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Receptor tyrosine kinase genes respond transcriptionally to sublethal doses of five insecticides by a mode-of-action independent way in *Leptinotarsa decemlineata* (Say)

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ABSTRACT

Sublethal effects of insecticides on insects have been widely documented. However, the underlining mechanisms remain largely unclear. Insecticides may be as ligands and disruptors to affect the expression and function of tyrosine kinase receptors (RTKs). Based on the transcriptome and the genomic data of *Leptinotarsa decemlineata*, 15 novel RTK members were identified and annotated in the present paper. These RTKs showed distinct 1:1 orthology relationships with *Tribolium castaneum* ones, suggesting functional conservation of RTKs in insects. The expression responses of the 16 RTKs to sublethal exposure to two juvenile hormone analogs (methoprene and pyriproxyfen) and three γ-aminobutyric acid receptor blockers (fipronil, butene-fipronil and endosulfan) were determined. Each of the 5 insecticides changed the expression levels of a specific subset of RTK genes, demonstrating that RTK genes respond transcriptionally to insecticides by a mode-of-action independent way. Our results raise the possibilities that insecticides may exert their sublethal effects through affecting the expression of RTK genes.

Introduction

Due to degradation by processes such as photolysis and hydrolysis, the dose of insecticide gradually decreases after spraying to a plant. As a result, insect pests are exposed to sublethal doses of insecticides for longer periods than to lethal concentrations (Desneux et al., 2005). Consequently, sublethal effects of insecticides on insect performance, physiology and behavior may strongly affect population dynamics and deserve investigation in detail (Desneux et al., 2007).

Up to now, the sublethal effects on insects have been documented in several classes of insecticides, including organophosphates, pyrethroids, neonicotinoids, biological insecticides, and insect growth regulators (Huang et al., 2016; Peng and Yang, 2016; Wang et al., 2016). In general, sublethal insecticide exposure prolongs insect developmental periods and decreases survival rates. The exposure sometimes reduces adult emergence, longevity, fecundity and fertility (Stark and Banks, 2003; Wang et al., 2016). Some insecticides causes behavioral abnormalities such as a disruption of perceive ability to host plant odors, or a decrease of the olfactory learning ability (Dively et al., 2015; Peng and Yang, 2016). Moreover, a sublethal dose of insecticides increases susceptibility to pathogen infection (Wu et al., 2012). In some cases, however, sublethal effects of insecticides also include an increase of fitness by stimulation of reproduction (Guedes and Cutler, 2014).

Further researches reveal that a sublethal dose of insecticides disrupts hormone signaling, affects immune system, or changes enzymatic activities (Xia et al., 2015; Yang and Zhang, 2015). In spite of these studies, the mechanisms underlining the sublethal effects of insecticides have not been fully uncovered. One possible mechanism is that insecticides may act as chemical ligands and/or disruptors to affect cell communication, which is mediated through the attachment of the ligands to their protein receptors (Ségaliny et al., 2015). Among these receptors, tyrosine kinase receptors (RTKs) comprise a large family that mediate cellular transmembrane communication to regulate critical cellular processes, such as proliferation and differentiation, survival and metabolism, cell migration and cell cycle (Lemmon and Schlessinger, 2010).

Human has 58 RTKs, which fall into 20 subfamilies (Lemmon and Schlessinger, 2010; Ségaliny et al., 2015). *Caenorhabditis elegans* genome contains 40 RTKs, of which only 13 can be classified in 10 out of the 20 human RTK subfamilies (Plowman et al., 1999). In insect species, the RTK genes are fewer. Among the fully sequenced insect genomes, the RTK numbers are 21 in *Drosophila melanogaster*, 12 in *Drosophila mojavensis*, 15 in *Aedes aegypti*, 12 in *Anopheles gambiae*, 14 in *Culex*

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quinquefasciatus, and 16 in *T. castaneum* (Sopko and Perrimon, 2013; Vogel et al., 2013).

In the present paper, according to amino acid sequences of *D. melanogaster* and *T. castaneum* RTKs (Sopko and Perrimon, 2013; Vogel et al., 2013), the *L. decemlineata* official gene set (Baylor College of Medicine Human Genome Sequencing Center) and transcriptome (Shi et al., 2013) data were mined. Subsequent examination and analyses led us to successfully identify and define 16 RTK members in *L. decemlineata*. Furthermore, we determined the expression responses of the 16 RTKs to a sublethal dose of five insecticides and found that these RTK genes responded transcriptionally to insecticides by a mode-of-action independent way.

Materials and methods

Insect rearing

Insects were routinely kept in an insectary as previously described (Shi et al., 2013), and were supplied with potato foliage at vegetative growth or young tuber stages in order to assure sufficient nutrition.

Chemicals

Insecticides included two juvenile hormone analogs methoprene (isopropyl (2E,4E,7S)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate) (Shanghai Kewelchem Company, Shanghai, China) and pyriproxyfen (2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy) pyridine) (Ivy Fine Chemicals Corporation, USA), two phenylpyrazole fipronil (81% powder, Bayer, Leverkusen, Germany) and butene-fipronil (90.0%, Daliang Ruize Agro-chemical Co., Ltd.) and an organochlorine endosulfan (96.0%, Nanjing Red-sun Chemical Co., Ltd., Nanjing, China). They were kept in a refrigerator between the experimental sessions.

Homology searches

The RTKs from *D. melanogaster* and *T. castaneum* were downloaded from the National Centre for Biotechnology Information (NCBI). Those protein sequences were used for TBLASTN (e-value < 0.00001) searches of *L. decemlineata* official gene set (Baylor College of Medicine Human Genome Sequencing Center) and transcriptome data (Shi et al., 2013), respectively. Each hit was further screened using BLASTX (e-value < 0.00001) against the non-redundant database at the NCBI to confirm its identity with other insect RTK genes. The resultant sequences were considered as *L. decemlineata* RTK candidates.

Molecular cloning and phylogenetic analysis

The sequence correctness of *L. decemlineata* RTK candidates was substantiated by polymerase chain reaction (PCR) using primers in Table 1. For the RTK fragments, 5′- or 3′-RACE was performed to obtain 5′- and 3′- untranslated region (primers listed in Table 1), with SMARTer RACE cDNA amplification kit (Takara Bio., Dalian, China). Thermal cycling conditions for 5′-RACE and the first-round PCR of the 3′-RACE were 94 °C for 3 min; followed by five cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min; and another five cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, then followed by 25 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 2 min. The last cycle was followed by a final extension at 72 °C for 10 min. The product from the first-round PCR of the 3′-RACE was then diluted 100 times, and 1 μL diluted product was used as the template to perform the second-round PCR using 3′-NGSP and nested universal primer A (NUP). Thermal cycling conditions of the second-round PCR were 94 °C for 3 min; followed by five cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min, then followed by 25 cycles of 94 °C for 30 s, 65 °C for 30 s and 72C for 2 min. The last cycle was followed by a final extension at 72 °C for 10 min.

For the complete RTK sequences, the complete open reading frame (ORF) was verified using primers in Table 1. The resulting sequences were submitted to GenBank.

The annotated RTKs from *T. castaneum* and *D. melanogaster* were respectively aligned with the predicted *L. decemlineata* RTKs using ClustalW v.2.1. The alignments were used to construct the maximum-likelihood (ML) trees using RAxML v.8 (Stamatakis, 2014) to select the best-fitting model (LG + γ, with estimated frequencies) after estimation by ProtTest 3.2.1 (Darriba et al., 2011). The reliability of ML tree topology was evaluated by bootstrapping a sample of 1000 replicates.

Insecticide treatment

In order to find the inducible RTK transcripts by methoprene and pyriproxyfen, ten newly-eclosed fourth-instar larvae were fed for 1 day on foliage immersed with water (control), 100 ng/mL methoprene, or 100 ng/mL pyriproxyfen. Each treatment was replicated three times.

A topical application was used to apply fipronil, butene-fipronil or endosulfan to *L. decemlineata* fourth instar larvae. Insecticide was dissolved in analytical-grade acetone. Larvae were treated individually with 0.22 μL of insecticide solution (0.024 μg/μL cyhalothrin, 0.0073 μg/μL fipronil, 0.0081 μg/μL endosulfan), respectively, a dose approximately equivalent to the estimated LD_{20} value. The insecticide solution was applied to the dorsal abdominal segment using a 10-μL microsyringe connected to a microapplicator (Hamilton Company, Reno, NV). After treatment, 10 insects were placed in Petri dishes (9 cm in diameter and 1.5 cm in height) containing fresh potato leaves. Both control and treatment were repeated three times and fed for 24 h.

Real-time quantitative PCR (qRT-PCR)

After the newly-ecdysed fourth-instar larvae were exposed to insecticide for 24 h, total RNA samples from insecticide-treated and control larvae were prepared from whole bodies of the survivors, using SV Total RNA Isolation System Kit (Promega). Each sample contained 5–8 larvae and repeated in biological triplicate. Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer's instruction. The mRNA abundance of selected RTK genes was estimated by qRT-PCR using SYBR Premix Ex TaqTM (Perfect Real Time) (Takara Bio Inc., Dalian, China) and ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction, using the primer pairs listed in Table 2. A reverse transcription negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. Each sample was repeated in technical triplicate. The reaction mixture, PCR protocol and four internal controls (*LdRP4*, *LdRP18*, *LdARF1* and *LdARF4*, the primers listed in Table 1) were the same as previously described (Shi et al., 2013).

All methods and data were confirmed to follow the MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines (Bustin et al., 2009). The generation of specific PCR products was confirmed by gel electrophoresis. The primer pair for each gene was tested with a 10-fold logarithmic dilution of a cDNA mixture to generate a linear standard curve (crossing point [CP] plotted vs. log of template concentration), which was used to calculate the primer pair efficiency. Data were analyzed by the $2^{-\Delta\Delta CT}$ method, using the geometric mean of internal control genes (Shi et al., 2013) for normalization. All primer pairs amplified a single PCR product with the expected sizes, showed a slope less than −3.0, and exhibited efficiency values ranging from 2.0 to 2.1.