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No cross-resistance between imidacloprid and pymetrozine in the brown planthopper: status and mechanisms

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ABSTRACT

Cross-resistance between insecticides, especially from different groups, can be extremely unpredictable, and it has been a serious concern in pest control. Pymetrozine has been widely used to control *Nilaparvata lugens* with the suspension of imidacloprid for the resistance, and *N. lugens* has showed obvious pymetrozine resistance in recent years. To investigate the possible cross-resistance between imidacloprid and pymetrozine is very important to avoid the adverse effects on resistance development and pest control. Bioassays of two field populations in five consecutive years showed that imidacloprid resistance decreased greatly, while pymetrozine resistance increased significantly. The synergist piperonyl butoxide (PBO) could synergize both imidacloprid and pymetrozine in all field populations, which indicated the importance of P450s in the resistance to two insecticides. Imidacloprid resistance was reported to be associated with two P450s, CYP6AY1 and CYP6ER1, which could metabolize imidacloprid efficiently. However, the recombinant proteins of these two P450s did not show any enzymatic activity to metabolize pymetrozine. The pymetrozine susceptibility did not change when CYP6AY1 and CYP6ER1 mRNA levels were reduced by RNA interference (RNAi), although which could obviously decrease imidacloprid resistance. *In vivo* and *in vitro* studies provided evidences to demonstrate that there was no cross-resistance between imidacloprid and pymetrozine in *N. lugens*, which was different from the findings in *Bemisia tabaci*.

1. Introduction

The brown planthopper, *Nilaparvata lugens*, is one of the most important pests on rice in through Asian countries and areas. It has caused enormous economic losses and developed high resistances to all kinds of chemical insecticides, including organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids [1]. As the first neonicotinoid insecticide, imidacloprid was introduced to China in the early 1990s and soon became the most important insecticide to control *N. lugens* because of its efficacy and long-lasting effect [2]. Due to extensive use of imidacloprid in rice paddy fields, an increasing resistance level in *N. lugens* is inevitable. It caused the suspension of imidacloprid in the control of *N. lugens* in China since 2006 [3–5]. In recent years, pymetrozine, an azomethine pyridine insecticide, shows extreme effects against sucking pest insects, and has been commonly used for *N. lugens*' control [6,7]. It is reported that some field populations of *N. lugens* in China still remained susceptible to pymetrozine in 2010 [7], but the resistance increased to medium or high levels in 2011 and 2012 [8]. These reports suggested that the increasing resistance of *N. lugens* to pymetrozine was relevant with the wide use of this insecticide to control rice planthoppers. However, other possible explanations still could not be excluded, such as cross-resistance between different insecticides.

It has been reported that there was obvious cross-resistance between imidacloprid and pymetrozine in some insects. In *Bemisia tabaci*, pymetrozine could be hydroxylated by CYP6CM1, a P450 monooxygenase, which conferred imidacloprid resistance in this pest [9]. Reciprocal selection experiments confirmed the cross-resistance between pymetrozine and thiamethoxam in *B. tabaci* [9], and cross-resistance relationships between most neonicotinoids were well established for *B. tabaci* [10]. The similar circumstance was also described in the greenhouse whitefly, *Trialeurodes vaporariorum* [11]. Cross-resistance between neonicotinoids and pymetrozine is most likely conferred by the same detoxification mechanism, such as the overexpression of cytochrome-P450-dependent monooxygenases capable of metabolizing both insecticides in spite of their apparent structural dissimilarity [12–14]. Two P450 genes, CYP6AY1 and CYP6ER1, have been reported to confer imidacloprid resistance in *N. lugens* [15,16]. Here, the resistances in two field populations in five consecutive years were monitored, and a series of researches were performed to explore whether the cross-resistance between imidacloprid and pymetrozine existed in *N. lugens*. Clarifying the cross-resistance between imidacloprid and pymetrozine could provide essential information to underpin product choice within resistance management strategies.

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2. Materials and methods

2.1. Insect, insecticides and reagents

The susceptible strain (Sus) of *N. lugens* was a laboratory strain originally collected from China National Rice Research Institute in September 2001. The resistant strain (Res) was originally collected from paddy field in Anqing (Anhui, China) in September 2005 and successively selected by imidacloprid in laboratory. Two field populations were collected from Anqing and Huzhou (Zhejiang, China) in September from 2010 to 2014. Insects were kept in a constant temperature incubator with rice plants at 25 ± 1 °C, 70–80% relative humidity and 16-h light/8-h dark photoperiod.

Imidacloprid and pymetrozine were purchased from Red Sun Corporation (Nanjing, China). N, N-Dimethylformamide (DMF, reagent grade), piperonyl butoxide (PBO, reagent grade) and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Bioassays and synergistic experiment

The bioassay was performed according to the rice seedling dip method [17]. Insecticides were dissolved in DMF, and then diluted in distilled water containing 0.05% Triton X-100 to generate a series of concentrations. Four rice seedlings were immersed in a concentration of insecticide for 15 s. After air-drying, the seedlings were placed in plastic cups with 1.5% agar to maintain moist conditions. Thirty insects (3rd instar nymph) were treated at each concentration, and every treatment was repeated at least three times. Rice seedlings dipped in the distilled water containing 0.05% Triton X-100 were served as the control. In the synergism analysis, 2 μg PBO in 0.08 μL acetone was topically delivered on to the prothorax notum of the test insects 1 h before the insecticide application. All treated insects were maintained on rice plants in the constant temperature incubator as mentioned above. The mortalities were recorded after 48 h for imidacloprid and 120 h for pymetrozine. The bioassays data were analyzed using Data Processing System (DPS) software [18].

2.3. RNA interference

The target sequences of CYP6AY1 (GenBank accession number: AJ852423.2) and CYP6ER1 (GenBank accession number: FJ928994.1) were amplified by RT-PCR using specific primers conjugated with 23 bases of the T7 RNA polymerase promoter (Table 1). The PCR products were used as templates for dsRNA synthesis using the T7 Ribomax Express RNAi Systme (Promega, Madison, WI, USA). After synthesis, the dsRNA was isopropanol precipitated, resuspended in ultra-pure water, quantified spectrophotometrically at 260 nm and its purity and integrity were determined by agarose gel electrophpresis. It was kept at −80 °C until use. As a control, bacterial Lac-Z (GenBank accession number: AJ308295) dsRNA was also produced as described above. *N. lugens* were immobilized on ice and 50 nL of purified dsRNA (5 μg/μL) was injected at slow speed at the conjunctive between prothorax and mesothorax [19]. The bioassay and mRNA levels of CYP6AY1 and CYP6ER1 were determined in 96 h after injection.

Table 1
Primers used in this study.

Gene	Primer(i, q)[a]	Sequence(5′-3′)
CYP6AY1	F (i)	CCAATCACCGCACACCTGGTCAACC
	R (i)	GCTTGAGCTGCTATAACACTCTCTG
	F (q)	GCTGTTTCACTTCTTGAGACTCCG
	R (q)	GCTTGAGCTGCTATAACACTCTCTG
CYP6ER1	F (i)	GTCAACTTCTACGTTTACTCCTATTG
	R (i)	ATCACATTCAGCCCGTAGTTGTTTG
	F (q)	TGGCTGTTAATCAAGAGATGCAGC
	R (q)	CTGAAGCGCATAGACCGGAATC
Lac-Z	F (i)	GGAAGATCAGGATATGTGG
	R (i)	CTTCATCAGCAGGATATCC
β-actin	F (q)	CTGGACTTCGAGCAGGAAATGGC
	R (q)	CGACGTCGCACTTCATGATCGAG
GAPDH	F (q)	GTGATGTGAAAGCCGAAGGAAACT
	R (q)	GCGTTGGAGATGACCTTGTAAGAGG

[a] i: primers used for RNA interference; q: primers used for qPCR.

2.4. Quantitative real-time reverse transcriptase polymerase chain reaction

N. lugens were collected and RNA was extracted using TRIzol reagent (Invirogen, USA). Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed in a 25-μL total reaction volume containing 5 ng of total RNA, a 0.5-μL primer mix containing 10 μM each of forward and reverse gene specific primer, 0.5 μL of Ex TaqTM HS (5 U/μL), 0.5 μL of PrimeScript RT Enzyme Mix, 12.5 μL of 2 × One Step SYBR RT-PCR Buffer and 8.5 μL of H_2O. The PCR was done with the following cycling regime initial incubation of 42 °C for 5 min and 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 60 °C for 20 s and 72 °C for 15 s. Each qRT-PCR experiment was performed in three independent biological replicates and analyzed in three technical replications. The relative expression of each P450 gene was calculated according to the $2^{-\Delta\Delta CT}$ method [20]. For normalization, two reference genes (β-actin and GAPDH) were validated experimentally for each generation and treatment, with the geometric mean of the selected genes then used for normalization according to the strategy described previously [21]. The specific primers pair for each gene was provided in Table 1.

2.5. Functional expression of P450s in Escherichia coli

The cDNA sequences encoding CYP6AY1 and CYP6ER1 were subcloned into the expression vector pCWori at *NdeI* and *HindIII* sites. Then the treated and purified PCR product was subcloned into vector pCWori. The plasmid was verified by nucleotide sequencing.

The pCWori vector plasmid containing CYP6AY1 or CYP6ER1 was transformed into DH5α (an *E.coli* competent cell) in the presence of ampicillin. The cells were grown in TB culture with some modification (1 mM thiamin, 100 mM potassium phosphate, 1 mM δ-aminolevulinic acid, pH 7.4) at 37 °C for 12 h at 250 rpm. Then 0.5 mL cultured medium was added into 50 mL fresh modified TB medium, and the cells were grown at 250 rpm and 37 °C till the OD_{600} value reached 0.55. Isopropyl β-D-thiogalactoside (IPTG, with final concentration of 0.4 mM) was added into cultured medium, and the cells were grown at 170 rpm and 28 °C for 48 h. Then the cells were collected by centrifugation at 2800 ×*g* and 4 °C for 5 min. The resulting pellets were resuspended in 0.1 M Tris–HCl lysis buffer (pH 7.4), containing 10 mM 3-[(3-cholamidopropyl) dime-thylammonio]-1-propanesulf-sonate (CHAPS), 20% glycerol, and 1 mM EDTA. CO-difference spectroscopy was used to determine the expression levels [22].

The cell pellets expressing CYP6AY1 and CYP6ER1 were resuspended in 50 mM Tris-acetate buffer (pH 7.6) containing 250 mM sucrose and 0.25 mM EDTA. Freshly prepared lysozyme solution was added at the final concentration of 0.25 mg/mL, and the mixture was then gently shaken at 4 °C for 45 min. After centrifugation at 2800 ×*g* for 15 min at 4 °C, the resulting spheroplasts were suspended in 0.1 M sodium phosphate buffer (pH 7.4), containing 20% glycerol, 1 mM PMSF, 0.1 g/mL leupeptin, and 0.04 units/mL aprotinin, and disrupted by sonication at 4 °C. After centrifugation at 4000 ×*g* for 10 min at 4 °C, the supernatant was centrifuged at 100,000 ×*g* for 1 h. The resulting membrane pellet was suspended in 0.1 M sodium phosphate buffer (pH 7.4) containing 20% glycerol and used for enzymatic characterization.

2.6. Enzyme activity determination and HPLC analysis of insecticides metabolism

The activity of P450 was assayed at 30 °C in a 0.1 M Tris–HCl buffer (pH 7.4) containing 0.25 mM $MgCl_2$. The reaction system contained