Effects of Nucleopolyhedrovirus Infection on the Development of Helicoverpa armigera (Lepidoptera: Noctuidae) and Expression of Its 20-Hydroxyecdysone—and Juvenile Hormone—Related Genes

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Effects of nucleopolyhedrovirus infection on the development of Helicoverpa armigera (Lepidoptera: Noctuidae) and expression of its 20-hydroxyecdysone– and juvenile hormone–related genes

Songdou Zhang, Fengming Wu, Zhen Li, Zhenqiang Lu, Xinfeng Zhang, Qingwen Zhang, and Xiaoxia Liu*

Abstract

In recent years, the interactions between baculoviruses and their insect hosts have become a research focus because baculoviruses can suppress the development and manipulate the behavior of insects. Many studies reported that nucleopolyhedrovirus (NPV) infection might disrupt the hormone balance in insects, but the effect of NPV infection on the development and expression of hormone-related genes in larvae of Helicoverpa armigera Boddie (Lepidoptera: Noctuidae) remains unclear. In this study, the mortality, development time, and pupal weight of H. armigera were recorded after 4th and 5th instars had been treated per os with different concentrations of Helicoverpa armigera single NPV (HaSNPV). Results showed that mortality increased and development time was prolonged to different degrees along with increasing concentrations of HaSNPV. The pupal weight did not differ between the HaSNPV-infected and control insects when 4th instars were infected but was significantly reduced when 5th instars were infected with HaSNPV at concentrations of 108 and 109 polyhedral inclusion bodies (PIB) per milliliter. Compared with the healthy control group, larval body weight was significantly reduced from the 3rd day after infection when 4th instars had been treated with HaSNPV at concentrations of 108 and 109 PIB/mL. Results from quantitative reverse-transcriptase polymerase chain reaction assays revealed that 20-hydroxyecdysone–related genes (ECR, USP, E75, and NTF2) were down-regulated and juvenile hormone–related genes (MET, JHi, and HSP90) were up-regulated after HaSNPV infection. This study improves our understanding of the interactions between baculoviruses and host insects.

Key Words: NPV infection; insect hormone; transcript level; qRT-PCR; development

Palabras Clave: infección VAN; hormona de insectos; nivel de transcripción; qRT-PCR; desarrollo

Baculoviruses are a class of large, double-stranded DNA viruses that infect only invertebrate hosts and have been developed as environmentally safe biological control agents (Park et al. 1993). In recent years, with the improving of people’s living standards and the growing environmental consciousness, use and development of high-efficiency, low-toxicity, and pollution-free pesticides have become more
The expression levels of ecdysone-related genes (ECR, USP, E75, BR, HR3, and NTF2) and JH-related genes (MET, JHEH, HSP90, and JHH) [Liu et al. (2011)] were determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The ribosomal protein L32 (RPL32) gene in *H. armigera* was used as an internal control for qRT-PCR normalization. The selected gene sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI). The primers used in qRT-PCR were designed with DNAClub software (http://www.softpedia.com/get/Science-CAD/DNA-Club.shtml) according to gene sequences. All primer pairs were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table 3).
Table 1. Effect of different HaSNPV concentrations on the mortality, development time, and pupal weight of Helicoverpa armigera larvae treated in the 4th instar.

<table>
<thead>
<tr>
<th>Concentration (PIB/mL)</th>
<th>Mortality (%)</th>
<th>Development time (d)</th>
<th>Pupal weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4th instar</td>
<td>5th instar</td>
</tr>
<tr>
<td>0</td>
<td>3.33</td>
<td>2.58 ± 0.07 a</td>
<td>5.40 ± 0.62 a</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>18.33</td>
<td>2.73 ± 0.08 a</td>
<td>5.92 ± 0.91 b</td>
</tr>
<tr>
<td>10⁻³</td>
<td>45.00</td>
<td>3.15 ± 0.08 b</td>
<td>5.82 ± 0.73 b</td>
</tr>
<tr>
<td>10⁻²</td>
<td>78.33</td>
<td>3.33 ± 0.10 b</td>
<td>6.46 ± 0.52 c</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>100</td>
<td>3.55 ± 0.13 bc</td>
<td>n/a</td>
</tr>
<tr>
<td>10⁻⁰</td>
<td>100</td>
<td>3.78 ± 0.15 c</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Summary statistics

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/a</td>
<td>300 (5, 295)</td>
<td>20.77</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>152 (3, 149)</td>
<td>9.34</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>152 (3, 149)</td>
<td>17.32</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>152 (3, 149)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

In total, 60 larvae were treated per concentration in 3 replicate experiments.

The data in the table are means ± SE. Means within the same column followed by a different letter are significantly different at P ≤ 0.05, Turkey’s HSD test.

SAMPLE COLLECTION AND TOTAL RNA EXTRACTION

To analyze the temporal expression profile of 20E- and JH-related genes in H. armigera larvae upon HaSNPV infection, larvae were treated with 10 μL HaSNPV suspension (10³ PIB/mL) according to the above method. Then, at least 10 larvae were collected at each of 6 time points (0, 24, 48, 72, 96, and 120 h), quickly frozen in liquid nitrogen, and immediately placed at −80 °C for later use. The larvae fed with artificial diet pretreated with an equal amount of sterile water were simultaneously collected as controls.

To avoid contamination with RNase, thawed larvae were placed into RNase-free micro tissue grinders that contained 1 mL Trizol reagent (Invitrogen, Gaithersburg, Maryland, USA) and ground for 5 min until the samples were completely homogenized. Then the total RNA was extracted by transferring 400 μL larval homogenate into a 2 mL RNase-free centrifuge tube that contained 600 μL Trizol reagent and following the manufacturer’s instructions (Zhang et al. 2015). The purity and concentration of RNA samples were determined twice with an ultraviolet spectrophotometer (Thermo Scientific NanoDrop 2000, Rockford, Illinois, USA). The 1st-strand complementary DNA (cDNA) was synthesized in triplicate from 1 μg total RNA of each sample according to PrimeScript RT reagent kit (TaKaRa, Kyoto, Japan), and the resulting products were immediately stored at −80 °C for later use (Bustin et al. 2009; Zhang et al. 2015). The quality and concentration of RNA samples were determined twice with an ultraviolet spectrophotometer (Thermo Scientific NanoDrop 2000, Rockford, Illinois, USA). The 1st-strand complementary DNA (cDNA) was synthesized in triplicate from 1 μg total RNA of each sample according to PrimeScript RT reagent kit (TaKaRa, Kyoto, Japan), and the resulting products were immediately stored at −80 °C for later use (Bustin et al. 2009; Zhang et al. 2015).

QUANTITATIVE REAL-TIME PCR ANALYSIS

Real-time PCR amplification and analysis were performed using SYBR green supermix (TaKaRa) following the manufacturer’s instructions on a Bio-Rad CFX Connect™ Real-Time PCR System (Bio-Rad, Hercules, California, USA), and the final reaction volume obtained was 20 μL. The real-time PCR was run in triplicate for each cDNA sample (Zhang et al. 2015). The amplification conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The specificity of amplified products was further confirmed by melting curve analysis from 65 to 95 °C and 2% agarose gel electrophoresis. The mRNA expression of target genes was quantified using the comparative Cross Threshold (CT, the PCR cycle number that crosses the signal threshold) method (Livak & Schmittgen 2001). The CT value of the reference gene was subtracted from the CT value of the target gene to obtain ΔCT. The normalized fold changes of the target gene mRNA expression were expressed as 2⁻ΔΔCT, where ΔΔCT is equal to ΔCTtarget sample − ΔCTcontrol sample.

STATISTICAL ANALYSES

All experiments were performed in triplicate, and the results were expressed as the means ± standard error (SE). The results of development time and pupal weight were analyzed by ANOVA followed by Turkey’s HSD multiple comparison test in SPSS 17.0 software for statis-
Results

MORTALITY, DEVELOPMENT TIME, AND PUPAL WEIGHT AFTER VIRUS INFECTION

When 4th instars were treated with HaSNPV at different concentrations, the mortality of control larvae (fed artificial diet with sterile water) was 3.3% and that of virus-treated larvae increased with increasing HaSNPV concentrations; all larvae treated with 10³ and 10⁴ PIB/mL died before pupation (Table 1). The development time of larvae treated with HaSNPV increased significantly compared with control larvae. When the larvae were infected with HaSNPV at 10³ and 10⁴ PIB/mL, the development time of 4th instars increased by 37.6 and 46.5%, respectively. At 10⁵ PIB/mL, the development time of 4th and 5th instars to pupation increased by 2.1 and 1.8 d, respectively, compared with control larvae (Table 1), whereas the pupal weight was similar between HaSNPV-infected and control insects.

When 5th instars were treated with HaSNPV at different concentrations, mortality increased with increasing HaSNPV concentration, the development time increased after treatment with 10³, 10⁴, and 10⁵ PIB/mL compared with control larvae, and the weight of pupae infected with 10³ and 10⁴ PIB/mL was significantly less than that of control pupae (Table 2).

FLUCTUATION OF BODY WEIGHT IN 4TH INSTARS UPON INFECTION

In general, the body weight of 4th instars gradually increased, and fast growth occurred from the 2nd day after treatment in every group (Fig. 1A). The body weight of infected larvae decreased with increasing HaSNPV concentration. Weight (Fig. 1A) and size (Fig. 1B) of larvae treated with 10³ and 10⁴ PIB/mL were significantly reduced from the 3rd day onward.

TRANSCRIPTION ANALYSIS OF 20E-RELATED GENES

Effects of HaSNPV infection on 20E-related genes in H. armigera larvae at the transcript level were analyzed by real-time PCR. The results showed that HaSNPV infection significantly inhibited the expression levels of 20E receptor ECR and its copartner USP after virus infection at 48, 72, 96, and 120 h, but had no obvious effect at 24 h (Fig. 2). The transcript levels of E75, a 20E early responsive gene, significantly decreased after virus infection at 24, 72, 96, and 120 h but was not different from controls at 48 h (Fig. 2). HaSNPV infection induced the expression of the two 20E early responsive genes BR and HR3 (Fig. 2), which significantly increased 48, 96, and 120 h after virus infection. The transcript levels of NTF2 increased after virus infection at 24 and 48 h, but decreased at 96 and 120 h (Fig. 2).

TRANSCRIPTION ANALYSIS OF JH-RELATED GENES

As shown by real-time PCR, MET, which is a JH candidate receptor gene, was significantly up-regulated at 24, 72, and 120 h after virus infection, with no noticed expression difference to controls at 48 and 96 h (Fig. 3). The transcript levels of JHi and HSP90 were significantly up-regulated at 24, 48, 72, and 120 h and at 24, 48, 72, and 96 h, respectively, after virus infection (Fig. 3). The JHEH gene was significantly up-regulated by virus infection at 24 and 120 h and down-regulated at 48 and 96 h (Fig. 3).

Discussion

In order to enhance their transmission, baculoviruses cause the host insects to develop slower or to change their behavior (Kamita et al. 2005; Liu et al. 2006; Hoover et al. 2011). Parasites of invertebrates and vertebrates mainly target 4 physiological systems (endocrine,
neural, immunomodulatory, and neuromodulatory) to induce behavioral changes (Beckage 1993; Adamo 2002; Thomas et al. 2005; Helluy 2013). Understanding how these systems connect and communicate is important for theoretical as well as practical reasons.

In our study, HaSNPV showed a high virulence and pathogenicity to 4th and 5th instars of H. armigera. The development time of virus-infected larvae was prolonged compared with larvae of the healthy control group. Our results are consistent with former studies showing that the molting and pupation of larvae were blocked by virus infection via controlling host insect ecdysone levels (O’Reilly & Miller 1989; Liu et al. 2006). Although the levels of ecdysone, which regulates the molting and metamorphosis in insects, have been shown to decline after

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**Fig. 1.** Effect of HaSNPV infection on the larval body weight of Helicoverpa armigera. (A) ♦, ■, ▲, ×, —, and ● indicate larvae infected with HaSNPV at the concentrations 0, 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ PIB/mL, respectively. (B) a, b, c, d, e, and f show H. armigera larvae on the 5th day after infection with HaSNPV at the concentrations 0, 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ PIB/mL, respectively.
virus infection, the expression changes of ecdysone-related genes are little investigated. Therefore, we selected 6 ecdysone-related genes, namely ECR, USP, E75, BR, HR3, and NTF2, to study the effect of HaSNPV infection on the larval 20E signal in H. armigera. Transcript levels of two 20E receptors (ECR and USP) and an early transcription factor (E75) were down-regulated after virus infection, which agrees with former results that HaECR transcript levels declined 72 h after HaSNPV infection (Jayachandran et al. 2013). Interestingly, the transcript levels of BR and HR3 genes were up-regulated after virus infection, which is consistent with previous research showing that HR3 was up-regulated nearly 8-fold in response to baculovirus infection (Breitenbach et al. 2011). The vital roles of BR have been demonstrated in metamorphic processes and embryogenesis of insects, but whether it is involved in neural, endocrine, and muscular coordination remains unclear (Piulachs et al. 2010). HR3, which is a probable nuclear hormone receptor and metamorphosis-related gene, plays key roles during metamorphosis (Xiong et al. 2013), but whether the up-regulation of HR3 after virus infection implies other functions remains to be investigated. The transcript levels of NTF2 markedly increased after virus infection at 24 and 48 h, but then decreased at 96 and 120 h. The reason for this fluctuation may be that NTF2 and small GTPase Ran are involved not only in the 20E signal transduction pathway but also in the nucleo-cytoplasm transport of macromolecules (He et al. 2010). Hence, our results showed that virus infection altered the transcription of 20E-related genes that may relate to the biological and physiological changes observed in infected larvae.

From the 3rd day of virus infection, the body weight and size of larvae treated with 10⁷ and 10⁸ PIB/mL were reduced compared with healthy larvae. The fluctuation of body weight was closely related to molting and development time because the exoskeleton limits the continuous growth of insects (Riddiford et al. 2003). It is possible that virus infection may suppress the growth and development of host insects by disturbing the hormone balance via influence of the viral Egt gene. The virus Egt gene encodes an enzyme that modifies a hydroxyl group on 20E, thereby inactivating the molting hormone and resulting in a delay or in the absence of molting in infected larvae (O’Reilly et al. 1992; Chen et al. 1997; Slavicek et al. 1999).

It is known that JH is a central hormone that regulates insect development and growth (Dubrovsky 2005), but the specific interactions between JH and virus infection remain unclear. Generally, it is hypothesized that inactivating 20E and maintaining the JH titer at status quo level are beneficial to the reproduction of the virus because the infected insects continue to feed and produce more occlusion bodies (polyhedra) (Chen et al. 1997). In Adoxophyes honmam (Lepidoptera: Tortricidae), JH esterase activity had no peak in the final instar of entomopoxvirus-infected larvae, suggesting that JH titers in virus-infected larvae remained high (Nakai et al. 2004). In Apis mellifera L. (Hymenop-
tura: Apidae), nurse-aged bees had an elevated JH titer that peaked at 8 d of age after infection with *Nosema ceranae* Fries, Feng, Feng, da Silva, Slemenda & Pieniazek (Dissociodihaplophasida: Nosematidae) (Gobirsch et al. 2013). We selected 4 JH-related genes, namely *MET*, *JHEH*, *HSP90*, and *JHEH*, to study the effect of *HaSNPV* infection on *H. armigera*. The transcript levels of *MET*, *JHEH*, and *HSP90* were significantly up-regulated following virus infection, implying that virus infection might induce a JH titer that prolongs the larval stage. *JHEH* was up-regulated after virus infection at 24 and 120 h but down-regulated at 48 and 96 h. The role of *JHEH* in the JH metabolic reaction is to decrease JH levels (Yang et al. 2011). Altered expression of a gene involved in the removal of JH would contribute to delayed pupation and allow the virus to propagate (Breitenbach et al. 2011).

Overall, this study directly determined the transcript levels of several genes involved in the 20E and JH pathways in *H. armigera* larvae after *HaSNPV* infection and investigated the inhibitory effect of *HaSNPV* on the growth and development of *H. armigera* larvae. We hypothesized that the expression levels of most 20E-related genes would decrease to varying degrees after virus infection, possibly because the viral *Egt* gene inactivated the normal ecdysone hormone metabolism in the diseased host larvae. Why JH-related genes were up-regulated after virus infection remains unclear. It is possible that JH may be in involved in the host defense against virus infection to suppress the viral life cycle. The particular mechanism by which 20E and JH jointly respond to virus infection is worth of further study.

The authors have declared that no conflict of interest exists. Author contributions: SZ and XL conceived and designed the experiments; SZ performed the experiments; SZ, FW, and ZL analyzed the data; FW, ZL, and XZ contributed reagents, materials, and analysis tools; SZ, ZL, QZ, and XL wrote the paper.

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