Research Article

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Super-*kdr* mutation M918L and multiple cytochrome P450s associated with the resistance of *Rhopalosiphum padi* to pyrethroid

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Abstract

BACKGROUND: *Rhopalosiphum padi* is an important pest affecting cereal crops worldwide. Pyrethroid, including lambda-cyhalothrin, has been widely used to control *R. padi* in the field. This work investigated the resistance levels of *R. padi* field populations to lambda-cyhalothrin, and analysed biochemical and molecular mechanisms of aphid resistance to the insecticide pyrethroid.

RESULTS: A lambda-cyhalothrin-resistant field population (JY) was sampled, and a super-*kdr* mutation, M918L, in the voltage-gated sodium channel (VGSC) was identified in the population. The lambda-cyhalothrin-resistant strain (LC-R) was subsequently established by selecting the field population with lambda-cyhalothrin. All individuals of the *R. padi* LC-R strain showed the M918L heterozygous mutation in the VGSC IIS4–IIS6 region. Cross-resistance profiles of the LC-R strain to nine insecticides were detected. Both synergistic and enzyme activity studies indicated that cytochrome P450 monooxygenase played an important role in this resistance. Further gene expression analysis showed that seven P450 genes were significantly upregulated in the LC-R strain compared with the susceptible strain.

CONCLUSION: Field-evolved resistance to pyrethroid insecticides has been found in *R. padi*. The M918L (super-*kdr*) mutation in the VGSC was documented for the first time in field samples obtained from an important wheat-growing area. The super-*kdr* mutation, as well as metabolic resistance mediated by P450 genes, was determined to contribute to the lambda-cyhalothrin resistance in *R. padi*.

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Keywords: *Rhopalosiphum padi*; lambda-cyhalothrin; cytochrome P450; voltage-gated sodium channel; M918L; pyrethroid resistance

1 INTRODUCTION

The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), is a prevalent pest affecting wheat worldwide.[1,2] In addition to direct feeding damage, this aphid also transmits barley yellow dwarf virus (BYDV), leading to reduced quality and yield.[1,3] Control of *R. padi* is primarily dependent on repeated sprays with chemical insecticides. Pyrethroid insecticides are one main option for controlling aphids on cereals, including *R. padi*. Unfortunately, resistance to pyrethroid has been recorded in some field populations.[4] To date, the underlying mechanisms governing resistance in *R. padi* have not been documented.

Insecticide resistance has been attributed to two major causes: target-site insensitivity and increased metabolic detoxification.[5–8] Target-site mutations of the voltage-gated sodium channel (VGSC) gene are also linked to pyrethroid resistance in aphids.[5,9] Knock-down resistance (*kdr*) was first reported in *Myzus persicae* in 1997 when a leucine-to-phenylalanine replacement (L1014F) in transmembrane segment IIS6 was detected in some pyrethroid-resistant clones.[10] Subsequently, the super-knockdown resistance (super-*kdr*) substitution of methionine for leucine at position 918 (M918L) has been reported within the nearby IIS4–S5 intracellular linker.[11] In addition, Fontaine *et al.* identified an alternative super-*kdr* variant (M918L) in *M. persicae* from France and demonstrated that the variant was linked to resistance to lambda-cyhalothrin.[12] Chen *et al.* found that the mutation M918L contributed to pyrethroid resistance in field populations of *Aphis gossypii* from Bt cotton growing

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regions of China.[13] The L1014F heterozygous mutation in *Sitobion avenae* was associated with pyrethroid resistance.[14] Detoxifying enzymes that mediate metabolic resistance, including cytochrome monooxygenases (P450s) and carboxylesterases, have been shown to be important in pyrethroid resistance in aphid species, such as *A. gossypii*,[15] *M. persicae*[16] and *Aphis glycines*.[17] In a previous study, we found that carboxylesterase is associated with cyhalothrin resistance in *R. padi*.[18] Insect P450 genes fall into four major clades,[19] and members of the CYP3 and CYP4 clades, particularly those of the CYP4, CYP6 and CYP9 families, have been most frequently linked to xenobiotic detoxification across a range of insect species.[20] The functions of P450 genes in CYP2 and mitochondrial clades have been mostly linked to physiological functions.[21] With the availability of the whole genome sequence of *R. padi*, it becomes possible to characterize the P450 associated with insecticide resistance in this species.[22]

In this study, an *R. padi* field population resistant to lambda-cyhalothrin was collected from an important wheat-growing region of China. A resistant strain was further established by selecting the field strain with lambda-cyhalothrin. The susceptibility of the strain to insecticides frequently used for aphid control in cultivated crops was tested. Finally, the biochemical and molecular mechanisms governing the resistance of *R. padi* to lambda-cyhalothrin were investigated.

2 MATERIALS AND METHODS

2.1 Insects

The susceptible *R. padi* strain (SS) was collected from a wheat field population in Gansu Province in China in 2013. The susceptible strain was maintained on wheat seedlings (cultivar Xiaoyan 22) in mesh cages (41 × 41 × 41 cm) in the laboratory without contacting any insecticides at 23 ± 1 °C under a 16:8 h light/dark photoperiod.

Five geographical populations were collected from wheat plants in different regions of an important wheat-growing area (Shaanxi Province) of China in May 2019 (Table 1). Field generations were reared in the laboratory for one generation on wheat seedlings (cultivar Xiaoyan) to obtain enough apterous adult aphids for bioassays. For gene sequencing, apterous adults from the field were stored in 95% ethanol until DNA extraction. The lambda-cyhalothrin-resistant strain (LC-R) was obtained from the JY field population by laboratory selection with lambda-cyhalothrin for 21 generations. To normalize the bioassay and insecticide selection time, we considered 7 days to be the generation time in this study. During the insecticide selection process, the toxicity of imidacloprid was evaluated every three generations to confirm the median lethal concentration (LC_{50}) value,[23] which was used as the selected concentration for the following four generations. The LC-R strain was reared under the conditions mentioned above.

2.2 Insecticides and chemicals

The insecticides used for bioassays included lambda-cyhalothrin (96% purity; Yancheng Nongbo Bio-technology Co., Ltd., Yancheng, China), deltamethrin (99% purity; Nanjing Red Sun Co., Ltd., Nanjing, China), fenvalerate (96% purity; Shandong Sheda Crop Science Co., Ltd., Shouguang, China), bifenthrin (95% purity; Nanjing Red Sun, Nanjing, China), imidacloprid (95% purity; Jiangsu Changlong Chemical Co., Ltd., Taixing, China), thiamethoxam (96% purity; Shandong Sino-Agri United Biotechnology Co., Ltd., Jinan, China), chlorpyrifos (96% purity; Shangdong Moderne Chemical Co., Ltd., Jinan, China), malathion (95% purity; Tianjin Aigefu Co., Ltd., Tianjin, China), indoxacarb (95% purity; Anhui Huaxing Chemical Industry Co., Ltd., Hexian, China), and methomyl (97% purity; Shandong Huyang Technology Co., Ltd, Jinan, China).

The chemicals used for synergistic bioassays were piperonyl butoxide (PBO; Sigma-Aldrich, St. Louis, MO, USA), triphenyl phosphate (TPP; Shanghai Chemical Reagent Co., Ltd, Shanghai, China) and diethyl maleate (DEM; Shanghai Chemical Reagent Co, Shanghai, China). Chemicals used for enzyme activity analyses were: α-naphthol, fast blue B salt (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and α-naphthyl acetate (α-NA; Solarbio, Beijing, China); eserine, EDTA, 1,4-dithiothreitol (DTT), NADPH, *p*-nitroanisole, reduced glutathione (GSH) and Coomassie Brilliant Blue G250 (all Sigma); and 1-chloro-2,4-dinitrochlorobenzene (CDNB) and bovine serum albumin (BSA) (Roche, Mannheim, Germany).

2.3 Bioassays

The LC_{50} of insecticides was tested using the leaf-dipping bioassay method.[23] Technical-grade insecticides were dissolved in acetone and diluted to 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 and then placed in a Petri dish containing moistened filter paper. The control was treated with distilled water containing 0.01% (v/v) Triton X-100 and 0.01% acetone alone. Some 50–60 apterous adults were treated at each concentration with three replicates, and aphid mortality was assessed after 24 h. The LC_{50} and 95% confidence limits (CL) of the LC_{50} were calculated using DPS software (Zhejiang University, Hangzhou, China).

2.4 Mutation detection of *R. padi* voltage-gated sodium channel gene fragments

The IIS4–IIS6 region contains the mutation sites (M918, L925, T929, F932 and L1014) previously demonstrated to confer

Table 1. Susceptibility to lambda-cyhalothrin of *Rhopalosiphum padi* from five field populations and a laboratory susceptible strain

Population code (region)	Origin	Slope ± SE	LC_{50} (mg L^{-1}) (95% CL)	RR[a]
SS	Lanzhou, Gansu	1.889 ± 0.168	0.646 (0.552–0.768)	
QX	Qianxian, Shaanxi	1.920 ± 0.183	1.709 (1.399–2.031)	2.65
CH	Chunhua, Shaanxi	1.594 ± 0.170	1.042 (0.7645–1.312)	1.61
JY	Jingyang, Shaanxi	1.665 ± 0.160	15.596 (13.002–19.329)	24.14
QS	Qishan, Shaanxi	1.686 ± 0.168	2.307 (1.915–2.762)	3.57
LQ	Liquan, Shaanxi	1.709 ± 0.176	2.289 (1.889–2.752)	3.54

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resistance to pyrethroids in many insect species.[5,9,13,14] Eight apterous adults from each of the five field populations and the laboratory strain of *R. padi* were genotyped for the presence of mutation sites at the IIS4–IIS6 region of the *RpVGSC* gene. Genomic DNA (gDNA) was extracted using an EZNA® Tissue DNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's recommended protocol. Using the gDNA as a template, the IIS4–IIS6 region of the *RpVGSC* gene was amplified with the primer pair *RpVGSC*-F and *RpVGSC*-R using LA *Taq* polymerase (Takara, Dalian, China) (Table S1). Polymerase chain reaction (PCR) conditions were: denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and finally, 72 °C for 10 min. The PCR products were visualized by 1.0% agarose gel electrophoresis and directly sequenced in both directions using primers described as above for PCR application at Shanghai Sangon Biotech Co., Ltd., Shanghai, China. Sequences were analysed using Chromas and Clustal X.[24,25]

2.5 Synergistic bioassays

For analysis of the enzyme inhibitor synergism effect, DEM, PBO and TPP were dissolved in acetone. In accordance with the methods of Elzaki *et al*. and Wang *et al*.,[23,26] 2 h before insecticide treatments, aphids were treated individually with 100 mg L^{-1} of TPP, PBO or DEM. Procedures followed the leaf-dipping bioassay protocol.[23] The synergism ratio was calculated by dividing the LC_{50} value of insecticide alone by the LC_{50} value of insecticide with a synergist.

2.6 Enzyme activity analysis

The Bradford method using BSA as the standard was used to determine the enzyme solution total protein content, and the optical density (OD) was recorded at 562 nm. The enzyme source was prepared by homogenizing 40 apterous adult aphids with 1 mL of pre-chilled sodium phosphate buffer (0.1 mM, pH 7.6, containing 1 mM of EDTA and 1 mM of DTT) on ice and then centrifuged at 12 000 *g* for 30 min at 4 °C. The supernatant was recentrifuged at 12 000 *g* for 10 min at 4 °C. The clear supernatant was used to determine the activity of P450 monooxygenases, carboxylesterases and glutathione *S*-transferases (GST).

The activity of P450 monooxygenases was measured using the method described by Elzaki *et al*. with slight modifications.[26] Ten microlitres of the enzyme solution and 180 μL of 2 mM *p*-nitroanisole were pipetted into each well of a 96-well microplate, and the reaction was initiated by adding 10 μL of 9.6 mM NADPH and incubated for 2 h at 30 °C. The OD at 405 nm was recorded using an iMark Microplate Reader (Bio-Rad, Hercules, CA, USA).

Carboxylesterase activity was analysed according to the method of Wang *et al*.[23] One hundred microlitres of phosphate buffer (0.04 M, pH 7.0) containing 3.0×10^{-4} mol L^{-1} α-NA and 10^{-4} mol L^{-1} physostigmine, and 75 μL of enzyme solution was incubated at 30 °C for 15 min. The reaction was then stopped by adding 25 μL of fast blue B salt solution (1% fast blue B salt and 5% sodium dodecyl sulfate). The OD at 600 nm was recorded at 30 °C with the same microplate reader as above.

The GST activity test was performed using the method described by Rodríguez *et al*. and Elzaki *et al*.[26,27] The reaction solution contained 10 μL of enzyme solution, 100 μL of 1.2 mM CDNB and 100 μL of 6 mM GSH. The OD at 340 nm was monitored over 6 min at 10-s intervals at 27 °C using the same microplate reader as above.

2.7 RNA extractions and cDNA synthesis

Total RNA was extracted from apterous adults using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was treated with DNase I (Takara, Kyoto, Japan) for digesting DNA contamination. The quality and quantity of the RNA were measured using a Nano-Drop spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and 1% gel electrophoresis. First-strand cDNA was synthesized from the total RNA (1.0 μg) using the M-MLV reverse transcriptase cDNA Synthesis Kit (Promega, Madison, WI, USA), with oligo$(dT)_{18}$ serving as a primer.

2.8 Quantitative RT-PCR and analysis

Quantitative real-time PCR was performed on the LightCycler 480 system (Roche) with Fast Start SYBR Green I Master Mix (Roche) in accordance with the manufacturer's instructions. Gene-specific primers (Table S1) were synthesized by Sangon Biotech Co. The thermal cycling protocol was initiated at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s. The melting curve was obtained by raising the temperature from 65 to 95 °C in 0.5 °C increments. The glyceraldehyde-3-phosphate dehydrogenase (GADPH) and *β-actin* genes of *R. padi* were used as the internal reference genes to normalize the target gene expression levels.[23] The experiment included at least three independent biological replicates for each sample. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.9 Statistical analysis

The statistical significance of differences in enzyme activities and qRT-PCR in the two strains was calculated by Student's *t*-test with the level of significance at $P < 0.05$. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used to perform all statistical analyses.

3 RESULTS

3.1 Susceptibility of *R. padi* field populations to lambda-cyhalothrin

The susceptibilities of five field populations of *R. padi* from Shaanxi Province, China were tested compared with the SS strain maintained in the laboratory for > 6 years without any insecticide (Table 1). The results indicated that all field populations were less susceptible to lambda-cyhalothrin than the SS laboratory strain. The largest resistance ratio (24.14-fold) corresponded to the field population from Jingyang (population code JY), and the other field populations were susceptible [resistance ratio (RR) ≤3) or minor resistance (3 < RR ≤5) to lambda-cyhalothrin.

3.2 Sequencing of *R. padi* voltage-gated sodium channel gene fragments

An ~ 1600 bp fragment was amplified from the IIS4–IIS6 region of the *R. padi* VGSC using the *RpVGSC* primers (Table S1). Compared with the susceptible strain genotype, two individuals of the JY field population (2 of 8) showed a single base difference in their sequencing traces (Fig. 1A), which resulted in a non-synonymous substitution of methionine with leucine corresponding to the amino acid residue 918 (M918L), a same substitution as the super-*kdr* mutation reported in several aphid species, such as *A. gossypii*[13,28] and *M. persicae*.[29] This base change (ATG to TTG) appeared as a mixed peak in the sequence chromatograms (Fig. 1B), indicating that individuals contained heterozygous genotypes. None of the other mutation sites (L925, T929, F932

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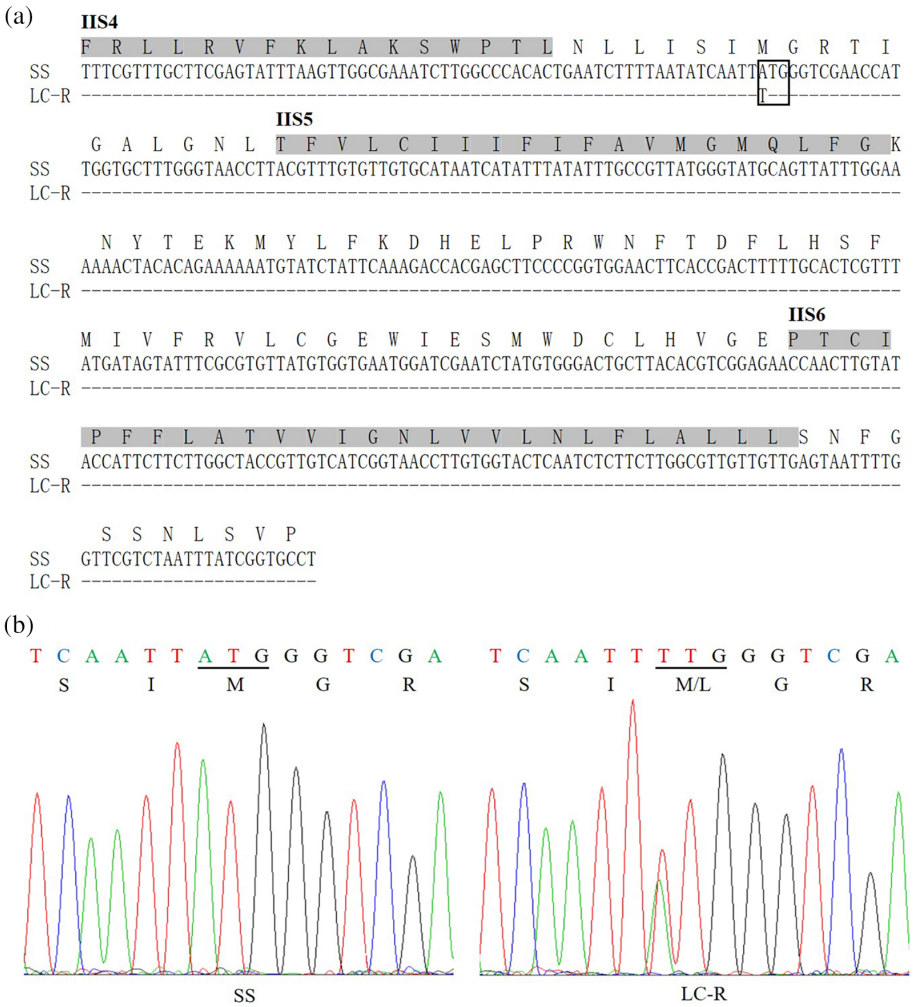

Figure 1. (A) Partial nucleotide and deduced amino acid sequences of the *para*-like sodium channel gene from the susceptible strain (SS) and lambda-cyhalothrin-resistant strain (LC-R). The single codon change ATG to TTG, causing the super-*kdr* substitution M918L, is boxed. IIS4–IIS6 regions are highlighted with a grey background. (B) Partial nucleotide sequence chromatograms of the voltage-gated sodium channel across the super-*kdr* mutation site M918L. Two peaks (representing ATG and TTG) are observed in the lambda-cyhalothrin-resistant strain (LC-R) of *Rhopalosiphum padi*, indicating a heterozygous mutation.

and L1014) previously reported in VGSCs of other insect species were detected in JY and other field populations of *R. padi*.

3.3 Lambda-cyhalothrin resistance development and synergism

To systematically explore the molecular mechanisms of lambda-cyhalothrin resistance in *R. padi*, a lambda-cyhalothrin resistant strain (LC-R) was obtained by continuous selection with lambda-cyhalothrin from the JY field population for 21 generations. As outlined in Table 2, LC_{50} increased from 17.596 to 102.561 mg L^{-1}. The resistance increased steadily from 24.14-fold at F_1 to 152.70-fold at F_{17} and then fluctuated ~ 150-fold between F_{17} and F_{21} (Table 2). Twenty aphids were taken randomly from the F_{21} of the LC-R strain and tested individually using DNA sequencing; all individuals showed the M918L heterozygous mutation in the IIS4–IIS6 region of the *R. padi* VGSC.

Table 2. Resistance development of *Rhopalosiphum padi* LC-R strain to lambda-cypermethrin under chemical selection in the laboratory

Generation	Slope ± SE	LC_{50} (mg L^{-1}) (95% CL)	RR[a]
1	1.665 ± 0.160	15.596 (13.002–19.329)	24.14
3	1.497 ± 0.159	17.282 (14.040–22.356)	26.75
5	1.299 ± 0.153	19.437 (15.333–26.554)	30.09
7	1.563 ± 0.175	27.309 (21.164–38.988)	42.27
9	1.702 ± 0.170	33.154 (26.851–43.602)	51.32
11	1.744 ± 0.186	46.000 (35.670–65.685)	71.21
13	1.476 ± 0.172	63.399 (47.756–95.509)	98.14
15	1.539 ± 0.178	79.472 (60.028–119.309)	123.02
17	2.132 ± 0.190	98.642 (83.302–121.906)	152.70
19	2.158 ± 0.191	99.650 (84.115–123.230)	154.26
21	1.961 ± 0.187	102.561 (85.095–130.213)	158.76

[a] Resistance ratio (RR) = LC_{50} of lambda-cypermethrin resistant strain/ LC_{50} of the susceptible strain (0.646 mg L^{-1}).

3.4 Cross-resistance patterns

The cross-resistance patterns for different insecticides, including pyrethroids, neonicotinoids, organophosphates and carbamates,

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were determined with F_{21} generation apterous adults. The highest level of cross-resistance to deltamethrin (108.33-fold) was found in the LC-R strain. The strain showed obvious cross-resistance to fenvalerate (36.99-fold) and bifenthrin (37.23-fold). Compared with the SS strain, the LC-R strain also showed different levels of cross-resistance to thiamethoxam (12.33-fold), malathion (22.81-fold), indoxacarb, (17.61-fold) and methomyl (16.67-fold), but no obvious resistance to imidacloprid and chlorpyrifos (Table 3).

3.5 Synergistic effect to toxicity in susceptible and lambda-cyhalothrin resistant strains

The synergistic effects of PBO, TPP and DEM on lambda-cyhalothrin toxicity to LC-R and SS strains are shown in Table 4. The oxidase inhibitor PBO showed 2.51- and 1.26-fold synergism to lambda-cyhalothrin in the LC-R and SS strains, respectively. TPP had a synergism ratio value of 1.39 and 1.06 on lambda-cyhalothrin in the LC-R and SS strains, respectively. DEM showed a synergism ratio of 0.98 and 1.11 on lambda-cyhalothrin in the respective two strains (< 1.20).

3.6 Metabolic enzyme activities

To evaluate the role of metabolic detoxification mechanisms in the LC-R strain, the activities of P450 monooxygenases, carboxylesterases and GSTs in LC-R and SS strains were detected and are shown in Table 5. The results indicated that cytochrome P450 activity was significantly higher in LC-R than in SS with an enzyme ratio of 1.78. The carboxylesterase and GST activities in LC-R did not increase significantly in comparison with the SS strain (1.14- and 0.94-fold, respectively). These results suggested that lambda-cyhalothrin resistance might primarily be associated with cytochrome P450s.

3.7 Expression profiling of *R. padi* P450 genes in lambda-cyhalothrin resistant and susceptible strains

The expression of P450 genes[22] from clade 3 and clade 4 in apterous adult aphids from the LC-R strain was analysed by comparison with the expression in the SS strain. Of the 39 CYP genes tested, seven (*CYP6CY19*, *CYP6CZ1*, *CYP6CY51*, *CYP6DA1*, *CYP6DC1*, *CYP4CH1* and *CYP4CJ5*) were significantly upregulated in the LC-R strain compared with the SS strain, among which *CYP6DC1* and *CYP4CJ5* showed remarkably high expression levels with 4.31- and 4.08-fold overexpression, respectively (Fig. 2).

4 DISCUSSION

Since their development in the 1970s, pyrethroids have become some of the most widespread insecticides employed for the control of agricultural pests and insect vectors on a global scale due to their low cost, low mammalian toxicity and high insecticidal capability.[30–33] Unfortunately, widespread use of pyrethroids has resulted in a high level of resistance in some insect species.[33] A number of mechanisms have been proposed for this resistance, including target site insensitivity and metabolism by carboxylesterases[18,34] and P450s.[17,35] In the present study, we documented a super-*kdr* mutation (M918L) in the VGSC of the *R. padi* field population from a major wheat-growing area. The molecular mechanisms governing the lambda-cyhalothrin resistance of a strain selected from the *R. padi* field population were analysed. The results indicated that the super knockdown resistance (super-*kdr*/M918L) and constitutive overexpression of P450 genes were involved in the resistance to the chemical.

We monitored insecticide resistance levels of the *R. padi* field populations collected from an important wheat-growing area. The results showed that most *R. padi* field populations were still susceptible or had minor resistance to lambda-cyhalothrin. Although *R. padi*, one of the most important cereal aphids, has

Table 3. Cross-resistance patterns of the LC-R strain of *Rhopalosiphum padi* to different insecticides

Insecticide	Strain	Slope ± SE	LC_{50} (mg L^{-1}) (95% CL)	RR[a]
Deltamethrin	SS	1.905 ± 0.183	0.585 (0.470–0.700)	108.33
	LC-R	2.478 ± 0.260	63.374 (51.648–84.409)	
Fenvalerate	SS	1.399 ± 0.160	0.590 (0.449–0.734)	36.99
	LC-R	1.22 ± 0.159	21.827 (16.69–28.001)	
Bifenthrin	SS	1.742 ± 0.174	0.579 (0.459–0.700)	37.23
	LC-R	1.240 ± 0.148	21.554 (17.157–26.621)	
Imidacloprid	SS	1.631 ± 0.157	0.641 (0.539–0.777)	4.96
	LC-R	1.420 ± 0.163	3.182 (2.398–4.777)	
Thiamethoxam	SS	1.567 ± 0.157	0.844 (0.693–1.013)	12.33
	LC-R	1.444 ± 0.153	10.404 (8.332–12.777)	
Chlorpyrifos	SS	1.402 ± 0.152	0.663 (0.519–0.815)	3.48
	LC-R	2.767 ± 0.220	2.310 (2.037–2.610)	
Malathion	SS	1.665 ± 0.158	1.000 (0.839–1.193)	22.81
	LC-R	1.859 ± 0.256	22.806 (15.816–41.773)	
Indoxacarb	SS	1.745 ± 0.164	1.124 (0.951–1.339)	17.61
	LC-R	1.435 ± 0.159	19.792 (14.511–30.699)	
Methomyl	SS	1.830 ± 0.171	0.874 (0.735–1.031)	16.67
	LC-R	2.396 ± 0.265	14.571 (11.544–20.435)	

[a] Resistance ratio (RR) = LC_{50} of lambda-cyhalothrin-resistant (LC-R) strain /LC_{50} of the susceptible (SS) strain.

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Table 4. Synergistic effects of triphenyl phosphate (TPP), diethyl maleate (DEM) and piperonyl butoxide (PBO) in lambda-cyhalothrin-resistant and -susceptible strains of *Rhopalosiphum padi*

Strain	Insecticide/synergist	Slope ± SE	LC_{50} (mg L^{-1}) (95% CL)	SR[a]
SS	lambda-cyhalothrin	1.889 ± 0.168	0.646 (0.552–0.768)	–
	lambda-cyhalothrin +TPP	2.053 ± 0.176	0.610 (0.526–0.715)	1.06
	lambda-cyhalothrin +DEM	1.919 ± 0.168	0.656 (0.563–0.778)	0.98
	lambda-cyhalothrin +PBO	1.989 ± 0.173	0.521 (0.447–0.4609)	1.26
LC-R	lambda-cyhalothrin	1.961 ± 0.187	102.561 (85.095–130.213)	–
	lambda-cyhalothrin +TPP	1.943 ± 0.180	73.540 (62.566–88.741)	1.39
	lambda-cyhalothrin +DEM	1.617 ± 0.165	92.711 (75.842–119.309)	1.11
	lambda-cyhalothrin +PBO	1.658 ± 0.162	40.871 (33.634–48.897)	2.51

[a] Synergism ratio (SR) = LC_{50} (insecticide alone)/LC_{50} (insecticide with synergist). LC-R, lambda-cyhalothrin-resistant strain; SS, susceptible strain.

been considered to be at low risk of developing resistance to pesticides, mainly because of the abundance of untreated alternative hosts that act as susceptible refuges[14,36]; unfortunately, the LC_{50} value of lambda-cyhalothrin against the JY field population was significantly higher than that in the laboratory susceptible strain, and this lethal concentration was also significantly higher than that found in a field population by Huang *et al*. in 2017.[4] Foster *et al*. found that *S. avenae*, another important pest of cereal crops, displayed ~40-fold resistance to lambda-cyhalothrin.[14] Our results suggest that the development of pyrethroid resistance in *R. padi* represents a significant new threat to cereal production.

Sequencing of the VGSC identified a single mutation, M918L, in the JY population compared with the susceptible strain and the other field populations. VGSCs, the primary target of pyrethroids, have been studied in many insects.[5,10,12,14] The L1014F *kdr* mutation, a leucine-to-phenylalanine replacement, in transmembrane segment II6 of VGSC was first demonstrated in several pyrethroid-resistant insect species, including *M. persicae* and *S. avenae*.[10,14] Subsequently, a super-*kdr* mutation, M918L, alongside the L1014F mutation within the nearby IIS4–S5 intracellular linker, has been identified in *M. persicae* and was linked to resistance to lambda-cyhalothrin.[12] Considering these reports, the homologous regions were amplified from gDNA from the field samples, the LC-R and SS strains, and a single M918L mutation was found in the field samples of the JY field population, as well as in the LC-R resistant strain originating from the field population. In *A. gossypii*, the M918L mutation is associated with cypermethrin, a pyrethroid insecticide.[13,37] Fontaine *et al*. also noted a significant increase in resistance to lambda-cyhalothrin due to the presence of the M918L mutation.[12] Wu *et al*. found two non-synonymous mutations, M918L and V1010A, which are expected to be responsible for the high resistance of *Thrips tabaci* to lambda-cyhalothrin.[38] Panini *et al*. studied the impact of allelic variations of two super-*kdr* mutations, M918T and M918L, and found that the M918L mutation strongly affects pyrethroid efficacy, particularly of type II pyrethroids, such as lambda-cyhalothrin.[29] The presence of M918L is always heterozygous, and no data concerning the influence of the homozygous genotype for the M918L mutation are available.[25] This mutation may have high fitness costs, and as a consequence, the mutant could be less competitive than other genotypes in the field.[29]

Table 5. Detoxifying enzyme activities in the lambda-cyhalothrin-resistant and -susceptible strains of *Rhopalosiphum padi*

Enzyme	Strain	Specific activity of enzyme (nmol mg^{-1} min^{-1} protein)	Ratio (LC-R/SS)
CES	SS	1909.89 ± 169.14	1.00
	LC-R	2174.96 ± 84.61	1.14
GST	SS	14.43 ± 0.41	1.00
	LC-R	13.62 ± 0.34	0.94
P450	SS	1.11 ± 0.09	1.00
	LC-R	1.97 ± 0.07*	1.78

*Means are significantly different at the 0.05 level according to the *t*-test. LC-R, lambda-cyhalothrin-resistant strain; SS, susceptible strain.

To further reveal the evolution of lambda-cyhalothrin resistance, cross-resistance patterns and other resistance mechanisms, the JY population was set up under continuous selection with lambda-cyhalothrin for 21 generations. The resistance level of the strain to lambda-cyhalothrin rapidly increased, and finally, a resistance strain (LC-R) that contained the M918 heterozygous in all individuals conferred high-level resistance to lambda-cyhalothrin compared with the SS strain (RR = 158.76). Similarly, the house fly *Musca domestica* developed 113.57-fold resistance to lambda-cyhalothrin compared with the susceptible population after 11 generations of selection.[39] *A. glycines* developed 43.42- and 76.67-fold resistance to lambda-cyhalothrin quickly after 25 and 40 generations of resistance selection.[17,40] These studies indicate that pests have the potential to evolve resistance to lambda-cyhalothrin under selection pressure. Integrating data from field and laboratory studies, our results reveal that the resistance risk of lambda-cyhalothrin exists in the *R. padi* field population, suggesting that the application of lambda-cyhalothrin should be undertaken with greater care.

Studies concerning resistance mechanisms and cross-resistance patterns in *R. padi* are important to develop sustainable management tactics in the field. Selection with lambda-cyhalothrin resulted in cross-resistance to an array of pesticides. In pyrethroids, the relative order of the resistance developed in the LC-R strain was deltamethrin > bifenthrin > fenvalerate. Deltamethrin is highly similar to lambda-cyhalothrin in structure (i.e. only the chlorine and fluorine atoms at the vinyl of lambda-cyhalothrin is substituted by the bromine in deltamethrin).[34,41] In addition, the LC-R strain showed a low level of cross-resistance to organophosphates (chlorpyrifos and malathion) and carbamates (isoprocarb and methomyl). Xi *et al*. found that the resistance ratio was 11.66 and 9.32 for chlorpyrifos and methomyl in a lambda-cyhalothrin-resistant strain of *A. glycines*.[17] Interestingly, no cross-resistance

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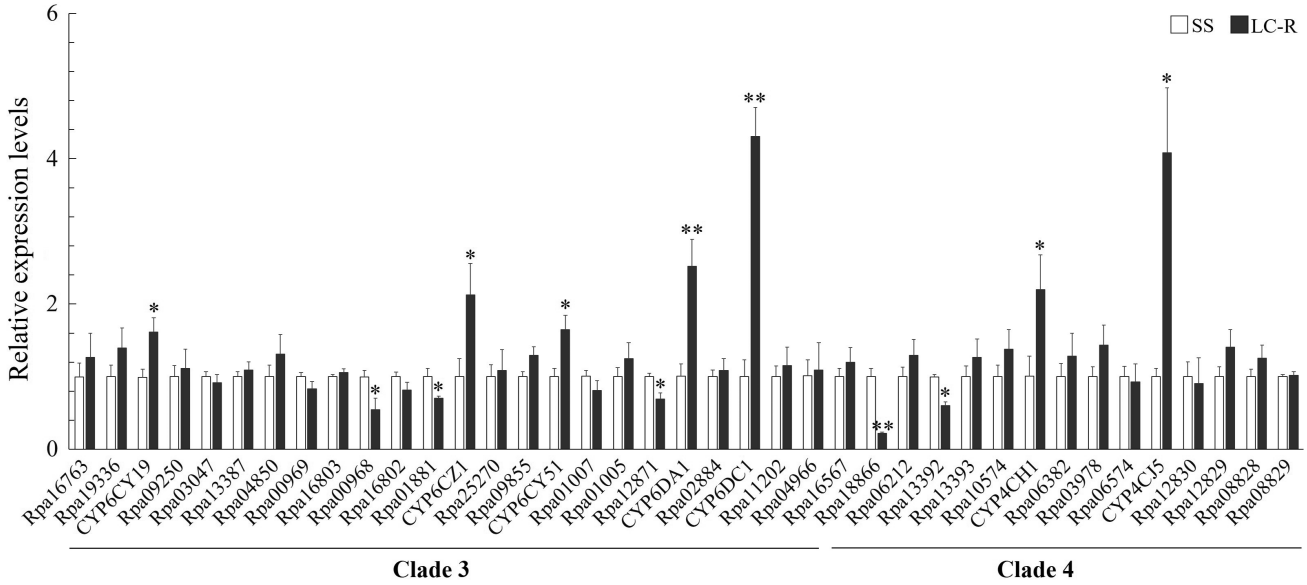

Figure 2. Expression levels of P450 genes in the lambda-cyhalothrin-resistant (LC-R) and susceptible (SS) strains of *Rhopalosiphum padi*. Data are shown as the mean ± SEM. Significant difference between samples by Student's *t*-test $*P < 0.05$, $**P < 0.01$.

to imidacloprid and thiamethoxam was detected. Previously, we reported that an imidacloprid resistance strain of *R. padi* showed cross-resistance to pyrethroids.[23] Similarly, the lambda-cyhalothrin-resistant *A. glycines* developed no cross-resistance to imidacloprid.[17] In terms of the control strategy, these results suggest that lambda-cyhalothrin and imidacloprid can be used in rotation in the field, which will reduce the selective pressure of a specific product and ultimately delay the development of resistance to both products.

Synergist bioassays are commonly used to test for metabolic mechanisms in insecticide resistance.[31] In the present study, PBO showed a significant effect in the resistant strain with increasing lambda-cyhalothrin toxicity by 2.51-fold. Accordingly, a high level of P450 activity was also observed in the LC-R strain compared with that in the SS strain. These results indicated that the resistance to lambda-cyhalothrin in *R. padi* was partially due to enhanced P450 monooxygenase activity. Cytochrome P450 monooxygenases are involved in pyrethroid resistance and have been demonstrated in many insect species, such as *A. glycines*,[17] *Apis mellifera*,[42] *Meligethes aeneus*[43] and *Helicoverpa armigera*.[44] In addition to P450, carboxylesterase is another important detoxification enzyme involved in pyrethroid resistance. Zhang *et al*. found that carboxylesterase activities in the beta-cypermethrin strain were significantly higher than those in the *M. domestica* susceptible strain.[34] The specific activities of carboxylesterases indicated significant differences between the lambda-cyhalothrin-resistant and -susceptible strains of *A. glycines*.[11] In a previous study, we found that *R. padi* carboxylesterase had hydrolase activity against pyrethroid cyhalothrin.[12] In this study, carboxylesterase showed highly limited effects in the LC-R strain.

P450s are the main enzymes involved in conferring resistance to various types of insecticides, and P450-medaited resistance typically results from overexpression of one or multiple P450 genes.[45–47] Previous studies indicated that CYPs belonging to clade CYP3 (including CYP3s, CYP6s and CYP9s) and clade CYP4 are most frequently linked to xenobiotics including chemical insecticide detoxification in different insect species.[20] In the current study, five CYP6s and two CYP4s were significantly upregulated in the LC-R strain, of which *CYP6DC1* and *CYP4CJ5* were remarkably overexpressed. Increasing levels of one or multiple P450 genes appear to be a general molecular mechanism in chemical insecticide-resistant insects.[26,46] Overexpressed P450 *CYP6A2-like* and *CYP6A14-like* were involved in lambda-cyhalothrin resistance in *A. glycines*.[17] Zhen *et al*. found that six CYP6s were highly increased in a lambda-cyhalothrin resistance population of *Apolygus lucorum*.[48] Brun-Barale *et al*. showed that five CYPs (*CYP4L5*, *CYP4L11*, *CYP6AE11*, *CYP332A1* and *CYP9A14*) were significantly overexpressed in the *H. armigera* deltamethrin-resistant strains from Kaya and Seville.[21] Three CYP (*CYP9A12*, *CYP9A14* and *CYP6B7*) genes belonged to clade CYPs were overexpressed in the fenvalerate-resistant Chinese YGF strain of *H. armigera* with 1690-fold resistant level.[49]

In summary, a lambda-cyhalothrin-resistant *R. padi* field population was collected from a major wheat-growing region. The population carried the super-*kdr* mutation M918L in some individuals and evolved high-level resistance (158.76-fold) after laboratory selection with lambda-cyhalothrin. Our results strongly suggest that the super-*kdr* M918L mutation in VGSCs and constitutive expression of multiple P450s are expected to have an important role in *R. padi* lambda-cyhalothrin resistance. Further studies on resistance-related genes are warranted to confirm whether these multiple P450s could metabolize pyrethroids, which may be important for *R. padi* control programmes and resistance prediction worldwide.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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