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Characterization of Heliothis Virescens Ascovirus 3h *orf21* that Encodes a Virion Protein

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ABSTRACT

Homologues of Heliothis virescens ascovirus 3h (HvAV-3h) *orf21* are found in 9 completely sequenced members of the ascoviruses, but so far their functions are unknown. Here, *orf21* (*3h-21*) was cloned in-frame into a pET-28a bacterial expression vector. The fusion protein produced by this construct was used for the preparation of a polyclonal antiserum. RT-PCR analysis showed a single transcript of *3h-21* of approximately 0.7kb was transcribed beginning at 24h post-infection in infected *Helicoverpa armigera* larvae. Western blot analysis of extracts from HvAv-3h-infected *Helicoverpa armigera* larvae detected a 25.6 kDa protein late in infection. This antiserum also reacted with a 25.6 kDa protein in purified virions of HvAv-3h. The protein was not extensively modified post-translation. Immunoelectron microscopy confirmed that the 3H-21 is associated with the structure of HvAV-3h virions.

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Abbreviations: dsDNA, double-stranded circular DNA; ORFs, Open Reading Frames; MCP, Major Capsid Protein; HvAV-3h, Heliothis virescens ascovirus 3h SDS-PAGE,SDS-Polyacrylamide Gel Electrophoresis; TEM, Transmission Electron Microscopy .

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Introduction

The *Ascoviridae* are a family of large, enveloped virions containing double-stranded circular DNA (dsDNA) genomes of 110 to 200kbp[1,2]. Ascoviruses are infectious to the lepidopteran insects transmitted by parasitoid wasps, causing chronic infections with symptoms of stunted growth and milk-coloured haemolymph[3]. Some of them are a natural control agent of *Helicoverpa armigera* (Hübner), *Spodoptera exigua* (Hübner) and *Spodoptera litura* (Fabricius) such insect pests in agriculture and forestry[4].

The *Ascoviridae* family has been divided into two genera: *Ascovirus*, which infects various members of the family Noctuidae (Insecta: Lepidoptera), and *Toursvirus*, which replicates in both its lepidopteran and parasitoid vector hosts[5]. Eleven isolates of ascoviruses have been completely sequenced to date[1,2,6-14]. Further researches reported that 25 ascovirus open reading frames (ORFs) were found to be conserved in all ascoviruses except the members of *Toursvirus* [2]. So far, researches of major capsid protein (MCP, TnAV-2a) and DNA-binding protein (P64, SfAV-1a) and ORF 117 (3H-117, HvAV-3h) has been carried out, but almost the viral genes remain to be explored [14-16].

Heliothis virescens ascovirus 3h (HvAV-3h) belongs to the Ascovirus genus. It cause high mortality among economically important insect pests, thereby controlling insect populations. We sequenced the HvAV-3h genome which is 190,519 bp in length with 185 ORFs[3]. HvAV-3h *orf21* (*3h-21*) is located between nts 27,539 and 28,225 in the HvAV-3h genome, encodes a putative 228 aa with predicted molecular weight of 25.6 kDa.

Homologs of 3H-21 have been identified in 9 completely sequenced ascoviruses suggesting that it maybe involved in some basic process of viral life cycle, but are not present in *Toursvirus* which includes two species (*Diadromus pulchellus toursvirus 1*, DpTV-1a and *Dasineura jujubifolia toursvirus 2*, DjTV-2a). Thus far it is uncertain whether 3H-21 and its homologs are functional genes. *3h-21* is one of conserved gene which homologues encoding structural protein in TnAV-6a (ORF 4), SfAV-1a (ORF 17) and HvAV-3i (ORF 22)[17-19]. In this study, we analyzed the *3h-21* gene by examining transcription of the gene and expression of its protein product in HvAV-3h infected *Helicoverpa armigera* larvae, finding that the *3h-21* is indeed expressed and that the 3H-21 protein is located on the virion of HvAV-3h.

Materials and Method

1. Insects and viruses

HvAV-3h was isolated by Huang *et al*. and stored in Huang's laboratory. HvAV-3h was propagated in *H. armigera* larvae as described previously[20]. A laboratory colony of *H. armigera* insects

was cultured on an artificial diet at 27 ± 1 °C and a 16/8 h (light/dark) photoperiod according to Yu *et al*. [21].

2. Computational analysis

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 prediction of gene homologues, conserved domains, motifs, signal peptide and nuclear localization signals[22]. 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 alignment was performed with CLUSTALX (version 1.83) and Gene Doc.

3. Generation of anti-3H-21 antiserum

The *3h-21* was amplified from the HvAV-3h genomic DNA by PCR using an upstream primer 3h-21-F (5'-GGATCCATGTCAATATCCAGTGTATCCG-3', with a BamH I site underlined) and a downstream primer 3h-21-R (5'-CTCGAGTCAGTCGGTTGGTAATCTACAC-3', with a Xho I site underlined). The PCR product was inserted into pGEM-T Easy vector (Promega, USA), then digested with BamH I and Xho I, and was ligated into the expression vector pET-28a(+) (Novegen, GER).

The 3H-21 fusion protein with a 6× His tag was expressed in E. coli BL21(DE3) by inducing with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 12 h , and the 6× His-tagged recombinant 3H-21 protein was purified on proteinlso Ni-NTA resin code column (TransGen Biotech, CHN). The purified protein was injected subcutaneously to immunize New Zealand white rabbits[23]. The prepared polyclonal rabbit antiserum against 3H-21 was used for the immunoassays.

4. Virion purification

Newly molted (6-12 h) third-instar *H. armigera* larvae were inoculated with HvAV-3h as described previously[24]. The hemolymphs of morbid larvae were collect 7 days post infection and suspended in ice-cold TE buffer (10 Mm Tris, 1.0mM EDTA, pH 7.4) in the presence of protease inhibitors. The suspension was solicited for 15s at 10 W, layered onto a 25 to 55% (w/v) sucrose gradient and then centrifuged at 4 °C for 1.5h at 72,100g. The virion bands were collected, diluted fivefold with TE buffer and centrifuged for 1h at 4°C at 110,000 g to sediment the virions (Hou *et al.*, 2013). The purity of virions in negative staining was checked by transmission electron microscopy (TEM).

5. Western blotting

Protein concentrations were determined by the method of Bradford. Virion proteins were fractionated by SDS-PAGE, blotted on Nitrocellulose membranes (NC) by semi-dry electrophoresis transfer. Using an anti-3H-21 antiserum as the primary antibody (diluted 1:5000) for 1 h at room temperature, followed by alkaline

phosphatase conjugated anti-rabbit antibodies (1:5000). Further treatments as described previously[14]. Lysates of mock-infected third-instar *H. armigera* larvae were used as a negative control, and infected with HvAV-3h served as a positive control.

6. Tanstription analysis

To confirm expression and analyze temporal expression of *3h-21* in *H. armigera* larvae, total RNA was isolated from mock-infected and third-instar *H. armigera* larvae infected with HvAV-3h for different time points (0, 3, 6, 12, 24, 48, 72, 96, 120 and 168 hpi) using the Trizol RNA extraction kit (TaKaRa, JPN) according to the manufacturer's protocol.

The cDNA was synthesized with the total RNA by using a PrimeScript II 1st Strand cDNA Synthesis kit (TaKaRa) following the protocol specified by the manufacturer. The primers used were the *3h-21*-specific primers (as above), the mcp-specific primers mcp-F (5'-GGATCCATGACTTCAAACA CAGAAACGC-3', BamH I site underlined) and mcp-R(5'-CTCGAGTTAATTGAAA TCGCCTCCG-3', Xho I site underlined), and the *gapdh*-specific primers *gapdh*-F (5' -ATGTCCAAAATCGGTATCAACG-3') and *gapdh*-R (5'-TTAATCCTTGGTCTG GATGTACT-3'). The subsequent PCR was performed with 35 cycles and then the PCR products were analyzed on a 1.0 % agarose gel.

7. Expression analysis

Early third-instar *H. armigera* larvae were inoculated with HvAV-3h as described previously. Mock-infected larvae were taken as a control and the GAPDH as a positive control. The morbid larvae were collected at various time points (0, 3, 6, 12, 24, 48, 72, 96, 120 and 168 hpi) post infection and extracted by RIPA (Solarbio, CHN) according to manufacturer's suggested protocol. Protein samples were separated by SDS-PAGE after lysed in SDS-PAGE loading buffer by boiling for 10min and the procedures of western blot analysis as described above.

8. Fluorescence microscopy analysis

The third-instar *H. armigera* larvae inoculated with HvAV-3h were collected at different time points (48, 96, and 120 hpi). The abdominal segments of the larval body were cut and fixed with 4% paraformaldehyde. The samples were then dehydrated and embedded in paraffin. The paraffin sections immersed in the distilled water after removed 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. The next steps were carried out according to standard methods[25]. The stained sections were observed under the microscope and were photographed. The mock-infected larvae served as control.

Results

1. Sequence analysis of 3H-21 and its homologues

The *3h-21* gene is 687 nucleotides in length, theoretically encoding a protein with molecular weight of 25.6kDa and pI of 5.86. BLAST search of GenBank indicated that the predicted protein was present in all ascovirus except the members of *Toursvirus* and most homologous to HvAVs and SfAV-1a proteins respectively. (See figure 1).

2. Transcription analysis of 3h-21

To see whether *3h-21* was transcribed, RT-PCR analysis was performed with total RNA purified from mock-infected and HvAV-3h-infected *H.armigera* larvae at various times. This analysis served to provide information about temporal regulation and the sizes of *3h-21* transcripts. A single band of the expected size (approximate 0.7 kb) was detected as early as 6 hpi, which remained stably detectable to 168 hpi. The transcript size

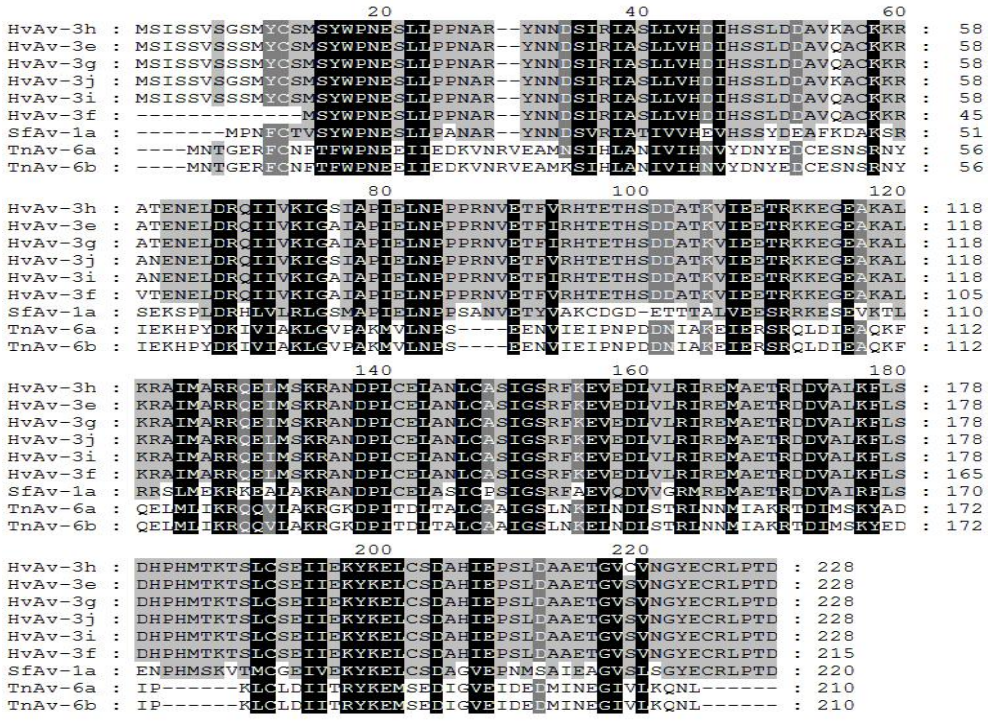

Figure 1. Multiple sequence alignment of 3H-21 and its homologues.

Black shading: 100% identity. Dark grey shading: 85% identity. Light grey shading: 70% identity.

Alignment of 3H-21 homologs encoded by Spodoptera frugiperda ascovirus 1a (SfAV-1a), and various isolates of Heliothis virescens ascovirus (HvAV-3e, -3f,-3g, -3i, -3j). Abbreviations and data sources with GenBank accession numbers in parentheses: HvAV-3h, ORF 21 (AYD68152.1); HvAV-3j, ORF 23 (BBB16493.1); HvAV-3e, ORF 23 (YP_001110876.1); HvAV-3g, ORF 24 (YP_009701680.1); HvAV-3i, ORF 22 (AXN77205.1); HvAV-3f, ORF 22 (YP_009701488.1); SfAV-1a ORF 17 (YP_762372.1).

in tune with predicted size of 687 bp. In contrast, the mcp fragment (1368 bp) was detectable at 3-96 hpi. No signal was amplified from the RNA isolated from mock-infected larvae.

3. Immunodetection of the 3H-21 protein in vivo and in virus particles

Protein extracts of mock-infected and third-instar *H.armigera* larvae infected with HvAV-3h isolated at different time points (0, 3, 6, 12, 24, 48, 72, 96, 120 and 168 hpi) were separated by SDS-PAGE and subjected to western blot analysis using the 3H-21-specific rabbit antiserum. This polypeptide was first detected at 48 hpi and remained detectable up to 168 hpi (See figure 2). No specific immuno--reactive band was detected from mock--infected control larvae. The protein size of 25.6 kDa was in agreement with the predicted molecular weight, suggesting that no major post translational modification of the 3H-21 protein occurred.

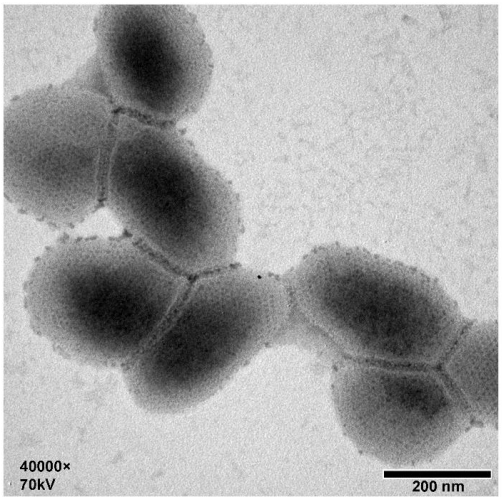

Figure 2. Purification of HvAV-3h virions were examined by TEM analysis.

To investigate whether the 3H-21 protein is a structural protein, western blot analysis was carried out on purified HvAV-3h virions (See figure 3 and 4). 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-21-specific rabbit antiserum (See figure 5). The antibody did not react to mock-infected larvae but reacted strongly with a 25.6 kDa protein at virions and 120 hpi, suggesting that the 3H-21 protein is a constituent of HvAV-3h virions. MCP was a widely identified structural protein among ascoviruses, in tune with the 3H-21. As a reference protein in host, GAPDH react to extract of mock-infected and infected larvae but did not reacted with virions.

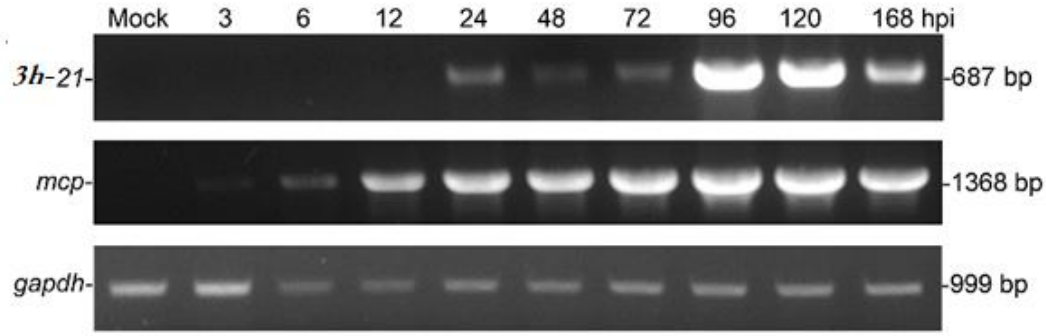

Figure 3. Transcription analysis of *3h-21* in HvAV-3h infected *H. armigera* larvae.

Total RNA was extracted from HvAV-3h infected H. armigera larvae at 3, 6, 12, 24, 48, 72, 96, 120 and 168 hpi or mock-infected larvae. PCR was performed, and the amplification products were subsequently analyzed electrophoresis in a 1.0 % agarose gel. Mcp is a positive control for structural protein genes, and *gapdh* is a internal loading control.

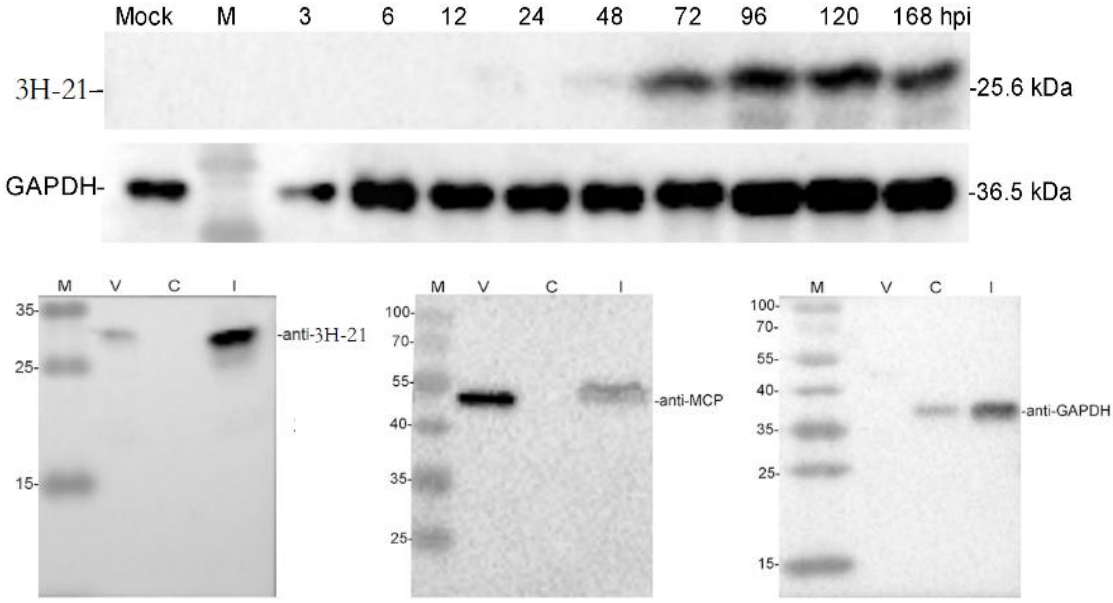

Figure 4. Western blot analysis of 3H-21.

(a) Western blot analysis of 3H-21 in HvAV-3h-infected *H. armigera* larvae from 3 to 168 hpi lane numbers correspond to time p.i..Mock-infected larvae and GAPDH used as controls. (b) Western blot analysis of 3H-21 in the purified 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 h p.i.) (I lane 4) were analysed by SDS-PAGE and western blot analysis. Size standards are indicated on the left; the single 25.6 kDa immunoreactive protein is indicated on the right.

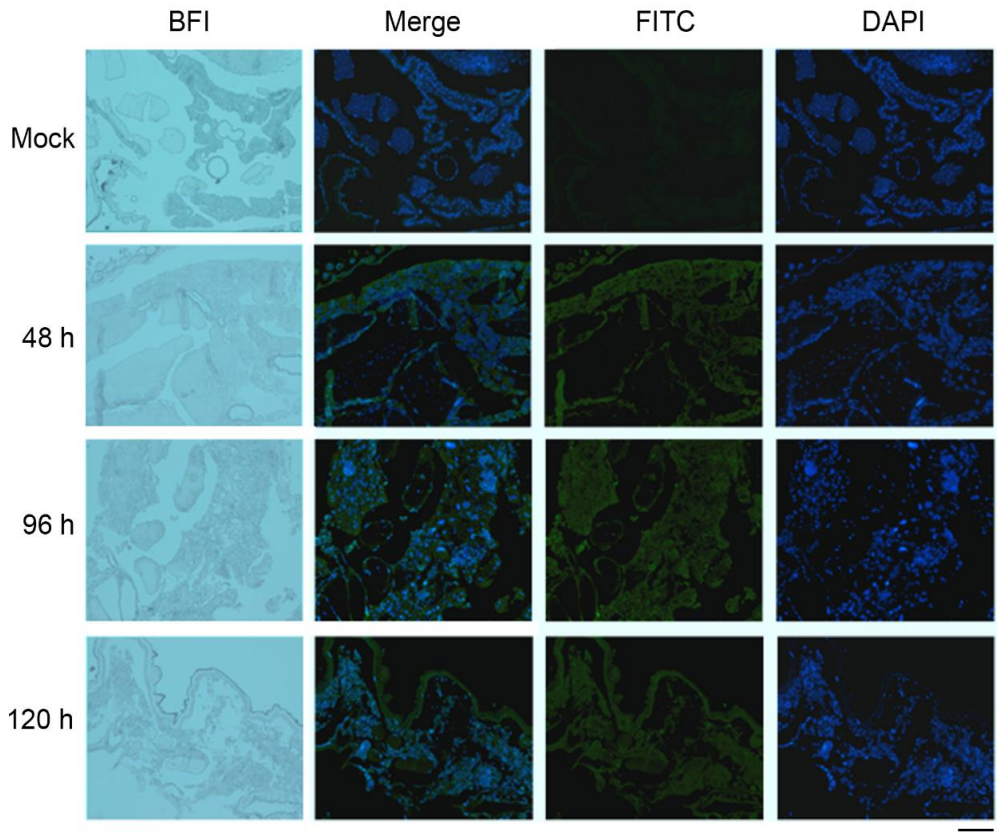

Figure 5. Immunohistochemistry analysis.

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 3H-21 proteins in larvae from 48 to 120 hpi. Scale bar 100 nm.

4. Localization of 3H-21 proteins in insect

Immunohistochemical staining was carried out with against 3H-21 rabbit antiserum as the primary antibody after paraffin-embedded tissue sections removing paraffin from and retrieving antigenicity. The immunohistochemistry results showed that no strong green fluorescence signal was detected in all tissues of mock-infected larvae, indicating that no expression of 3H-21 protein. The positive signal was observed in most fat body cells 48 hpi, indicating the expression of 3H-21 protein. At 92 hpi, the nucleus of fat body became swollen and the positive signal distributed both inside and outside the nucleus; 120 hpi, the constitution of fat body were completely destroyed, most of the cells showed positive signal, and 3H-21 protein was largely expressed.

Disccusions

In this study, we have described the identification a novel structural protein of HvAV-3h. This protein, encoded by 3H-21, is hitherto unique to *Ascovirus* and has not been found in any other *Toursvirus* to date. The coding region of 3H-21 potentially encodes a 25.6 kDa protein, which is close to the actual size of the protein confirmed by western blot analysis with the prepared 3H-21 antiserum. This suggests that the protein is not extensively modified post-translation, for example, by glycosy--lation. This is agree with other characteristics of 3H-21, as it does not have a signal sequence or a Golgi retention signal were predicted with Scratch protein predictor.

3h-21 is a conserved gene with homologues in all *Ascovirus*, specifically various isolates of HvAV (-3j, -3e, -3g, -3i, -3f) and SfAV-1a. The ORF 22 from HvAV-3i identified as a structural protein share 97% homology with 3H-21[17], the ORF 17 from SfAV-1a (now the type species of *Ascoviridae*) share 61% homology[18] , and the ORF 4 from TnAV-6a share 30% homology[19].

To elucidate the function of 3H-21, we analyzed the transcription and expression of 3H-21 in HvAV-3h-infected *H. armigera* larvae. RT-PCR showed that *3h-21* transcription started at 6hpi, but western blot analysis suggested that 3H-21 was first detected at 48h and continued to be present at 168 hpi. This type of temporal expression also exit in our other study of structural protein in HvAV-3h [16]. Ascoviruses have unique structural and biological characteristic which distinguish from other insect virus. Considering this, this type of temporal different from transcription and expression may cause by the unique feature of ascovirus. The functions and precise location of 3H-21 in the virion have not been elucidated fully. This may suggest that the 3H-21 protein either plays a role in the assembly of ascovirus virion.

So far, three structural proteins, MCP, P64 and 3H-117 have been investigated to be essential factors for ascoviruses which is highly conserved among *Ascoviridae, Iridoviridae* [15,16,18,19]. MCP has been described previously in TnAV-2a as one of major structural proteins. And used for the evolutional study of several related dsDNA viruses [15]. P64 is another most abundant protein in SfAV-1a as a structural protein required for packaging of the large viral genome into the virion during assembly[14]. An intriguing finding suggests that P64 interacting with other structural proteins as a unit could condense and release gDNA in the virions [26]. 3H-117 is conserved among all sequenced ascoviruses which have identified as a structural protein in HvAV-3h with a typical sub-cellular transport from nucleus to cytoplasm [16].

As reported previously, HvAV-3h infection leads to destructive pathological

changes in the host larval fat bodies. Hematoxylin-eosin staining of larval transverse sections from HvAV-3h infected *H. armigera* larvae, illustrating the pathological morphology among different tissues. The fat bodies of the HvAV-3h infected larvae had disintegrated into debris in 72 hpi but no distinct differences were found in the muscle and gut tissues during the same time period [27]. In this report, immunohistochemical analysis were carried out to suggest the expression of structural proteins or virions in larvae tissues represented by 3H-21, which reveals the pathogenic process of HvAV-3h in the fat bodies of infected *H. armigera* larvae. Our results are consistent with the H.E staining analysis by Li *et al.*.

In conclusion, *3h-21* was identified as a conserved gene in Ascovirus encoding a component protein of HvAV-3h virion. Further experiments would allow the determination of possible interactions of these structural proteins and how these proteins enable HvAV-3h to set up a successful infection *in vivo*, and to elucidate the structure and organization of ascoviral virions. This study establish a foundation for further investigation of ascoviral virions.

Statement of Author Contributions

Y.Z., H.Y., N.L., G.H.H. conceived and designed the experiments. Y.Z. performed the experiments and wrote the manuscript. All the authors discussed and commented on the manuscript.

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References

[1]. Liu YY, Xian WF, Xue J, et al. Complete genome sequence of a renamed isolate, Trichoplusia ni ascovirus 6b, from the United States[J]. Genome Announcements, 2018, 6(10): UNSP e00148-18.

[2]. Huang GH, Hou DH, Wang M, et al. Genome analysis of Heliothis virescens ascovirus 3h isolated from China[J]. Virologica Sinica, 2017, 32 (2):147-154.

[3]. Arai E, Ishii K, Ishii H, et al. An ascovirus isolated from *Spodoptera litura* (Noctuidae: Lepidoptera) transmitted by the generalist endoparasitoid *Meteorus pulchricornis* (Braconidae: Hymenoptera)[J]. Journal of General Virology, 2018,99 (4):574-584.

[4]. Li SJ, Wang X, Zhou ZS,et al. A comparison of growth and development of three major agricultural insect pests infected with Heliothis virescens ascovirus 3h (HvAV-3h) [J]. PLoS ONE, 2013, 8(12): e85704.

[5]. Asgari S, Bideshi DK, Bigot Y, et al. ICTV Virus Taxonomy Profile: Ascoviridae[J]. Journal of General Virology, 2017, 98 (1): 4-5.

[6]. Bideshi DK, Demattei MV, Rouleux-Bonnin F, et al. Genomic sequence of Spodoptera frugiperda ascovirus 1a, an enveloped, double--stranded DNA insect virus that manipulates apoptosis for viral reproduction[J]. Journal of Virology, 2006, 80 (23): 11791-11805.

[7]. Bigot Y, Rabouille A, Sizaret PY, et al. Particle and genomic characteristics of a new member of the Ascoviridae: Diadromus pulchellus ascovirus[J]. Journal of General Virology, 1997, 78 (5): 1139-1147.

[8]. Wang L, Xue J, Seaborn CP, et al. Sequence and organization of the Trichoplusia ni ascovirus 2c (Ascoviridae) genome [J]. Virology, 2006, 354 (1): 167-177.

[9]. Asgari S, Davis J, Wood D, et al. Sequence and organization of the Heliothis virescens ascovirus genome[J]. Journal of General Virology, 2007, 88: 1120-1132.

[10]. Wang J, Yang M, Xiao H, et al. Genome Analysis of Dasineura jujubifolia Toursvirus 2, A Novel Ascovirus[J]. Virologica Sinica, 2019: 1-9..

[11]. Smede M, Furlong MJ, Asgari S. Effects of Heliothis virescens ascovirus (Hvav-3e) on a novel host, *Crocidolomia pavonana* (Lepidoptera: Crambidae)[J]. Journal of Invertebrate Pathology, 2008, 99 (3): 281-285.

[12]. Huang GH, Wang YS, Wang X, et al. Genomic sequence of Heliothis virescens ascovirus 3g isolated from *Spodoptera exigua*[J]. Journal of Virology, 2012, 86 (22): 12467-12468.

[13]. Chen ZS, Cheng XW, Wang X, et al. Genomic analysis of a novel isolate Heliothis virescens ascovirus 3i (Hvav-3i) and identification of ascoviral repeat orfs (aros)[J]. Archives of Virology, 2018, 163 (10): 2849-2853.

[14]. Tan Y, Spears T, Bideshi DK, et al. P64, a novel major virion DNA-binding protein potentially involved in condensing the Spodoptera frugiperda ascovirus 1a genome[J]. Journal of Virology, 2009, 83 (6): 2708-2714.

[15]. Zhao K, Cui L. Molecular characterization of the major virion protein gene from the Trichoplusia ni ascovirus[J]. Virus Genes, 2003, 27 (1): 93-102.

[16]. Zhao Y, Yu H, He L, et al. 3H-117, a structural protein of Heliothis virescens ascovirus 3h (HvAV-3h) [J]. Virus Genes, 2019, 55 (5): 688-695.

[17]. Chen ZS, Cheng XW, Wang X, et al. Proteomic analysis of the Heliothis virescens ascovirus 3i (HvAV-3i) virion [J]. Journal of General Virology, 2018, 100 (2): 301-307.

[18]. Tan Y, Bideshi DK, Johnson JJ, et al. Proteomic analysis of the Spodoptera frugiperda ascovirus 1a virion reveals 21 proteins [J]. Journal of General Virology, 2009, 90: 359-365.

[19]. Cui L, Cheng X, Li L, et al. Identification of Trichoplusia ni ascovirus 2c virion structural proteins. Journal of General Virology, 2007, 88: 2194-2197

[20]. Huang GH, Garretson TA, Cheng XH, et al. Phylogenetic position and replication kinetics of Heliothis virescens ascovirus 3h (Hvav-3h) isolated from *Spodoptera exigua* [J]. PLoS ONE, 2012,7 (7): e40225.

[21]. Yu H, Li ZQ, He L, et al. Response analysis of host *Spodoptera exigua* larvae to infection by Heliothis virescens ascovirus 3h (HvAV-3h) via transcriptome [J]. Scientific Reports, 2018, 8: 5367.

[22]. Kosugi S, Hasebe M, Tomita M, et al. Systematic identification of cell cycle--dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs[J]. Proceedings of the National Academy of Sciences of the United States of America,2009, 106 (25):10171-10176.

[23]. Li XF, Yu H, Zhang CX, et al. *Helicoverpa armigera nucleopolyhedrovirus orf81* is a late gene involved in budded virus production[J]. Archives of Virology, 2014,159 (8): 2011-2022.

[24]. Federici B A, Vlak JM, Hamm JJ. Comparative study of virion structure, protein composition and genomic DNA of three ascovirus isolates [J]. Journal of General Virology, 1990, 71: 1661-1668.

[25]. Boenisch T. Pretreatment for immunohisto--chemical staining simplified [J]. Applied Immunohistochemistry & Molecular Morphology, 2007, 15 (2): 208-212.

[26]. Bideshi DK, Demattei MV, Rouleux--Bonnin F, et al. Genomic sequence of Spodoptera frugiperda ascovirus 1a, an enveloped, double-stranded DNA insect virus that manipulates apoptosis for viral reproduction [J]. Journal of Virology, 2006, 80 (23): 11791-11805.

[27]. Li ZQ, Yu H, Huang GH. Changes in lipid, protein and carbohydrate metabolism in *Spodoptera exigua* larvae associated with infection by Heliothis virescens ascovirus 3h[J]. Journal of Invertebrate Pathology, 2018, 155: 55-63.