels of resistance in the field.[9–11,45,46] *Rhopalosiphum padi* mainly damages wheat and other gramineous crops. Unlike *R. padi*, *A. gossypii* and *M. persicae* are highly polyphagous species that are often treated

Table 3. Toxicity of different insecticides to imidacloprid-resistant (IM-R) and susceptible (SS) strains of *R. padi*

Insecticide	Strain	Slope ± SE	LC_{50} (mg L^{-1}) (95% confidence limit)	RR[a]
Imidacloprid	SS	1.650 ± 0.231	0.646 (0.469-0.834)	40.35
	IM-R	1.590 ± 0.127	26.069 (18.290-47.102)	
Thiamethoxam	SS	3.517 ± 0.349	0.743 (0.666-0.814)	10.72
	IM-R	1.683 ± 0.174	7.968 (4.094-61.932)	
Acetamiprid	SS	3.572 ± 0.145	0.981 (0.903-1.062)	6.21
	IM-R	0.892 ± 0.412	6.095 (2.581-20.280)	
Chlorpyrifos	SS	2.711 ± 0.110	0.456 (0.367-0.631)	2.48
	IM-R	1.923 ± 0.102	1.131 (0.883-1.602)	
Malathion	SS	2.001 ± 0.301	2.915 (2.568-3.258)	3.42
	IM-R	2.218 ± 0.106	9.973 (7.075-15.551)	
Beta-cyhalothrin	SS	2.100 ± 0.074	0.465 (0.402-0.535)	1.35
	IM-R	2.085 ± 0.132	0.630 (0.493-0.764)	
Isoprocarb	SS	1.375 ± 0.222	0.061 (0.039-0.082)	6.30
	IM-R	2.139 ± 0.135	0.384 (0.309-0.466)	
Sulfoxaflor	SS	1.248 ± 0.170	0.488 (0.355-0.704)	1.50
	IM-R	1.292 ± 0.251	0.730 (0.523-1.158)	

[a] RR = resistance ratio = LC_{50} of the IM-R strain/LC_{50} of the SS strain.

Table 4. Synergistic effects of TPP, DEM and PBO on the imidacloprid-resistant (IM-R) and susceptible (SS) strains of *R. padi*

Strain	Imidacloprid/ synergist	Slope ± SE	LC_{50} (mg/L) (95% confidence limit)	SR
SS	Imidacloprid	1.650 ± 0.121	0.6460 (0.4688-0.8344)	–
	Imidacloprid+TPP	2.533 ± 0.267	0.5429 (0.3886-0.6852)	1.19
	Imidacloprid+DEM	1.978 ± 0.193	0.6396 (0.5224-0.8178)	1.01
	Imidacloprid+PBO	2.132 ± 0.272	0.3481 (0.2986-0.4256)	1.86
IM-R	Imidacloprid	1.590 ± 0.155	26.0691 (18.2895-47.1018)	–
	Imidacloprid+TPP	2.013 ± 0.234	10.6243 (8.7881-13.3056)	2.45
	Imidacloprid+DEM	1.561 ± 0.274	23.1614 (16.4089-41.2462)	1.13
	Imidacloprid+PBO	1.256 ± 0.321	7.2553 (5.4405-9.5641)	3.59

SR, synergism ratio.

Table 5. Detoxifying enzyme activities in the imidacloprid-resistant (IM-R) and susceptible (SS) strains of *R. padi*

Enzyme	Strain	Specific activity of enzyme	Ratio (IM-R/SS)
CarE[a]	SS	1.943 ± 0.041 a	
	IM-R	2.674 ± 0.041 b	1.38
GST[b]	SS	0.486 ± 0.008 a	
	IM-R	0.569 ± 0.020 a	1.17
P450s[c]	SS	1.739 ± 0.015 a	
	IM-R	3.269 ± 0.042 b	1.88

Different letters indicate that the means are significantly different by Student's *t*-test ($P < 0.05$).
[a] CarE activity = nmol μg^{-1} min^{-1}.
[b] GST activity = nmol μg^{-1} min^{-1}.
[c] P450 activity = EU of P450 μg^{-1}.

intensively with neonicotinoids, resulting in widespread resistance in different countries.[10] To preserve the quality and yield of wheat crops, the central government of China has extended the national range of the integrated pest management of wheat, with the rotational use of different types of insecticides as a strategy adopted in the last decade, which could be effective for managing insecticide resistance of wheat aphids. Our life-table analysis showed that the *R. padi* imidacloprid-resistant strain had lower fecundity and shorter longevity, indicating a fitness cost in the resistant strain. In the field, susceptible insects with higher fitness were able to show a quick recovery of the population when the use of imidacloprid was suspended or rotated, which can also explain the slow development of the resistance of *R. padi* rather than the rapid emergence of high resistance in the field when area-wide wheat pest control strategies are applied. In another study with 3-h exposure to a sublethal concentration of imidacloprid on residual film in glass tubes for three generations, the fecundity and longevity of *R. padi* were deleteriously affected.[47] However, the fecundity of *R. padi* essentially recovered by the F5 generation, although its longevity was still reduced. Both the short-term exposure to imidacloprid on residual film in glass tubes in that analysis and the continuous selection with imidacloprid on wheat seedlings in the current study had deleterious effects on *R. padi*, but the duration and the extent of the effects varied with different selection pressures.

Patterns of cross-resistance in the IM-R strain have important implications for resistance management. Our results suggest that imidacloprid resistance in *R. padi* involved cross-resistance to two neonicotinoids (thiamethoxam and acetamiprid), and did not show obvious cross-resistance to sulfoxaflor. Sulfoxaflor is an nAChR agonist that is structurally distinct from neonicotinoids.[10] Similar to our findings, previous studies found that aphids and

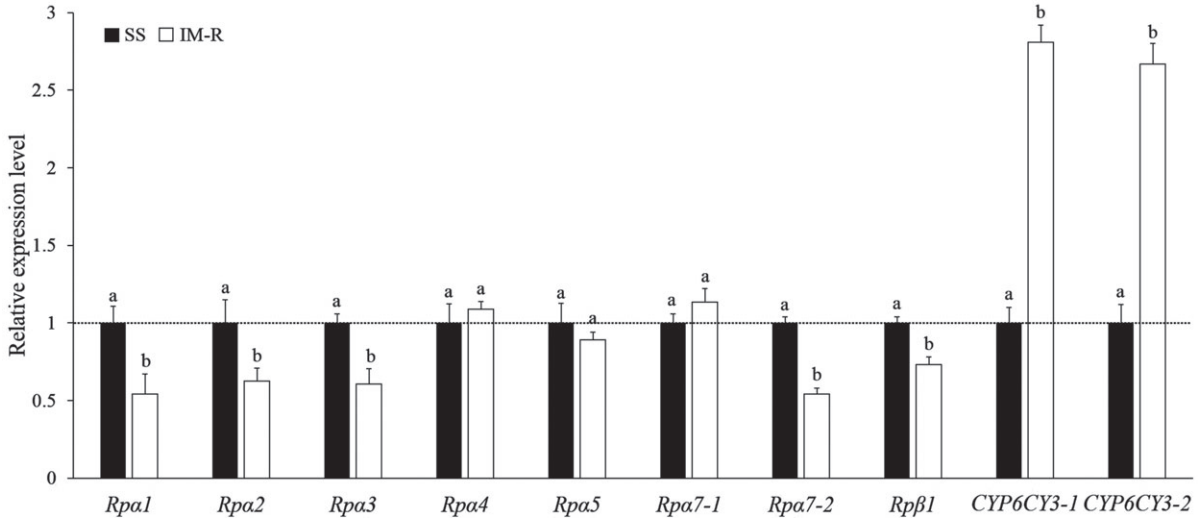

Figure 2. The relative transcript level of *R. padi* nAChR subunits and *CYP6CY3* genes in the imidacloprid-resistant (IM-R) and susceptible (SS) strains. Data are shown as the mean ± standard error of the mean; different letters denoted a significant difference among samples by Student's *t*-test ($P < 0.05$). The expression of each target gene was normalized to the SS strain.

whiteflies with metabolic resistance were almost completely susceptible to sulfoxaflor.[48–50] It is conceivable that the structural differences between sulfoxaflor and imidacloprid can explain the lack of cross-resistance of the imidacloprid-resistant insect strains to sulfoxaflor.[48–50] Several factors can affect the response slope value of toxicity, including the mode of action of the insecticide and the homogeneity of insects within a population.[51,52] However, a higher slope value typically suggests a more homogeneous response to an insecticide within populations and less risk of resistance development, and vice versa.[51,52] In this study, the slopes observed for imidacloprid and sulfoxaflor in both the susceptible and the imidacloprid-resistant strains were relatively low compared with those for other insecticides, which indicates a more heterogeneous response to imidacloprid and sulfoxaflor within the aphid populations and a greater possibility that the aphid strains would develop higher resistance to these two chemicals. The rotational use of chemicals with different targets could play an important role in the management of *R. padi* resistance to neonicotinoids. However, it is important to anticipate the risks of cross-resistance involving novel members of a broad mode-of-action group because these risks can be mechanism-specific.[10] For example, an *M. persicae* field population with an R81T mutation in the β1 subunit of the nAChR showed cross-resistance to both neonicotinoids and sulfoxaflor, which differed from the nonexistent cross-resistance pattern in strains with no neuro-target mutation.[10]

Typically, the enhanced metabolic capability of detoxification systems and/or reduced insecticide target-site sensitivity contributes to insecticide resistance.[19] The metabolic detoxification system in insects consists of major groups of enzymes, such as cytochrome P450, esterases, and glutathione-S-transferases. Synergistic bioassays are commonly used to test for the involvement of metabolic mechanisms in insecticide resistance.[21] In our study, synergism by PBO, an inhibitor of the P450-monooxygenase oxidative metabolic system, had a significant effect on imidacloprid toxicity in the IM-R strain. A CarE inhibitor (TPP) also had some effects in the IM-R strain, but no obvious synergism with DEM was observed. The synergistic analyses were consistent with the enzyme activity assays in showing that P450 and esterase detoxification could be important biochemical mechanisms for imidacloprid resistance in *R. padi*. GST had no obvious contribution to resistance, which was similar to that found in *N. lugens*[53] and *M. persicae*.[31,33]

Enhanced P450 activity is an important mechanism for imidacloprid resistance in many insects. Although some additional mechanisms may have contributed, the enhanced activity of P450s was the major mechanism of imidacloprid resistance in *M. persicae*.[31,33] Over-expression of *CYP6CY3-1* contributes to imidacloprid resistance in *A. gossypii*.[54] Microarray analysis revealed the constitutive over-expression of a single P450 gene, *CYP6CY3*, and quantitative PCR showed that the over-expression is attributable, at least in part, to gene amplification.[31] Imidacloprid resistance in the brown planthopper, *N. lugens*, has been comprehensively studied. Analysis of detoxification enzyme activity demonstrated that the mechanism for imidacloprid resistance in a field population of *N. lugens* was mainly enhanced by P450 detoxification.[53] Bass *et al.*[26] found that a single P450 gene (*CYP6ER1*) was highly overexpressed in all resistant strains, and the expression pattern was significantly correlated with the resistance phenotype of the *N. lugens* IM-R strain. Ding *et al.*[27] showed that *CYP6AY1* expression was increased 17.9 times in the *N. lugens* IM-R strain compared with SS. Additionally, *CYP4CE1* and *CYP6CW1* were shown to play roles in imidacloprid resistance in *N. lugens*.[29] In our study, expression of the cytochrome P450s *CYP6CY3-1* and *CYP6CY3-2* was significantly higher in the IM-R strain than in SS. Further analysis is needed to confirm the roles of these two genes in the resistance of *R. padi* to imidacloprid.

CarEs have been implicated in insect metabolic resistance mainly to chemicals with ester bonds, including pyrethroids, organophosphates, and carbamates.[55,56] Enhanced CarE activity was occasionally linked to resistance to some chemicals that lack ester bonds, but the evidence for involvement of CarEs in this resistance requires further investigation, and the biochemical basis for this resistance remains to be elucidated.[55] Increased production of detoxifying CarEs has been found in imidacloprid-resistant strains of *M. persicae* and *A. gossypii*; however, this enhanced activity may have resulted from previous selection by other chemicals such as pyrethroids.[31] The imidacloprid-resistant strain in this study originated from a field population in China. Organophosphates, carbamates and pyrethroids were widely used to control *R. padi*

for a long time in China before neonicotinoid insecticides were applied instead.[57] Previous field selection by other chemicals with ester bonds could possibly explain the increased activity of CarEs in this study, although further investigations are needed to determine the detailed biochemical mechanisms of the resistance. Actually, the resistant strain IM-R in this study showed different levels of cross-resistance to other chemicals, including organophosphates and carbamate.

An R81T (arginine to threonine substitution) mutation in the loop D region of the β1 subunit mediating neonicotinoid resistance was found in some aphid species.[58,59] Loop D is one of three regions (D, E, and F) in the β1 subunit that combines with loops A, B, and C of the α subunit to form the binding site for the natural ligand acetylcholine and certain antagonists, including neonicotinoids.[60,61] The R81T mutation resulted in reduced sensitivity of nAChR to neonicotinoids through the loss of the direct electrostatic interaction of the electronegative pharmacophore with the basic arginine residue at this key position within loop D.[12] In this study, we did not identify any amino acid mutation in nAChRs, although three single nucleotide mutations were detected in the nucleotide sequence. Moreover, our previous study also did not detect this point mutation in *R. padi* field populations in China.[42]

Some studies have shown that the down-regulation of the nAChR subunit may be associated with neonicotinoid resistance.[17,18,62,63] In *A. gossypii*, the transcript level of the nAChR β1 subunit decreased significantly in the IM-R strain compared with SS.[17] RNA interference of the *L. decemlineata* nAChR α1 (*Ldα1*) subunit significantly reduced its mRNA level and greatly decreased insect sensitivity to imidacloprid.[18] The expression of nAChR α2 (*Mdα2*) in the neonicotinoid-resistant field population and an imidacloprid-selected strain of *M. domestica* was 60% lower than that in the susceptible strain. Moreover, sequence data found no mutation explaining the increased resistance.[62] Zhang *et al.* found that the reduced transcripts of nAChR α8 (*Nlα8*) were associated with imidacloprid resistance in *N. lugens*, and an electrophysiological study showed that decreased *Nlα8* cRNA injection in *Xenopus* oocytes significantly reduced imidacloprid potency at recombinant receptors.[63] Interestingly, we found that the transcript levels of the α1, α2, α3, α7-2, and β1 subunits decreased significantly in the IM-R strain of *R. padi*. The down-regulation of the nAChR subunits could be a possible mechanism of resistance of *R. padi* to neonicotinoids, although more studies need to be performed to confirm this mechanism. Previously, we found that the ATP-binding cassette (ABC) transporter *ABCC1* was up-regulated in the imidacloprid-resistant *R. padi* strain, and imidacloprid induced the transcription of *ABCC1*, indicating a role of ABCC1 in the efflux of insecticides in *R. padi*; nevertheless the precise role of ABCC1 in the insecticide resistance remains to be further determined.[64]

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