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Leptinotarsa cap 'n' collar isoform C/Kelch-like ECH associated protein 1 signaling is critical for the regulation of ecdysteroidogenesis in the larvae

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ABSTRACT

Drosophila cap 'n' collar isoform C (CncC) and Kelch-like ECH associated protein 1 (Keap1) regulate metamorphosis by transcriptional control of a subset of genes involved in ecdysteroidogenesis, 20-hydroxyecdysone (20E) signaling, and juvenile hormone (JH) degradation. In the present paper, we found that prothoracicotropic hormone signal was required for the activation of *LdCncC* and *LdKeap1* in *Leptinotarsa decemlineata*. Moreover, RNA interference of *LdCncC* or *LdKeap1* in the fourth-instar larvae delayed development. As a result, the treated larvae obtained heavier larval and pupal fresh weights and had larger body sizes than the controls. Furthermore, knockdown of *LdCncC* or *LdKeap1* significantly reduced the mRNA levels of four ecdysone biosynthetic genes (*Ldspo*, *Ldphm*, *Lddib* and *Ldsad*), lowered 20E titer and decreased the transcript levels of five 20E response genes (*LdEcR*, *LdUSP*, *LdE75*, *LdHR3* and *LdFTZ-F1*). However, the expression of two JH epoxide hydrolase genes and JH contents were not affected in the *LdCncC* and *LdKeap1* RNAi larvae. Dietary supplementation with 20E shortened the developmental period to normal length, rescued the larval and pupal body mass rises, and recovered or even over-compensated the expression levels of the five 20E response genes in either *LdCncC* or *LdKeap1* RNAi hypomorphs. Therefore, *Ld*CncC/*Ld*Keap1 signaling regulates several ecdysteroidogenesis genes, and consequently 20E pulse, to modulate the onset of metamorphosis in *L. decemlineata*.

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1. Introduction

In insects, the combination of a high titer of 20-hydroxyecdysone (20E) and a low level of juvenile hormone (JH) triggers larval-pupal metamorphosis. Ecdysone is synthesized in insect prothoracic glands (PGs) from cholesterol, under the catalyzation of a series of cytochrome P450 monooxygenases (CYPs) encoded by Halloween genes such as *spook* (*spo*), *phantom* (*phm*), *disembodied* (*dib*) and *shadow* (*sad*). Ecdysone is then released from PGs into hemolymph. It is transported to peripheral tissues, and is converted to 20E by another CYP, the product of a Halloween gene *shade* (*shd*) (Iga and Kataoka, 2012; Niwa and Niwa, 2014). The expression of these Halloween genes and consequently the timing of pupation are regulated by prothoracicotropic hormone (PTTH)-Torso receptor-mitogen activated protein kinase (MAPK) pathway (consisting of four core components Ras, Raf, MEK and ERK) (McBrayer et al., 2007; Rewitz et al., 2009).

A timely decrease in JH is also crucial for metamorphosis. JH is degraded mainly by two hydrolases, JH epoxide hydrolase (JHEH, EC 3.3.2.3) and JH esterase (JHE, EC 3.1.1.1) (Gu et al., 2015; Lü et al., 2015). JHEH falls into the microsomal epoxide hydrolase family (Arand et al., 2005; Morisseau and Hammock, 2005), and JHE belongs to the carboxylesterase family (Share and Roe, 1988).

In *Drosophila melanogaster*, cap 'n' collar isoform C (CncC) and Kelch-like ECH associated protein 1 (Keap1), the homologs of mammalian nuclear factor erythroid 2 related factor 2 (Nrf2) and Keap1, act as transcription activators of a subset of Halloween and JH hydrolyzation genes (Deng, 2014; Deng and Kerppola, 2013,

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2014). Up to now, CncC and Keap1 homologs have been found in other insect species in both holometabolans and hemimetabolans (Deng and Kerppola, 2013; Grimberg et al., 2011; Kalsi and Palli, 2015; Karim et al., 2015; Misra et al., 2011, 2013; Peng et al., 2016; Sykiotis and Bohmann, 2008). An interesting question then arises: are CncC and Keap1 the conserved transcription activators of both ecdysteroidogenesis and JH hydrolyzation genes among insect species?

Upon biosynthesis and release, 20E, acting through its cognate receptor, a dimer of ecdysone receptor (EcR)/ultraspiracle (USP), triggers a conserved transcriptional cascade including early genes such as *Broad-Complex* (*BR-C*), *Ecdysone-induced protein 75* (E75) and *E74*, early-late genes such as *hormone receptor 3* (*HR3*), and late genes such as *Fushi tarazu factor 1* (*FTZ-F1*), to stimulate metamorphosis (Iga and Kataoka, 2012; Luan et al., 2013). In *Drosophila*, CncC/Keap1 signaling has been proven to activate several early ecdysone-response genes in the salivary glands (Deng, 2014; Deng and Kerppola, 2013, 2014). Is CncC/Keap1 signaling involved in the activation of these early ecdysone-response genes in other insects?

The Colorado potato beetle *Leptinotarsa decemlineata* (Say) has a robust RNA interference (RNAi) response to double stranded RNAs (dsRNAs) (Guo et al., 2015, 2016; Kong et al., 2014; Liu et al., 2014; Shi et al., 2016a, 2016b, 2016c; Zhu et al., 2015). Using *in vivo* RNAi, we previously demonstrated that the ecdysteroidogenesis and 20E signaling genes were conserved in *L. decemlineata* (Guo et al., 2015, 2016; Kong et al., 2014; Liu et al., 2014; Zhu et al., 2015). In the work presented here, we knocked down either *LdCncC* or *LdKeap1* to study its roles in the larval-pupal metamorphosis in *L. decemlineata*.

2. Materials and methods

2.1. Experimental animal

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. Upon reaching full size, the fourth-instar larvae stopped feeding, dropped to the ground, burrowed to the soil and entered the prepupae stage. The prepupae spent an approximately 3 days to pupate. The pupae lasted about 5 days and the adults emerged.

2.2. Molecular cloning

The putative *LdCncC* and *LdKeap1* isoforms were obtained from the genome (https://www.hgsc.bcm.edu/arthropods/colorado-potato-beetle-genome-project) and transcriptome data (Shi et al., 2013). The correctness of the sequences was substantiated by polymerase chain reaction (PCR) using primers in Table S1. The full-length cDNAs were obtained by 5'- and/or 3′-RACE, using SMARTer RACE kit (Takara Bio.), with specific primers listed in Table S1. After obtaining full-length cDNAs, primer pairs (Table S1) were designed to verify the complete open reading frames. All of the sequenced cDNAs were submitted to GenBank (accession numbers: *LdCncA*, KY458169; *LdCncB*, KY458170; *LdCncC1*, KY458171; *LdCncC2*, KY458172; *LdKeap1A*, KY458173; *LdKeap1B*, KY458174).

2.3. Preparation of dsRNAs

The same method as previously described (Zhou et al., 2013) was used to express ds*PTTH* (214 bp), ds*Torso* (302 bp), ds*Ras* (327 bp), ds*phm* (345 bp), ds*shd* (438 bp), ds*EcR* (344 bp), ds*E75* (361 bp), ds*CncC*-1 (200 bp), ds*CncC*-2 (411 bp), ds*Keap1*-1 (373 bp), ds*Keap1*-2 (302 bp) and ds*egfp* (a 414 bp fragment of enhanced green fluorescent protein gene). The twelve dsRNAs were individually transcribed with specific primers in Table S1, using *Escherichia coli* HT115 (DE3) competent cells lacking RNase III. 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.

2.4. Dietary introduction of dsRNA

The same method as previously reported (Fu et al., 2015) was used to introduce dsRNA into larvae. The newly-ecdysed fourth-instar larvae were allowed to feed foliage immersed with bacterial suspension containing ds*PTTH*, ds*Torso*, ds*Ras*, ds*phm*, ds*shd*, ds*EcR*, ds*E75*, or each of the two dsRNAs of *LdCncC* (ds*CncC*-1 and ds*CncC*-2) and *LdKeap1* (ds*Keap1*-1 and ds*Keap1*-2) for 3 days (replaced with freshly treated ones each day). The PBS- and ds*egfp*-dipped foliage were used as controls. The larvae were then transferred to untreated foliage if necessary.

The beetles were weighed twice during trial period. The adult emergence was recorded during a 2-week trial period. The samples on day 3 after the initiation of the experiments were collected. The effects of gene silencing, the levels of five Halloween genes (*Ldspo*, *Ldphm*, *Lddib*, *Ldsad*, and *Ldshd*), five 20E response genes (*LdEcR*, *LdUSP*, *LdE75*, *LdHR3* and *LdFTZ-F1*), a total of 14 *LdGST* transcripts and two *Jheh* genes, and 20E and JH titers were determined.

To test the rescuing effects of 20E in larval development, two additional experiments were performed using foliage dipped with ds*CncC*-1, ds*CncC*-1+10^{-6} M 20E, or ds*Keap1*-1, ds*Keap1*-1+10^{-6} M 20E, a concentration used in our previous research (Guo et al., 2016). The PBS- and ds*egfp*-immersed leaves were used as controls.

For above experiments, three biological replicates were carried out.

2.5. Real-time quantitative PCR (qRT-PCR)

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 internal control genes (the primers listed in Table S1) 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. Data were analyzed by the $2^{-\Delta\Delta CT}$ method, using the geometric mean of internal control genes for normalization. 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).

2.6. Quantitative determination of 20E and JH

20E was extracted according to a ultrasonic-assisted extraction method (Liu et al., 2014), and its titer (ng per g body weight) was analyzed by a liquid chromatography tandem mass spectrometry-mass spectrometry (LC-MS/MS) system using a protocol the same as described (Zhou et al., 2011).

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