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The roles of *CYP6AY1* and *CYP6ER1* in imidacloprid resistance in the brown planthopper: Expression levels and detoxification efficiency

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ABSTRACT

Two P450 monooxygenase genes, *CYP6AY1* and *CYP6ER1*, were reported to contribute importantly to imidacloprid resistance in the brown planthopper, *Nilaparvata lugens*. Although recombinant *CYP6AY1* could metabolize imidacloprid efficiently, the expression levels of *CYP6ER1* gene were higher in most resistant populations. In the present study, three field populations were collected from different countries, and the bioassay, RNAi and imidacloprid metabolism were performed to evaluate the importance of two P450s in imidacloprid resistance. All three populations, DOT (Dongtai) from China, CNA (Chainat) from Thailand and HCM (Ho Chi Minh) from Vietnam, showed high resistance to imidacloprid (57.0-, 102.9- and 89.0-fold). *CYP6AY1* and *CYP6ER1* were both over expressed in three populations, with highest ratio of 13.2-fold for *CYP6ER1* in HCM population. Synergism test and RNAi analysis confirmed the roles of both P450 genes in imidacloprid resistance. However, *CYP6AY1* was indicated more important in CNA population, and *CYP6AY1* and *CYP6ER1* were equal in HCM population, although the expression level of *CYP6ER1* (13.2-fold) was much higher than that of *CYP6AY1* (4.11-fold) in HCM population. Although the recombinant proteins of both P450 genes could metabolize imidacloprid efficiently, the catalytic activity of CYP6AY1 (K_{cat} = 3.627 pmol/min/pmol P450) was significantly higher than that of CYP6ER1 (K_{cat} = 2.785 pmol/min/pmol P450). It was supposed that both P450 proteins were important for imidacloprid resistance, in which CYP6AY1 metabolized imidacloprid more efficiently and CYP6ER1 gene could be regulated by imidacloprid to a higher level.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens*, is a severe insect pest of rice crops all over Asian countries and areas. This insect pest causes damage not only by sucking directly on the phloem of rice resulting in undergrowth, even death of the plants [1], but also by transmitting plant viruses that bring serious diseases to rice plants [2,3]. Chemical control is the main way to control BPH, which inevitably causes insecticide resistances in this insect pest. In recent years, along with the extensive use of neonicotinoid insecticides, especially imidacloprid, to control BPH, high resistance to insecticides was detected in the lab-selected strains and field populations [4–6].

Well understanding the insecticide resistance mechanisms is a key step for the resistance management, such as the set-up of resistance detection method. So far, two main mechanisms have been reported which underlay insecticide resistance in BPH: target mutation and detoxifying enzymes. A point mutation in nicotinic acetylcholine receptors, the target of imidacloprid, was identified in the lab-selected resistant strain, which significantly reduced imidacloprid affinity on receptors and caused high resistance to imidacloprid in BPH [7,8]. In spite of that, this mutation was not prevalent in BPH field populations [4,9,10]. In contrast, changes in detoxifying enzymes, especially P450 monooxygenases (P450s) were found as the primary mechanisms for imidacloprid resistance in BPH field populations [10–12].

Although many P450 genes were found to be over-expressed in resistant strains or field populations of BPH [4,12], two genes, *CYP6AY1* and *CYP6ER1*, were thought most important in imidacloprid resistance [6,10,12]. The recombinant *CYP6AY1* could metabolize imidacloprid efficiently, while *CYP6ER1* showed the biggest increase in expression levels in resistant populations, for example up to 90-fold in some populations compared to a susceptible strain [12]. Thus, it is interesting to find out the importance of these two P450 gene/proteins in imidacloprid resistance in BPH, from their expression levels and also the imidacloprid metabolism efficiencies. In the present study, RNAi and functional expression experiments were performed to uncover the roles of *CYP6AY1* and *CYP6ER1* in imidacloprid resistance: similarity and difference.

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2. Materials and methods

2.1. Chemicals

Clothianidin and dinotefuran were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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. Insects

The susceptible strain (Sus) of BPH was collected from China National Rice Research Institute in September 2001 and reared on untreated rice under controlled conditions (27 ± 1 °C, 16 h photoperiod) as a laboratory strain. The three BPH field populations analyzed in this study were collected from Dongtai (DOT, China), Chainat (CAN, Thailand) and Ho Chi Minh (HCM, Vietnam) in August 2013.

2.3. Bioassay

The bioassay and synergism analysis were performed as the previous description [13]. Two- to three-day old macropterous adult females (unmated) were used in the present study. Insecticides were diluted to a series of concentrations with acetone. After carbon dioxide anesthesia, a droplet (0.08 μL) of insecticide solution was applied topically to the prothorax notum of tested BPH with a hand microapplicator (Burkard Manufacturing Co., Ltd., Rickmansworth, UK). Thirty insects were treated at each concentration, and every treatment was repeated at least three times. As controls, instead of insecticide solution, acetone was used. The treated insects were reared on seedlings cultured soilless in the rearing box (20 × 20 × 10 cm) at 27 ± 1 °C and 16 h photoperiod. The results were checked after 48 h. In the synergism analysis, 2 mg 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 [14].

2.4. Detection of mRNA levels

The mRNA levels of *CYP6AY1* and *CYP6ER1* genes were measured by qRT-PCR (quantitative real-time reverse transcriptase polymerase chain reaction) using the One Step SYBR PrimeScript RT-PCR Kit (Takara) as previously [10]. The specific primers for *CYP6AY1* was identical to that used in the previous study [10], and the specific primers for *CYP6ER1* were TGGCTGTTAATCAAGAGATGCAGC (forward) and CTGAAGCGCA TAGACCGGAATC (reverse). *CYP6AY1* and *CYP6ER1* mRNA levels of BPH in field populations and the Sus strain were compared and analyzed. In this section, the single band amplified by the gene specific primers was confirmed by nucleotide sequencing. 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 [15].

2.5. RNA interference

The target sequences of *CYP6AY1* and *CYP6ER1* were amplified by RT-PCR using specific primers 6AF: CCA ATC ACC GCA CAC CTG GTC AAC C, 6AR: GCT TGA GCT GCT ATA ACA CTC TCT G, and 6EF: GTC AAC TTC TAC GTT TAC TCC TAT TG, 6ER: ATC ACA TTC AGC CCG TAG TTG TTT G. As control, bacterial Lac-Z (AJ308295) fragment sequence was amplified as previously described [10,16]. The dsRNA preparation, dsRNA injection were performed as the previous description [10]. In brief, the dsRNA was synthesized using the T7 Ribomax Express RNAi System (Promega, USA). For injection, 50 nL dsRNA(5 mg/mL) for one gene was injected into the brown planthopper (soon after ecdysis of the 5th instar larvae of macropterous females) at low speed at the conjunctive between prothorax and mesothorax, as described previously [16]. mRNA level determination was performed as described in *2.4*.

2.6. Functional expression of CYP6AY1 and CYP6ER1 and membrance fraction isolation

The full length cDNA of *CYP6AY1* and *CYP6ER1* were subcloned into the expression vector pCWori at sites *NdeI* and *HindIII* respectively. After verified by nucleotide sequencing, the plasmids were transformed to *Escherichia coli* which was used to express the recombinant proteins. The cells were grown in modified TB medium (pH 7.4, 1 mmol thiamin, 100 mmol potassium phosphate, 1 mmol δ-aminolevulinic acid) stirred at 250 rpm at 37 °C for 12 h. Then added 0.5 mL cultured medium to 50 mL fresh modified TB medium, and cultured at 250 rpm at 37 °C till the OD_{600} value of the medium reached 0.55. Isopropyl β-D-thiogalactoside (IPTG, final concentration 0.4 mmol) was then added, and the cells were grown at 170 rpm and 28 °C. 48 h later, cells were harvested by centrifugation (2800 ×*g*, 5 min, 4 °C). The pellet was suspended in tris–acetate buffer (pH 7.6, 50 mmol tris–acetate, 250 mmol sucrose and 0.25 mmol EDTA). After added freshly prepared lysozyme (final concentration 0.25 mg/mL), the mixture was gently shaken at 4 °C for 45 min. Then centrifuged at 2800 ×*g* for 15 min at 4 °C, the pellet were resuspended in 0.1 mol sodium phosphate buffer (pH 7.4, 0.1 mmol sodium phosphate, 20% glycerol, 1 mmol PMSF, 0.1 g/mL leupeptin, 0.04 units/mL aprotinin) and disrupted by sonication at 4 °C. After centrifugation (4000 ×*g* for 10 min at 4 °C), the supernatant was centrifuged at 100,000 ×*g* for 1 h. The pellet was resuspended in sodium phosphate buffer and used for enzymatic characterization.

2.7. Enzymatic activities determination

The determination of enzymatic activities of CYP6AY1 and CYP6ER1 was performed as described previously (10). In brief, the reaction mixture contained 0.2 mmol P450 protein, 2 mmol house fly P450 reductase, NADPH-regenerating system and 3 mg/L imidacloprid. Instead of the recombinant protein, the membrane fraction of untransfected *E. coli* cells was used as control. Each interval of 20 min, 200 μL solution was extracted from the reaction mixture, and the imidacloprid concentration was determined by reversed-phase HPLC on a 5-μm SupelCosil LC18-DB column (4.6 cm × 250 cm, Supelco) using a solvent mixture composed of 20% CH_3CN, 80% H_2O, which was isocratic for 5 min, followed by a linear gradient to 80% CH_3CN, 20% H_2O over 40 min (flow rate 1 mL/min). Imidacloprid elution was monitored by absorption at 270 nm by Spectroflow UV/Vis Detector (Kratos Analytical Instruments, USA). Meanwhile, the main metabolites of imidacloprid by recombinant protein were identified by UPLC-MS. The extracted solutions were subjected to Ultra Performance LC (Waters Acquity UPLC System, Waters, Eschborn, Germany). Samples were eluted by a mobile phase (HPLC water containing 0.1% (*w*/*v*) formic acid and acetonitrile 0.1% (*w*/*v*) formic acid) at rate of 0.6 mL/min followed by mass spectrometer in a TSQ Vantage triple quadrupole instrument with H-ESI II source (Thermo, Dreieich, Germany) operating in positive ion mode. High purity argon was used as the collision gas and nitrogen at 450 °C as the sheath/auxiliary gas. The capillary temperature was 270 °C.

2.8. Data analysis

All data were analyzed using Data Processing System (DPS) software [17]. Differences in mortality and mRNA levels were analyzed by one-way ANOVA with at least three repeats. Differences between values were analyzed using an LSD pair wise comparison of means. The level of significance for results was set at $p < 0.05$.