Research Article

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Cross-resistance and possible mechanisms of chlorpyrifos resistance in *Laodelphax striatellus* (Fallén)

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Abstract

BACKGROUND: *Laodelphax striatellus* (Fallén) is a major pest of cultivated rice and is commonly controlled in China with the organophosphate insecticides. To develop a better resistance management strategy, a chlorpyrifos-resistant strain of *L. striatellus* was selected in the laboratory, and its cross-resistance to other insecticides and possible mechanisms of the chlorpyrifos resistance were investigated.

RESULTS: After 25 generations of selection with chlorpyrifos, the selected strain of *L. striatellus* developed 188-fold resistance to chlorpyrifos in comparison with the susceptible strain, and showed 14- and 1.6-fold cross-resistance to dichlorvos and thiamethoxam respectively. There was no apparent cross-resistance to abamectin. Chlorpyrifos was synergised by the inhibitor triphenyl phosphate; the carboxylesterase synergistic ratio was 3.8 for the selected strain, but only 0.92 for the susceptible strain. The carboxylesterase activity of the selected strain was approximately 4 times that of the susceptible strain, whereas there was no significant change in the activities of alkaline phosphatase, acid phosphatase, glutathione *S*-transferase and cytochrome P450 monooxygenase between the strains. The Michaelis constant of acetylcholinesterase, maximum velocity of acetylcholinesterase and median inhibitory concentration of chlorpyrifos-oxon on acetylcholinesterase were 1.7, 2.5 and 5 times higher respectively in the selected strain.

CONCLUSION: The high cross-resistance to the organophosphate dichlorvos in the chlorpyrifos-resistant strain suggests that other non-organophosphate insecticides would be necessary to counter resistance, should it arise in the field. Enhanced activities of carboxylesterase and the acetylcholinesterase insensitivity appear to be important mechanisms for chlorpyrifos resistance in *L. striatellus*.

Keywords: cross-resistance; chlorpyrifos; resistance; *Laodelphax striatellus*; carboxylesterase; acetylcholinesterase; Homoptera; P450 monooxygenase

1 INTRODUCTION

The small brown planthopper, *Laodelphax striatellus* (Fallén) (Homoptera: Delphacidae), has a wide distribution from south-east Asia to Siberia and Europe, mainly in the temperate zone.[1] As a pest of rice and wheat, it causes serious damage to rice by the transmission of stripe virus and black streaked dwarf virus. In China, *L. striatellus* is found in all rice-growing areas, and has been considered a serious problem since 1999. The density of the *L. striatellus* population has increased dramatically in recent years, especially in the middle and downstream Yangtze River, and it is believed that the resistance of *L. striatellus* to pesticides is one of the important factors behind this increase.[2]

Laodelphax striatellus populations have been discovered with resistance to organophosphate (OP), carbamate and neonicotinoid insecticide classes.[3–8] In Japan, resistance of *L. striatellus* to the OP malathion was recognised as early as 1965,[3] and since then the resistance to diazinon, fenobucarb and carbaryl has been reported.[4,5] Recently, failure in field control of *L. striatellus* with insecticides has been reported in some areas of China, due to apparent resistance to members of the carbamate, neonicotinoid and organophosphate insecticide classes.[5,7,8]

Chlorpyrifos is a broad-spectrum and contact-effective organophosphate insecticide that has been used against pests such as leaf folder, planthoppers, aphides, bollworm and cabbage butterfly in China. Along with fipronil and nitenpyram, chlorpyrifos is one of the few insecticides that provide effective control of *L. striatellus* in China.[8,9] Since October 2009 in China, the use of fipronil in rice paddies has been discontinued, and therefore it is

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likely that chlorpyrifos will be used more frequently and extensively. Low to moderate levels of resistance to chlorpyrifos have already been identified in some field populations.[7,8] However, so far the mechanisms of chlorpyrifos resistance in *L. striatellus* remain unclear. In this study, the role of metabolic enzymes and insensitive acetylcholinesterase (AChE) in *L. striatellus* resistance was evaluated.

2 MATERIALS AND METHODS

2.1 Insects

The susceptible YN strain of *L. striatellus* was collected from Yunnan, China, in July 2001 and has been reared without contact with any insecticides. The YN-CPF strain was derived from the YN strain by continuous selection with chlorpyrifos for 25 generations in the laboratory. In each generation, about 2000 third-instar nymphs were bioassayed by the rice seedling dip method. Average mortality was 68%. The insects were reared on rice seedlings at $27 \pm 1\ ^{\circ}C$ under a 14 : 10 h light : dark regime.

2.2 Insecticides and chemicals

The insecticides and chemicals used in this study are listed as follows. Chlorpyrifos 480 g L^{-1} emulsifiable concentrate (EC) was obtained from Dow AgroSciences, dichlorvos 800 g L^{-1} EC came from Hubei Xianlong Chemical Company (Hubei, China), abamectin 20 g L^{-1} EC came from Heartale Agrochemical Co. Ltd (Hunan, China) and technical thiamethoxam (94.5%) was obtained from Jiangsu Kesheng Group Corporation (Jianhu, China). Triphenyl phosphate (TPP) and piperonyl butoxide (PBO) were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). Diethyl maleate (DEM) was purchased from Acros Organic. TPP, DEM and PBO were formulated separately with 100 mL L^{-1} Triton X-100 as the emulsifier and acetone as the solvent, while *N*,*N*-dimethylformamide was used as the solvent for thiamethoxam. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), acetylthiocholine iodide (ATChI), glutathione reductase, oxidised glutathione and 7-ethoxycoumarin were purchased from Sigma. β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) was purchased from Roche Diagnostics (Indianapolis, IN). α-Naphthyl acetate (α-NA) was purchased from Sinopharm Chemical Reagent Company (Shanghai, China). Chlorpyrifos-oxon was purchased from laboratories of Dr Ehrenstorfer. Fast Blue RR salt, bovine serum albumin, Triton X-100, eserine and the reduced glutathione (GSH) were all from Shanghai Chemical Reagent Co., Ltd, China. Coomassie Brilliant Blue G-250 was purchased from Fluka, and 4-nitrophenyl phosphate disodium salt (PNPP) from Shanghai Lanji Technology Company (Shanghai, China).

2.3 Bioassays

Bioassays using the rice seedling dip method were conducted as previously described.[8] The formulated insecticides were diluted to generate six serial dilutions with distilled water. Three rice seedlings as a group were immersed individually into each of these insecticide dilutions for 10 s. After natural air drying, the seedlings were placed into disposable plastic cups with moistened paper to maintain wetness of the rice seedling roots. Ten third-instar nymphs were placed into each treated plastic cup. Rice seedlings dipped in distilled water were used as control. For each dilution, 30 insects were treated. All tests were maintained at $27 \pm 1\ ^{\circ}C$, and mortality was recorded after 48 h for chlorpyrifos and dichlorvos, 72 h for thiamethoxam and 120 h for abamectin. For analysis of the synergistic effect of the enzyme inhibitors on chlorpyrifos, 10 mg L^{-1} TPP, DEM or PBO was added in each dilution. Bioassay data were analysed by POLOPLUS® software.

2.4 Enzyme assays

2.4.1 Enzyme preparation

Laodelphax striatellus nymphs (third instar) were homogenised in ice-cold buffer and centrifuged at 4 °C and 12 000 rpm for 20 min, and the supernatant was then used as the enzyme source. The buffers used were 0.1 M sodium phosphate buffer (pH 7.5) for carboxylesterase, 0.05 M Na_2CO_3–$NaHCO_3$ buffer (pH 10.0) for alkaline phosphatase (ALP), 0.2 M HAc–NaAc buffer (pH 4.6) for acid phosphatase (AP), 0.02 M sodium phosphate buffer [pH 7.2, containing 10 mL L^{-1} Triton X-100 and 1 mM ethylenediaminetetraacetic acid (EDTA)] for AChE and 0.05 M Tris–HCl buffer (pH 7.5) for glutathione *S*-transferase (GST). Thirty nymphs were homogenised for AP and ALP, and 20 nymphs for carboxylesterase, AChE and GST. For AChE assay, the homogenate was filtered through cotton wool pledget before centrifugation.

For cytochrome P450 monooxygenase (P450) activity assays, 50 third-instar nymphs were homogenised in 1 mL of 0.1 M phosphate buffer [pH 7.8, containing 1 mM EDTA, 1 mM α-phenyl-2-thiourea, 1 mM phenylmethanesulfonyl fluoride and 0.1 mM dithiothreitol (DTT)]. The homogenates were centrifuged at 4 °C and 5000 × *g* for 5 min, the resulting supernatant was filtered through cotton wool, the filtrate was centrifuged at 10 000 × *g* for 20 min and the filtration step was repeated. After 100 000 × *g* centrifugation for 60 min, the microsomal pellet was collected and resuspended in 250 µL of 0.1 M phosphate buffer (pH 7.8, containing 1 mM EDTA, 1 mM α-phenyl-2-thiourea and 0.1 mM DTT) and then used as the enzyme source.

2.4.2 Carboxylesterase assay

Carboxylesterase assays were performed according to Stumpf and Nauen[10] with minor modification. The stock enzyme source was first diluted tenfold, and then 80 µL was added into each well of a 96-well microtitre plate. The assay was started by adding 120 µL of substrate solution into each well. The substrate solution consisted of 2 mM Fast Blue RR salt, 10 mM α-NA and 1 µM eserine in 0.2 M sodium phosphate buffer (pH 6.0). Changes in absorbance values were measured continuously for 10 min at 450 nm and 27 °C using a microplate reader (Elx808; BioTek). The non-enzymatic reactions of substrate solution were measured as controls.

2.4.3 AP and ALP assay

AP and ALP activities were tested using PNPP as substrate.[11] The total reaction volume per well was 200 µL, consisting of 100 µL enzyme source and PNPP (giving a final concentration of 1 mM) in 0.05 M Na_2CO_3–$NaHCO_3$ buffer (pH 10.0) for ALP or 0.2 M HAc–NaAc buffer (pH 4.6) for AP. Changes in absorbance values were measured continuously for 15 min at 405 nm and 30 °C. The hydrolysis of PNPP in the absence of enzyme was measured as a control.

2.4.4 GST assay

GST activity was determined using CDNB and GSH as substrates. GST assays were performed according to Rauch and Nauen[12] with slight modifications. The total reaction volume per well was 300 µL, consisting of 100 µL enzyme source plus 200 µL of

Table 1. The level of resistance to chlorpyrifos and cross-resistance to other insecticides in the YN-CPF strains of *Laodelphax striatellus*

	YN		YN-CPF			
Insecticide	Slope (± SE)	LC_{50} (mg L^{-1}) (95% CL)	Slope (± SE)	LC_{50} (mg L^{-1}) (95% CL)	RR[a]	CR[b]
Chlorpyrifos	2.04 (±0.28)	8.09 (6.24–10.89)	2.24 (±0.30)	1525 (1199–2011)	188.55	
Dichlorvos	1.99 (±0.30)	11.31 (7.74–14.99)	4.93 (±0.75)	159.63 (136.1–187.1)		14.11
Thiamethoxam	2.31 (±0.28)	4.57 (3.53–5.83)	3.94 (±0.73)	7.30 (6.08–9.25)		1.60
Abamectin	1.82 (±0.29)	0.30 (0.22–0.40)	2.04 (±0.31)	0.24 (0.18–0.31)		0.80

[a] RR (resistance ratio) = LC_{50} of YN-CPF strain/LC_{50} of YN strain.
[b] CR (cross-resistance ratio) = LC_{50} of YN-CPF strain/LC_{50} of YN strain.

Table 2. Synergistic effect of TPP, PBO and DEM on the toxicity of chlorpyrifos to the YN and YN-CPF strains of *Laodelphax striatellus*

Strain	Chlorpyrifos/synergist	Slope (± SE)	LC_{50} (mg L^{-1}) (95% CL)	SR[a]
YN	Chlorpyrifos	2.04 (±0.28)	8.09 (6.24–10.89)	
	Chlorpyrifos + TPP	1.90 (±0.29)	8.82 (6.71–12.16)	0.92
	Chlorpyrifos + PBO	2.34 (±0.29)	9.41 (7.48–11.90)	0.86
	Chlorpyrifos + DEM	2.52 (±0.35)	7.87 (6.16–9.84)	1.03
YN-CPF	Chlorpyrifos	2.24 (±0.30)	1525 (1199–2011)	
	Chlorpyrifos + TPP	1.60 (±0.23)	393.8 (174.2–802.8)	3.87
	Chlorpyrifos + PBO	1.77 (±0.32)	413.6 (295.7–644.7)	3.69
	Chlorpyrifos + DEM	2.76 (±0.34)	547.6 (444.6–677.8)	2.79

[a] SR (synergism ratio) = LC_{50} of chlorpyrifos/LC_{50} of chlorpyrifos + synergist.

CDNB and GSH in 0.1 M sodium phosphate buffer (pH 6.5) (giving final concentrations of 0.4 mM CDNB and 4 mM GSH). Changes in absorbance values were measured continuously for 10 min at 340 nm and 30 °C. The hydrolysis of CDNB and GSH in the absence of enzyme was measured as a control.

2.4.5 *P450 assay*

Cytochrome P450 monooxygenase *O*-deethylase activity towards the substrate 7-ethoxycoumarin was measured using the methods of Rauch and Nauen.[13] Quantities of 70 µL of the microsomal fraction and 40 µL of 7-ethoxycoumarin (2 mM in 0.1 M, pH 7.8 phosphate buffer) were added to each well of a 96-well microplate. The reaction was started by adding 10 µL of aqueous NADPH (9.6 mM in 0.1 M, pH 7.8 phosphate buffer) to the well. The plate was incubated while shaking for 30 min at 30 °C. The background fluorescence was removed by the addition of 10 µL of 30 mM oxidised glutathione and glutathione reductase (10 µL, 0.5 U). After 10 min at 25 °C, the reaction was stopped with 140 µL of 50% acetonitrile in Tris-base buffer (0.05 M, pH 10.0). The volume of 7-hydroxycoumarin produced during incubation was quantified with a spectrofluorometer (Spectramax Geminixs; Molecular Devices) at an emission wavelength of 465 nm and an excitation wavelength of 390 nm. The assay was replicated at least 3 times with each strain of *L. striatellus*. Wells without any microsomal pellet served as controls.

2.4.6 *Kinetics and sensitivity of AChE*

The analysis of AChE kinetics was performed as previously reported.[14] The enzyme source was diluted fivefold before use. To determine the K_m and V_{max} values, 100 µL of DTNB (1 mM) and 100 µL of ATChI solution (0.023–6.00 mM) were mixed, and the reaction was initiated by the addition of 100 µL of enzyme solution. The reaction was monitored at 405 nm and 25 °C for 20 min.

For AChE sensitivity analysis, chlorpyrifos-oxon was first dissolved in acetone as stock solution and diluted to a series of concentrations (0.0015–0.094 mM for the YN strain and 0.0029–0.19 mM for the YN-CPF strain) with sodium phosphate buffer (0.02 M, pH 7.2, containing 1 mL L^{-1} Triton X-100). AChE was first inhibited by mixing 97 µL of enzyme solution with 3 µL of the above chlorpyrifos-oxon solution at 25 °C for 25 min. The residual enzyme activity was tested by the addition of 100 µL of DTNB (1 mM) and 100 µL of ATChI (4.5 mM) as above. The final concentration of acetone in the reaction mixture was always below 1%. The median inhibitory concentration (IC_{50}) of chlorpyrifos-oxon on AChE was calculated by DPS software.

2.5 Protein assay

The total protein content of the enzyme solution was determined by the Bradford[15] method using bovine serum albumin as the standard.

3 RESULTS

3.1 Resistance level and cross-resistance

The toxicity of several insecticides against the YN and YN-CPF strains of *L. striatellus* is shown on Table 1. Compared with the YN strain, the YN-CPF strain showed high resistance to chlorpyrifos (188-fold), significant cross-resistance to dichlorvos (14-fold) and minor resistance to thiamethoxam (1.6-fold). No cross-resistance to abamectin was seen.

3.2 Synergistic effect evaluated

There was no significant synergistic effect of TPP, PBO and DEM on chlorpyrifos in the YN strain (Table 2). However, significant synergism was seen for all three synergists in the YN-CPF strain,

Table 3. Metabolic enzyme activities in the YN and YN-CPF strains of *Laodelphax striatellus*

	Enzyme activity[a]		
Substrate	YN strain	YN-CPF strain	YN-CPF/YN
COE/α-NA	42.13 (±3.72) a	164.68 (±35.12) b	3.91
ALP/PNPP	0.19 (±0.076) a	0.16 (±0.029) a	0.84
AP/PNPP	0.15 (±0.071) a	0.14 (±0.044) a	0.93
P450/ECOD	195.96 (±32.99) a	136.19 (±18.79) a	0.70
GST/CDNB	2.36 (±0.24) a	2.10 (±0.038) a	0.89

[a] Enzyme activity is shown as mean (± SD) (mOD min^{-1} μg^{-1} protein, P450 is mRfu 30 min^{-1} μg^{-1} protein). Means in a row followed by different letters are significantly different ($P < 0.05$, Student's *t*-test); the same applies to the following tables.

Table 4. Kinetic parameters of AChE in the YN and YN-CPF strains of *Laodelphax striatellus*

Kinetic parameter	YN	YN-CPF	YN-CPF/YN
K_m (mM)	0.083 (±0.0065) a	0.14 (±0.0082) b	1.69
V_{max} (mOD min^{-1} μg^{-1} protein)	1.41 (±0.48) a	3.55 (±0.87) b	2.52

Table 5. IC_{50} of chlorpyrifos-oxon on AChE in the YN and YN-CPF strains of *Laodelphax striatellus*

	YN strain	YN-CPF strain	YN-CPF/YN
IC_{50} (μM)	0.038 (±0.018) a	0.19 (±0.045) b	5.00

with the LC_{50} of chlorpyrifos decreasing from 1525 to 393, 413 and 547 mg L^{-1} respectively. The results suggested that metabolic enzymes were involved in the chlorpyrifos resistance in the YN-CPF strain of *L. striatellus*.

3.3 Metabolic enzymes activities

In order to provide further evidence for the enhanced metabolic activities in chlorpyrifos resistance, carboxylesterase, AP, ALP, P450 and GST activities were measured in both the YN and YN-CPF strains (Table 3). Compared with that in the YN strain, the carboxylesterase activity in the YN-CPF strain was elevated approximately fourfold, but for other metabolic enzymes (P450, GST, AP and ALP) there was no significant difference between the YN and YN-CPF strains.

3.4 Kinetics and sensitivity of AChE

The kinetics of AChE from the YN and YN-CPF strains was tested to determine the relationship between AChE sensitivity and resistance (Table 4). Compared with the YN strain, the turnover of AChE in the YN-CPF strain was increased 2.5-fold, but the affinity of AChE for the substrate was decreased (K_m increased 1.7-fold). The IC_{50} of chlorpyrifos-oxon on AChE was fivefold higher in the YN-CPF strain (Table 5), suggesting that the AChE enzymes in the resistant strain were significantly more tolerant to the OP insecticide.

4 DISCUSSION

In this study, the esterase inhibitor TPP showed a significant synergistic effect on chlorpyrifos in the resistant strain of *L. striatellus*, and biochemical assays showed an increase in carboxylesterase activity. Together, this suggests that one or more carboxylesterases are upregulated in the resistant strain and may contribute to resistance by hydrolysis or sequestration of chlorpyrifos, as shown for OPs in other insect species. Other esterase AP and ALP activities were similar in the YN and YN-CPF strains, which suggested that the two enzymes were unimportant to chlorpyrifos resistance in the YN-CPF strain of *L. striatellus*.

It is difficult to conclude the actual role of P450 and GST on chlorpyrifos resistance in *L. striatellus* from this study. Although the inhibitors of these enzymes showed significant synergism on chlorpyrifos toxicity in the YN-CPF strain, the enzyme activities to synthetic substrates were similar between the resistant and susceptible strains. Chlorpyrifos can be bioactivated by P450 through a desulfuration reaction to form chlorpyrifos-oxon, and also be detoxified by P450 through a dearylation reaction.[16] Conceivably, P450-mediated resistance could be due to an increase in the activity or proportion of the latter, without affecting the overall P450 activity towards synthetic substrates. Similarly, the significant synergistic effect of DEM on chlorpyrifos but the same level of GST activity in the resistant strain, compared with the susceptible strain, suggest that GST-mediated resistance is not simply the result of upregulation or mutations of the genes encoding but also of cooperation among the GST superfamily members.[17] As AChE is the target of organophosphate and carbamate insecticides, AChE can dramatically enhance the resistance level even in the presence of other resistance mechanisms.[18] The fact that chlorpyrifos resistance could not completely be overcome by the synergists inhibiting metabolic enzymes in the YN-CPF strain suggests that target-site resistance may also play a role in the resistance of *L. striatellus* to chlorpyrifos.

Both increased AChE activity and insensitive AChE alteration can contribute to organophosphate resistance in insects.[19,20] The similar resistance mechanisms of target-site resistance are likely to be involved in the resistance of *L. striatellus* to chlorpyrifos. The K_m and IC_{50} values are significantly higher in the YN-CPF strain, indicating that AChE insensitivity is probably involved in the resistance of *L. striatellus* to chlorpyrifos. In addition, the V_{max} of the AChE is also higher in the YN-CPF strain, suggesting that increased activity and/or overexpression of AChE could also contribute to the observed resistance. Alternatively, the carboxylesterases upregulated in the resistant strain may have had the ability to hydrolyse AChE substrates to some extent, and this would also explain the apparent decrease in affinity (i.e. high K_m) that was measured. Further studies are needed to confirm the increased amount or structural modification of AChE in the YN-CPF strain.

This work also confirmed that the resistant *L. striatellus* to chlorpyrifos had little or no cross-resistance to other classes of insecticides such as thiamethoxam and abamectin, but significant cross-resistance to other organophosphates such as dichlorvos, which probably resulted from distinct resistance mechanisms for those insecticides. As carboxylesterases are thought to play an important role in insects resistant to dichlorvos,[21] the cross-resistance of *L. striatellus* to the insecticide could result from the upregulation of these enzymes. Alternatively, as both chlorpyrifos and dichlorvos have the same target enzyme (AChE), cross-resistance of *L. striatellus* to dichlorvos could also be due to target-site resistance.

In this study, the YN-CPF strain showed 188-fold higher resistance to chlorpyrifos after 25 generations of selection compared with the non-selected YN strain, while the non-selected YN strain had unchanged susceptibility to chlorpyrifos after 25 generations (LC_{50}: 8.09 or 6.24–10.89 mg L^{-1}) compared with that before the 25 generations (LC_{50}: 7.84 or 5.86–11.06 mg L^{-1}),[8] suggesting that no other unexpected changes occurred during 25 generations of selection except treatment with chlorpyrifos. It is reasonable to assume that the resistance of the YN-CPF strain results from the selective stress of chlorpyrifos. Although a chance event of selecting a low-level mutation from a largely susceptible background may frequently result in alternative outcomes if selections are performed in duplicate, there are approximately 600 survivable individuals in every generation following chlorpyrifos selection, which is large enough to represent a chlorpyrifos-resistant strain in this study. In addition, the appropriate level of statistical power during post-selection analytical work is the point at which repetition becomes critical. All data in this study represent mean values of three independent experiments performed in duplicate, so the authors believe that the laboratory-based selections do not necessarily replicate field-based selections, and these results have important implications for using chlorpyrifos to control *L. striatellus*. Although chlorpyrifos is effective in controlling *L. striatellus* at present, the risk of resistance formation to chlorpyrifos is high.[8] The present results suggest that, to slow the development of chlorpyrifos resistance in *L. striatellus*, rice-growers could rotate chlorpyrifos with insecticides of other classes such as thiamethoxam and abamectin.

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