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Research article

Dynamics of CYP6ER1 expression in brown planthopper (*Nilaparvata lugens* Stål) under exposure to imidacloprid

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Abstract

Importance of the work: The brown planthopper (*Nilaparvata lugens* Stål) causes severe damage to rice crops in Indonesia. *CYP6ER1* contributes to *N. lugens* resistance against imidacloprid.

Objectives: To investigate the relative roles of the cytochrome P450 monooxygenase, *CYP6ER1*, in determining a resistance phenotype to imidacloprid.

Material and Methods: The relative expression was investigated of *CYP6ER1* in *N. lugens* at various developmental stages, various imidacloprid concentrations and various exposure times using real-time quantitative polymerase chain reaction. The *N. lugens* population from Pringsewu Regency, Lampung Province, Indonesia was exposed to imidacloprid for two generations (Psw2) and six generations (Psw6). The relative gene expression of *CYP6ER1* in the laboratory, Psw2 and Psw6 populations were determined at the first and fifth instars and in male and female adults.

Results: The resistant ratios of Psw2 and Psw6 were 3.7-fold and 5.4-fold, respectively, higher than that of the laboratory population. The *CYP6ER1* expression of Psw2 was up-regulated in all developmental stages; however, in Psw6, it was up-regulated only in the early stages. In Psw6, *CYP6ER1* was up-regulated after the population had been exposed to 350 parts per million (ppm) and 1,050 ppm imidacloprid with exposure times of 24, 72 and 96 hr after treatment using the third instars, relative to the before exposure levels.

Main finding: Up-regulation of *CYP6ER1* occurred only in the first instars of Psw6 and in other stages of Psw2. In Psw6, *CYP6ER1* was rapidly up-regulated starting from the first 24 hr after treatment and increased with time until 96 hr after treatment. These findings showed the involvement of *CYP6ER1* in imidacloprid detoxification. These *CYP6ER1* expression patterns may be used to develop rapid diagnosis for resistance management purposes.

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Introduction

Nilaparvata lugens Stål (Hemiptera: Delphacidae), known commonly as the rice brown planthopper, infests rice crops in almost all provinces in Indonesia (CABI, 2020; EPPO, 2024). In 2021/2022, infestations of *N. lugens* were reported in more than 9,319 ha of rice during the rainy season and 12,825 ha during the dry season (BBPOPT, 2023). Thus, *N. lugens* is a major threat to rice production in Indonesia. In Java, the level of damage caused by *N. lugens* in 2013/2014 varied, with *N. lugens* outbreaks being related to the use of pesticides (Triwidodo, 2021).

Neonicotinoids are neuroactive insecticides that have been widely used to manage *N. lugens* for more than 20 yr due to their persistent residual effect and high selectivity (Gorman et al., 2008). In Indonesia, imidacloprid, a neonicotinoid, has been used to manage *N. lugens* since 1992 (Ditjen PSP, 2016). Imidacloprid resistance has been detected across various *N. lugens* populations in Indonesia. For example, low levels of resistance have been reported based on the resistance ratio (RR) in Juwiring populations (RR = 7.0-fold), while moderate levels of resistance were reported in Sukamandi populations (RR = 12.7-fold) and high levels of resistance were reported in the Karawang populations (RR = 108.1-fold) (Baehaki et al., 2016; Surahmat et al., 2016). There was moderate resistance to imidacloprid in Banyumas (RR = 46.2-fold; Diptaningsari et al., 2020) and Cilacap (RR = 29.4-fold; Londingkene et al., 2016). In addition, *N. lugens* resistance to imidacloprid has been reported in several countries in Asia, such as China, Japan, the Republic of Korea and Vietnam, with RR values ranging from 24-fold to 2,106-fold (Matsumura et al., 2008; Gorman et al., 2008; Wang et al., 2008; Wang et al., 2009; Zhang et al., 2014; Wu et al., 2018). The development of *N. lugens* resistance should be monitored to mitigate crop failure events and to minimize pest management costs.

N. lugens resistance to imidacloprid is autosomal and dominant, manifesting through high expression of detoxification enzymes (Diptaningsari et al., 2019; Morimura et al., 2019). Detoxification by cytochrome P450 monooxygenases (P450s) has been reported in both laboratory and field populations (Wen et al., 2009; Puinean et al., 2010; Bass et al., 2011; Bass et al., 2014). Further research demonstrated overexpression of multiple P450 genes in laboratory and field populations (Liu et al., 2003; Puinean et al., 2010; Bass et al., 2011; Bass et al., 2014). *CYP6ER1* and *CYP6AY1* are up-regulated in imidacloprid-resistant *N. lugens* populations (Bass et al., 2011; Ding et al., 2013; Garrood et al., 2015; Wu et al., 2018**).** *CYP6ER1* confers imidacloprid resistance in *N. lugens* during all developmental stages, whereas other P450s have effects only at some developmental stages (Zhang et al., 2016a). *CYP6ER1* up-regulation is associated with the metabolism of xenobiotics, including detoxification of insecticide, which leads to insecticide resistance.

Information is still limited on the occurrence and expression of *CYP6ER1* in populations of *N. lugens* in Indonesia. Garrood et al. (2015) showed that during the period 2005−2012, several *N. lugens* populations in Indonesia exhibited substantial up-regulation of *CYP6ER1*. *CYP6ER1* detected in a *N. lugens* population from Indramayu, Indonesia, is a variant of *CYP6ER1*vA that causes imidacloprid resistance in *N. lugens* from Thailand and Vietnam (Zimmer et al., 2018). The current study aimed to determine *CYP6ER1* relative expression levels in laboratory and field populations that were exposed to imidacloprid at various developmental stages, concentrations and exposure times. The findings from this study should provide insights that can help in improving the application of imidacloprid and possible insecticide rotation to mitigate development of *N. lugens* resistance. Additionally, *CYP6ER1* can be used as a candidate molecular barcoding gene for rapid resistance diagnosis.

Materials and Methods

Origin of Nilaparvata lugens

Two *N. lugens* populations (laboratory and field) were used. The laboratory population (Lab) has been reared and maintained since 1986 without any exposure to insecticides. This population was originally collected from the Regency of Sleman, Special Region of Yogyakarta, Indonesia. The field population (about 200 adults) was collected in 2019 from the Regency of Pringsewu, Lampung Province, Indonesia (referred to below as Psw).

Rearing and selection of Nilaparvata lugens

The laboratory and field populations of *N. lugens* were reared based on an established laboratory procedure using rice seedlings of Ciherang rice. The rice seeds were washed, placed on a tray and covered with a cloth for 48 hr. After germination, the seeds were moved into a plastic container (diameter 16 cm, height 18 cm). Seedlings aged 7–10 d after planting (DAP) were used as *N. lugens* feed. About 100 individuals of

N. lugens were placed in plastic containers to allow them to oviposit. After 4–5 d, the *N. lugens* adults were moved to new containers to increase the *N. lugens* population size. After the eggs had hatched and the rice seedlings had turned yellow, the nymphs were moved to a new container containing new 7 DAP rice seedlings. All populations were reared under laboratory conditions at 27°C, air humidity at 70%–80% relative humidity and a photoperiod of 12 hr light and12 hr darkness.

Selection using imidacloprid commercial formulation (Confidor extra 350SC; PT. Bayer; Indonesia) began at the 22nd to 27th generation (G_{22}–G_{27}). The field populations (Psw) were reared without exposure to imidacloprid due to the COVID-19 pandemic for the 21 generations after collection. Imidacloprid was applied using the leaf dipping method (a modified method from IRAC No. 005 standardized methods (IRAC, 2012)) in which 5–7 DAP rice seedlings were immersed in pesticide solutions for 15 s before they were air-dried. The treated rice seedlings were placed in plastic cups (diameter 5.5 cm, height 11 cm) with water to ensure the seedling's health. Third instars of G_{22}–G_{27} were selected by exposing them to imidacloprid-treated rice seedlings. Imidacloprid concentrations were increased from 175 ppm to 490 ppm at the sixth selection. As many as 150–660 of the third instars were selected by exposure to imidacloprid at each generation and mortality was observed 168 hr after treatment (HAT). The exposure to imidacloprid for six generations caused 34%–50% mortality. Then, the surviving insects were moved and reared on 7–10 DAP seedlings in similar plastic containers under similar conditions. After two (Psw2) and six (Psw6) generations of selection, these two field populations were examined for resistance bioassay and gene expression.

Resistance bioassay

The third instar of *N. lugens* from the Psw and Lab populations was used in the resistance bioassay. A series of imidacloprid concentrations was prepared ranging from 0 ppm to 2,100 ppm for challenging the Psw and between 0 ppm and 613 ppm for the Lab, using the leaf dipping method. Seven to eight series of concentrations were used to determine the dose lethal to 50% of the population (LC_{50}) for each population, using 50 of the third instars of *N. lugens* for each concentration. Mortality was observed and recorded at 96 HAT.

Probit analysis was conducted using the JMP Pro software (version 13; SAS Inc.; Cary, NC, USA) to determine the LC_{50} values, with the mortality rate of exposed insects being corrected using Abbott's formula (Abbott, 1925). The RR was calculated by comparing the LC_{50} of Psw to the LC_{50} of Lab. LC_{50} values were considered significantly different if their 95% confidence intervals (CIs) did not overlap (Marçon et al., 1999). Resistances levels were divided into several categories: no resistance, RR < 2-fold; low resistance, RR = 2–10-fold; moderate resistance, RR = 11–30-fold; high resistance, RR = 31–100-fold; and very high resistance, RR = > 100-fold (Vila et al., 2002).

Stage-dependent expression of CYP6ER1

CYP6ER1 expression was determined for the Psw2, Psw6 and Lab populations based on differential expression at various developmental stages. Total RNA was isolated from the first and fifth instar nymphs and male and female adults with three biological replicates and one insect used for each replication. Three *N. lugens* individuals at each stage were sampled and preserved using RNAlater® solution (Sigma-Aldrich; Burlington, MA, US), before they were stored at −20°C in a freezer for later RNA extraction.

Total RNA was extracted using a Total RNA Mini Kit (Tissue) from Geneaid (New Taipei City, Taiwan) according to the manufacturer's protocols. Reverse transcription to cDNA was done using RaverTra Ace α (Toyobo U.S.A. Inc.; New York, NY, USA) according to the manufacturer's protocols. The quality and concentration of RNA and cDNA were calculated using a spectrophotometer (MaestroGen Inc.; Hsinchu, Taiwan), they were stored at −20°C in a freezer until further processing.

Gene expression of *CYP6ER1* was determined using real-time quantitative PCR (RT-qPCR) (Biorad CFX96 *Real-Time System*) and using a SensiFAST™ SYBR® No-ROX Kit (Bioline; London, UK) with a total reaction of 20 µL containing 2 µL (100 ng) of cDNA, 10 µL of reaction buffer (SensiFAST™ SYBR®), 0.8 µL each of the reverse and forward primers and 6.4 µL of nuclease-free water by following the manufacturer's protocols. The RT-qPCR primers used were those developed by Wu et al. (2018), *CYP6ER1* (NlCYP6ER1-RTF:5′-ATTCCGGTCTATGCGCTTC-3′; NlCYP6ER1-RTR:5′-TGGATTGGCGCTCTCTTACT-3′), with β-Actin used as a housekeeping gene (Nlactin-F: -5′TGGACTTCGAGCAGGAAATGG-3′; Nlactin-R: 5′-ACGTCGCACTTCAGATCGAG-3′). The RT-qPCR thermocycle regime was: initial denaturation at 95°C for 2 min, followed by 40 amplification cycles of denaturation at 95°C for 5 s, annealing at 58°C for 10 s and extension at 72°C

for 5 s. Gene expression was calculated by comparing Ct or $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), with β-Actin used for the normalization of each treatment and the control treatment as the second normalization. The RT-qPCR was performed with three biological replicates and two technical replicates; the results were expressed as -fold changes in the *CYP6ER1* gene.

The analysis of variance of stage-dependent expression of *CYP6ER1* expression data was performed using the R software (version 4.3.2; R Core Team, 2023) following a completely randomized design experiment, which in turn was followed by Tukey's honest significant test for significant differences among treatments at $p < 0.05$. The data were expressed as mean ± SD values for all parameters. The figure was built using the GraphPad Prism software (version 8; San Diego, CA, USA).

Concentration and time-dependent expression of CYP6ER1

The effects of concentration and exposure duration of imidacloprid on *CYP6ER1* expression were determined by exposing the third instar of the Psw6 population to imidacloprid solutions of 350 ppm and 1050 ppm for five durations (0 hr, 24 hr, 48 hr, 72 hr or 96 hr). After exposure to imidacloprid, three surviving insects from each treatment were preserved using RNAlater® and stored at −20°C in a freezer for later RNA extraction. Three biological replicates were used for each treatment and one insect was used for each replication. The RNA extraction, cDNA synthesis and RT-qPCR of *CYP6ER1* were performed using similar procedures to those described above (see the Stage-dependent expression of *CYP6ER1* section). The concentration and time-dependent expression of *CYP6ER1* data were analyzed using multivariate principal component analysis (Jolliffe and Cadima, 2016) in the R software (version 4.3.2; R Core Team, 2023) packages FactoMineR and factoextra.

Results

Rates of resistance to imidacloprid

After two generations of selection, Psw2 had an RR 3.7-fold relative to that of the Lab (susceptible) population. The RR increased to 5.4-fold after six generations of selection (Table 1). The LC_{50} values for Psw2 and Psw6 were significantly higher than for Lab, as indicated by the non-overlapping 95% CIs of the LC_{50} values (Marçon et al., 1999).

Stage-dependent expression of CYP6ER1

CYP6ER1 expression fluctuated in *N. lugens* across developmental stages. Up-regulation occurred in Psw6 at the first instar but not in the other stages. *CYP6ER1* expression was up-regulated in Psw2 male and female adults (Fig. 1).

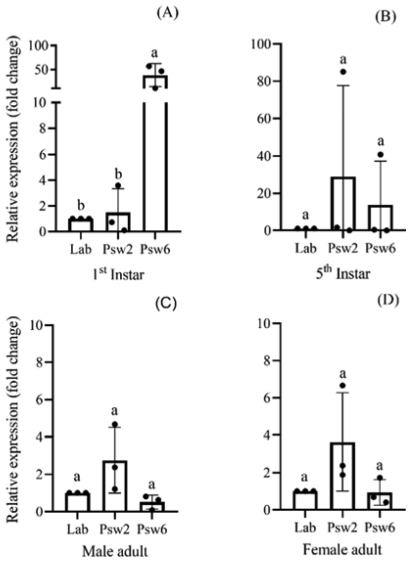

Fig. 1 Relative expression of *CYP6ER1* in various developmental stages in *Nilaparvata lugens* laboratory (Lab) and Pringsewu (Psw) populations: (A) 1st instar; (B) 5th instar; (C) male adult; (D) female adult. Bars represent mean and error bars represent ± SD. Different lowercase letters above bars indicate significant differences ($p < 0.05$) among means.

Table 1 LC_{50} values of imidacloprid for laboratory and selected third-instar *Nilaparvata lugens* populations on rice seedlings

Population	*n*	Slope ± SE	LC_{50} (95% CI; ppm)	χ^2 (df)	RR
Lab	400	0.99 (±0.10)	203.87 (171.40–238.27)	10.25 (5)	1.0
Psw2	400	0.81 (±0.11)	762.13 (632.00–973.44)	1.55 (5)	3.7*
Psw6	450	1.12 (±0.13)	1,108.87 (967.55–1,246.58)	6.28 (6)	5.4*

ppm = parts per million; df = degrees of freedom; CI = confidence interval; *n* = numbers of insects exposed; RR = resistance ratio (LC_{50} selected strain/ LC_{50} of Lab); Lab = laboratory population; Psw2 and Psw6 = field population collected in 2019 from the Regency of Pringsewu, Lampung Province, Indonesia exposed to imidacloprid for two generations and six generations, respectively.
* = significant at $p < 0.05$.

Concentration and time-dependent expression of CYP6ER1

Levels of *CYP6ER1* expression in Psw6 exposed to different concentrations of imidacloprid over 0–96 hr increased with exposure times. Expression began to rise at 24 hr post-exposure and continued to increase through 96 hr. Fig. 2 shows that different concentration treatments gave different *CYP6ER1* expression responses. This could be seen in the relationship between the concentration of 350 ppm imidacloprid and the concentration of 1,050 ppm imidacloprid. *CYP6ER1* expression was high when the exposure time was 96 hr at both concentrations, whereas *CYP6ER1* expression was high at 72 hr exposure time only at a concentration of 1,050 ppm (Fig. 2). These results indicated that the up-regulation of *CYP6ER1* could be induced by imidacloprid and that it could reach a maximum within a certain time after exposure.

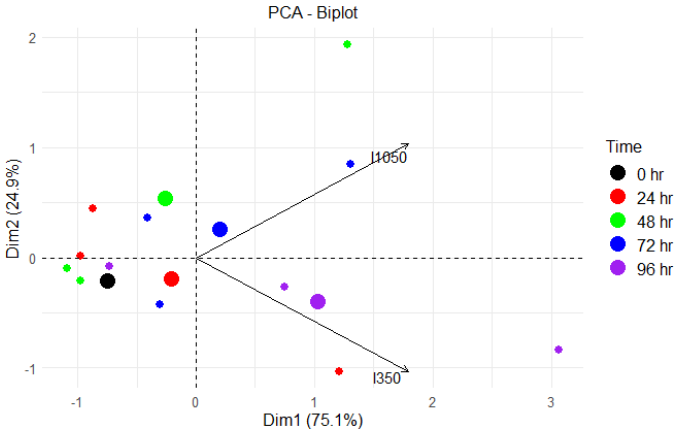

Fig. 2 Principal component analysis (PCA) of imidacloprid concentration and time-dependent relative expression of *CYP6ER1* in 3rd instar *Nilaparvata lugens* (Psw6) before and after exposure. I350 = imidacloprid at 350 ppm; I1050 = imidacloprid at 1,050 ppm.

Discussion

Continuous insecticide application can result in insecticide resistance in insect pests. *N. lugens* is highly adaptive and can develop resistance quickly. Imidacloprid has been used to manage *N. lugens* since 1992. In Indonesia, 47 registered insecticide brands contain imidacloprid (Republic of Indonesia Ministry of Agriculture, 2021). In Java, imidacloprid was used at various attack stages of *N. lugens* in 2013 and 2014 (Triwidodo, 2021). Insect resistance to imidacloprid has been reported in many countries, including Indonesia (APRD, 2024). The increasing RR after imidacloprid selection indicates that resistance management for this insect is needed.

Exposure of the Psw population to imidacloprid resulted in resistance and up-regulation of *CYP6ER1*, with the first instar of the Psw6 population having the highest *CYP6ER1* relative expression (Fig. 1). Imidacloprid selection reduces genetic heterogeneity within that population and results in imidacloprid-resistant insect pests. The increase in RR is due to high detoxification rate coupled with high detoxifying enzyme activity (Wen et al., 2009; Puinean et al., 2010; Zhang et al., 2016a. Furthermore, *CYP6ER1* is more easily induced in susceptible populations (Zhang et al., 2016b). The current results were consistent with those of other studies in which metabolism during early developmental stages contributed to imidacloprid resistance (Liu et al., 2003; Liu et al., 2005; Liu et al., 2006). A high level of transcription of P450 genes increases the level of encoded proteins which then detoxify xenobiotics within insects. The highest P450 expression was shown in body fat (Pang et al., 2016). Observed gene expression patterns indicate differences among developmental stages and differences in metabolic rates. Higher up-regulation of *CYP9M10* was observed in the larval stages of culex than in adults, whereas the levels of expression of *CYP6AA7*, *CYP9J34* and *CYP9J40* were similar across developmental stages (Liu et al., 2015), implying that several P450 genes are involved and respond to insecticides differently at various developmental stages.

CYP6ER1 expression during early developmental stages in populations with high RR affected *N. lugens* sensitivity to imidacloprid (Fig. 1). These results were supported by the findings of Zhang et al. (2016b). In addition, variable gene expressions and catalytic activity of multiple P450s across populations are important factors influencing insecticide resistance (Zhang et al., 2016a). Induction by imidacloprid and P450 overexpression in resistant populations increases the chances of adapting to stress from insecticides. Therefore, inducibility of enzymes catalyzing xenobiotic detoxification is a risk factor for insecticide resistance development (Zhang et al., 2016a; Zhang et al., 2016b).

The current demonstrated that *CYP6ER1* was rapidly up-regulated beginning 24 HAT and continually increasing over time until 96 HAT. These findings were consistent with those of other showing that *CYP6ER1* and *CYP6AY1* were the main metabolites to detoxify imidacloprid after the first 20 min and continually increased until 120 min (Bao et al., 2016). The current study also confirmed that *CYP6ER1* plays

an important role in imidacloprid resistance by *N. lugens* at all developmental stages after exposure for 25 generations (Zhang et al., 2016a). Mismatching or inconsistency between gene expression and RR could be caused by the complexity of P450 activity (Sun et al., 2018).

CYP6ER1 is expressed as a response of *N. lugens* to imidacloprid exposure. However, *CYP6ER1* up-regulation did not occur across all developmental stages, rather only during the 1st instar of Psw6 in the current study. *CYP6ER1* knockdown experiments using RNAi demonstrated that *CYP6ER1* played a role in resistance to imidacloprid, thiamethoxam, dinotefuran and clothianidin (Pang et al., 2016; Sun et al., 2018; Jin et al., 2019; Mao et al., 2019). The continuous use of various active ingredients at various developmental stages can result in the expression of multiple P450 genes and possibly cause cross resistance. The findings of the current study should contribute to understanding the dynamics of resistance of *N. lugens* and the involvement of *CYP6ER1* in imidacloprid detoxification. Additionally, the current findings suggested that expression of *CYP6ER1* in the early stages could be used for the development of rapid diagnostic tools to monitor the spread of insecticide-resistant *N. lugens*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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