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ORIGINAL PAPER

3H-117, a structural protein of Heliothis virescens ascovirus 3h (HvAV-3h)

Ying Zhao[1,2] · Huan Yu[1,2] · Lei He[1,2] · Ni Li[1,2] · Guo-Hua Huang[1,2]

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Abstract

The open reading frame 117 (*3h-117*) of Heliothis virescens ascovirus 3h (HvAV-3h), which is a conserved coding region present in all completely sequenced ascovirus members, was characterized in this study. By RT-PCR detection, *3h-117* transcription began at 6-h post-infection (hpi) and remained stable until 168 hpi in HvAV-3h-infected *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae. In addition, *3h-117* putatively encodes a 21.5-kDa protein (3H-117) predicted to be a CTD-like phosphatase. Western blot analysis using a prepared rabbit polyclonal antibody specific to 3H-117 showed that the product could be detected at 24 hpi, which remained stably detectable until 168 hpi. The same analysis also demonstrated that the 3H-117 protein localized in the virions of HvAV-3h. Immunofluorescence analysis showed that at 24 hpi, 3H-117 was mainly located in the nuclei of *H. armigera* larval fat body cells and later spread into the cytoplasm. In summary, our results indicate that 3H-117 is a structural protein of HvAV-3h.

Keywords Ascovirus · Structural protein · HvAV-3h · Phosphatases

Introduction

The *Ascoviridae* family is a group of double-stranded DNA insect viruses with numerous allantoid- and bacilliform-shaped virions enveloped in vesicles [1–4]. Ascoviruses are infectious to insect larvae transmitted by parasitoid wasps. Some of the viruses are considered potential natural control agents of pest species, such as *Helicoverpa armigera* (Hübner), *Spodoptera exigua* (Hübner), and *Spodoptera litura* (Fabricius) (all Lepidoptera: Noctuidae), because of their ability to cause retarded growth and loss of appetite in larvae [5].

Currently, 10 isolates of ascoviruses have been completely sequenced according to the NCBI database [3, 4, 6–13]. Further investigations have reported that 44 ascovirus open reading frames (ORFs) were conserved among all sequenced ascoviruses [3]. However, a majority of the viral genes remain unexplored. Among structural proteins, research only pertaining to major capsid proteins (MCP, TnAV-2a) and DNA-binding proteins (P64, SfAV-1a) has been conducted in attempts to understand the relationships involved in ascovirus evolution with other related insect DNA viruses and critical factors involved in virion assembly [14, 15]. With the development of proteomic detection systems, more comprehensive analyses of the protein composition of ascovirus virions will enable more detailed investigations into virion structure and the mechanisms associated with infection. Studies on structural proteins have become vital in understanding ascoviruses. Initially, 7 and 21 structural proteins were identified in the virion proteomic analyses of Trichoplusia ni ascovirus 6a (TnAV-6a, previously designated TnAV-2c) and Spodoptera frugiperda ascovirus 1a (SfAV-1a), respectively [16, 17]. Subsequently, 67 virion-associated proteins were identified from a sample containing all HvAV-3i virion proteins, which are involved in virion assembly and virus replication [18].

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✉ Guo-Hua Huang
ghhuang@hunau.edu.cn

[1] Hunan Provincial Key Laboratory for Biology and Control of Plant Diseases and Insect Pests, Changsha 410128, China

[2] College of Plant Protection, Hunan Agricultural University, Changsha 410128, China

3h-117 of HvAV-3h is one of the conserved coding regions whose homologs have been identified as structural proteins in SfAV-1a (ORF 109) and HvAV-3i (ORF 111) [17, 18]. In this study, we analyzed *3h-117* by transcriptional, expression, and localization analyses and demonstrated that the protein is present in HvAV-3h virions. This study provides a foundation for further investigation of the mechanisms underlying ascovirus morphogenesis and infection.

Materials and methods

Insects and viruses

HvAV-3h was propagated in *H. armigera* larvae as described previously [19]. The culture of *H. armigera* was maintained according to Li et al. [5].

Sequence and phylogenetic analysis

The full-length gene coding for *3h-117* was obtained from genome sequencing of HvAV-3h (GenBank: KU170628.1, direct submission by Huang et al.). The sequence was analyzed using the SCRATCH protein predictor (http://scratch.proteomics.ics.uci.edu/) and NLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) for the prediction of gene homologs, conserved domains, motifs, signal peptide, and nuclear localization signals [20]. Protein comparisons with entries in the updated GenBank/EMBL, SWISS-PROT and PIR databases were performed with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were performed with CLUSTALX (version 1.83) and GeneDoc. The phylogenetic tree was drawn using the maximum likelihood method based on the sequence alignment [21]. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X [22].

Generation of anti-3h-117 antiserum

The coding region was amplified from HvAV-3h genomic DNA by PCR using an upstream primer *3h-117*-F (5′-GGATCCATGTTTGCGAAACCAGAGC-3′, with a *BamH*I site underlined) and a downstream primer *3h-117*-R (5′-AAGCTTCTACGTATATCTATCGTGTAGGT-3′, with a *Hind*III site underlined). The PCR product was inserted into the pGEM-T Easy Vector (Promega, Madison, USA), digested with *BamH*I and *Hind*III, and then ligated into the expression vector pET-28a(+) (Novagen, Pfungstadt, GER). The 3H-117 fusion protein with a 6×His tag was expressed in *E. coli* BL21 (DE3) by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 12 h, and the 6×His-tagged recombinant 3H-117 protein was purified on a ProteinIso® Ni–NTA resin column (TransGen Biotech, Beijing, CHN). A New Zealand white rabbit was injected with 100 µg purified protein in Freund's complete adjuvant (Sigma-Aldrich, USA). This was followed by two booster injections in incomplete Freund's adjuvant (Sigma-Aldrich) at 2-week intervals before exsanguination. Ten days after the last injection, blood was recovered by cardiac puncture [23]. The prepared polyclonal rabbit antiserum against 3H-117 was used for the immunoassays.

Virion purification

Newly molted (6–12 h) third-instar *H. armigera* larvae were inoculated with HvAV-3h as described previously [24]. A sterile insect pin dipped into the HvAV-3h hemolymph was used to pierce the proleg of the third-instar larvae. Hemolymph samples (1 mL) from morbid larvae were collected with a pipette at 7 days post-infection and suspended in ice-cold TE buffer (10 mM Tris, 1.0 mM EDTA, pH 7.4) containing proteinase inhibitors (Roche, Shanghai, CHN). The suspension was sonicated (SCIENTZ, Ningbo, CHN) for 15 s at 10 W, layered onto a 25–55% (w/v) sucrose gradient and then centrifuged at 4 °C for 1.5 h at 72,100 g. The virion bands were collected, diluted fivefold with TE buffer, and centrifuged for 1 h at 4 °C at 110,000 g to pellet the virions. The formvar-coated grid containing the purified virions was negatively stained with 2% sodium phosphotungstate and checked by transmission electron microscopy (TEM) (H-7650; Hitachi, Japan).

Western blot analysis

Protein concentrations were determined by the Bradford method. Virion proteins were fractionated by 12% SDS-PAGE and blotted on nitrocellulose (NC) membranes by semidry electrophoretic transfer. The membranes were incubated for 1 h in 5% skimmed milk powder in TBST at room temperature. An anti-3H-117 antiserum was used as the primary antibody (diluted 1:5000) for 4 h at room temperature, followed by alkaline phosphatase conjugated anti-rabbit antibodies (1:5000) as secondary antibodies for 1 h at room temperature. Further detailed treatments were conducted as described previously [14]. The proteins were visualized with an enhanced chemiluminescence system (ChemiDoc™ XRS+; Bio-Rad), according to the manufacturer's instructions.

Transcription analysis

To analyze the temporal expression of *3h-117* in HvAV-3h-infected *H. armigera* larvae, total RNA was isolated from mock-infected and third-instar *H. armigera* larvae infected

with HvAV-3h at different time periods (3, 6, 12, 24, 48, 72, 96, 120, and 168 hpi) using the TRIzol RNA extraction kit (TaKaRa, Kusatsu, Japan) according to the manufacturer's protocol. The cDNA was synthesized with total RNA using a PrimeScript II 1st Strand cDNA Synthesis kit (TaKaRa) following the protocol specified by the manufacturer. The primers used were *3h-117*-F and *3h-117*-R, the *mcp*-specific primers *mcp* -F (5′-GGATCCATGACTTCAAACACAGAA ACGC-3′, *BamH*I site, underlined) and *mcp*-R (5′-CTCGAG TTAATTGAAATCGCCTCCG-3′, *Xho*I site, underlined), and the *gapdh*-specific primers *gapdh*-F (5′-ATGTCCAAA ATCGGTATCAACG-3′) and *gapdh*-R (5′-TTAATCCTTGGT CTGGATGTACT-3′). The subsequent PCR using the Taq™ enzyme (TaKaRa) was performed for 35 cycles, and then the PCR products were analyzed on 1% agarose gels.

Expression analysis

Early third-instar *H. armigera* larvae were inoculated with HvAV-3h as described above. Mock-infected larvae were used as a control, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control. The morbid larvae were collected at various time points (3, 6, 12, 24, 48, 72, 96, 120, and 168 hpi) and extracted by RIPA lysis buffer (Solarbio, Beijing, CHN) according to the manufacturer's suggested protocol. Protein samples (3 μg in each lane) were separated by 12% SDS-PAGE after being lysed in SDS-PAGE loading buffer by boiling for 10 min, followed by Western blot procedures as described above.

Immunohistochemical analysis

Third-instar *H. armigera* larvae inoculated with HvAV-3h were collected at different times (24, 72, and 120 hpi). The abdominal segments of the larval body were cut and fixed with 4% paraformaldehyde. Samples were then dehydrated and embedded in paraffin. Paraffin-embedded tissue sections designated for immunohistochemical staining require several pretreatment steps, including the removal of paraffin from and the rehydration of tissue sections and the retrieval of antigenicity. Sections were rinsed several times in xylene, dehydrated through a graded series of ethanol solutions, and cleared in distilled water. The remaining steps were carried out according to standard procedures [25]. The stained sections were observed under a microscope (Axio Vert A1; ZESSIS, GER) and photographed. The final image was analyzed with ZEN (ZESSIS, GER). The mock-infected larvae served as the control.

Results

Sequence and phylogenetic analyses

3h-117 is 570 nucleotides (nt) long and theoretically encodes a protein with a molecular weight of 21.5 kDa and a pI of 4.71. A search of the protein databases GenBank and SWISS-PROT predicted that the putative 3H-117 protein contains conserved domains in protein superfamilies known to be involved in nucleic acid metabolism, including the catalytic domain of CTD-like phosphatases (CPDc, smart00577), nuclear interacting factor-like phosphatases (NIF, NLI, P03031), and transcription factor TFIIF-interacting CTD phosphatases (FCP1, COG5190). According to PSORT II and NLS Mapper analyses, the 3H-117 protein was predicted to be localized in both the cytoplasm and nucleus.

A BLAST search of GenBank indicated that the predicted protein was present in all ascoviruses and most homologous to HvAVs and SfAV-1a (Fig. 1). Furthermore, 3H-117 shares 52% identity with the putative proteins of TnAV-6a and TnAV-6b. It is least homologous to the putative protein encoded by Diadromus pulchellus ascovirus 4a (DpAV-4a), with only 34% identity. In addition, *3h-117* showed more than 30% identity to the homologs from iridoviruses.

A phylogenetic tree was derived using the maximum likelihood method to compare the putative protein sequences from 10 AVs and 5 iridovirus species (Fig. 2). Our analysis indicated that HvAV-3h is most closely related to the HvAV-3j isolated in Japan. Notably, AVs are closely related to the Turbot reddish body iridovirus (TRBIV), which contains a CTD-like phosphatase. This result is consistent with the phylogeny of AVs based on the sequences of DNA polymerase and MCP [15, 26] and further strengthens the speculation that AVs and iridoviruses may share a common origin.

Immunodetection of 3H-117 in HvAV-3h virions

To determine if the 3H-117 protein is a structural protein, Western blot analysis was carried out on purified HvAV-3h virions (Fig. 3). Protein extracts of HvAV-3h-infected larvae isolated at 120 hpi were separated by SDS-PAGE and subjected to Western blot analysis using the 3H-117-specific rabbit antiserum (Fig. 4). The antibody did not react to mock-infected larvae but reacted strongly with a 21.5-kDa protein in the virions at 120 hpi, suggesting that the 3H-117 protein is a constituent of HvAV-3h virions. MCP is a commonly identified structural protein in ascoviruses, consistent with 3H-117. As a reference protein in its host, GAPDH reacted to extracts of mock-infected and infected larvae but did not react with virions.

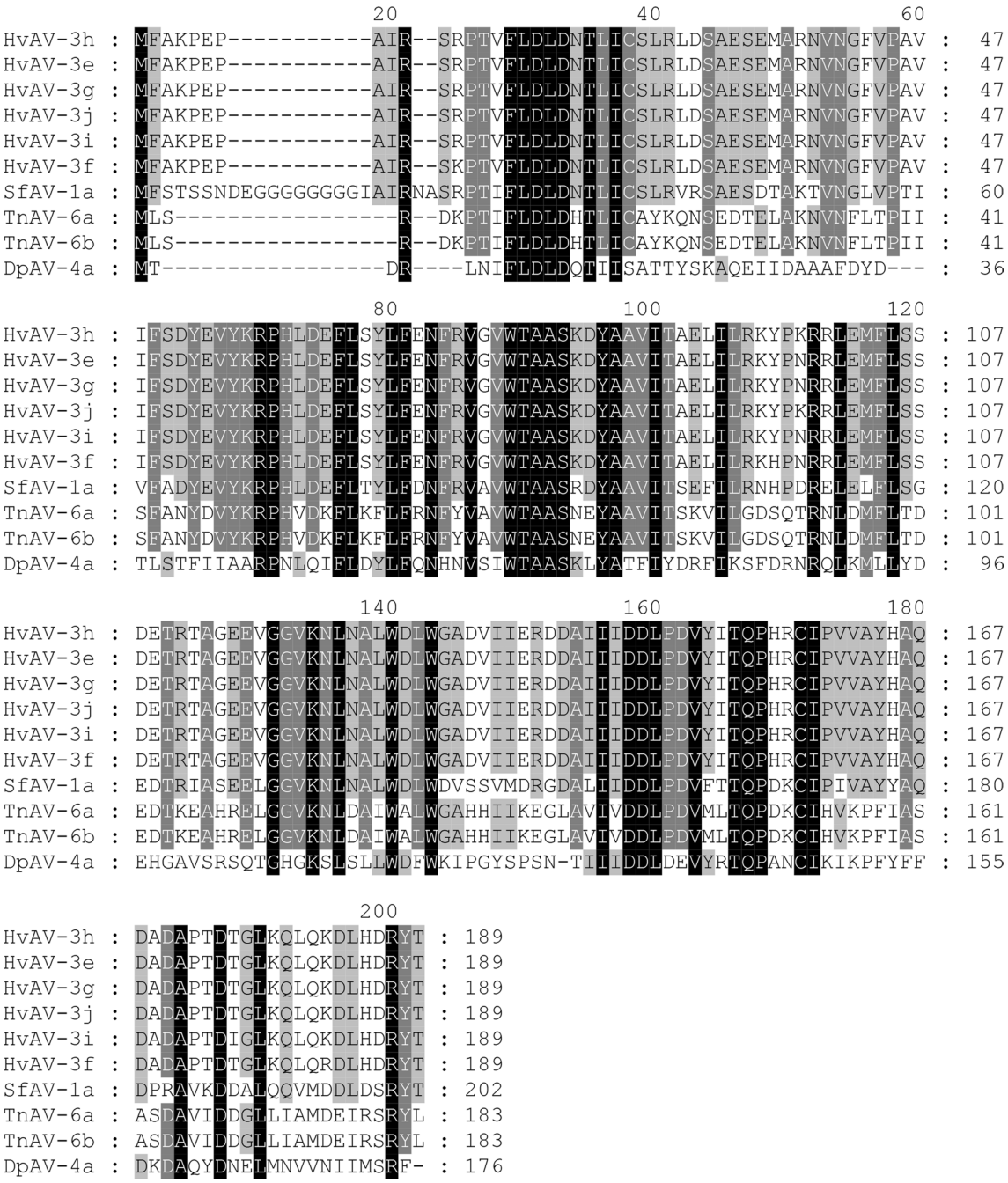

Fig. 1 Multiple sequence alignment of 3H-117 and its homologs. Numbers on the right show the amino acid position of different sequences. Black shading: 100% identity, dark gray shading: 90% identity, light gray shading: 70% identity. Abbreviations and data sources with GenBank accession numbers in parentheses: HvAV-3h, APM84218.1; HvAV-3j, BBB16589.1; HvAV-3e, YP_001110961.1; HvAV-3 g, AFV50375.1; HvAV-3i, AXN77294.1; HvAV-3f, AJP09081.1; SfAV-1a, YP_762464.1; TnAV-6a, YP_803316.1; TnAV-6b, YP_803316.1; DaPV-4a, YP_009220737

Transcription and expression analyses of *3h-117* in vivo

To establish whether *3h-117* was transcribed, RT-PCR analysis was performed with total RNA purified from mock-infected and HvAV-3h-infected *H. armigera* larvae at various time intervals. This analysis provided qualitative information regarding the temporal regulation of *3h-117* transcripts. A single band (approximately 0.6 kb) was detected as early as 6 hpi (Fig. 5), which remained stably detectable until 168 hpi. In contrast, the *mcp* fragment (1368 bp) was detectable at 3–96 hpi. No signal

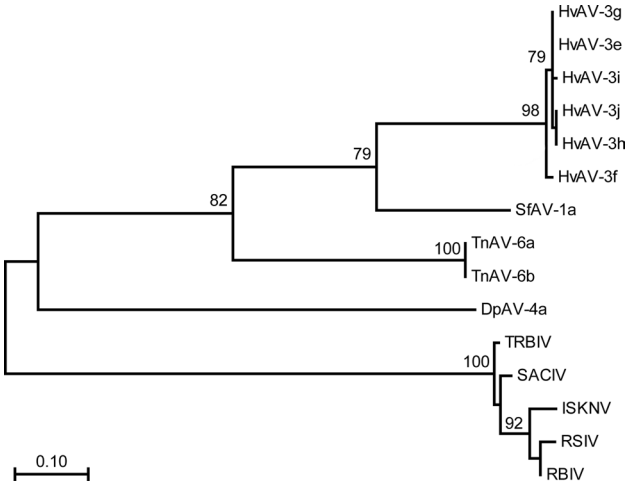

Fig. 2 Phylogenetic tree of ascoviruses and iridoviruses based on the comparison of 3H-117 protein homologs. Bootstrap values > 75% are shown at nodes (1000 replicates). Iridoviruses include the following: Infectious spleen and kidney necrosis virus (ISKNV), NP_612227.1; Rock bream iridovirus (RBIV), AAT71821.1; Turbot reddish body iridovirus (TRBIV), ADE34350.1; South American cichlid iridovirus (SACIV), AVR29663.1; Red seabream iridovirus (RSIV), BAZ95674.1

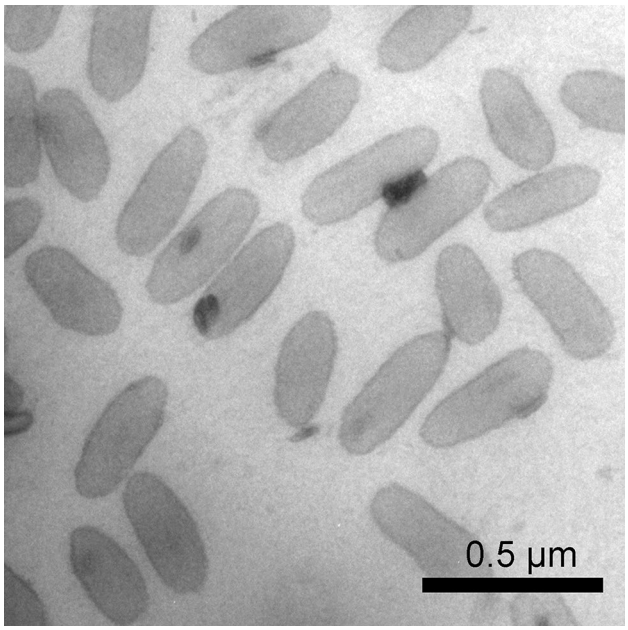

Fig. 3 Purification of HvAV-3h virions examined by TEM analysis

was amplified from the RNA isolated from mock-infected larvae.

Protein extracts of mock-infected and third-instar *H. armigera* larvae infected with HvAV-3h isolated at different times (0, 3, 6, 12, 24, 48, 72, 96, 120, and 168 hpi) were separated by 12% SDS-PAGE and subjected to Western blot analysis using the 3H-117-specific rabbit antiserum. This polypeptide was first detected at 24 hpi and remained detectable until 168 hpi (Fig. 6). No specific immunoreactive band was detected in mock-infected control larvae. The protein size of 21.5 kDa was in agreement with the predicted molecular weight, suggesting that no major post-translational modification of the 3H-117 protein occurred.

Localization of the 3H-117 protein in *H. armigera*

Immunohistochemical staining was carried out using anti-3H-117 rabbit antiserum as the primary antibody after removal of the paraffin from the paraffin-embedded tissue sections. The immunohistochemical results showed that green fluorescence was detected in the fat body at 24 hpi, verifying the presence of the 3H-117 protein. The positive signal localized primarily in the nucleus, although no apparent pathogenesis could be detected; at 72 hpi, the nucleus of the fat body became swollen, and positive signals were emitted from both inside and outside the nucleus; at 120 hpi, the integrity of the fat body was completely disrupted, emitting strong positive signals. There was no green fluorescence signal observed in the fat bodies of mock-infected larvae (Fig. 7).

Discussion

In the present study, we described and identified a structural protein from the HvAV-3h virus. The coding region potentially encodes a 21.5-kDa protein, which was confirmed by Western blot analysis with 3H-117 antiserum. This suggests that the 3H-117 protein is not extensively modified post-translationally, which is in line with other characteristics of 3H-117, as no signal sequence or disulfide bonds were predicted with the SCRATCH protein predictor.

3h-117 is a conserved coding region with homologs present in all ascoviruses, specifically various isolates of HvAV (-3j, -3e, -3g, -3i, -3f) and SfAV-1a. ORF 109 from SfAV-1a (now the type species of *Ascoviridae*), identified as a structural protein, shares 71% homology with 3H-117 [17], while ORF 111 from HvAV-3i shares 98% homology [18].

3H-117 contains domains conserved with the catalytic domain of CTD-like phosphatases, transcription factor TFIIF-interacting CTD phosphatases, and nuclear interacting factor-like phosphatases. Recently, Meinhart et al. [27] identified the function of CTD phosphatases in integrating nuclear events by binding proteins involved in mRNA biogenesis, which have roles in transcription, chromatin remodeling and modification, editing and nuclear export, DNA repair, and mRNA packaging [27]. Therefore, we speculated that 3H-117 has multiple functions in the metabolism of nucleic acids.

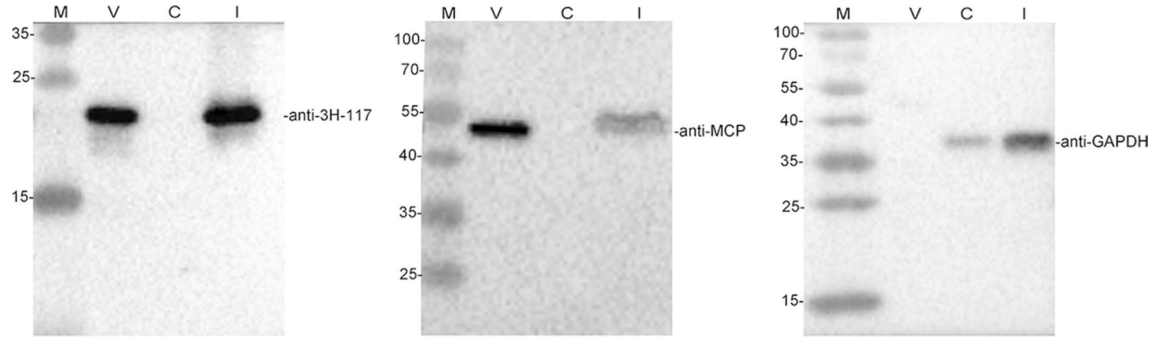

Fig. 4 Immunodetection of the 3H-117 protein in HvAV-3h virions. Virions (V lane 2) (2 mg per lane each), mock-infected third-instar *H. armigera* larvae (C lane 3), lysates of HvAV-3h-infected third-instar *H. armigera* larvae (120 hpi) (I lane 4) were analyzed by SDS-PAGE and Western blot analysis. Size standards are used. Molecular mass standards of marker (M) are shown on the left. The antisera used are shown on the right

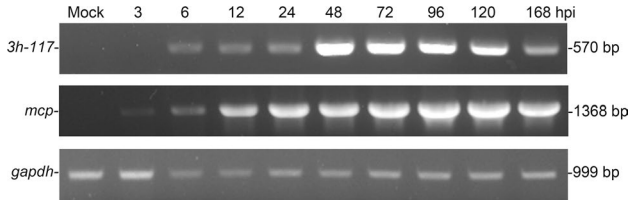

Fig. 5 Transcription analysis of *3h-117* in HvAV-3h-infected *H. armigera* larvae. Total RNA was extracted from HvAV-3h infected *H. armigera* larvae at 3, 6, 12, 24, 48, 72, 96, 120, and 168 hpi and in mock-infected larvae. PCR was performed, and the amplification products were subsequently analyzed by electrophoresis in a 1% agarose gel. *mcp* is a positive control for structural protein genes, and *gapdh* is an internal loading control

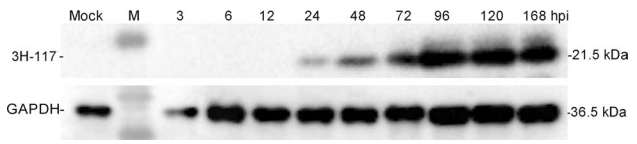

Fig. 6 Expression analysis of 3H-117 in HvAV-3h-infected *H. armigera* larvae. Protein samples were harvested from 3 to 168 hpi, separated by 12% SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with anti-3H-117 polyclonal antiserum. Mock-infected larvae and GAPDH were used as controls

To date, two of the most abundant structural proteins, MCP and P64, have been investigated and shown to be essential factors in ascoviruses [15–17]. MCP is highly conserved among *Ascoviridae*, *Iridoviridae*, *Phycodnaviridae*, and African swine fever virus [28]. This protein, first identified and reported from TnAV-2a and named TnAV-cp, and its homologs in other ascoviruses are the predominant structural proteins in virions used to the study of the evolution of several related dsDNA viruses [15]. P64 has been described previously in SfAV-1a as a structural protein required for packaging the large viral genome into the virion during assembly. In this regard, an intriguing finding suggests that the interaction between the CTD-like phosphatase, serine/threonine kinase, and DNA-binding protein could facilitate the phosphorylation/dephosphorylation of DNA-binding proteins to allow for the condensing and releasing of gDNA in the virions [14]. However, further studies are required to confirm whether the three proteins function as a unit.

To elucidate the function of 3H-117, we analyzed the transcription and expression of *3h-117* in HvAV-3h-infected *H. armigera* larvae. RT-PCR showed that *3h-117* transcription started at 6 hpi, but Western blot analysis suggested that 3H-117 was first detected at 24 h and continued to be present at 168-h post-infection. This was somewhat unexpected because the *3h-117*-specific transcript was detected as early as 6 hpi. This may be due to the low level of 3H-117 protein present at earlier time points or the low affinity of the 3H-117 antiserum and may relate the unique structural and biological characteristics of ascoviruses, which distinguish them from other insect viruses.

As reported previously, HvAV-3h infection leads to destructive pathological changes in the host larval fat bodies. Hematoxylin–eosin staining of transverse sections from HvAV-3h-infected *S. exigua* larvae illustrated the pathological morphology among different tissues. The fat bodies of the HvAV-3h-infected larvae had disintegrated into fragments by 72 hpi, although no observable differences were found in the muscle and gut tissues during the same time period [29]. In this report, immunohistochemical analyses were conducted to verify the expression of the structural or virion protein, 3H-117, in larval tissues. The analyses demonstrated the pathogenic process HvAV-3h caused in the fat bodies of infected *H. armigera* larvae.

In conclusion, *3h-117* is conserved among AVs that encode a component protein in the HvAV-3h virion. Future studies will allow the identification of possible interactions between these structural proteins and how these proteins enable HvAV-3h to manipulate a successful infection

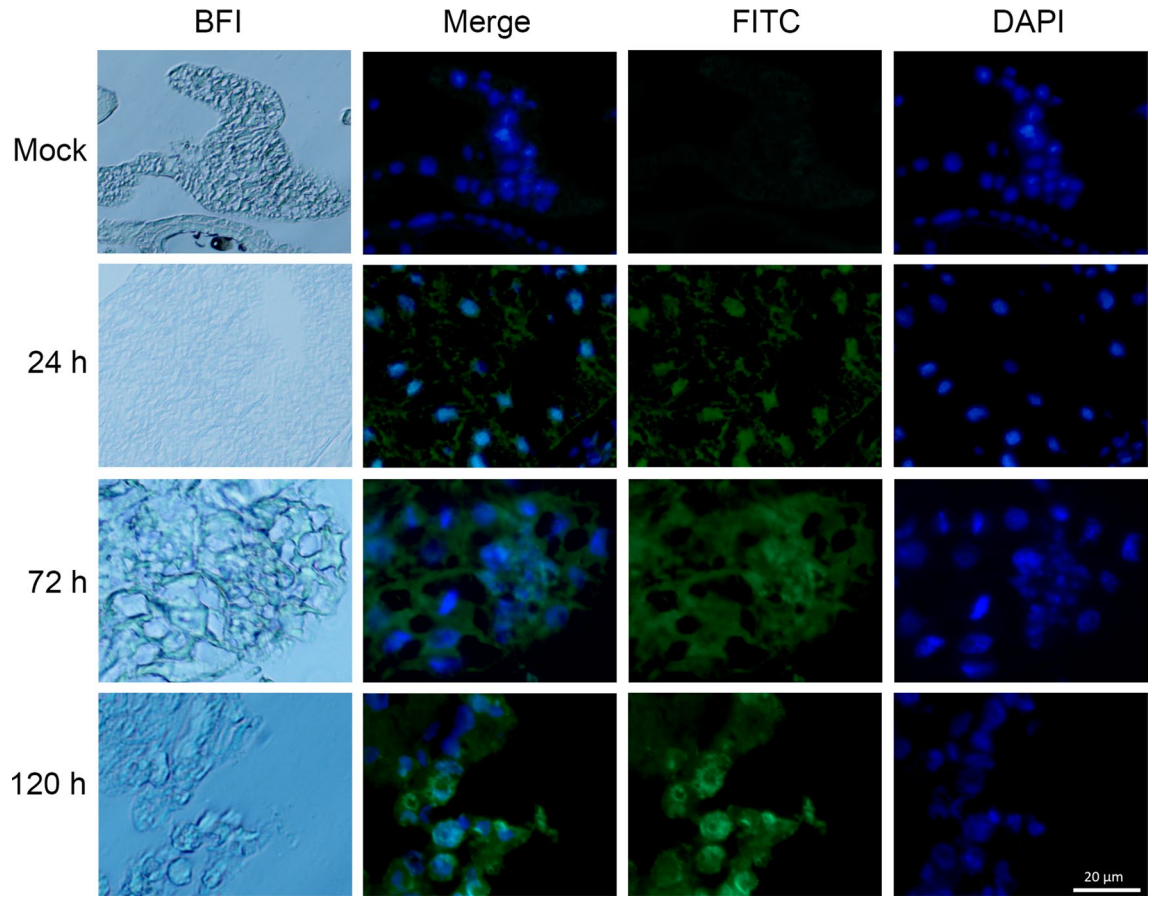

Fig. 7 Immunohistochemistry analysis. The fat body tissues from third-instar *H. armigera* larvae infected by HvAV-3h at different points post-infection (shown at the left). Fluorescence microscopy shows the progression of the 3H-117 protein in larvae from 24 to 120 hpi. All the pictures have the same magnification, and the scale bar represents 20 μm

in vivo and to elucidate the structure and organization of AV virions. This study establishes a foundation for further investigation of ascoviral virions.

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Author contributions YZ, HY, and GHH conceived and designed the experiments. YZ, NL, and LH performed the experiments. YZ, GHH wrote the manuscript. All the authors discussed and commented on the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Research involving human participants and/or animals This study does not contain any studies with human participants performed by any of the authors.

Informed consent This study does not involve any human subject and therefore, there is no need of informed consent.

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