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Expression induction of P450 genes by imidacloprid in *Nilaparvata lugens*: A genome-scale analysis

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A B S T R A C T

The overexpression of P450 monooxygenase genes is a main mechanism for the resistance to imidacloprid, a representative neonicotinoid insecticide, in *Nilaparvata lugens* (brown planthopper, BPH). However, only two P450 genes (CYP6AY1 and CYP6ER1), among fifty-four P450 genes identified from BPH genome database, have been reported to play important roles in imidacloprid resistance until now. In this study, after the confirmation of important roles of P450s in imidacloprid resistance by the synergism analysis, the expression induction by imidacloprid was determined for all P450 genes. In the susceptible (Sus) strain, eight P450 genes in Clade4, eight in Clade3 and two in Clade2 were up-regulated by imidacloprid, among which three genes (CYP6CS1, CYP6CW1 and CYP6ER1, all in Clade3) were increased to above 4.0-fold and eight genes to above 2.0-fold. In contrast, no P450 genes were induced in Mito clade. Eight genes induced to above 2.0-fold were selected to determine their expression and induced levels in Huzhou population, in which piperonyl butoxide showed the biggest effects on imidacloprid toxicity among eight field populations. The expression levels of seven P450 genes were higher in Huzhou population than that in Sus strain, with the biggest differences for CYP6CS1 (9.8-fold), CYP6ER1 (7.7-fold) and CYP6AY1 (5.1-fold). The induction levels for all tested genes were bigger in Sus strain than that in Huzhou population except CYP425B1. Screening the induction of P450 genes by imidacloprid in the genome-scale will provide an overall view on the possible metabolic factors in the resistance to neonicotinoid insecticides. The further work, such as the functional study of recombinant proteins, will be performed to validate the roles of these P450s in imidacloprid resistance.

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1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is a notorious pest of rice crops throughout Asia. Through direct sucking, oviposition and virus disease transmission, BPH causes a dramatic reduction in rice yield and great economic losses [1–3]. Imidacloprid is a representative of neonicotinoid insecticides acting on insect nicotinic acetylcholine receptors (nAChRs) [4]. Because of the efficacy, long-lasting effect and harmless to mammals, imidacloprid has been used extensively in BPH control [5]. However, imidacloprid resistance has been developed in BPH field populations from several countries in Asia [1,3,6]. Thus, it is significant to reveal the underlying resistance mechanisms.

Insecticide resistance may evolve along several mechanisms, including enhancement of drug excretion, up-regulated enzymes of metabolic detoxification, and target insensitivity [7]. Although target-site resistance to imidacloprid has been reported in a laboratory-selected resistant strain of BPH, this mechanism is not prevalent in field populations [7,8]. In contrast, cytochrome P450 monooxygenases (P450s) were found to play important roles in imidacloprid resistance in both selected strains and field populations [6]. P450s are a multigenic superfamily of haem-thiolate enzymes with lots of functions, including nutrition and xenobiotic detoxification through metabolizing a wide range of endogenous and exogenous compounds [9–11]. In insects, the over-expression of some P450 genes has been proved to be associated with the increased metabolism of neurotoxic insecticides in resistant populations, including *Drosophila melanogaster* (CYP6G1), *Musca domestica* (CYP6D1), *Bemisia tabaci* (CYP6CM1), *Culex pipiens pallens* (CYP6F1), *Culex quinquefasciatus* (CYP9M10), and *N. lugens* (CYP6ER1, CYP6AY1) [12–18]. Therefore, the over-expression of P450s is a common mechanism for neonicotinoid insecticide resistance.

However, the studies of metabolic resistance to imidacloprid are always restricted to a few special kinds of up-regulated P450s because of the limit of P450 gene information, which always do not give a full view on metabolic resistance to insecticides [17–19]. Fortunately, the completion of BPH genome sequencing gave an opportunity to overall elucidate the metabolic factors in imidacloprid resistance [20]. In this study, the expression induction by imidacloprid was determined for all P450 genes in BPH, which may identify all putative P450 genes in response to imidacloprid stress.

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Table 1
Imidacloprid toxicities in the susceptible strain and eight field populations.

Population	LD_{50} (ng/pest)	Slope	Resistance ratio
Sus	0.112 (0.098–0.130) a	3.142 ± 0.285	1.00
Nanning	2.639 (2.281–2.641) c	2.303 ± 0.318	23.56
Guilin	2.174 (1.815–2.230) b	2.401 ± 0.444	19.41
Hangzhou	4.306 (3.540–5.393) d	1.874 ± 0.329	38.45
Huzhou	8.045 (7.125–10.240) f	1.720 ± 0.533	71.83
Wuxi	9.213 (7.618–11.567) f	1.596 ± 0.501	82.26
Nanjing	6.735 (5.158–7.333) e	2.142 ± 0.366	60.13
Anqing	6.281 (5.106–7.266) e	1.803 ± 0.479	56.08
Jiujiang	4.654 (3.722–5.324) d	1.754 ± 0.560	41.55

Different letters in LD_{50} column showed the significant differences at 0.05 level.

2. Materials and methods

2.1. Insecticides and chemicals

Imidacloprid (97%) was provided by Prof. Li Zhong from East China University of Science and Technology (Shanghai, China). Acetone (reagent grade), piperonyl butoxide (PBO, reagent grade), triphenyl phosphate (TPP, reagent grade), and diethyl maleate (DEM, reagent grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Insect

The susceptible strain (Sus) of BPH was obtained from China National Rice Research Institute (Zhejiang, China) in September 2001 and had been reared as a laboratory strain without any contact with insecticides. Eight field populations were collected from paddy fields in Nanning (Guangxi, China), Guilin (Guangxi, China), Hangzhou (Zhejiang, China), Huzhou (Zhejiang, China), Wuxi (Jiangsu, China), Nanjing (Jiangsu, China), Anqing (Anhui, China), and Jiujiang (Jiangxi, China) in September 2014. Insects were reared on rice plants at the tillering stage at 27 ± 1 °C, humidity 70%–80% and 16 h light/8 h dark photoperiod in laboratory cages.

2.3. Toxicity bioassay and expression induction

The bioassay and synergism experiment were performed by topical application method [21]. The 4th instar nymphs were used as test insects. Imidacloprid were diluted to six concentrations with acetone. After anesthetized by carbon dioxide, a droplet (0.04 μL) of imidacloprid solution was applied topically to the prothorax notum of BPH with a hand microapplicator (Burkard Manufacturing Co. Ltd., Rickmansworth, UK). Insects only treated with 0.04 μL acetone was as control. Thirty insects were treated in each concentration, and every treatment was repeated at least three times. The treated insects were reared at 27 ± 1 °C, humidity 70%–80% and 16 h light/8 h dark photoperiod in laboratory cages. The mortality was checked in 48 h. For synergism bioassays, 2 μg of synergist (PBO, TPP or DEM) in 0.04 μL acetone was topically applied on the prothorax notum of each insect 1 h before the imidacloprid application.

For expression induction, the 4th instar nymphs were exposed to imidacloprid at the dose of LD_{50}, and the survivals were collected at 48 h after imidacloprid application, then frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Insects treated with acetone were used as control.

2.4. RNA isolation and cDNA synthesis

Total RNA was extracted from at least three pools of 20 individuals at the 4th instar using Trizol reagent (Life Technologies, USA) following the manufacturer's instructions. DNA contaminations were removed by treating RNA extractions products with RNase-free DNase (Ambion, Austin, TX, USA) and purified by phenol-chloroform. Agarose gel electrophoresis (1%) and spectrophotometery (Nanodrop Technologies, Wilmington, DE, USA) were used to assess the quantity and quality of the RNAs. cDNA was synthesized by Superscript III and random hexamers (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.5. Detection of P450s mRNA levels

Fifty-four P450 gene sequences obtained from BPH genome (GenBank accession number AOSB00000000, BioProject PRJNA177647) were provided by Prof. Zhang Chuanxi from Zhejiang University (Hangzhou, China) [20]. Quantitative real-time PCR (qRT-PCR) was performed to determine the mRNA levels of P450s using the One Step SYBR PrimeScript RT-PCR Kit (Takara, China) following the user manual. Each qRT-PCR experiment was performed 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 [22]. All primer pairs for qRT-PCR were listed in Table S1. 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 [23].

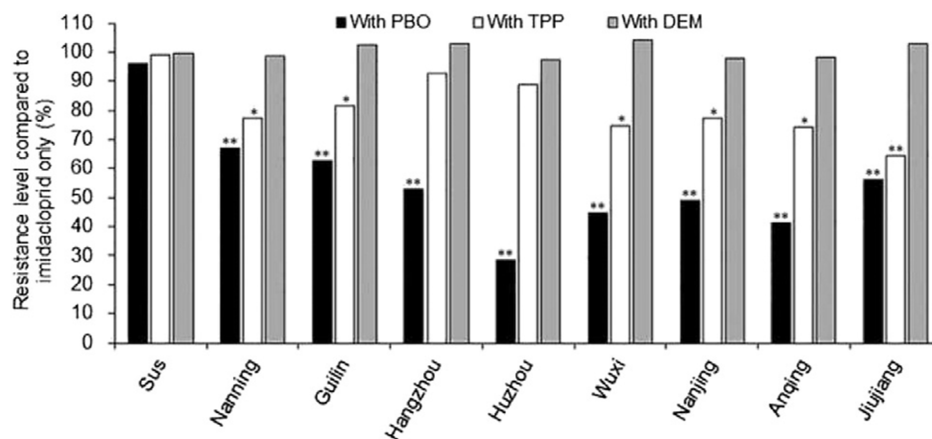

Fig. 1. The effects of three synergists on imidacloprid toxicities in the susceptible strain and field populations. The significant differences were compared among different treatments, with imidacloprid only or with imidacloprid plus a synergist, in each population based on LD_{50} data. * and ** showed significant differences from the treatment by imidacloprid only at 0.05 and 0.01 level, respectively.

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