



Overexpression of a virus-like particle influenza vaccine in Eri silkworm pupae, using *Autographa californica* nuclear polyhedrosis virus and host-range expansion

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Abstract

Ecological investigations of silkworms have revealed that Eri silkworms (*Samia cynthia ricini*) possess useful morphological and ecological characteristics for virus-like particle (VLP) production, namely non-seasonal breeding, longer lengths, and heavier weights than *Bombyx mori* silkworms. Furthermore, when vector DNA from *Bombyx mori* nuclear polyhedrosis virus (BmNPV), which is unable to replicate in Sf9 cells from Eri silkworms, was replaced with the *Autographa californica* nuclear polyhedrosis virus (AcNPV) vector, three improved AcNPV influenza virus recombinants capable of replication in Sf9 cells were obtained. Although VLP antigens produced previously in silkworms were not evaluated individually, the present recombinant Fukushima (FkH5) and Anhui (AnH7) VLP antigens were detected in tissue fluids and fat bodies of Eri silkworms. Here, we aimed to determine the function of the AcNPV vector and *P143* gene by expressing recombinants in Sf9 cells and eri silkworm pupae. The FkH5 recombinant produced high yields of haemagglutinin (HA)-positive VLPs, showing a mean HA titre of 1.2 million. Similarly, high production of H7 HA VLPs was observed in the fat bodies of eri silkworm pupae. Antigenic analysis and electron microscopy examination of Eri-silkworm-produced H5 HA VLPs showed characteristic antigenicity and morphology similar to those of the influenza virus. Although FkH5 recombinants possessing the AcNPV vector did not replicate in Bm-N cells, the introduction of the *helicase* p143 gene from BmNPV resulted in their production in Bm-N and Sf9 cells.

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Introduction

Development of influenza vaccines first began after the pandemic of 1918–1919 and the isolation of the causative agent, influenza A virus [1, 2]. The severe outbreak, designated the Spanish influenza epidemic, caused 20–40 million deaths worldwide and drove rapid development of the influenza vaccine [3–5]. Then, the first licenses for civilian vaccines were authorised, and full-scale production of influenza vaccines was performed using embryonated chicken eggs, resulting in 70–80% effectiveness [6, 7]. Despite these efforts, and although egg-grown influenza vaccines are traditionally used in many parts of the world, new antigenic variants have recently been observed following passage of mammalian-derived vaccine viruses in embryonated chicken eggs [8]. To overcome this problem, mammalian-cell-derived influenza vaccines produced in Madin–Darby canine kidney (MDCK) cells have been used in the United States of America (USA) and Europe [9]. Additionally, recent circulation of the potentially pandemic H5 and H7 avian influenza

viruses has also promoted the development of human and avian influenza vaccines [10–14].

Previously, to improve immune responses to inactivated influenza vaccines, we developed artificial ribosomal vaccines using muramyl-dipeptide; this vaccine is morphologically similar to current virus-like particle (VLP) vaccines and induces higher levels of haemagglutination-inhibition (HI) antibodies and cellular immunity in mice and humans [15–17]. Researchers have investigated the efficacy of current VLP vaccines in different field settings [18–21]. We have also developed influenza VLP vaccines, using single-codon-optimized H5 and H7 haemagglutinin (HA) genes in *Bombyx mori* silkworms, and have demonstrated extremely high yields of these gene products [22, 23]. In a recent report on the development of a Japanese encephalitis vaccine [24], the use of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) showed enhanced expression levels in Bm-N cells [25]. For example, a BmNPV H5 Fukushima (Fk) recombinant produced 800,000 HA units per pupa when a single-codon-optimized HA gene was used, and a BmNPV Anhui (An) H7 recombinant showed an HA titre of approximately 400,000 per pupa. In contrast, Pushko et al. [26] produced moderate yields of H5, H7, and H9 VLP HA antigens in Sf9 cells originating from *Spodoptera frugiperda*. Furthermore, high expression levels of the *Escherichia coli lacZ* gene were confirmed in Sf-21 and Bm-N cells coinfecting with *Autographa californica* nuclear polyhedrosis virus (AcNPV) and BmNPV [27]. Kumar et al. [28] successfully produced large amounts of foot-and-mouth disease VLP protein in eri silkworms using an AcNPV vector. Interestingly, hybrid baculoviruses containing sequences from *B. mori* and *A. californica* NPVs can grow well in both Bm-N and Sf-21 cells, and Maeda et al. [29] and Kim et al. [27] demonstrated expansion of the host range by constructing recombinants between Bm-NPV and AcNPV. Based on these studies, the 143-kDa protein P143 was found to be necessary for viral DNA synthesis and therefore important for host range specificity [30, 31].

The aim of this study was to determine the function of the above-described AcNPV vector and the P143 gene by expressing the recombinants in Sf9 cells and Eri silkworm pupae. We describe dramatic overexpression of an avian influenza HA VLP vaccine (H5 subtype), as well as the morphological antigenic characteristics of the vaccine.

Materials and methods

Comparison of the ecological characteristics of silkworms

In this study, 10 *B. mori* silkworms and 10 Eri silkworms were collected at the fifth-instar phase of 5-day-old larvae to

measure their lengths and body weights. Similarly, 10 female pupae of each species of silkworm were also collected for body weight measurements. *B. mori* silkworms were purchased from Baculotechnologies Co., Ltd. (Kagawa, Japan), and Eri silkworms were provided by Okinawa UKAMI Sericulture Co. Ltd. (Okinawa, Japan).

Cells and viruses

Sf9 cells (originating from *S. frugiperda*) were purchased from Sigma–Aldrich (Tokyo, Japan), and Bm-N cells (originating from *B. mori*) were provided by Baculotechnologies Co. Ltd. Both cell lines were maintained in Grace's insect medium (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum. MDCK cells were maintained in TC-MEM containing 10% foetal bovine serum and used to produce influenza virus.

Generation of recombinant viruses

The construction of recombinant expression plasmids using the BmNPV vector was described previously [22, 23]. DNA sequences encoding FkH5 HA, AnH7 HA, and A/duck/Korea/A76/2010 H7N7 (KoH7) HA DNA were synthesised by Dragon Genomics (Shiga, Japan) and inserted into the pFastBac1 vector using a Gibson Assembly Cloning Kit (NEB). The resultant constructs were used to transform DH10Bac cells (purchased from Thermo Fisher Scientific, K.K., Japan). Subsequently, the recombinant AcNPV DNA was purified and used to transfect Sf9 cells. One week after transfection, supernatants from the infected cells were recovered to obtain the AcNPV recombinants. The helicase gene from the BmNPV vector was also inserted into the region between the V-cath and chitinase structural genes of the AcNPV vector. Recombinant DNA encoding FkH5, KoH7, or AnH7 HA proteins was extracted from the BmNPV vector using a High Pure Viral Nucleic Acid Kit (Roche, Switzerland), and HA DNA was amplified by PCR. The resultant PCR products were inserted downstream of the pFastbac1 polyhedrin promoter, and the resultant plasmids were used to transform DH10Bac. Subsequently, the recombinant AcNPV DNA was purified and used to transfect Sf9 cells. One week later, culture supernatants containing the recombinant AcNPV were collected (Fig. 2a).

Preparation of viral antigens and their antibodies

Low-pathogenic avian influenza viruses were kindly provided by Dr. R. G. Webster, St. Jude Children's Research Hospital, Memphis, TN, USA (Import Permit No.: 29 douken 322, issued on 12 June 2017 by the Ministry of Agriculture, Forestry and Fisheries, Japan). These included RG-A/Barn Swallow/Hong Kong/1161/2010-A/PR/8/34 H5N1 [R]

(6+2): (Swallow HK H5:SwHKH5), RG-A/Anhui/2013-A/PR/8/34 H7N9 [R] (6+2): (Anhui H7:AnH7), and A/Hong Kong/308/2014 -A/PR/8/34 H9N2 [R] (6+2): (HKH9). The SwHKH5, AnH7, and HKH9 viruses were produced in 10-day-old embryonated chicken eggs. After a 2-day incubation, the infected allantoic fluids were harvested and concentrated by centrifugation at $110,000\times g$ for 2 h. Antisera were prepared from mice after three intraperitoneal immunisations conducted at 1-week intervals. The above-mentioned viruses were used to characterise the antigenicity of H5 and H7 VLP antigens.

Fluorescent antibody (FA) test

The recombinant AcNPV-FkH5, AcNPV-AnH7, and AcNPV-KoH7 viruses were inoculated into Bm-N or Sf9 cells on glass coverslips. Three days later, the cells were washed with phosphate-buffered saline (PBS). The cells were then treated with mouse anti-influenza antibodies against H5 and H7 viruses and subjected to subsequent procedures, as described previously [22, 23]. The samples were observed under a fluorescent microscope (Olympus, Tokyo, Japan) at $400\times$ magnification.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests

Test samples were serially diluted twofold in 50 μ L of PBS in 96-well U-bottom plastic plates, and 50 μ L of 0.5% (v/v) chicken erythrocytes in PBS was placed in all wells and mixed well. After a 30-min incubation at room temperature (23–25 °C), the HA titres were estimated based on the last complete HA [22, 23]. For HI testing, similar twofold dilution series of antiserum were prepared in 25 μ L of PBS, and an equal volume of four units of test HA antigen was added to each well and mixed. After the addition of 50 μ L of 0.5% chicken erythrocytes and thorough mixing, the plates were incubated at room temperature for 30 min, and their HI profiles were determined as the highest inhibition titres.

Haemadsorption (HAD) tests

The culture medium was removed from infected cells, and the cells were washed once with PBS. Subsequently, a 0.5% chicken erythrocyte suspension was added to the cells. After a 30-min incubation at room temperature, the test cells were washed in PBS and observed under a stereoscopic microscope (Olympus, Tokyo, Japan) at $400\times$ magnification.

Production and purification of the HA VLP antigen in eri silkworm pupae

Two recombinants, AcNPV-AnH7 and AcNPV-FkH5, were inoculated into Eri silkworm pupae. Nine days later, the

infected pupae were homogenized in PBS containing 0.5% sodium thiosulfate as an antioxidant. Homogenization was performed using a UR-20P Handy Sonic Ultrasonic Disruptor (Tomy Digital Biology, Tokyo, Japan). Sonication was performed on ice with six pulses, each lasting 2 min. Each homogenate was then centrifuged at $3,800\times g$ for 30 min. The supernatant was further centrifuged at $72,000\times g$ for 2 h on a 20% (w/w) sucrose gradient in a SW28 swing rotor (Beckman Coulter, Japan). The resulting pellet was resuspended in PBS for subsequent experiments.

Histopathological examination of eri silkworm pupae infected with AcNPV-FkH5 and AcNPV-AnH7 recombinants

Pupa samples were collected, fixed in methanol-Carnoy's solution, processed for paraffin embedding, sectioned at 3 μ m, and stained with haematoxylin and eosin (HE). Immunohistochemistry was performed with DYKDDDDK-tagged antibodies (Wako, Osaka, Japan) and mouse antibodies against reference strains of SwHKH5 and AnH7 influenza viruses.

Electron microscopy

The purified homogenate of AcNPV-FkH5-infected Eri silkworm pupae was centrifuged through a 10–50% (w/w) sucrose density gradient at $141,000\times g$ for 120 min in an SW28 swing rotor. The gradient was fractionated, and the HA activity and protein concentration of each fraction were determined. Six fractions showing high HA activity (greater than 8,192) were collected, and sucrose was removed from the samples using column filtration (PD-10 Desalting Columns; GE Healthcare Bio-Sciences AB, Sweden). The filtrates were examined under an H-7600 electron microscope (Hitachi, Japan) after staining with 2% phosphotungstic acid.

Results

Comparison of the physical characteristics of Eri and *B. mori* silkworms

As shown in Fig. 1a, the face morphology, larva shape (with many spines and black spots), pupa size, and wing colour (black wings with white stripes) of Eri silkworms are quite different from those of *B. mori* silkworms (Fig. 1b). Eri silkworm larvae were longer and approximately two times heavier than *B. mori* silkworms (Fig. 1c). These differences may have influenced the yield of functional VLP proteins of the viruses.

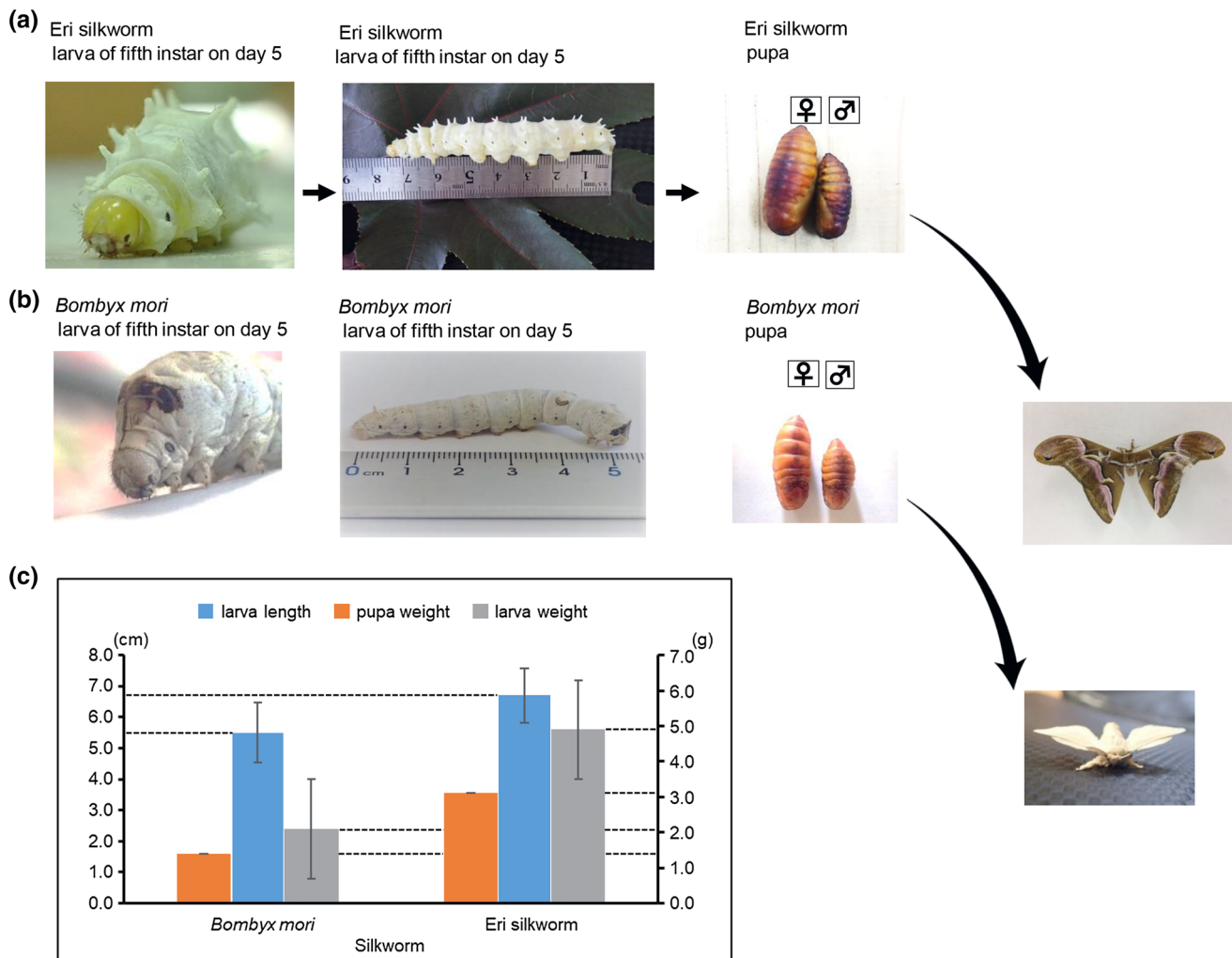


Fig. 1 Ecological comparisons of *B. mori* and Eri silkworms. (a, b) Head appearance (left) and comparison of the sizes and weights of larvae and pupae (centre and right). Comparison of wing shapes

and the colours of eri silkworm moths and *B. mori* silkworms (lower right). (c) Summary of data comparing the physical attributes of *B. mori* and Eri silkworms

Comparison of the replication of BmNPV and AcNPV recombinants

First, three recombinant viruses were examined in growth tests in Bm-N and Sf9 cells. The results of FA tests showed that the recombinant AcNPV-FkH5, AcNPV-KoH7, and AcNPV-AnH7 viruses failed to express HA genes in Bm-N cells (Fig. 2b). In contrast, the same H5 and H7 HA genes encoded within the AcNPV vector showed clear FA reactions in Sf9 cells (Fig. 2b). Similarly, all three recombinants adsorbed to Sf9 cells infected with the recombinant AcNPV-FkH5 and AcNPV-AnH7 viruses. These results were confirmed by performing HAD tests (Fig. 2b). Consistent with the FA and HAD results, we found that the AcNPV-FkH5, AcNPV-KoH7, and AcNPV-AnH7 recombinant viruses produced HA antigen, with titres of 1024–2048 (Table 1).

Histopathological examination of AcNPV-recombinant-infected eri silkworm pupae

To investigate the distribution of VLP HA antigens in Eri silkworm pupae, AcNPV-FkH5 and AcNPV-AnH7 recombinants were inoculated into Eri silkworm pupae, and whole thin sections were examined 9 days later using an anti-DYKDDDDK-tag monoclonal antibody and mouse antibodies against SwHKH5 and AnH7 influenza viruses. Numerous vacuoles were detected in the fat body tissues of pupae infected with FkH5 and AnH7 recombinant viruses. As shown in Fig. 3a and b, fat body cells were well stained with HE reagents. Further, the vacuoles showed prominent staining with the anti-DYKDDDDK-tag monoclonal antibody (Fig. 3c and d).

Fig. 2 Construction and confirmation of replacement expression plasmids. (a) Separation model of the H5 and H7 HA target genes from the BmNPV vector (left). Insertion model of the AcNPV H5 and H7 target genes into the pFastBacTM1 plasmid (right). (b) Expression of the FkH5, KoH7, and AnH7 recombinant viruses inserted in the AcNPV vector. FA tests in Bm-N and Sf9 cells (left and centre). HAD test with the above recombinants in Sf9 cells (right)

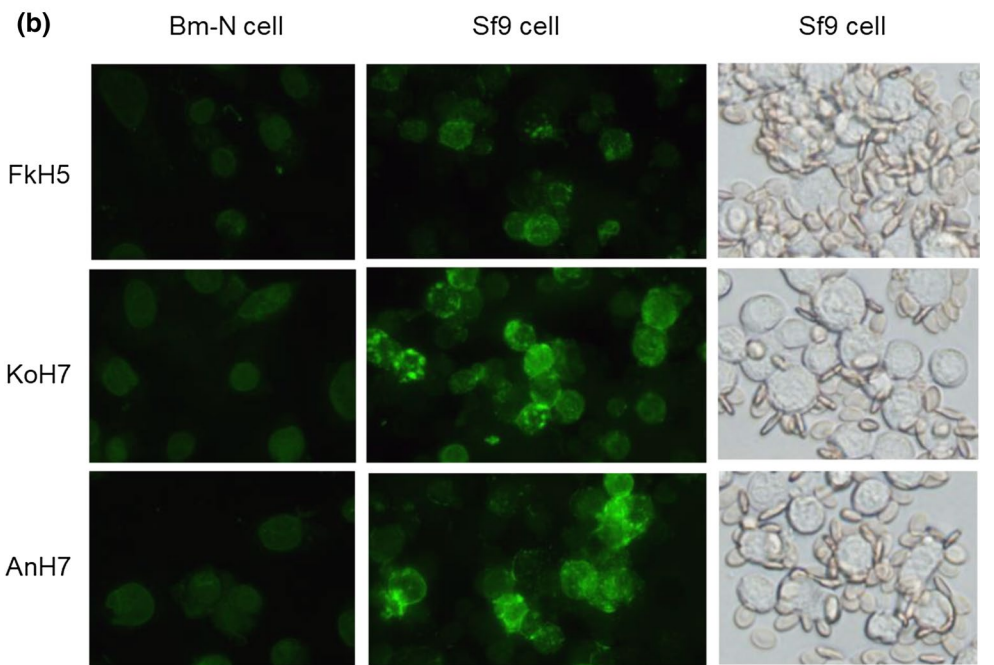
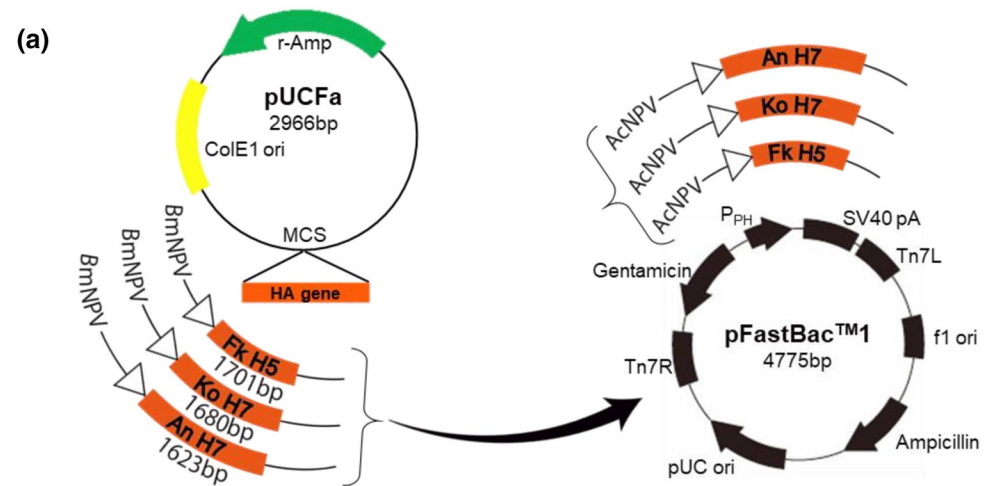


Table 1 HA activity of AcNPV antigens produced in Bm-N and Sf9 cells infected with H5 and H7 AcNPV recombinant viruses

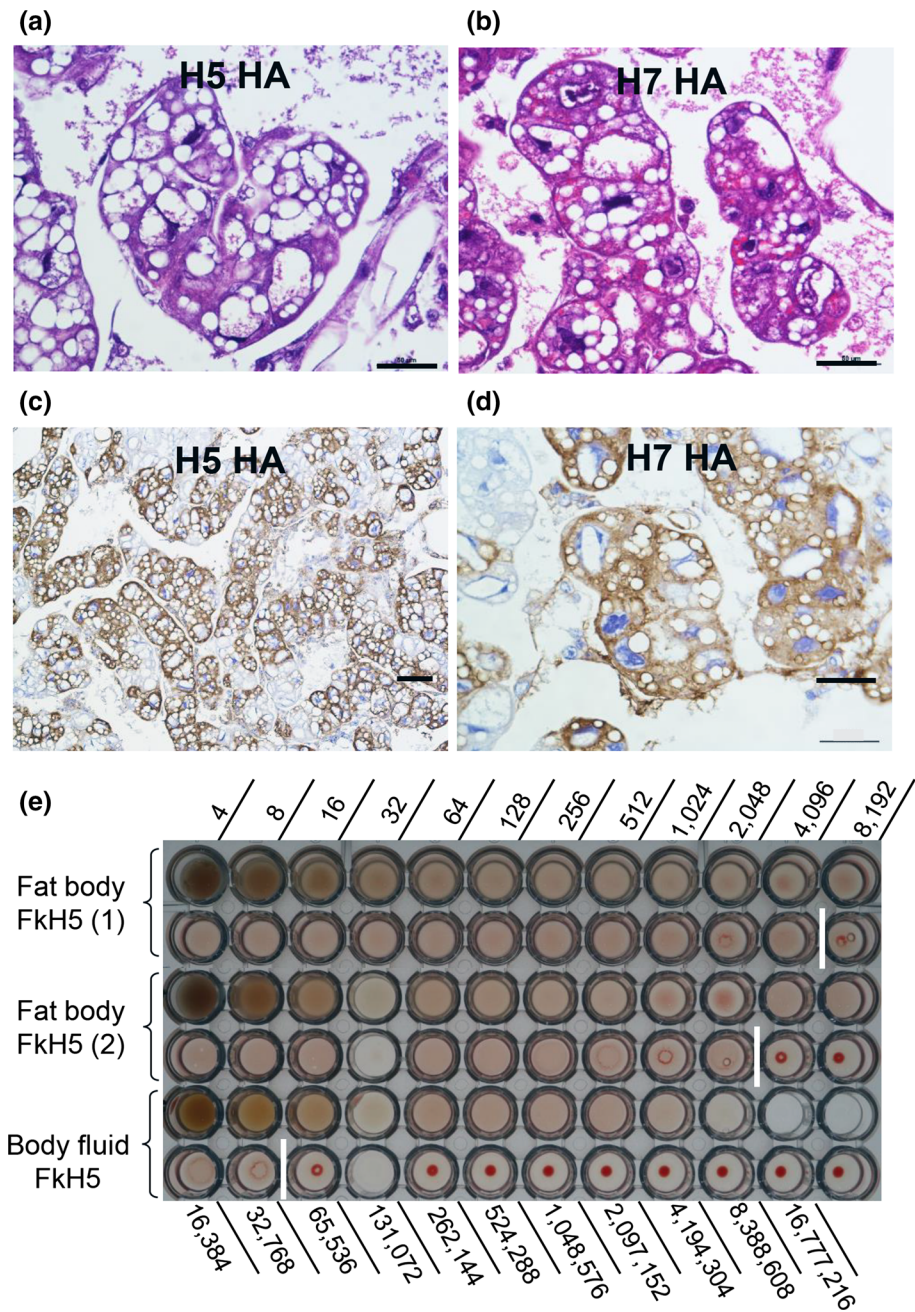
Recombinant	Cell line	
	Bm-N	Sf9
AcNPV-FkH5	<4	2,048
AcNPV-KoH7	<4	1,024
AcNPV-AnH7	<4	1,024

Production of an influenza virus HA VLP antigen in eri silkworms and examination by electron microscopy

The AcNPV-FkH5 and AcNPV-AnH7 recombinant viruses were then inoculated into Eri silkworm pupae. AcNPV-FkH5 recombinant-inoculated Eri silkworm pupae were

separated into two groups containing 22 and 15 pupae. Since extremely high HA titres were measured in each homogenate, a representative HA titration microplate (Fig. 3e) is shown to illustrate this. Importantly, HA VLP antigens appeared to be produced in both the fat body and body fluids (Fig. 4a and b). This may be the first evidence of the production of VLP antigens in both tissues in Eri silkworm. H5 VLP antigens produced in the fat body tissues of Eri silkworm pupae showed extremely high HA titres (16.7 and 8.3 million in the first (1) (n=22) and second (2) (n=15) group (Fig. 3e and Fig. 4a). The mean HA titre was 12.5 million (Fig. 4a). Interestingly, the HA VLP yield of FkH5 VLPs per pupa was 39.2 million. However, the AcNPV-AnH7 recombinant still produced 0.58 million HA VLP antigens, similar to previous reports (Fig. 4a). For reference, the titres of H5

Fig. 3 (a, b) HE staining of fat body tissues in Eri silkmoth pupae infected with H5 HA (a) and H7 HA (b). Scale bar (a, b): 50 μ m. (c, d) Immunohistochemical staining with an anti-DYKDDDDK-tag monoclonal antibody showing widespread cytoplasmic staining in silkmoth pupae infected with H5 HA (c) and H7 HA (d). Scale bar: 100 μ m (c) and 50 μ m (d). (e) An HA-titration plate used to estimate the HA activity of fat body homogenates inoculated with FkH5 HA recombinants and body fluids of pupae infected with the recombinants. Numbers on the top and bottom of the plate indicate the HA titre of the well, and white lines show the endpoint of HA titration



VLP and H7 VLP produced in *B. mori* silkmoth infected with FkH5 BmNPV were 0.8 and 0.4 million [22, 23].

Morphological and antigenic analysis of influenza HA VLP antigens produced in Eri silkworms

The structure of the authentic influenza virus (Fig. 4c) and H5 HA VLP antigen obtained from Eri silkworms (Fig. 4d) were fundamentally similar, based on the clear projections arising from the surface of authentic influenza virus and VLPs. For example, approximately 50–200 nm of the surface of several H5 VLPs was covered with 14-nm-long

spikes (Fig. 4d). Although individual projections were not clearly recognized, projections detected on the surface of native virions were more densely arranged than those of H5 VLP. In contrast, the individual H5 HA projections were clearly separated from each other. Although different sizes of VLP were observed in the VLP samples (data not shown), overall, all of the HA projections on the VLPs were similar in length. The results of the antigenic analysis of the FkH5 HA antigen are shown in Fig. 5a. Although antigenic analysis of silkworm-derived AcNPV H5 VLP and BmNPV H5 VLP was performed with antisera prepared in mice against the reference strains of H5 and H7 influenza viruses, AcNPV

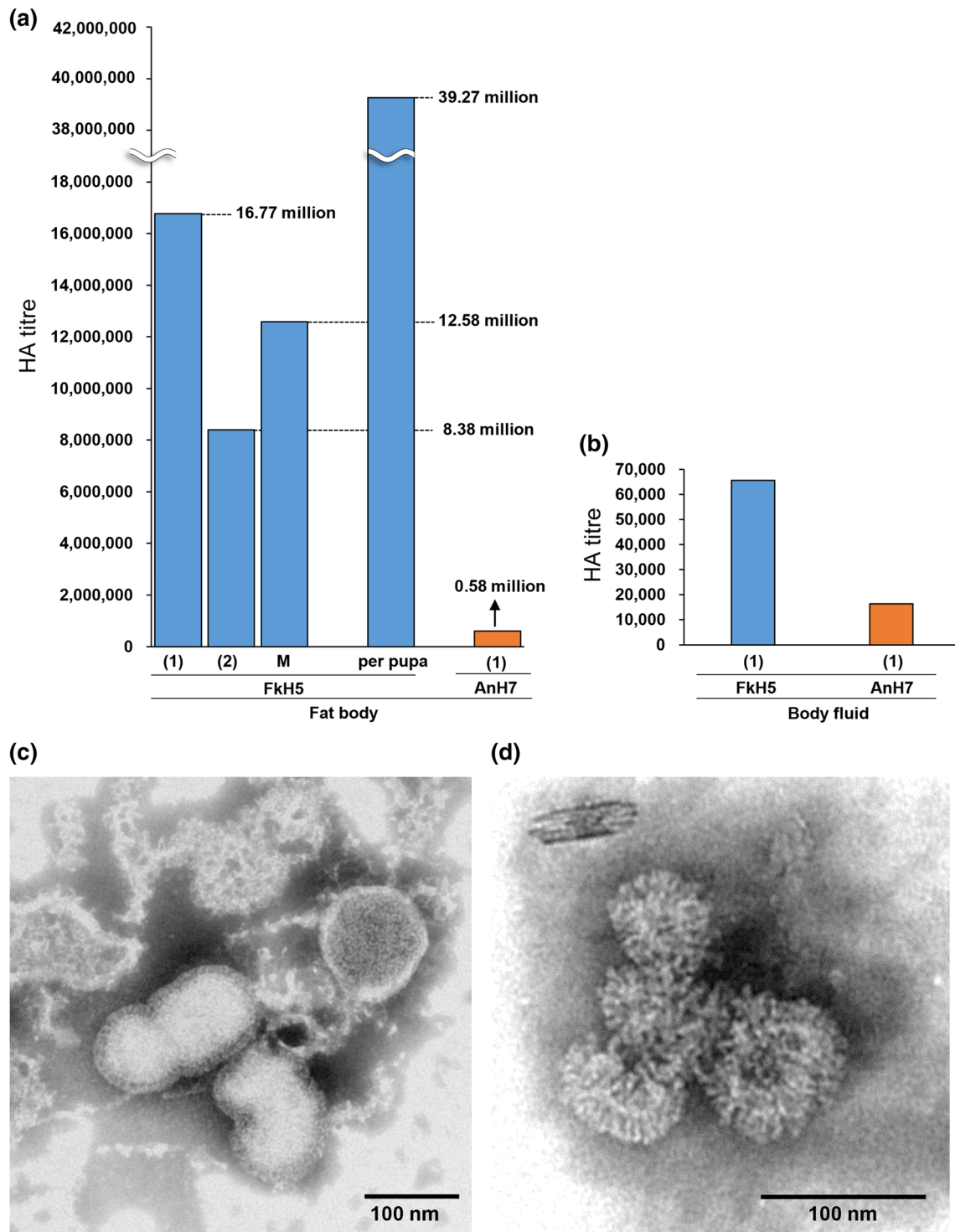


Fig. 4 Production of two VLP antigens (FkH5 VLP and AnH7 VLP) in Eri silkworm pupae and electron microscopic examination. (a) Comparison of the HA VLP yield produced in Eri silkworm pupae. The yields of FkH5 VLPs produced in the fat bodies of Eri silkworm pupae are shown. The AcNPV-FkH5 recombinant was inoculated into 37 pupae. Nine days after infection, pupae were separated

(first group, (1), n=25; second group, (2), n=15). M, mean HA titre of group 1 and group 2 pupae homogenates. (b) HA titre of group 1 and group 2 body fluids. (c, d) Electron micrographs of H5 influenza virus and FkH5 VLP antigens. (c) Purified SwHKH5 influenza virus. (d) Purified FkH5 VLP antigens produced in the fat bodies of Eri silkworm pupae

H5 VLP and BmNPV H5 VLP antigens appeared to react specifically with the antisera to the H5 reference strain and failed to react with the anti-H7 antibody. Similarly, haemagglutination of AcNPV H7 antigen was inhibited with H7 antibody, but not with H5 antiserum. Additionally, antiserum against reference H7 virus did inhibit the haemagglutination of AcNPV H7 VLP antigen, showing a high HI titre of 4,096. It was also interesting that both AcNPV FkH5 and AcNPV AnH7 VLP fat antigens produced in tissue fluids reacted specifically with H5 and H7 antibodies prepared in mice.

Host-range expansion by introducing the helicase gene from BmNPV into the AcNPV recombinant

Functionally important sites are distributed throughout the AcNPV genome [36], and we targeted the site between the V-cath and chitinase gene regions for insertion of foreign genes (Fig. 5b). We inserted the helicase gene from BmNPV into that region (Fig. 5c), and as shown in Fig. 5b–e, the improved AcNPV-FkH5 and AcNPV-AnH7 recombinants contained two different helicase genes in each vector. As a result, the two

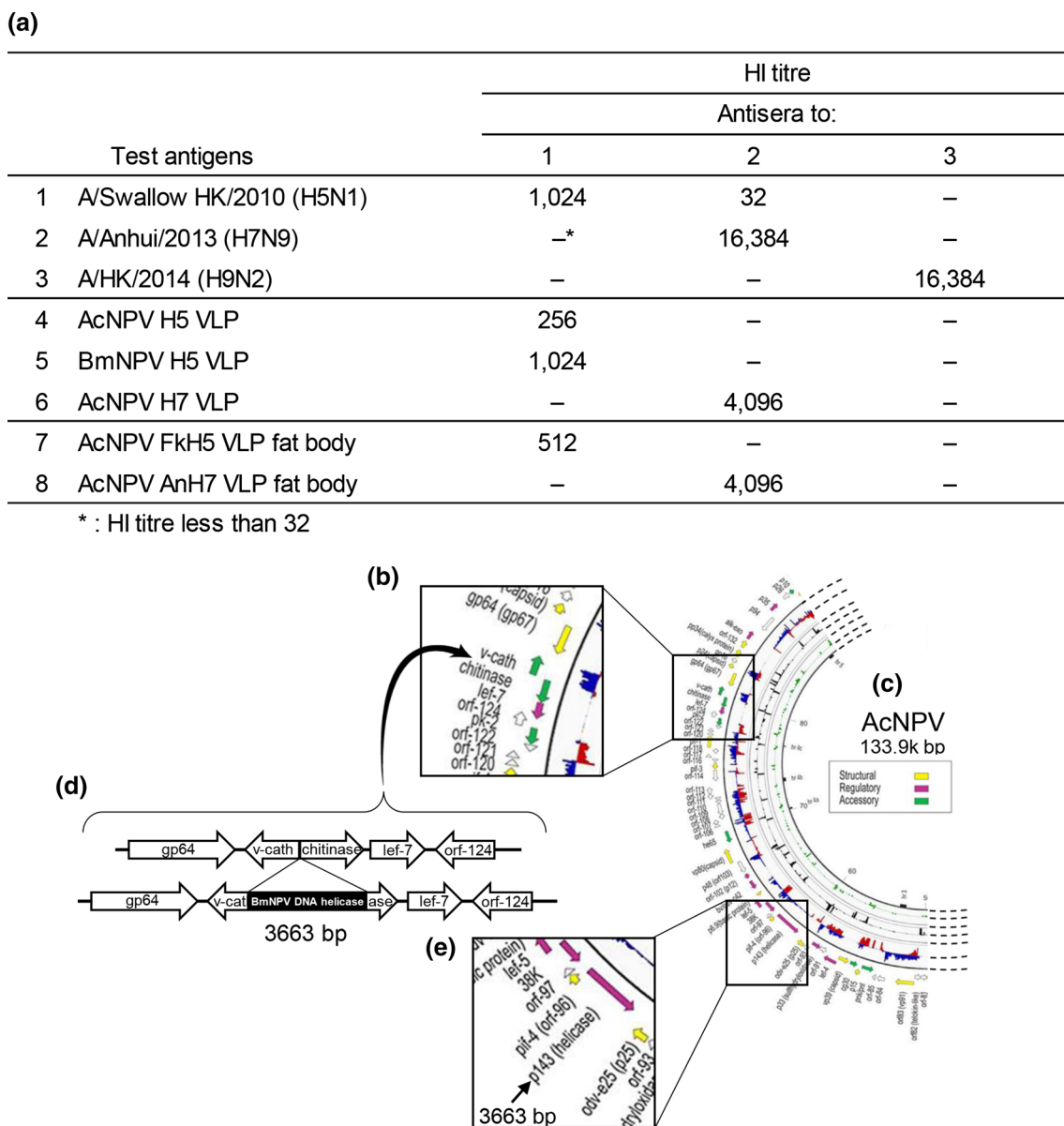


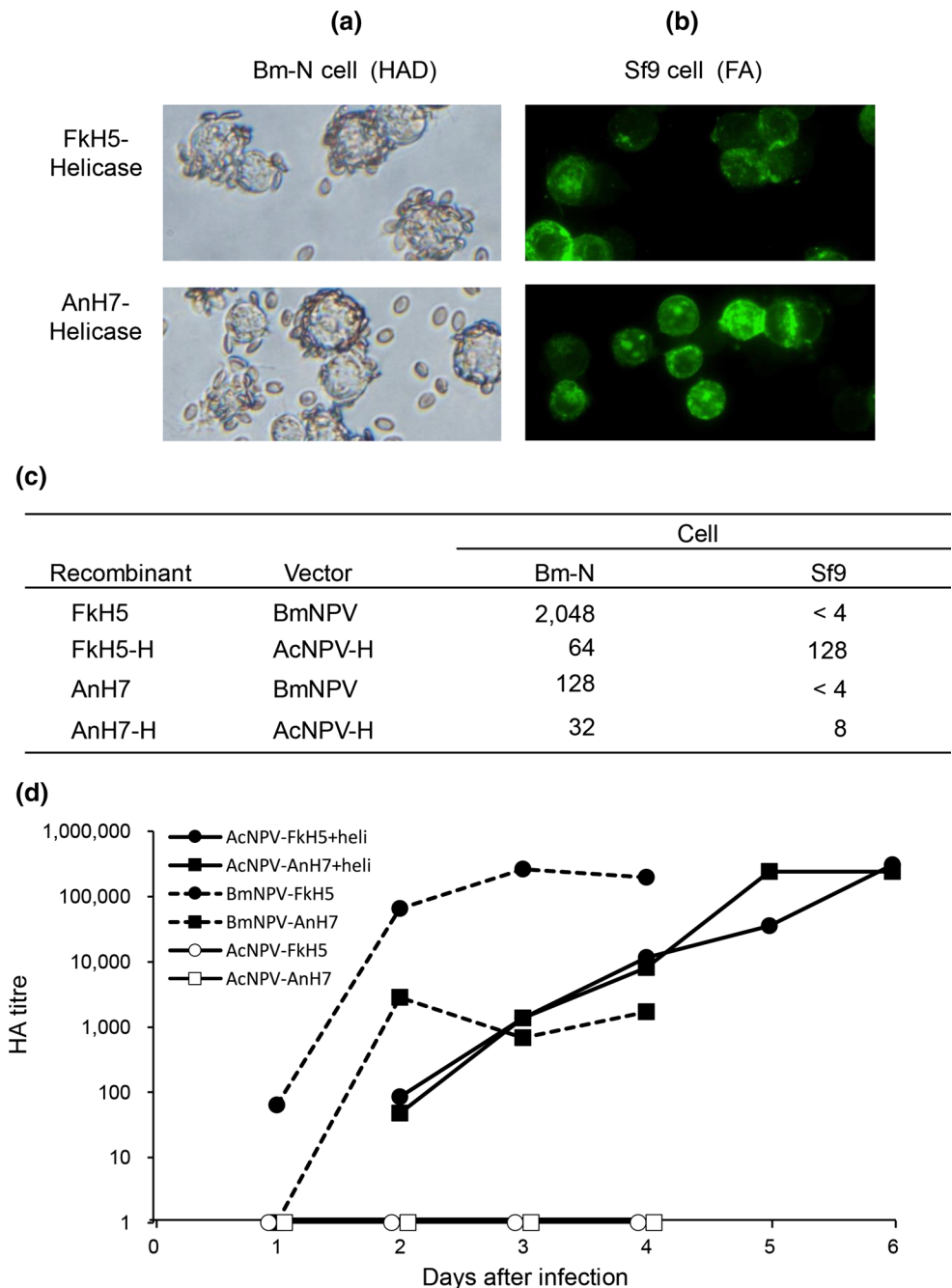
Fig. 5 Antigenic analysis of the influenza VLP antigen produced in Eri silkworms and construction of the AcNPV expression plasmid with introduction of the helicase gene. (a) Antigenic analysis of the VLPs. (b) The presence and insertion locus of the helicase gene in the

AcNPV vector (c). (d) Expansion of the helicase gene area from the AcNPV plasmid. (e) The helicase gene already present in the AcNPV vector, resulting in the AcNPV H5 and H7 recombinants having two helicase genes

helicase-containing recombinants (AcNPV-FkH5+heli and AcNPV-AnH7+heli) expressed their structural genes in both Bm-N and Sf9 cells, as indicated by positive HAD (Fig. 6a) and FA (Fig. 6b) reactions. Additionally, AcNPV-FkH5+heli and AcNPV-AnH7+heli recombinants appeared to produce HA VLP antigens in Bm-N and Sf9 cells (Fig. 6c). When these recombinants were inoculated into *B. mori* pupae, they both produced high HA titres even in *B. mori* silkworm systems (Fig. 6d). These HA

titres increased linearly over time. The mean HA activity titres of FkH5 and AnH7 VLP were approximately 300,000 and 240,000, respectively (Fig. 6d). BmNPV-FkH5 and BmNPV-AnH7 replicated well in *B. mori* silkworms (Fig. 6d). However, the AnH7 HA titre was lower than those of FkH5 in *B. mori* silkworms, which were similar to those produced using the BmNPV recombinant in the *B. mori* silkworm system (Fig. 6d). Thus, AcNPV recombinants containing two helicase genes could replicate well in both silkworm cells and silkworm hosts.

Fig. 6 Replication of AcNPV recombinants containing two helicase genes and the BmNPV recombinant in two different insect cells from *B. mori* and *S. frugiperda*. Replication of two recombinants with two helicase genes in Bm-N (a) and Sf9 (b) cells. Replication was examined by performing HAD (a) and FA (b) tests. (c) Results of a growth activity assay in Bm-N and Sf9 cells infected with FkH5 and AnH7 recombinants, with (FkH5-H and AnH7-H) or without (FkH5 and AnH7) an additional helicase gene. (d) Replication kinetics of the indicated recombinants and the effects of the helicase genes. BmNPV-FkH5 and BmNPV-AnH7 were also produced in *B. mori* silkworms



Discussion

Here, we describe the dramatic overexpression of an avian influenza (subtype H5) HA VLP vaccine and its morphological antigenic characteristics.

Baculoviruses have been used to produce various influenza VLP vaccines in response to the appearance of possible pandemic H5 and H7 avian influenza viruses [10–14]. For example, a single injection of the H7 avian VLP vaccine produced in Sf9 cells increased protective immunity against H7N9 influenza virus in a mouse model [32]. Smith et al. [33] developed an H7 VLP vaccine using Sf9 cells, and its immune responses in mice reached protective levels against the homologous human Anhui 2013 and heterologous Jalisco 2012 chicken isolates. Moreover, a recent report showed that a suitable amount of H7 VLP vaccine was produced in Sf9 cells, with an HA titre of 2,048. Additionally, immunisation in ferrets using 15 µg of the H7N9 VLP vaccine reduced replication of the challenge H7N9 virus to approximately 10^4 – 10^5 plaque-forming units (PFU)/mL [34], and H5 and H7 VLP vaccines prepared using the AcNPV vector protected chickens against infection with highly pathogenic H5 and H7 viruses [35]. In previous studies, influenza vaccine development was evaluated using a combination of the AcNPV vector and insect cells.

In addition to an increase in the HA VLP yield, we were interested in a report by Maeda et al. [29], who isolated a hybrid baculovirus from Sf-21 cells co-infected with BmNPV and AcNPV that could replicate in both Bm-N and Sf9 cells. A similar report also described host-range expansion for the *E. coli lacZ* gene and showed increased expression by a recombinant baculovirus [27].

In summary, we demonstrated that the use of two helix genes from BmNPV and AcNPV in the recombinant AcNPV-FkH5 and AcNPV-AnH7 vectors promoted the replication in both Bm-N and Sf9 cells. Our experimental results suggest that different combinations of structural genes from the influenza virus genome, codon optimisation, usage of the AcNPV DNA vector, and usage of Eri silkworm as the host may be helpful in increasing the production of AcNPV-influenza VLP recombinants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving animal and human participants Mouse experiments were approved and performed in accordance with the Fundamental

Rules for Animal Experiments and the Guidelines for Animal Experiments Performed at The Institute of Biological Resources published by the Animal Welfare and Animal Care Committee, including the Animal Ethics Committee of the Institute of Biological Resources, Okinawa, Japan.

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