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Transcriptional response of Methoprene-tolerant (Met) gene to three insect growth disruptors in *Leptinotarsa decemlineata* (Say)

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ABSTRACT

Some insect growth disruptors (IGDs), such as pyriproxyfen and halofenozide, may be used to control *Leptinotarsa decemlineata*. However, their mechanism of action remains elusive. Methoprene-tolerant (Met) mediates juvenile hormone (JH) signal to control numerous essential physiological processes. In the present paper, we identified a *Met* gene (*LdMet*). *Ld*Met protein was a typical basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) transcription factor with a bHLH domain, two PAS domains (PAS-A and PAS-B) and a region called PAS associated C terminal (PAC). Eight conserved amino acids critical for JH binding were located in PAS-B and PAC domains. The temporal expression pattern of *LdMet* was in accordance with the variation of circulating JH titers. Feeding of juvenoid methoprene or pyriproxyfen, or provide for JH dose-dependently stimulated the expression of *LdMet*. RNA interference-mediated knockdown of two JH degradation genes increased the transcription of *LdMet*, while silencing of a JH biosynthesis gene repressed the transcription. Conversely, ingestion of an ecdysteroid agonist halofenozide or 20-hydroxyecdysone (20E) reduced the mRNA levels of *LdMet*, in a dose-dependent manner, whereas knockdown of either ecdysteroidogenesis or 20E signaling genes increased the mRNA accumulation. Providing that the expression of *LdMet* can be disturbed by methoprene, pyriproxyfen and halofenozide, *LdMet* may be a potential target of these IGDs in L. *decemlineata* larvae.

Introduction

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is a notorious defoliator of potato in most potato-growing areas of the world. Chemical control inevitably leads to the development of insecticide resistance in this beetle (Alyokhin, 2009; Alyokhin et al., 2008; Cingel et al., 2016; Jiang et al., 2011; Jiang et al., 2012; Jiang et al., 2010; Lu et al., 2011; Shi et al., 2012). The use of insect growth disruptors (IGDs) may represent an alternative method. Some IGDs such as juvenoids (for instance hydroprene, methoprene and pyriproxyfen) and non-steroidal bisacylhydrazine compounds (for example halofenozide, tebufenozide, methoxyfenozide, chromafenozide, fufenozide) can disturb juvenile hormone (JH) signal (Pener and Dhadialla, 2012; Ramaseshadri et al., 2012; Smagghe et al., 2012). As a result, these IGDs can affect numerous essential physiological processes regulated by JH, such as molting, metamorphosis, reproduction, diapause, and even behavior (Wang et al., 2017; Zhu and Noriega, 2016; Zhu et al., 2017).

Among these IGDs, methoprene is a synthetic JH analog. In *Drosophila melanogaster*, mutation in Methoprene-tolerant (Met) causes around 100 times more resistant to methoprene, as well as JH III (Ashok et al., 1998; Wilson and Fabian, 1986). Further researches verify that Met is the intracellular nuclear receptor of JH in insects (Charles et al., 2011; Jindra et al., 2015). It belongs to a basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family (Ashok et al., 1998; Furness et al., 2007). A bHLH-PAS nuclear receptor contains several relatively well-conserved domains. The bHLH is typically responsible for DNA binding and dimerization; PAS-A and PAS-B are crucial for selecting dimerization partner and ensuring the specificity of target gene activation (Zelzer et al., 1997); and the PAS associated C terminal (PAC) fragment is usually responsible for the regulation of protein complex activity (Kewley et al., 2004).

In the absence of JH, Met is present as an inactive homodimer. Upon JH binding to the PAS-B domain, Met undergoes a conformational change that liberates it from the homodimer complex and allows it to bind a new partner, Taiman (Tai) (Li et al., 2014; Lozano et al., 2014). Met/Tai heterodimer then binds to an E box-like sequence (5′-CACGTG-3′) in the promoter region of the JH response genes, and activates their transcription to transduce JH signals in both holometabolans and hemimetabolans (Li et al., 2014; Lozano and Belles, 2014; Lozano et al., 2014).

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As a synthetic JH analog, methoprene may bind to Met, triggers continuous potent JH signal to disturb larval development (Ashok et al., 1998; Wilson and Fabian, 1986). To date, however, the mode of action of most other IGDs remains elusive. In L. *decemlineata*, pyriproxyfen exposure delays digging behavior, induces severe pupal malformations and causes the failure of adult emergence (Koopmanschap et al., 1989). Moreover, halofenozide ingestion causes larval lethality (Smagghe et al., 1999). Do the two IGDs disturb the expression of *Met* gene to exert their toxicities in *Leptinotarsa*? In the present paper, we first identified *LdMet*, and then carried out two experiments to confirm that methoprene, pyriproxyfen and halofenozide played a disruptive role in the transcription of *LdMet*. It appears that *LdMet* is a potential target of these IGDs in L. *decemlineata*.

Materials and methods

Insect rearing and chemical

The L. *decemlineata* beetles were kept in an insectary according to a previously described method (Shi et al., 2013), with potato foliage at the vegetative growth or young tuber stages in order to assure sufficient nutrition. At this feeding protocol, the larvae progressed through the first, second, third, and fourth instars at an approximate period of 2, 2, 2 and 4 days, respectively.

Ecdysteroid 20-hydroxyecdysone (20E) (Sigma-Aldrich, USA), an ecdysteroid agonist halofenozide (Hal) (ChemService, West Chester, USA), juvenile hormone (JH) (Sigma-Aldrich, USA), and two juvenoids methoprene (Shanghai Kewelchem Company, Shanghai, China) and pyriproxyfen (Ivy Fine Chemicals Corporation, USA) were purified by a reverse phase high performance liquid chromatography or a high performance liquid chromatography before experiments.

Molecular cloning and phylogenetic analysis

In a previous study, we obtained *LdMet* cDNA (Fu et al., 2015b). To verify the complete open reading frame, total RNA was extracted from the fourth larval instars and first-strand cDNA was synthesized using the reverse transcriptase (M-MLV RT) (Takara Bio., Dalian, China) and an oligo (dT) 18 primer. Polymerase chain reaction (PCR) was performed with a pair of primers shown in Table 1. The full-length *LdMet* cDNA was available in GenBank (accession number KP147911).

Thirteen Met/the germ cell-expressed (Gce) proteins from six holometamorphosis insect species, four hemimetamorphosis insects and one crustacea species were obtained from NCBI. Among these proteins two paralogs were found in *D. melanogaster* (*DmMet* and *DmGce*), *Bombyx mori* (*BmMet1* and *BmMet2*) and *Danaus plexippus* (*DpMet1 and DpMet2*). These proteins were respectively aligned with *LdMet* protein using ClustalW 2.1 (Larkin et al., 2007). A neighbor-joining (NJ) tree of the 14 Met/Gce proteins was constructed using MEGA 6 (Tamura et al., 2013) by the Poisson correction method. The reliability of NJ tree topology was evaluated by bootstrapping a sample of 1000 replicates.

Preparation of dsRNAs

Specific primers used to clone the fragments of dsRNAs were listed in Table 1. These dsRNAs were individually expressed using *Escherichia coli* HT115 (DE3) competent cells lacking RNase III following the established method (Zhou et al., 2013). Individual colonies were inoculated, and grown until cultures reached an OD600 value of 1.0. The colonies were then induced to express dsRNA by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.1 mM. The expressed dsRNA was extracted and confirmed by electrophoresis on 1% agarose gel (data not shown). Bacteria cells were centrifuged at 5000 × *g* for 10 min, and resuspended in an equal original culture volume of 0.05 M phosphate buffered saline (PBS, pH 7.4). The bacterial solutions (at a dsRNA concentration of about 0.5 μg/mL) were used for experiment.

Dietary introduction of dsRNA

The same method as previously reported (Zhou et al., 2013) was used to introduce dsRNA into larvae. Potato leaves were immersed with a bacterial suspension containing a dsRNA for 5 s, removed, and dried for 2 h under airflow on filter paper. The PBS- and ds*egfp* (*enhanced green fluorescent protein*)-dipped leaves were used as controls. Five treated leaves were then placed in Petri dishes (9 cm diameter and 1.5 cm height). The newly-ecdysed third-instar larvae were starved for at least 4 h prior to the experiment. Then, ten larvae were transferred to each dish as a repeat. For each treatment, three repeats were set. The larvae were allowed to feed treated foliage for 3 days (replaced with freshly treated ones each day).

Influence of insect growth disruptors and hormones on the expression of LdMet

Chemicals were solved in distilled water with added surfactant (Tween 20, 1 g/L) to give stock solutions of 100 ng/mL. They were further serially diluted ten folds with distilled water before bioassay.

A leaf-dip method (Shi et al., 2012) was adopted to carry out two independent bioassays as previously described (Liu et al., 2014) using newly-ecdysed fourth-instar larvae. The first bioassay was to determine the influences of methoprene, pyriproxyfen and JH on the expression of *LdMet*. Potato leaves were dipped with water (control), 10 and 100 ng/mL methoprene; 10 and 100 ng/mL pyriproxyfen; or 10 and 100 ng/mL JH. The second was to test the influence of Hal and 20E on the transcription of *LdMet*. Potato foliage was immersed with water (control), 10 and 100 ng/mL Hal; or 10 and 100 ng/mL 20E. For each treatment, three repeats were set. The larvae were allowed to feed the foliage for a day. The resultant larvae were used for real-time quantitative PCR (qRT-PCR).

For each bioassay, three biological replicates were carried out.

Real-time quantitative PCR (qRT-PCR)

For temporal expression analysis, RNA templates were derived from eggs, the larvae from the first through fourth instars, the prepupae (PP, 1 to 4 days after burring into soil) and the pupae (P, 7 days after burring into soil). For analysis of the effects of treatments, total RNA was extracted from treated larvae. Each sample contained 5–10 individuals and repeated three times. The RNA was extracted using SV Total RNA Isolation System Kit (Promega). Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer's instructions. Quantitative mRNA measurements were performed by qRT-PCR in technical triplicate, using 4 internal control genes (*LdRP4*, *LdRP18*, *LdARF1* and *LdARF4*, the primers listed in Table 1) according to our published results (Shi et al., 2013). An RT 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.

According to a previously described method (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. 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. Data were analyzed by the $2^{-\Delta\Delta CT}$ method, using the geometric mean of the four internal control genes for normalization.