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Research paper

Juvenile hormone regulates the differential expression of putative *juvenile hormone esterases* via *methoprene-tolerant* in non-diapause-destined and diapause-destined adult female beetle

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ABSTRACT

Juvenile hormone (JH) plays an essential role in regulating molting, metamorphosis, reproduction, and diapause (dormancy), in many insects and crustaceans. JH esterases (JHEs) can control JH titer by regulating JH degradation. Although the biochemistry and structure of JHEs have been well studied, regulation of their expression remains unclear. We identified three putative *JHEs* (*JHE1*, *JHE2*, *JHE3*) in the cabbage beetle *Colaphellus bowringi*, and investigated the regulation of their expression by JH signaling in non-diapause-destined (NDD, reproductive) and diapause-destined (DD) female adults. Sequence and phylogenetic tree analyses indicate that the three putative JHEs shared conserved motifs with the JHEs of other insects and one crustacean, and were similar to Coleopteran, Dipteran, Orthopteran, Hymenopteran, and Decapodan JHEs. They were, however, less closely related to Hemipteran and Lepidopteran JHEs. *JHEs* were more highly expressed in NDD female adults than in DD female adults. JH analog induction in DD female adults significantly upregulated the expression of *JHE1* and *JHE2*, but had no effect on the expression of *JHE3*. Knockdown of the JH candidate receptor *methoprene-tolerant* (*Met*) in NDD female adults downregulated the expression of all three *JHEs*. These results suggest that *JHE* expression is positively correlated with JH signaling, and that *Met* may be involved in the JH-mediated differential expression of *JHE* in DD and NDD adult female *C. bowringi*.

1. Introduction

In many insects and crustaceans, juvenile hormone (JH) is considered one of the most important hormones regulating molting, metamorphosis, reproduction, diapause, and even behavior (Chang, 1993; Giray et al., 2005; Jindra et al., 2013; Denlinger and Armbruster, 2014). Accurate regulation of JH levels is therefore critical and achieved by biosynthesis and degradation (De Kort and Granger, 1996; Belles et al., 2005). Previous studies in insects have demonstrated that a specific carboxylesterase, JH esterase (JHE), can downregulate JH titer by converting active JH (JH III) to inactive JH acid and JH acid diol (Kamita and Hammock, 2010). Although crustaceans do not produce JH III, they use the JH III precursor, methyl farnesoate, to regulate development. It has been suggested that JHE may also degrade crustacean JH by hydrolyzing methyl farnesoate into farnesoic acid (Homola and Chang, 1997; Lee et al., 2011; Sin et al., 2015). The conserved function of JHE suggests that it has a similarly conserved structure among arthropods (Lee et al., 2011; Sin et al., 2015), but this requires confirmation.

Site-directed mutagenesis, biochemistry analysis, and multiple sequence alignment shows that the JHEs of insects have five conserved functional motifs; RF, DQ, GQSAG, E, and GxxHxxD/E (Kamita and Hammock, 2010). Moreover, because JHE generally plays a role in hemolymph (Vince and Gilbert, 1977; Kamita and Hammock, 2010), the presence of the N-terminal signal peptide is another diagnostic indicator of JHE. The structure of JHE has been well studied, at least in insects. However, how JHE expression is regulated remains unclear. Previous studies of *Drosophila melanogaster* (Kethidi et al., 2005), the cabbage looper *Trichoplusia ni* (Venkataraman et al., 1994; Jones et al., 1998), and the diamondback moth *Plutella xylostella* (Duan et al., 2016) found that either JH, or JH analog (JHA), could induce *JHE* expression, but the regulatory mechanism responsible for this was not determined. Recent research on JH signaling has demonstrated that the bHLH-PAS transcription factor methoprene-tolerant (Met) is the intracellular

Abbreviations: DD, diapause-destined; dsRNA, double-stranded RNA; JH, Juvenile hormone; JHA, JH analog; JHE, JH esterase; LD, long-day; Met, methoprene-tolerant; NDD, non-diapause-destined; RT-qPCR, reverse transcription-quantitative PCR; RNAi, RNA interference; SD, short-day

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Table 1
Primers for gene cloning, RT-qPCR, and RNAi.

Genes	Forward primers (5′–3′)	Reverse primers (5′–3′)	PCR efficiency (%)	Standard curve R^2
Gene cloning				
JHE1	accgccagatagtgactgac	caataattactagtgaggta	n.a.	n.a.
JHE2	cttgtttgaatcatgttgct	tctgcgattagttttatttc	n.a.	n.a.
JHE3	caatgcttccgaagaatcta	tttctatttattctcaaatagg	n.a.	n.a.
qRT-PCR				
RPL19	gtaatgcgatgcggcaagaa	aaacctgtagcggtgcactc	102.2	0.998
JHE1	ggtagtgccagcatgacgaa	gctttgcaatccgtttgtcg	109	0.978
JHE2	cctgccagtgacattttggc	tcatttggcgcaactttcgg	99.9	0.997
JHE3	gtccgcatttgttcccgttt	tgccagatcttctacgtgcc	109.2	0.991
Met	caattgctcaacacccagcc	ccttcgttgagcgacagtct	98.2	0.998
RNAi				
Met	gcgtaatacgactcactataggatgattgaggaagtgtcggg	gcgtaatacgactcactatagggattctcgtggtggaccagt	n.a.	n.a.
GFP	gcgtaatacgactcactataggtggtcccaattctcgtggaac	gcgtaatacgactcactataggcttgaagttgaccttgatgcc	n.a.	n.a.

Note: n.a., not applied.

nuclear receptor of JH in many insects (Charles et al., 2011; Jindra et al., 2015). This new finding prompted us to investigate whether Met is involved in the regulation of JH-mediated JHE expression. High JHE expression is considered vital for the photoperiodic downregulation of JH titer in some insects (Kramer and De Kort, 1976; Vermunt et al., 1999; Ishikawa et al., 2012), but whether this true of insects in general is not clear.

The beetle *Colaphellus bowringi* is a serious pest of cruciferous vegetables in Asia. At 25 °C under short-day (SD) conditions, adult female *C. bowringi* prepare for reproduction and do not enter diapause, which is arrested reproduction. Such females are therefore termed non-diapause-destined (NDD), or reproductive, females. However, at 25 °C under long-day (LD) conditions, adult females enter reproductive diapause and are consequently termed diapause-destined (DD) females (Xue et al., 2002; Wang et al., 2004). JH stimulates NDD females to express vitellogenin and complete ovarian development within 4 days of eclosion, and the absence of JH allows DD females to complete preparation for diapause within the same period (Liu et al., 2016; Tan et al., 2016). Therefore, the JH levels of NDD and DD females would be expected to differ significantly. Using *C. bowringi* as a model insect, we identified three putative *JHEs* in this species and investigated how their expression was regulated by JH-Met signaling in NDD and DD females. The results suggest that *JHE* expression is positively correlated with JH signaling in both NDD and DD females, and that *Met* may be involved in JH-induced *JHE* expression in *C. bowringi*. This suggests that JH-Met signaling regulates the differential expression of *JHEs* in DD and NDD adult female *C. bowringi*.

2. Materials and methods

2.1. Insect rearing

The founders of our laboratory colony of *C. bowringi* were collected as larvae in Xiushui County (29°10N, 114°40E), Jiangxi Province, China and fed on radish *Raphanus sativus* L. var. *longipinnatus* (Brassicales: Brassicaceae) (Tan et al., 2015). Offspring of this population were maintained in our lab and used for this study. NDD female adults were produced by keeping larvae at 25 °C under a 12:12 h light:dark photoperiod (SD), and DD female adults were produced by keeping larvae at 25 °C under a 16:8 h light:dark photoperiod (LD).

2.2. cDNA cloning and sequence analysis

Based on the gene annotation of our *C. bowringi* transcriptome (Tan et al., 2015), we found three unigenes that could be putative *JHEs*. These three genes were amplified via PCR with corresponding primers, and inserted into the pMD™-18 T Vector (TaKaRa, Japan) for sequencing. Predicted amino acid sequences were deduced with the ExPASy Translate tool (http://web.expasy.org/translate/) and the sequences were aligned and compared with JHE sequences from other insect taxa and a shrimp using MEGA 4.1 software (Fig. S1). Conserved JHE motifs were identified by comparing the results with previous studies (Ward et al., 1992; Kamita and Hammock, 2010). The sequence identity and similarity of the three putative *C. bowringi* JHEs with those from different insect taxa and the shrimp were determined by pairwise sequence alignment using EMBOSS Matcher (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). A rooted phylogenetic tree was constructed using the neighbor-joining method in MEGA 4.1 software with the carboxylesterase 4A-like protein of *Tribolium castaneum* as the outgroup. The cDNA sequences of the three putative JHEs of *C. bowringi* are available from GenBank (*JHE1*, KY229689; *JHE2*, KY229690; *JHE3*, KY229691).

2.3. mRNA expression analysis

Reverse transcription-quantitative PCR (RT-qPCR) was used to determine the mRNA abundance in this study. We performed RT-qPCR following the Minimum Information for publication of Quantitative real time PCR Experiments (MIQE) guidelines (Bustin et al., 2010) and our previous protocol (Liu et al., 2016). Because JHE generally is high expressed in the fat body (similar to vertebrate liver) (Vermunt et al., 1999), we investigated the regulation of its expression by using this tissue in this study. Briefly, total RNA was extracted from the fat body of a pool of 15 female adults using RNAiso Plus (TaKaRa, Dalian, China). One μg of RNA was treated with DNAase to remove residual genomic DNA and then used for first-strand cDNA synthesis using the PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. One μL of a 20-fold diluted cDNA solution was subjected to RT-qPCR reactions with corresponding primers (Table 1) and SYBR® Premix ExTaq™ II (TaKaRa, Japan) using a Bio-Rad Detection iQ2 System. Based on the evaluation of reference genes in *C. bowringi* (Tan et al., 2015), *ribosomal protein L19* (*RPL19*) was used as the reference gene to normalize the *JHEs* expression. Relative expression was analyzed by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008) based on three independent biological replicates and three technical replicates.

2.4. JHA induction in DD female adults

DD females were treated with JHA methoprene as per our previous protocol (Liu et al., 2016). Briefly, 15 μg of methoprene in 200 nL was injected into newly emerged DD female adults. The control group was a similar number of females that were injected with the same amount of acetone. Fat bodies were collected from females for RT-qPCR analyses