



Characterization and functional analysis of a carboxylesterase gene associated with chlorpyrifos resistance in *Nilaparvata lugens* (Stål)

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ABSTRACT

The widespread and extensive application of insecticides have promoted the development of resistance in the brown planthopper *Nilaparvata lugens* (Stål), one of the most important rice pests in Asia. To better understand the underlying molecular mechanisms of metabolic resistance to insecticides, a chlorpyrifos-resistant (CR) strain of *N. lugens* was selected and its possible resistance mechanism was investigated. Synergistic tests using carboxylesterases (CarEs) inhibitor triphenyl phosphate (TPP) decreased the resistance of *N. lugens* to chlorpyrifos, and CarE activities could be induced by low concentrations of chlorpyrifos. Subsequently, a gene putatively encoding CarE, namely *NlCarE*, predominant in the midgut and ovary was isolated and characterized. The expression levels of *NlCarE* were detected and compared between the CR and a susceptible (SS) strain of *N. lugens*. Consistent with the increased CarE activity, this gene was overexpressed in the CR strain compared to the SS strain. The transcript levels of *NlCarE* were up-regulated by chlorpyrifos exposure, showing dose- and time-dependent expression patterns. Furthermore, RNA interference (RNAi)-mediated knockdown of *NlCarE* followed by insecticide application significantly increased the susceptibility of *N. lugens* to chlorpyrifos. These results demonstrate that *NlCarE* plays an important role in chlorpyrifos detoxification and its overexpression may be involved in chlorpyrifos resistance in *N. lugens*.

1. Introduction

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) is one of the most serious rice pests in Asia which causes dramatic losses in rice yield (Hu et al., 2014). In addition to sucking the sap from the phloem tissues, oviposition and virus transmission by *N. lugens* also lead to great damage to rice plants (Wang et al., 2008). The widespread use of chemical insecticides has been the primary strategy to control rice planthoppers for many years (Matsumura and Sanadamorimura, 2010). *N. lugens* has developed high resistance to many kinds of the synthetic insecticides, including the pyrethroids (Dai and Sun, 1984), pymetrozine (X. Zhang et al., 2014), phenylpyrazole (Garrood et al., 2016; Zhao et al., 2011), neonicotinoids (Gorman et al., 2010; X. Zhang et al., 2014) and organochlorines (Davies et al., 2010). For example, due to the extensive use of the first neonicotinoid insecticide imidacloprid in rice fields, high levels of resistance to imidacloprid have been developed in many areas (Matsumura et al., 2014). Failure in field control of *N. lugens* using this insecticide has been reported, and imidacloprid application has been discontinued for *N. lugens* management in China (Wen et al., 2009). Chlorpyrifos, a broad-spectrum organophosphates

insecticide, shows contact-effective toxicity against sucking pests, and has increasingly been used for *N. lugens*'s control in recent years (Wang et al., 2010). However, resistance monitoring of *N. lugens* showed that low to moderate levels of resistance to chlorpyrifos have been emerged in several field populations (Lin, 2011; Wang et al., 2009). Extensive outbreaks of *N. lugens* have been increasing in recent years, and it is believed that the development of resistance to chemical pesticides is one of the major reasons behind this increase. Therefore, a better understanding of mechanisms underlying insecticide resistance is urgently needed for developing effective pest management strategies.

Insecticide resistance involves several different mechanisms including reduction of cuticular penetration rate, enhancement of metabolic detoxification enzymes and target-site insensitivity (Liu et al., 2006; Perry et al., 2011). Two main mechanisms of insecticide resistance in *N. lugens* involve increased metabolic detoxification by enzymes and point mutations in target sites (Liu et al., 2005; Zhang et al., 2016). For example, three point mutations in acetylcholinesterase (AChE1), the target of chlorpyrifos, was identified in the field populations and lab-selected resistant strains, which significantly decreased insecticide sensitivities and caused high resistance to chlorpyrifos in *N. lugens* (Zhang

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et al., 2017). In addition, the simultaneous application with carboxylesterase (CarE) specific enzyme inhibitor triphenyl phosphate (TPP) significantly decreased the resistance of the lab-selected and field-resistant strains to chlorpyrifos. These results indicate that the metabolic detoxification of metabolizing enzymes, such as CarEs, might be involved in conferring chlorpyrifos resistance in *N. lugens* (Bao et al., 2016; Zhang et al., 2017). However, the underlying mechanisms of metabolic resistance to chlorpyrifos in *N. lugens* remain obscure.

The enhanced detoxification of CarEs has been reported to be associated with the metabolic resistance to many kinds of insecticides including organophosphates, carbamates and pyrethroids in insects (Alon et al., 2008; Zhang et al., 2015). CarEs are a multigenic and ubiquitous superfamily of metabolic enzymes with various biological functions in many metabolic reactions, including metabolism of specific hormones, pheromone degradation, xenobiotic detoxification, as well as hydrolyzing carboxyl esters of insecticides (Kontogiannatos et al., 2011; Yu et al., 2009). As one of the most important metabolic detoxification enzymes, CarEs have been well studied in various insects (Grigoraki et al., 2017; Yang et al., 2017). In recent years, it has been found that the over-expression and elevated activities of some CarEs in insects could be associated with the increased resistance to insecticides, including *Plutella xylostella* (Xie et al., 2017), *Tetranychus cinnabarinus* (Peng et al., 2016), *Locusta migratoria* (Zhang et al., 2015; Zhang et al., 2013), *Bactrocera dorsalis* (Hsu et al., 2016; Wang et al., 2015; Wang et al., 2016), *Musca domestica* (Taşkin et al., 2004; Zhang et al., 2010), *Tribolium castaneum* (Haubruge et al., 2002), *Leptinotarsa decemlineata* (Lü et al., 2015), *Anisopteromalus calandrae* (Baker et al., 1998) and *Aedes aegypti* (Grigoraki et al., 2016). In *N. lugens*, a CarE gene (*NIEST1*) associated with organophosphate insecticides resistance was isolated (Small and Hemingway, 2010), however, the understanding of the underlying molecular mechanism of CarEs-mediated resistance is still unknown. Given the increasing development of chlorpyrifos resistance in *N. lugens*, identification and functional analyses of CarEs genes involved in insecticide metabolism in *N. lugens* is of great significance.

In the present study, a relative susceptible strain (SS) and a chlorpyrifos-resistant strain (CR) of *N. lugens* were obtained from the same field-originated population by the continuous selection with or without chlorpyrifos. The roles of CarEs in chlorpyrifos resistance were evaluated using the CarE enzyme specific inhibitor TPP in both SS and CR strains. A gene putatively encoding CarE (*NICarE*) in different strains, developmental stages and tissues was identified and characterized. RNA interference (RNAi)-based knockdown of *NICarE* combined with insecticide bioassays in CR strain adults were performed to analyze the functions of this CarE gene involved in chlorpyrifos resistance.

2. Materials and methods

2.1. Experimental insects

The susceptible strain (SS) of *N. lugens* was collected from the South China Agriculture University in September 2008, and has been reared without any exposure to insecticides for > 8 years (Lu et al., 2015). The chlorpyrifos-resistant strain (CR) was selected from SS strain with chlorpyrifos for 8 generations in the laboratory. All insects were reared on Taichuang Native 1 rice seedlings (*Oryza sativa* L.) at $26 \pm 1^\circ\text{C}$, relative humidity of $65 \pm 5\%$ and a 16-h light: 8-h dark photoperiod in a climate chamber.

2.2. Toxicity bioassay and synergist assay

N. lugens females were selected to detect the toxicity (LD_{50}) of chlorpyrifos using the topical application method. In brief, five serial concentrations of chlorpyrifos in acetone were generated and a droplet insecticide solution (0.25 μL) was topically applied onto the abdomen of the newly emerged females with a hand microapplicator (Burkard Manufacturing Co Ltd., Rickmansworth, UK). Females treated with

acetone were set as controls. The mortality of *N. lugens* was recorded 24 h after chlorpyrifos treatment, and thirty females were used for each dilution. The LD_{50} value was calculated via standard probit analysis using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). In the synergism analysis, 2 μg of the synergist (triphenyl phosphate, TPP) dissolved in 0.25 μL acetone was topically applied onto the abdomen of the test females 1 h before the bioassay.

2.3. CarE activity assay

CarE activity was measured according to the method of Van Asperen with minor modification (Asperen, 1962). Briefly, five females were homogenized in 500 μL phosphate buffer (0.04 mol L^{-1} , pH 7.0) using an electrically-driven tissue homogenizer (TGrinder, Tiangen, Beijing, China) on ice, then centrifugation at $14000 \times g$ for 20 min at 4°C . The supernatant was quantified for protein concentration using the BCA assay kit (Tiangen, Beijing, China). For CarE activity assay, 100 μL of diluted supernatant and 500 μL of 30 mM α -naphthyl acetate (substrate) were mixed and incubated for 30 min at 30°C kept in darkness, then a quantity of 100 μL of color developing agent (5% SDS: 1% fast blue B = 5: 2) was added. After incubation at 30°C for an additional 30 min, the optical density at 600 nm (OD_{600}) was measured using a microplate reader (Eon, BioTek, Winooski, VT, USA). Wells in the absence of any enzyme were used as controls. The specific activity of CarE was calculated on the basis of α -naphthol standard curve and normalized to the protein concentration of enzyme supernatant.

2.4. Identification and sequencing of *NICarE*

Total RNA was extracted from the whole body of three-day-old females using Eastep™ Universal RNA Extraction kit (Promega Corporation, Madison, WI, USA), and RNA concentrations were determined by spectrophotometer Nanodrop2000C (Thermo Fisher Scientific, West Palm Beach, FL, USA). The first-strand cDNA template for PCR was synthesized in a 20 μL reaction containing 1 μg total RNA using PrimeScript RT Reagent kit with gDNA Eraser (TaKaRa, Tokyo, Japan). A partial cDNA sequence of *NICarE* was identified from the *N. lugens* transcriptome database, and a pair of gene-specific primers (Table 2) were designed to amplify the partial cDNA fragment. PCR was carried out using GoTaq Master Mix (Promega Corporation, Madison, WI, USA) with the following amplification conditions: 95°C of initial denaturation for 2 min; 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min; and a final extension of 72°C for 10 min. Amplified PCR fragments were gel purified using the Gel Extraction kit (Tiangen, Beijing, China), and then subcloned into pGEM-T easy vector (Promega Corporation, Madison, WI, USA) for sequencing (Life Technologies Company, Guangzhou, China). The SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) was used to obtain the full-length cDNA sequence according to the manufacturer's protocol. Specific primers used for 5' RACE and 3' RACE (Table 2) were designed based on cDNA sequences obtained from the PCR products. Two rounds of nested PCR were performed for RACE using gene-specific primer combined with the adaptor primer by initially denaturing the cDNA for 2 min at 95°C , followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; and a final extension of 72°C for 10 min. The overlapping fragments were assembled to generate the full-length *NICarE* sequence after the 5'-end and 3'-end sequences were obtained.

2.5. Developmental and tissue-dependent expression analyses of *NICarE*

To analyze the developmental expression patterns of *NICarE* in *N. lugens*, five instar nymph stages and adults were collected for total RNA extraction. For the tissue-dependent study, five different tissues including head, epidermis, midgut, ovary and fat body were dissected from three-day-old females. RNA isolation and cDNA synthesis were conducted as described earlier. Relative expression levels of *NICarE*

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