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Biochemical mechanisms of imidacloprid resistance in *Nilaparvata lugens*: Over-expression of cytochrome P450 CYP6AY1

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ABSTRACT

Imidacloprid is a key insecticide extensively used for control of *Nilaparvata lugens*, and its resistance had been reported both in the laboratory selected strains and field populations. A target site mutation Y151S in two nicotinic acetylcholine receptor subunits and enhanced oxidative detoxification have been identified in the laboratory resistant strain, contributing importantly to imidacloprid resistance in *N. lugens*. To date, however, imidacloprid resistance in field population is primarily attributable to enhanced oxidative detoxification by over-expressed P450 monooxygenases. A resistant strain (Res), originally collected from a field population and continuously selected in laboratory with imidacloprid for more than 40 generations, had 180.8-fold resistance to imidacloprid, compared to a susceptible strain (Sus). Expression of different putative P450 genes at mRNA levels was detected and compared between Res and Sus strains, and six genes were found expressed significantly higher in Res strain than in Sus strain. CYP6AY1 was found to be the most different expressed P450 gene and its mRNA level in Res strain was 17.9 times of that in Sus strain. By expressing in *E. coli* cells, CYP6AY1 was found to metabolize imidacloprid efficiently with initial velocity calculated of 0.851 ± 0.073 pmol/min/pmol P450. When CYP6AY1 mRNA levels in Res strain was reduced by RNA interference, imidacloprid susceptibility was recovered. In four field populations with different resistance levels, high levels of CYP6AY1 transcript were also found. *In vitro* and *in vivo* studies provided evidences that the over-expression of CYP6AY1 was one of the key factors contributing to imidacloprid resistance in the laboratory selected strain Res, which might also be the important mechanism for imidacloprid resistance in field populations, when the target site mutation was not prevalent at present.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is a major insect pest of rice crops throughout Asia. *N. lugens* is a phloem feeder extracting nourishment directly from the plant, with direct and indirect deleterious effects including reduction in plant growth, wilting and leaf chlorosis (Gorman et al., 2008). Besides direct sucking, oviposition and virus disease transmission by *N. lugens* result in more severe damage (Wang et al., 2008). In recent years, *N. lugens* outbreaks have been more common in Asian countries because the pest has developed medium to high levels of resistance to major insecticides, including organochlorines, organophosphates, carbamates, insect growth regulators and neonicotinoids (Wang et al., 2009).

Broad-spectrum insecticides used to control *N. lugens* over several decades were eventually compromised by insecticide resistance and superseded by more selective insecticides including neonicotinoids. The first neonicotinoid insecticide introduced to the market was imidacloprid in 1991 followed by several others belonging to the same chemical class and with the same mode of action (Nauen and Denholm, 2005). When imidacloprid was introduced into China in the early 1990s, it became one of the most common insecticides used against *N. lugens*, principally owing to its efficacy and long-lasting effect. However, recent surveys of *N. lugens* field populations from several countries in Asia have shown that the intensity and geographical distribution of imidacloprid resistance in the field has increased substantially since 2006 (Gorman et al., 2008; Wang et al., 2008; Wen et al., 2009).

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Neonicotinoids act on insect nicotinic acetylcholine receptors (nAChRs), which mediate fast cholinergic synaptic transmission in insect central nervous system (Matsuda et al., 2001). Our previous studies of lab-selected imidacloprid resistant *N. lugens* have identified a single point mutation (Y151S) within the extracellular, agonist-binding domain of two nAChR α subunits (Liu et al., 2005). Radioligand ([^{3}H]imidacloprid) binding on native nAChRs and heterologous expression of recombinant nAChRs demonstrated that the Y151S mutation dramatically reduced imidacloprid affinity on receptors, which contributed directly to imidacloprid resistance in *N. lugens* (Liu et al., 2005, 2006). Interestingly, although studies are now underway to examine the prevalence of the Y151S mutation in field populations, to date there is no evidence to indicate that this laboratory-selected target-site mutation is responsible for resistance in field populations of *N. lugens* (Millar and Denholm, 2007; Wen et al., 2009; Liu et al., 2009).

In *N. lugens* and other imidacloprid resistant insects, piperonyl butoxide (PBO) could significantly synergize imidacloprid, which gave suggestions that imidacloprid resistances were attributable to enhanced oxidative detoxification by over-expressed P450 monooxygenases (Choi et al., 2001; Nauen et al., 2002; Liu et al., 2003; Karunker et al., 2008; Wen et al., 2009; Puinean et al., 2009). P450s (encoded by CYP genes) constitute a multigenic superfamily of enzymes, known for their ability to metabolize a wide range of endogenous and exogenous compounds thus contributing to numerous functions including growth, development, nutrition and xenobiotic detoxification (Feyereisen, 1999, 2005). In insects, CYP genes mostly belonging to microsomal CYP4, CYP6, CYP9 and mitochondrial CYP12 families, have frequently been associated with detoxification processes giving tolerance to insecticides (Scott, 1999; Feyereisen, 1999). Some specific P450 enzymes, such as the human CYP3A4, *Drosophila melanogaster* CYP6G1 and *Bemisia tabaci* CYP6CM1vQ have been found to metabolize imidacloprid (Schulz-Jander and Casida, 2002; Joußen et al., 2008; Karunker et al., 2009). Although some indirect evidence indicated that P450 monooxygenases are involved in imidacloprid resistance in *N. lugens*, no P450 member(s) responsible for imidacloprid resistance has been identified in *N. lugens*.

2. Materials and methods

2.1. Insecticides and synergists

Imidacloprid (97%) was purchased from Red Sun Group Corporation (Nanjing, China). Triphenyl phosphate (TPP, reagent grade) and diethyl meteate (DEM, reagent grade) were purchased from the Shanghai Chemical Reagent Co, TLD (Shanghai, China). Piperonyl butoxide (PBO, reagent grade) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Experimental insects

The susceptible strain (Sus) of *N. lugens* was a laboratory strain obtained 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. Four field populations were also included in this study. Aq-F, Jp-F, Hz-F and Gl-F were collected from paddy fields in Anqing (Anhui, China), Jiangpu (Jiangsu, China), Hangzhou (Zhejiang, China) and Guilin (Guangxin, China) in August 2010. Insects were kept in laboratory cages with rice plants at the tillering stage at 25 ± 1 °C, humidity 70–80% and 16 h light/8 h dark photoperiod.

2.3. Toxicity bioassay

The bioassay and synergism analysis were performed as the previous description (Liu et al., 2003). Two- to three-day old macropterous adult females (unmated) were used as test animals in this study. Insecticides were diluted to a series of concentrations with acetone. For Sus strain, the concentrations were 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 ng/μl. For Res strain, the concentrations were 100, 200, 300, 400, 500 and 600 ng/μl. For Gl-F populations, the concentrations were 10, 20, 30, 40, 50 and 60 ng/μl. For Aq-F, Jp-F and Hz-F populations, the concentrations were 25, 50, 100, 150, 200 and 250 ng/μl. Under carbon dioxide anaesthesia, a droplet (0.08 μL) of insecticide solution was applied topically to the prothorax notum of test hoppers with a hand microapplicator (Burkard Manufacturing Co. Ltd, Rickmansworth, UK). Thirty insects were treated at each concentration, and every treatment was repeated three times. The controls used acetone instead of insecticide solution. The treated insects were reared on seedlings cultured soilless in the rearing box (20 × 20 × 10 cm) at 25 ± 1 °C and 16/8 h light/dark. The results were checked after 48 h. In the synergism analysis, 2 μg of synergist (TPP, PBO or DEM) in 0.08 μL acetone was delivered on to the prothorax notum of each female adult 1 h before the insecticide application.

2.4. Detection of mRNA levels

The mRNA levels of different P450 genes were measured by qRT-PCR (quantitative real-time reverse transcriptase polymerase chain reaction) using the One Step SYBR PrimeScript RT-PCR Kit (Takara). Total RNA was isolated from the two- to three-day old macropterous adult females (unmated) using a Trizol® kit (Invitrogen). qRT-PCR was performed in a 25 μL total reaction volume containing 5 ng of total RNA, 0.5 μL primer mix containing 10 μM each of forward and reverse gene specific primers, 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. qRT-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 β-actin (EU179846) was used as an internal control (Liu et al., 2008). Because only a single gene was used for normalization, the experiment conditions here were not with the compliance with all the criteria of the MIQE guidelines for RT-qPCR (Bustin et al., 2010). mRNA levels for each gene in Sus and Res strains were quantified in relation to the expression of β-actin. Gene specific primers for P450 genes and β-actin were provided (Table 1). CYP6AY1 mRNA levels in Res and Sus strains were compared, and the significant differences between two strains were analysed. In this section, the single band amplified by the gene specific primers was confirmed by nucleotide sequencing.

2.5. Expression of CYP6AY1 in Escherichia coli and membrane fraction isolation

CYP6AY1 full-length cDNA was amplified from the plasmid pGEM-T/CYP6AY1 and subcloned into the expression vector pCWori at sites *NdeI* and *HindIII* (Helvig et al., 2004). The plasmid was verified by nucleotide sequencing.

DH5α *E. coli* cells were transformed with pCWori/CYP6AY1 in the presence of ampicillin. Cells were grown at 37 °C for 12 h at 250 rpm in modified TB medium (pH 7.4), containing thiamin (1 mM), potassium phosphate (100 mM) and δ-aminolevulinic acid (1 mM). Then 0.5 ml cultured medium was added to 50 ml fresh modified TB medium, and cells were grown at 37 °C and 250 rpm till the OD_{600} value of the cultured medium reached 0.55. Isopropyl β-D-thiogalactoside (IPTG, with final concentration of 0.4 mM) was

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