Analysis of Genes Expression of *Spodoptera exigua* Larvae upon AcMNPV Infection

Jae Young Choi1,3, Jong Yul Roh2,9, Yong Wang3, Zou Zhen4, Xue Ying Tao1, Joo Hyun Lee1, Qin Liu1, Jae Su Kim5, Sang Woon Shin6*, Yeon Ho Je1,7*

1 Department of Agricultural Biotechnology, College of Agriculture and Life Science, Seoul National University, Seoul, Korea, 2 Division of Medical Entomology, Korea National Institute of Health, Chungbuk, Korea, 3 School of Life Science, Lanzhou University, Lanzhou, China, 4 State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China, 5 Department of Agricultural Biology, College of Agricultural Life Science, Chonbuk National University, Jeonju, Korea, 6 Department of Entomology and Institute for Integrative Genome Biology, University of California Riverside, Riverside, California, United States of America, 7 Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Korea

Abstract

**Background:** The impact of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) infection on host gene expression in *Spodoptera exigua* 4th instar larvae was investigated through the use of 454 sequencing-based RNA-seq of cDNA libraries developed from insects challenged with active AcMNPV or heat-inactivated AcMNPV.

**Methodology/Principal Findings:** By comparing the two cDNA libraries, we show that 201 host genes are significantly up-regulated and 234 genes are significantly down-regulated by active AcMNPV infection. Down-regulated host genes included genes encoding antimicrobial peptides, namely three gloverin isoforms and an attacin, indicating that the viral infection actively repressed the expression of a portion of the host immune gene repertoire. Another interesting group of down-regulated host genes included genes encoding two juvenile hormone binding proteins and a hexamerin, all of which are involved in juvenile hormone regulation. The expression of these genes was enhanced by the topical application of Juvenile Hormone III (JHIII) in the insects challenged with heat-inactivated AcMNPV. However, infection with the active virus strongly suppresses the expression of these three genes, regardless of the absence or presence of JHIII.

**Conclusions/Significance:** Using RNA-seq, we have identified groups of immune-regulated and juvenile hormone-regulated genes that are suppressed by infection with active AcMNPV. This information and further studies on the regulation of host gene expression by AcMNPV will provide the tools needed to enhance the utility of the virus as an effective protein expression system and as an insecticide.

Citation: Choi JY, Roh JY, Wang Y, Zou Z, Tao XY, et al. (2012) Analysis of Genes Expression of *Spodoptera exigua* Larvae upon AcMNPV Infection. PLoS ONE 7(7): e42462. doi:10.1371/journal.pone.0042462

Editor: Subba Reddy Palli, U. Kentucky, United States of America

Received April 5, 2012; Accepted July 9, 2012; Published July 31, 2012

Copyright: © 2012 Choi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ008036), Rural Development Administration, Republic of Korea. HJS, YW, XYT, and QL were supported by the 2nd stage of the Brain Korea 21 project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: btrus@snu.ac.kr (YHJ); shinwoong@gmail.com (SWS)

† These authors contributed equally to this work.

Introduction

Baculoviruses are large DNA viruses that primarily infect insects. Most baculoviruses are quite host-specific, infecting only a single species or a few closely related species, except for *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), which can infect a wide range of lepidopteran insects [1]. The defining features of baculoviruses include circular and supercoiled double-stranded DNA genomes, rod-shaped enveloped nucleocapsids, the production of occluded virions, and the encoding of their own RNA polymerase, and they are obligate parasites of arthropod hosts. Baculovirus gene expression during the viral replication cycle is mediated by two types of RNA polymerases: host RNA polymerase II for the transcription of early and delayed early virus genes and a virus-encoded RNA polymerase for the transcription of late and very late genes. The viral genome sizes vary from approximately 80 to over 180 kb, and they encode between 90 and 180 genes. In general, the virions exist in two different morphological forms: occluded derived virus (ODV) and budded virus (BV). BV spreads the virus from cell to cell in infected insects, whereas ODV spreads the virus between insect hosts [2].

Baculoviruses have long been used for two major purposes: as viral insecticides to control insect pests in agriculture and forestry, and as the basis of a popular eukaryotic protein expression system [3]. They are natural pathogens of insects and have been used to control insect pests such as the codling moth (*Cydia pomonella*) [4], the velvet bean caterpillar (*Anticarsia gemmatalis*) [5], and the cotton bollworm (*Helicoverpa armigera*) [6]. The high level of very late viral gene expression makes baculoviruses highly suitable as vectors for eukaryotic gene expression. Proteins expressed by the baculovirus expression vector system under the control of the polyhedrin gene (polh) promoter (one of the very late genes), can reach levels of up to 50% of the total cellular protein under optimal conditions.
Baculovirus expression, in combination with insect cells or larvae, also results in appropriate posttranslational modifications, in contrast to proteins produced from prokaryotic expression systems. Foreign proteins expressed by baculoviruses have been used in a number of vaccines, such as the animal vaccines directed against classical swine fever, or hog cholera [7,8], and human vaccines against cervical cancer [9] and prostate cancer [10].

The remaining challenges for baculovirus expression systems include the need to improve protein quality by combining various post-translational modifications (such as folding, glycosylation, and preventing degradation) and the need to stabilise the viral genome and the expression of the heterogeneous genes over longer periods of time. In addition, the slow speed of host killing by baculovirus, host adaptations and the complexity of producing standardised viral preparations limit the usage of this virus for insect control. Understanding how baculoviruses interact with their host cells at a molecular level will make it possible to engineer these viruses in a way that will enhance their usefulness as effective insecticides and protein expression systems.

Many viral proteins have been reported or predicted to be involved in host-virus interactions, resulting in host morphological changes after viral infection, inhibition of host apoptosis or motling, regulation of host stress, and host disintegration. During the viral replication cycle, an electron-dense, chromatin-like structure, the virogenic stroma, can be found near the centre of the nuclei of infected cells [11,12]. In the case of AcMNPV, this host cell morphological change is attributed to two viral proteins: the single-stranded DNA binding protein dbp (Ac25) and PP31 (Ac36) [13,14]. These two proteins are predicted to be a superoxide dismutase (Ac31, vSOD) and a flavin adenine dinucleotide (FAD)-linked sulphhydryl oxidase (Ac92, p33), based on an HHPred program-based protein homology comparison, and they have been implicated in protection from oxidative stress. Inhibition of host cell apoptosis or host motling is thought to prolong the infection stage, thereby allowing the virus to replicate over a longer period. AcMNPV encodes two copies of a member of the inhibitor of apoptosis (iap) gene family, iap-1 (Ac27) and iap-2 (Ac71). P35 (Ac135) is also an inhibitor of apoptosis and is able to block AcMNPV-induced apoptosis in S. frugiperda cells [15]. A viral ubiquitin encoded by AcMNPV Ac35 may regulate host apoptosis to stabilise a short-lived viral protein [16]. When insects were infected with a virus that did not express viral chitinase (Ac126) or cathepsin (Ac127), they remained intact for several days after death, indicating that these viral proteins play a role in the dissemination of the virus by degrading the insect upon death [17].

Baculovirus infection is also reported to affect the expression of host genes. *Bombyx mori* NPV (BmNPV) infection triggered a global down-regulation of host gene expression in insect cells beginning at approximately 12-18 h post infection (hpi) [18]. Down-regulation of host mRNAs following AcMNPV infection in *Spodoptera frugiperda* (Sf9) cells has been reported in multiple studies [19,20,21]. A global analysis using a differential display method found that AcMNPV infection in Sf9 cells caused global down-regulation of host mRNA levels at later time points during the infection (12-24 hpi), but up-regulated the heat shock protein cognate 70 (hs70) at earlier points [22]. A comprehensive microarray analysis followed by qRT-PCR analysis identified the up-regulation of several host genes, including hs70 [23].

To identify the effect of AcMNPV infection on the expression of host transcripts in *Spodoptera exigua* larvae, we used 454 sequencing to analyse the transcriptome. This is a newer alternative to traditional EST sequencing and is a much more cost effective means of sequencing transcriptomes. In addition, this method allows de novo sequencing, assembly and annotation of expressed genes in a non-model organism for which genome sequences are currently unavailable. The 454 sequencing technique can also be used to investigate transcriptome-wide differential gene expression between differently treated samples. For this study, we sequenced the cDNA libraries from insects treated with active AcMNPV and heat-inactivated AcMNPV 12 h after treatment. The combined read sequences from the two transcriptomes were then used to construct a pool of contigs. The read numbers from the two transcriptomes were compared to identify host genes that were up- or down-regulated after viral infection. Out of 5,945 total contigs, 201 genes were significantly up-regulated and 234 genes significantly down-regulated by active AcMNPV infection, as compared to heat-inactivated AcMNPV infection. Two small groups of host genes, a group of genes encoding antimicrobial peptides/proteins (AMPs) (gloverins and an attacin) and a group of three juvenile hormone-related genes, were down-regulated. The genes encoding the AMPs were strongly induced by challenge with the heat-inactivated AcMNPV, but this induction was suppressed by active AcMNPV. The genes encoding the two juvenile hormone binding proteins and a hexamerin were induced by challenge with the heat-inactivated AcMNPV and additionally enhanced by the application of Juvenile Hormone III (JHIII). This up-regulation was not observed in insects infected with active AcMNPV. These results strongly suggest that the active virus can suppress the expression of specific host genes.

**Results and Discussion**

**454 sequencing results and contig assembly**

As described in the Methods, cDNA libraries from *Spodoptera exigua* larvae challenged with active AcMNPV or heat-inactivated AcMNPV were subjected to a 1/8-plate production run on the 454 GS-FLX sequencing instrument, resulting in 77,616 and 74,928 reads, respectively. Files containing these data were deposited in the Short Read Archive of the National Center for Biotechnology Information (NCBI) with accession numbers SRX110132 [AcMNPV-challenged] and SRX110248 [heat-inactivated AcMNPV-challenged], respectively. After the sequence reads of viral origin were removed, a total of 130,335 total reads were obtained from the two cDNA libraries, which were assembled to create 5,945 contigs (>100 bp, average length of 667 bp) (Table S1). A total of 3,607 contigs were at least 500 bp in length, a greater number than that obtained by 454 pyrosequencing in other insects such as *Anopheles funestus* [24], *Melitaea cinxia* [25] and *Zygiaena filipendulae* [26]. The average number of reads assembled into a contig was 21.9. All files of assembled contigs and singletons from AcMNPV-challenged, heat-inactivated AcMNPV-challenged, and combined EST libraries are available by request.

To obtain an overview of the functional categories represented by the *S. exigua* transcriptome, we compared the 5,945 contigs with a *Drosophila* database using BLASTX. A *Drosophila*-based gene ontology search categorised 2,699 hits into 15 functional groups (Figure 1A). Enzymes involved in the metabolism of secondary metabolites and xenobiotics represented the two largest groups, accounting for 30% of the total number of contigs with a putative function. Metabolism of carbohydrates (236), energy (150), amino acids (251), lipids (118), and nucleotides (56) accounted for another 30%. While genes related to metabolism represented the largest collection of contigs overall, the genes involved in transcription (123) and translation (191), which constituted the basic genetic information processing machinery, were also highly expressed. Relatively low abundance genes that were identified by this analysis included genes encoding proteins involved in membrane functions.
transport and other cellular processes, including genes related to cell mobility, growth, death, and communication. The apparent low abundance of these transcripts could be due to the low homology to *Drosophila* proteins or to an inherent bias in the library construction.

Comparison of transcriptome profiles between active AcMNPV-infected and heat-inactivated AcMNPV-treated samples

The significant (binomial probability of <0.1) differences in the expression of different contigs were determined by comparing the read number of each contig between the active AcMNPV-infected sample (A-read) and the heat-inactivated AcMNPV-treated sample (I-read). Using this method, we identified 201 host genes that are significantly up-regulated and 234 genes that are significantly down-regulated by infection with active AcMNPV (Table S2 & S3). The distribution of gene functions between these two groups is quite distinctive (Figure 1B). Genes related to carbohydrate and lipid metabolism were concentrated in the up-regulated gene (UP) cohort. Likewise, twice as many genes related to secondary metabolism and xenobiotic metabolism were present in the UP group as compared to the group of down-regulated genes (DOWN). The UP group includes eight genes encoding cytochrome P450 family proteins, and one gene each encoding glutathione S-transferase, thioredoxin peroxidase, superoxide dismutase, and methylenetetrahydrofolate dehydrogenase, which are key enzymes in the detoxification of xenobiotic compounds. However, genes related to amino acid and nucleotide metabolism, such as alanine aminotransferase, amidophosphoribosyltransferase, and glutamine synthetase, were more suppressed in the presence of the viruses. More genes involved in translation, degradation, and signal transduction were also down-regulated in the live virus-treated samples as compared to the heat-inactivated samples. Eight genes related to the host immune response are found in the suppressed gene repertoire, including the gene encoding attacin and three genes encoding gloverin, all of which are immune effector molecules.

Viral gene expression 12 h post infection

We characterised the sequences of viral origin from both the A-read and the I-read by performing a BLASTN homology search to AcMNPV open reading frame (ORF) sequences. The A-read from the active AcMNPV challenged-cDNA library included 614 read sequences that originated from AcMNPV, whereas the I-read from the heat-inactivated AcMNPV sample only produced one sequence (Table S4). Out of 155 ORFs, the expression of 93
AcMNPV ORFs was detected by RNA-seq in host insect larvae at 12 hpi (Table S5).

To obtain an overview of AcMNPV gene expression 12 hpi, we grouped the AcMNPV ORFs based on their known functions and on their abundance in the A-read (Table 1). Here, we found that 5 of 6 ORFs encoding viral per os infectivity factors, which are involved in the initiation of midgut infections [27], were not detected (i.e., belonged to the no frequency group). This was expected because the virus was directly injected into the insect hemocoel cavity. We also found that 22 of 25 ORFs encoding viral structural proteins belonged primarily to the low frequency (≥ 10) or no frequency (≠ 10) groups. In addition, two of three ORFs encoding the viral RNA polymerase belonged to the no frequency group. Both viral structural proteins and the viral RNA polymerase are involved in the late or very late stages of the viral replication cycle. Furthermore, we did not detect any expression of the two ORFs encoding a chitinase and a cathepsin, which are involved in insect disintegration. In contrast, 10 of 11 ORFs encoding viral proteins involved in DNA replication belonged to the high frequency or low frequency (> 10) groups. These results indicate that, at 12 hpi, the viruses are primarily in the early stages of the viral replication cycle.

**A global down-regulation of host mRNA levels was not observed**

The number of I-reads and A-reads for each contig were graphed on an x,y plot, showing that the expression of the majority of host genes is not significantly affected (p > 0.1) (Figure 2). Only approximately 7.3% of contigs were up- (UP) or down- (DOWN) regulated by active AcMNPV infection. To confirm the expression profiles of the UP and DOWN gene categories, total RNA was isolated from 5th instar larvae 12 hpi with active or heat-inactivated AcMNPV. When 10 UP and 10 DOWN genes were tested by quantitative real-time PCR (qPCR), their expression profiles all matched the results obtained by RNA-seq (Figure 3, indicated in red in Table S2 and S3).

A global down-regulation of host transcription at late time points of infection has been reported in several studies [19,20,21]. A differential display approach showed that the decrease in host mRNA levels began between 12 and 24 hpi in Sf9 cells [22]. By means of a microarray approach, transcripts for the majority of host genes in Sf9 cells were shown to decline substantially 12 hpi [23]. In our experimental approach, we infected the insect larvae with AcMNPV, and no global down-regulation of host gene expression was observed. Only a small number of genes were significantly down-regulated by active AcMNPV infection (234 DOWN of 5,945 total contigs). We detected a similar number of contigs in the DOWN and UP groups (234 and 201, respectively). When the number of I-reads and A-reads for the contigs encoding ribosomal proteins were graphed, we observed that the expression of the majority of genes encoding ribosomal proteins are not significantly affected (Figure 4). Out of 94 contigs encoding ribosomal proteins, only 4 host genes are significantly up-regulated and 8 are down-regulated by active AcMNPV infection. The down-regulation of 4 genes encoding ribosomal protein (RpS20, RpS12, RpL19 and RpS3A) in Sf9 cells at 18 h or 24 h after infection with AcMNPV has been reported as evidence of a global

| Table 1. An overview of AcMNPV gene expression 12 hpi. |
|---------------------------------|---------------------------------|---------------------------------|
| **High frequency (≠ of A-reads \(\geq 10\))** | **Low frequency (≠ of A-reads <10)** | **No frequency** |
| Structural proteins | | |
| Occlusion bodies | Ac8 (polyhedrin), Ac131 (polyhedrin envelope), Ac137 (p10) | |
| Baculovirus envelope proteins | Ac128 (Gp64), Ac16 (BV/ODV-E26), Ac23 (Fusion protein-F), Ac94 (ODV-E25), Ac143 (ODV-E18) | Ac46 (ODV-E66), Ac109 (ODV-EC43), Ac148 (ODV-E56) |
| Nucleocapsid associated factors | Ac100 (P6.9, Ac144 (ODV-EC27), Ac9 (PP78/83), Ac137 (p10) | Ac54 (vp1054), Ac66, Ac77 (VLF-1), Ac92 (P33), Ac98 (38K), Ac129 (P24), Ac141 |
| Viral transcription activators | Ac147 (IE1) | Ac151 (ie2/ie-n), Ac153 (pe38) |
| DNA replication (essential) | Ac65 (DNA poll), Ac95 (p143) | |
| DNA replication (influential) | Ac25 (DBP), Ac139 (M53) | Ac49 (PCNA), Ac125 (LEF-7) |
| Viral RNA polymerase | | Ac37 (LEF-11) |
| per os infectivity factors | | |
| Oxidative stress | Ac119 (pif-1) | |
| Prolonged infection (Inhibition of apoptosis or molting) | | |
| Insect disintegration | | |
| Actin assembly | Ac9 (pp78/83), Ac20/21 (arf1) | |
| Virogenic stroma | Ac25 (dbp), Ac36 (pp31) | |

*indicates that the viral protein is a baculovirus envelope protein, which has role in per os infectivity [46]. BLASTN analysis using the viral ORF sequences showed that 614 read sequences from the active AcMNPV challenged-cDNA library (A-read) originated from the virus itself. The viral ORFs are grouped based on their known functions and the number of A-reads.

doi:10.1371/journal.pone.0042462.t001
down-regulation in host gene expression [23]. However, the expression of these genes was not significantly altered in our experiments (Figure 4), clearly indicating that there is no global down-regulation of host transcripts.

Although there were previous reports that describe a global down-regulation of the host mRNA by AcMNPV infection, those studies were carried out in vitro system using Sf9 cells, which were infected with the virus at an MOI (multiplicities of infection) of 10.

Figure 2. Graph of the numbers of I-reads and A-reads for each contig. The number of I-reads and A-reads of each contig were graphed on an x,y plot. For convenience, contigs were plotted on two separate graphs: for contigs shown in the left panel, the number of I- or A-reads is smaller than 150; for contigs shown in the right panel, the number of I or A-reads is equal to or larger than 150. The linear trendline (with the intercept set as zero) and the slope are indicated by a line and an equation. UP and DOWN contigs are indicated as green circles and red boxes, respectively.

doi:10.1371/journal.pone.0042462.g002

Figure 3. Validation of the RNA-seq results by quantitative real-time PCR (qPCR). The expression profiles of 10 UP and 10 DOWN contigs (randomly selected) were analysed by qPCR to validate the RNA-seq results. The tested contigs are indicated in red in Tables S2 and S3.

doi:10.1371/journal.pone.0042462.g003
Because most cultured cells (over 99%) were infected with the virus simultaneously under these infection conditions, transcripts for the majority of host genes in Sf9 cells were shown to decline substantially between 12–24 hpi. In contrast, in this study, in vivo S. exigua larvae were used as host system, in which only several parts of tissues including hemocyte and fatbody could be actually infected with injected viruses and most of tissues were remained as non-infected at 12 hpi, at which time point total RNA was isolated from whole body of the infected larvae. This was further supported by the viral gene expression at 12 hpi (Table 1) suggesting that, in S. exigua larvae, the viruses are primarily in the early stages of the viral replication cycle at 12 hpi. More prolonged infection times are needed to observe global host gene down-regulation in S. exigua larvae.

**Suppression of host immune gene activation by AcMNPV**

When the number of I-reads and A-reads for the contigs encoding immune-related peptides/proteins were graphed, we observed that the expression of several genes involved in the host immune response was significantly down-regulated in larvae infected with active AcMNPV (Figure 5A). These down-regulated genes include genes encoding three gloverins and one attacin. Gloverin is a glycine-rich antibacterial protein found in lepidoptera species [28,29]. One recent report showed that S. exigua gloverin (GLV) acts as an antimicrobial peptide (AMP) against Bacillus thuringiensis [30]. Attacin is also a glycine-rich protein, originally isolated from the lepidopteran insect Hyalophora cecropia [31]. The expression of attacin cDNA has been reported in S. exigua [32].

We analysed the expression profiles of GLV1 and attacin by qPCR. Gene expression was slightly up-regulated at 6 hpi in the control insects, which were mock-injected with Sf9 growth medium. We observed 5–7 times greater expression of both AMP genes 6 hpi in the insects infected with active or heat-inactivated AcMNPV (Figure 6), as compared to mock injection, suggesting that AcMNPV infection elicits some type of immune response. Interestingly, the GLV1 and attacin expression decreased rapidly at 12 hpi with active AcMNPV, whereas these genes continued to be up-regulated until 12 hpi in the insects infected with heat-inactivated AcMNPV (Figure 6). This clearly indicates that the active virus can suppress the induction of these AMP genes. It has been reported that AMP genes are expressed as an acute immune response to bacterial challenge, and therefore are rapidly transcribed following challenge (1 to 5 h), the transcription rate increases over a period varying between 6 and 24 h, depending on the gene, and thereafter either stops or levels off [33]. Therefore, it could be postulated that these immune related genes were induced in S. exigua larvae upon hemocoelic injection of AcMNPV (regardless of active and heat-inactivated), but the further expression of them was suppressed by viral modulation of host immune mechanisms in S. exigua larvae injected with the active AcMNPV at 12 hpi. This result may also suggest a possible antiviral role for these glycine-rich AMPs, a hypothesis that will be addressed by further research.
AcMNPV infection affects the hormonal regulation of host gene expression

The group of DOWN contigs included three genes encoding proteins that belong to the juvenile hormone binding protein (JHBP) family and three genes encoding hexamerin proteins (Table S3). Low molecular weight JHBPs of approximately 30 kDa have specific affinity for the juvenile hormone (JH) [34]. Insect hexamers have been shown to be bona fide JH-binding proteins [35,36] and reportedly bind to JHBP [37]. Both JHBP and hexamerin are proposed hemolymph carriers, which are involved in protecting JH from hydrolysis by esterases during transport from its site of synthesis to target tissues.

We analysed the expression profile of two JHBP genes (JHBP1 and JHBP2) and one hexamerin gene (contig00429) by qPCR. The expression was strongly induced at 12 hpi with heat-
inactivated AcMNPV (Figure 7). This induction was further enhanced by JHIII treatment (Figure 7), demonstrating that JH was involved in the regulation of these JH carrier genes. Interestingly, up-regulation of these genes was not observed 12 hpi with live AcMNPV, suggesting that the active viruses inhibit JH-related regulation of host genes.

We propose that JH may be involved in the host defence against baculovirus infection by interfering in the viral life cycle. In order for successful viral replication to occur, the baculovirus needs to control host molting and pupation in infected larvae. Host metamorphosis is regulated by two hormones: ecdysone, which causes molting, and JH, which allows larval molting but prevents pupation. Host insects may respond to AcMNPV infection by increasing JH trafficking, which could explain the activation of JH carrier genes by heat-inactivated AcMNPV. Live AcMNPV counteracts this measure by suppressing gene activation, thereby controlling host molting and pupation to be more favourable for the viral replication cycle.

Host ecdysone levels have been reported to be controlled by viral ecdysteroid UDP-glucosyltransferase (EGT). Viral EGT functions to block molting and pupation in infected insect larvae by inactivating ecdysone hormone [38]. This is thought to cause abnormal larval growth and prolongation of the larval instar, resulting in a higher virus yield [39]. Because a reciprocal interaction between JH and ecdysone in gene regulation has been reported [40], it is also possible that baculovirus infection affects the host ecdysone level, consequently triggering the activation of JH-dependent genes encoding the JHBPs and hexamerin. To test this hypothesis, we surveyed the gene expression profiles after infection with an AcMNPV EGT deletion mutant (AcMNPV-ΔEGT). This mutant was still able to suppress the gene activation, clearly indicating that the suppression of JH-related genes was not mediated through host ecdysone levels induced by viral EGT.

In this paper, we describe the suppression of host JH-related genes by AcMNPV. We hypothesise that the host insect increases the level of available juvenile hormone after the viral infection, but that active AcMNPV counters this measure through an unknown mechanism that does not involve viral EGT regulation of host ecdysone levels.

Materials and Methods

Insect cells, insects and viruses

The S. frugiperda cell line, Sf9, was maintained in TC-100 medium (WelGene, Korea) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (WelGene, Korea) at 27°C. The S. exigua larvae were obtained from a laboratory colony and raised at 25°C under a 16 h:8 h light:dark cycle with an artificial diet [41]. The wild-type AcMNPV C6 strain was propagated in Sf9 cells maintained in TC-100 medium. The AcMNPV virus was heat-inactivated by incubating at 60°C for 24 h.

Viral infection, JHIII treatment and RNA extraction

Inoculation of the insect larvae was carried out as described previously [42]. Approximately 50 μl of a viral suspension (1×10^7 PFU/ml) (containing active or heat-inactivated virus) with 6 mg/ml kanamycin was injected just underneath the dorsal cuticle of each S. exigua 5th instar larva using a Hamilton digital syringe (Hamilton, USA) fitted with a 22-gauge needle. JHIII (Sigma, USA) was applied topically (10 mg/ml in acetone, 1 μl/larva) to S. exigua larvae. After incubation at 25°C for 12 h, total RNA was isolated from infected S. exigua larvae using TRIZOL Reagent (Invitrogen, USA) according to the manufacturer’s instructions. The extracted total RNA was first treated with DNaseI (TaKaRa, Japan) to remove contaminating genomic DNA.

454 sequencing and sequence analysis

From the total RNA extracted from whole body of S. exigua 5th instar larvae, mRNAs were purified using the Oligotex mRNA Midi Kit (QIAGEN, Germany). Next, double-stranded cDNA was synthesised using the SMART cDNA Library Construction Kit (Clontech, USA). The cDNA was further purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) and checked for quality using an Agilent 2100 Bioanalyzer. Approximately 5 μg of cDNA was used for sequencing on a Roche/454 GS-FLX Titanium sequencer (Roche, Germany). A 1/8 plate sequencing run was performed at MACROGEN Co. (Korea) according to the manufacturer’s instructions. The raw 454 sequence files were processed to remove low quality regions and adaptor sequences using the SeqClean program (http://compbio.dlci.harvard.edu/tgi/software). The resulting sequences were then screened against the NCBI UniVec database and E. coli genome sequences to remove contaminating sequences. Sequences shorter than 100 bp were discarded. The processed sequences were assembled into contigs using the iAssembler program (http://bioinfo.bti.cornell.edu/tool/iAssembler).

**Figure 7. The expression profiles of two JHBP genes and one hexamerin gene.** Relative expression levels of two JHBP genes and one hexamerin gene 12 h after each treatment were examined by qPCR. Mock, treatment with Sf9 growth medium; AcMNPV, culture medium with AcMNPV; Inactivated AcMNPV, culture medium with heat-inactivated AcMNPV; AcMNPV/ΔEGT, culture medium with AcMNPV-ΔEGT. JHIII was topically applied directly after each treatment. doi:10.1371/journal.pone.0042462.g007
Analysis of relative transcription levels by qPCR

The 28S rRNA gene was used as a reference gene [43]. Single-strand cDNA was synthesised from the total RNA using the SuperScript III First-Synthesis System for RT-PCR (Invitrogen, USA) according to the manufacturer’s instructions. Real-time PCR was conducted using the 2× DyNaMo™ HS SYBR® Green qPCR Kit (FINNZYMES, Finland) and a CFX96™ Real-Time System (BIO-RAD, USA). The cycling profile used for qPCR was as follows: a preheating step for enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec. The relative transcription levels were calculated using the 2^(-ΔΔCt) method [44]. The primers used for qPCR are listed in Table S6.

Identification of functional classes

The contigs were analysed by comparing to Drosophila proteins using BLASTX (with a cut-off value of 1e-05). The resulting data set was used to reconstruct functional class profiles and pathways using the KEGG (Kyoto Encyclopedia of Genes and Genomes), except for the Immune response category, which was directly defined by comparing to Drosophila immune genes using BLASTX. The annotation results of S. exigua contigs derived from BLASTX were subsequently analysed in PROSITE, SMART, PFAM to confirm conserved domain structures.

Supporting Information

Table S1 List of assembled contigs with total reads from the cDNA libraries from both the AcMNPV-challenged and the heat-inactivated AcMNPV-challenged S. exigua.

| References |
|-----------------------------------|
| 1. Guo T, Wang S, Guo X, Lu C (2005) Productive infection of Autographa californica nucleopolyhedrovirus in silkworm Bombyx mori strain Haoyue due to the absence of a host antiviral factor. Virology 341: 231–237. |
| 2. Pearson MN, Russell RL, Rohrmann GF (2001) Characterization of a baculovirus-encoded protein that is associated with infected-cell membranes and budded virions. Virology 291: 22–31. |
| 3. Summers MD (2006) Milestones leading to the genetic engineering of baculoviruses as expression vectors and viral pesticides. Adv Virus Res 66: 1–73. |
| 4. Bangels E, Gobin B (2008) Efficacy results of insecticides against S. exigua challenged and the heat-inactivated AcMNPV-challenged S. exigua. (DOC) |
| 5. Moscardi F (1999) Assessment of the application of baculoviruses for control of S. exigua, a major pest of cotton. J Invertebr Pathol 81: 63–69. |
| 6. Sun X, Chen X, Zhang Z, Wang H, Bianchi FJ, et al. (2002) Bollworm responses to release of genetically modified Helicoverpa armigera nucleopolyhedroviruses in cotton. J Invertebr Pathol 81: 63–69. |
| 7. Bouam A, de Smit AJ, de Krijger EP, Terpstra C, Moormann RJ (1999) Efficacy and stability of a subunit vaccine based on glycoprotein E2 of classical swine fever virus. Vet Microbiol 66: 101–114. |
| 8. van Rijn PA, van Gennip HG, Moormann RJ (1999) An experimental marker vaccine and accompanying serological diagnostic test both based on envelope glycoprotein E2 of classical swine fever virus (CSFV). Vaccine 17: 433–440. |
| 9. Harper DM (2009) Currently approved prophylactic HPV vaccines. Expert Rev Vaccines 8: 1663–1679. |
| 10. van Oers MM, van Lent JW, Vlak JM (2010) Baculovirus infection on the mRNA and protein levels of the Spodoptera frugiperda ryanodine receptor initiation factor 4E. Insect Mol Biol 19: 255–264. |
| 11. Fraser JR (1986) Epidemic polyarthritis and Ross River virus disease. Clin Exp Immunol 68: 3–73. |
| 12. Pearson MN, Russell RL, Rohrmann GF (2001) Characterization of a baculovirus-encoded protein that is associated with infected-cell membranes and budded virions. Virology 291: 22–31. |
| 13. Summers MD (2006) Milestones leading to the genetic engineering of baculoviruses as expression vectors and viral pesticides. Adv Virus Res 66: 1–73. |
| 14. Guarino LA, Dong W, Xu B, Broussard DR, Davis RW, et al. (1992) Characterization of a baculovirus gene during infection of insect cells. Science 254: 1388–1390. |
| 15. Haas-Kogan DA, Yount G, Haas M, Levi D, Kogan SS, et al. (1996) p53-dependent G1 arrest and p53-independent apoptosis influence the radiobiologic response of glioblastoma. Int J Radiat Oncol Biol Phys 36: 95–103. |
| 16. Katsuma S, Mita K, Shimada T (2007) ERK- and JNK-dependent signaling pathways contribute to Bombyx mori nucleopolyhedrovirus infection. J Virol 81: 13700–13709. |
| 17. Ovi BG, Miller JK (1985) Regulator of host RNA levels during baculovirus infection. Virology 166: 515–523. |
| 18. van Oers MM, Vlak JM, Voorma HO, Thomas AA (1999) Role of the 3′ untranslated region of baculovirus p10 mRNA in high-level expression of foreign genes. J Gen Virol 80: 2253–2262. |
| 19. van Oers MM, van der Veen LT, Vlak JM, Thomas AA (2001) Effect of baculovirus infection on the mRNA and protein levels of the Spodoptera frugiperda ryanodine receptor initiation factor 4E. Insect Mol Biol 10: 255–264. |
| 20. Zhou S, Mita K, Shimada T (2007) ERK- and JNK-dependent signaling pathways contribute to Bombyx mori nucleopolyhedrovirus infection. J Virol 81: 13700–13709. |
| 21. Hoein HD, Reilly DR, Ozerski JA (2003) Autographa californica nucleopolyhedrovirus infection of Spodoptera frugiperda cells: a global analysis of host gene regulation during infection, using a differential display approach. J Gen Virol 84: 3029–3039. |
| 22. Salem T, Zhang F, Xie Y, Thiem SM (2011) Comprehensive analysis of host gene expression in Autographa californica nucleopolyhedrovirus-infected Spodoptera frugiperda cells. Virology 412: 167–178. |
| 23. Gregory R, Darby AG, Irving H, Coulby MB, Hughes M, et al. (2011) A de novo expression profiling of Anopheles funestus, malaria vector in Africa, using 454 pyrosequencing. PLoS One 6: e17418. |
| 24. Vera JC, Wheat CW, Feschmyer HW, Friisler MJ, Crawford DL, et al. (2008) Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Mol Ecol 17: 1636–1647. |
| 25. Zagreblozny M, Schrabe-Asking K, Jensen NB, Moller BL, Gorodkin J, et al. (2009) 454 pyrosequencing based transcriptome analysis of Zygarna flavipendex with focus on genes involved in biosynthesis of cyanogenic glucosides. BMC Genomics 10: 574. |
| 26. Peng K, van Oers MM, Hu Z, van Lent JW, Vlak JM (2010) Baculovirus per os infectivity factors form a complex on the surface of occlusion-derived virus. J Virol 84: 4947–4950. |
| 27. Lautenbier A, Liu G, Kang D, Beimis K, Steiner H (2002) Trichoplusia ni lygopen, an inducible immune gene encoding an antibacterial insect protein. Insect Biochem Mol Biol 32: 795–801. |
29. Kawaoka S, Katsuma S, Daimon T, Imoto R, Omuro N, et al. (2008) Functional analysis of four Gloverin-like genes in the silkworm, *Bombyx mori*. Arch Insect Biochem Physiol 67: 87–96.

30. Hwang J, Kim Y (2011) RNA interference of an antimicrobial peptide, gloverin, of the beet armyworm, *Spodoptera exigua*, enhances susceptibility to *Bacillus thuringiensis*. J Invertebr Pathol 108: 194–200.

31. Engstrom Y, Kadaliyl I, Sun SC, Samakovlis C, Hultmark D, et al. (1993) kappa B-like motifs regulate the induction of immune genes in *Drosophila*. J Mol Biol 232: 327–333.

32. Bang K, Park S, Yoo JY, Cho S (2011) Characterization and expression of attacin, an antibacterial protein-encoding gene, from the beet armyworm, *Spodoptera exigua* (Hubner) (Insecta: Lepidoptera: Noctuidae). Mol Biol Rep 39: 5151–5159.

33. Hoffmann JA, Hetru C, Reichhart JM (1993) The humoral antibacterial response of Drosophila. FEBS Lett 325: 63–66.

34. Kramer KJ, Sanburg LL, Kezdy FJ, Law JH (1974) The juvenile hormone binding protein in the hemolymph of *Manduca sexta* Johannson (Lepidoptera: Sphingidae). Proc Natl Acad Sci U S A 71: 493–497.

35. Braun RP, Wyatt GR (1996) Sequence of the hexameric juvenile hormone-binding protein from the hemolymph of *Locusta migratoria*. J Biol Chem 271: 31756–31762.

36. Tawfik AI, Kellner R, Hoffmann KH, Lorenz MW (2006) Purification, characterization and titre of the haemolymph juvenile hormone binding proteins from *Schistocerca gregaria* and *Cicalus binaculatus*. J Insect Physiol 52: 255–268.

37. Zalewiska M, Kochman A, Estève JP, Lopez F, Chassé K, et al. (2009) Juvenile hormone binding protein traffic – Interaction with ATP synthase and lipid transfer proteins. Biochim Biophys Acta 1788: 1695–1705.

38. O’Reilly DR, Miller LK (1989) A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science 245: 1110–1112.

39. O’Reilly DR, Miller LK (1990) Regulation of expression of a baculovirus ecdysteroid UDP-glucosyltransferase gene. J Virol 64: 1321–1328.

40. Zou YR, Kottnauer AH, Kuroda M, Tanaischi I, Littman DR (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393: 595–599.

41. Ghio HG, Lee SG, Lee BP, Choi KM, Kim JH (1990) Simple mass-rearing of beet armyworm, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae), on an artificial diet. Kor J Appl Entomol 29: 180–183.

42. Choudary PV, Kamita SG, Maeda S (1995) Expression of foreign genes in *Bombyx mori* larvae using baculovirus vectors. In: Richardson CD, editor. Baculovirus expression protocols. Totowa: Humana Press Inc. pp. 243–264.

43. Xue JL, Salem TZ, Turney CM, Cheng SW (2010) Strategy of the use of 28S rRNA as a housekeeping gene in real-time quantitative PCR analysis of gene transcription in insect cells infected by viruses. J Virol Methods 163: 210–215.

44. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

45. Landais I, Ogliastro M, Mita K, Nohata J, Lopez-Ferber M, et al. (2003) Annotation pattern of ESTs from *Spodoptera frugiperda* Sf9 cells and analysis of the ribosomal protein genes reveal insect-specific features and unexpectedly low codon usage bias. Bioinformatics 19: 2243–2250.

46. Sparks WO, Harrison RL, Bonning BC (2011) *Autographa californica* multiple nucleopolyhedrovirus ODV-E56 is a per os infectivity factor, but is not essential for binding and fusion of occlusion-derived virus to the host midgut. Virology 419: 69–76.