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Juvenile hormone (JH) esterase activity but not JH epoxide hydrolase activity is downregulated in larval *Adoxophyes honmai* following nucleopolyhedroviruses infection

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ABSTRACT

Juvenile hormones (JHs) and ecdysteroids are critical insect developmental hormones. JH esterase (JHE) and JH epoxide hydrolase (JHEH) are JH-selective enzymes that metabolize JH and thus regulate the titer of JH. Baculoviruses are known to alter host endocrine regulation. The nucleopolyhedroviruses, AdhoNPV and AdorNPV, are known to have slow and fast killing activity against *Adoxophyes honmai* (Lepidoptera: Tortricidae), respectively. Here we found that when penultimate (4th) instar *A. honmai* are inoculated with AdhoNPV or AdorNPV, the mean survival time is 9.7 and 8.2 days, respectively. The larvae molted once but did not pupate. The AdhoNPV- or AdorNPV-infected larvae did not show a dramatic increase in JHE activity as was found in mock-infected larvae, instead they showed a marked decrease in JHE activity. In contrast, both viral infections had no effect on JHEH activity. In order to further characterize the JHE activity, the JHE-coding sequence of *A. honmai* (*ahjhe*) was cloned and confirmed to encode a biologically active JHE. Quantitative real-time PCR analysis of *ahjhe* expression in 4th and 5th instar *A. honmai* revealed that AdhoNPV and AdorNPV are able to reduce *ahjhe* expression levels.

1. Introduction

Insect metamorphosis is predominantly regulated by juvenile hormones (JHs) and ecdysteroids such as 20-hydroxyecdysone. JHs are considered to maintain larval feeding behavior. During the final instar, a dramatic decrease in JH titer and spikes in ecdysone titer are required for the induction of pupation. The dramatic decrease in JH titer results from both a decrease in JH biosynthesis and an increase in its metabolism (Hammock, 1985). JH is metabolized to JH acid or JH diol by two hydrolytic enzymes, JH esterase (JHE) and JH epoxide hydrolase (JHEH), respectively (Kamita and Hammock, 2010; Morisseau and Hammock, 2005).

Normal endocrinological regulation of insect development can be altered following infection by baculoviruses. Baculoviruses are large (genome size from 80 to 180 kbp), arthropod-specific, double-stranded DNA viruses (van Oers, 2011). The family *Baculoviridae* is traditionally composed of two groups, nucleopolyhedrovirus (NPV) and granulovirus (GV). Many baculoviruses carry a gene that encodes an ecdysteroid UDP-glucosyltransferase (EGT), an enzyme that conjugates sugar molecules to ecdysteroids and making them inactive (O'Reilly and Miller, 1989). The result of ecdysteroid inactivation is that the baculovirus-infected host is unable to complete the normal molting process. Baculoviruses (and other insect viruses) have also been shown to alter host development by downregulating JHE activity (Hajós et al., 1999; Nakai et al., 2002, 2004). In the case of the smaller tea tortrix *Adoxophyes honmai* (Lepidoptera: Tortricidae), downregulation of JHE activity has also been observed following infection with an entomopoxvirus (Nakai et al., 2004). Downregulation of JHE activity may keep a relatively high level of JH at the sensitive period to prevent pupal metamorphosis and leads to a *status quo* condition. It is hypothesized that both inactivation of ecdysteroids and maintaining JH titer at *status quo* levels are beneficial to the virus pathogen because the infected insect will likely continue to feed to facilitate the production of viral progeny.

A. honmai is a major pest of tea plants in Japan. The baculovirus *Adoxophyes orana* GV (AdorGV) has been used as a biological

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insecticide to control *A. honmai* in Japanese tea fields. AdorGV was originally found from *A. orana* (Lepidoptera: Tortricidae) and has subsequently been developed into the commercial product Hamaki-Tenteki® (Arysta LifeScience Corp., Tokyo, Japan) and registered as a biopesticide in Japan in 2003 (Kunimi, 2007). Other entomopathogenic viruses that infect *A. honmai* (e.g., *A. honmai* GV (AdhoGV), *A. honmai* NPV (AdhoNPV), and *A. orana* NPV (AdorNPV) from *Baculoviridae*; and *A. honmai* entomopoxvirus (AHEV) from *Entomopoxvirinae*) have been isolated and studied as potential biological insecticides against *A. honmai*. These studies have shown that, except for AdorNPV, these viruses are slow-killing by inhibiting the development to pupal stage and kill the host only during the final instar (Hilton and Winstanley, 2008a; Ishii et al., 2002; Nakai et al., 2004). In contrast, AdorNPV is relatively fast-killing (Takahashi et al., 2008). In this study, we investigate how the slow-killing AdhoNPV and fast-klling AdorNPV differently regulate JH metabolism by inhibiting JHE or JHEH. Furthermore, we identified the JHE-coding sequence of *A. honmai* (*ahjhe*) and investigate the expression levels of *ahjhe* in *A. honmai*.

2. Materials and methods

2.1. Insects and cells

Eggs of *A. honmai* (Lepidoptera: Tortricidae) were obtained from Agro-Kanesho Co. Ltd. (Saitama, Japan) and used to establish a continuous colony that was maintained at 25 °C under a 16 h light: 8 h dark photoperiod. Experiments using *A. honmai* were also performed under these rearing conditions. Larvae were reared on an artificial diet (Silkmate 2S; Nihon Nosan-Kogyo Co. Ltd., Kanagawa, Japan).

Sf-9 (Invitrogen, Carlsbad, CA) and High Five™ (Invitrogen) cells were cultured at 27 °C in ExCell 420 (SAFC Biosciences, Lenexa, KS) medium supplemented with 2.5% fetal bovine serum and serum free ESF921 medium (Expression Systems, Davis, CA), respectively.

2.2. Viruses and viral inoculation

Isolates of NPVs of *A. honmai* (AdhoNPV) (Ishii et al., 2003; Nakai et al., 2003) and *A. orana* (AdorNPV) (Hilton and Winstanley, 2008b) were used in this study. Larval *A. honmai* are permissive to both AdhoNPV and AdorNPV (Takahashi et al., 2008). Larval *A. honmai* were inoculated with AdhoNPV or AdorNPV using a modified droplet feeding method (Kunimi and Fuxa, 1996). Newly molted fourth instar larvae were allowed to feed on a liquid inoculum containing 1.0×10^8 occlusion bodies (OBs)/ml (a > LC_{95} dose), 10% (w/v) sucrose, and 5% (w/v) red food coloring. The same solution without virus was used as a mock inoculum in control experiments.

2.3. Measurement of JHE activity in hemolymph

Hemolymph samples were collected from virus- or mock-infected 4th instar larvae at 24 h intervals and diluted in 100 mM sodium phosphate buffer (pH 8.0). Hemocytes were removed from the diluted hemolymph by centrifugation (10,000×*g*, 1 min, 4 °C) and the supernatant from this centrifugation was diluted again in 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0). The clarified and diluted hemolymph (100 μl) was transferred to a 10 × 75 mm glass test tube and the JHE activity in the diluted hemolymph was determined by a partition assay as described previously (Hammock and Sparks, 1977). To initiate the enzyme reaction 1 μl of JH III substrate, a mixture of racemic, unlabelled JH III (Sigma–Aldrich, St. Louis, MO) and [^{3}H]-labelled JH III (PerkinElmer, Boston, MA), was added to the diluted hemolymph. The final concentration of JH III was 5.0 μM. Following the addition of substrate, the reaction mixture was incubated at 30 °C for 60–300 min so that no more than 20% of the JH III was hydrolyzed during the incubation. The reaction was stopped by the addition of 100 μl of stop solution (methanol:water:ammonium hydroxide, 10:9:1). Then, iso-octane (250 μl) was added to the stopped reaction in order to partition JH III in the organic phase and JH III acid in the aqueous phase. An aliquot (50 μl) of the aqueous phase was transferred to 1 ml of ScintiVerse BD liquid scintillation counting cocktail (Fisher Chemical, Fair Lawn, NJ). A Tri-Carb 2810 TR (PerkinElmer) scintillation counter was used to determine counts per minute (CPM) during a 2 min-long counting cycle. All of the assays were performed using hemolymph that was obtained from three to seven individual larvae.

2.4. Measurement of JHEH and JHE activity in fat body

Fat body tissues were collected in parallel from the same larvae that were used to collect the hemolymph samples (Section 2.3). The mass of the fat body tissues was determined and the tissues were homogenized in 300 μl of 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0). The homogenized tissues were then diluted in 100 mM sodium phosphate buffer containing 0.1 mg/ml BSA (pH 8.0). The diluted fat body homogenate (100 μl) was transferred to a 10 × 75 mm glass test tube and 1 μl of 1 mM OTFP (3-octylthio-1,1,1-trifluropropan-2-one), a JHE-selective inhibitor (Abdel-Aal and Hammock, 1985) or ethanol (EtOH) was added to each sample. The homogenized fat body sample was pre-incubated with the OTFP (or EtOH) at 30 °C for 10 min prior to the addition of JH III substrate as described above. The amount of hydrolysis of the ester and epoxide moieties of JH III was determined as described above. JH III-hydrolytic activity in the presence of OTFP was assumed to result from the activity of JHEH, whereas JHE activity was calculated by subtracting JHEH activity from the total JH III-hydrolytic activity in the absence of OTFP. All of the assays were performed using fat body homogenates prepared from four to nine individual larvae.

2.5. RNA isolation and cDNA synthesis

Total RNA was extracted from the fat body tissues of virus- or mock-infected *A. honmai* using ISOGEN (Nippon Gene, Tokyo, Japan). The total RNA samples were treated with RNase-free, recombinant DNase I (TaKaRa, Shiga, Japan) in order to remove contaminating chromosomal DNAs. The DNase I treated total RNAs were used as template for cDNA synthesis using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa).

2.6. Cloning and sequencing of ahjhe

A full-length cDNA encoding the JHE of *A. honmai*, *ahjhe*, was identified by 3′-RACE and 5′-RACE using a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa) and 5′-Full RACE Core Set (TaKaRa). In order to identify primers for 3′- and 5′-RACE, an internal region of *ahjhe* was first amplified using two degenerate primers, lepiJHEs-F and lepiJHEs-R (Table 1). These primers were designed on the basis of highly conserved regions that are found in five reported lepidopteran JHEs (Feng et al., 1999; Hanzlik et al., 1989; Hinton and Hammock, 2001; Shiotsuki et al., 2000; Teese et al., 2010). The internal sequence of *ahjhe* was obtained by RT-PCR using lepiJHEs-F and lepiJHEs-R and template cDNAs generated from fifth instar day 3 (L5D3) *A. honmai*. PCR conditions with TaKaRa ExTaq Hot Start (TaKaRa) were as follows: 94 °C 3 min; 30 cycles of 94 °C 30 s; 60 °C 30 s; 72 °C 30 s; and one cycle of 72 °C 5 min. This PCR generated a 0.3 kbp-long amplicon that was gel-purified using a QIAquick Gel Extraction Kit (Qiagen,