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Baculovirus ODV-E66 degrades larval peritrophic membrane to facilitate baculovirus oral infection

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ABSTRACT

ODV-E66 is a major envelope proteins of baculovirus occlusion derived virus (ODV) with chondroitinase activity. Here, we studied the roles of ODV-E66 during Helicoverpa armigera nucleopolyhedrovirus (HearNPV) primary infection. ODV-E66 is a late viral protein dispensable for BV production and ODV morphogenesis. Deletion of *odv-e66* had a profound effect on HearNPV oral infectivity in 4th instar larvae with a 50% lethal concentration (LC_{50}) value of 26 fold higher than that of the repaired virus, compared to in 3rd instar larvae. Calcofluor white, an agent which destroys the peritrophic membrane (PM), could rescue the oral infectivity of *odv-e66* deleted HearNPV, implying the PM may be the target of ODV-E66. *In vitro* assays showed HearNPV ODV-E66 has chondroitinase activity. Electron microscopy demonstrated that *odv-e66* deletion alleviated the damage to the PM caused by HearNPV infection. These data suggest an important role of ODV-E66 in the penetration of the PM during oral infection.

1. Introduction

Lepidopteran baculoviruses are enveloped double-stranded DNA viruses characterized by a biphasic life cycle with two phenotypically distinct progeny phenotypes (Jehle et al., 2006). One, the budded virus (BV) is responsible for cell-cell systemic infection *in vivo* and in tissue culture, while the other progeny phenotype, the occlusion-derived virus (ODV) is essential for oral infection in larval midgut cells and horizontal transmission among insects (Slack and Arif, 2007). During oral infection, ODVs are released from the occlusion bodies (OBs) in the alkaline environment of host's midgut to initiate infection in susceptible epithelial cells (Ohkawa et al., 2005). Many ODV envelope proteins are involved in oral infection, such as *per os* infectivity factors (PIFs) which are essential for successful establishment of oral infection. So far, 10 PIFs have been identified including P74 (PIF0) (Haas-Stapleton et al., 2004; Kuzio et al., 1989), PIF1, PIF2, PIF3 (Ohkawa et al., 2005), PIF4 (Fang et al., 2009; Huang et al., 2012), PIF5 (ODV-E56) (Harrison et al., 2010; Sparks et al., 2011; Xiang et al., 2011a), PIF6 (Nie et al., 2012), PIF7 (Liu et al., 2016), PIF8 (VP91) (Javed et al., 2017; Zhu et al., 2013) and PIF9 (Boogaard et al., 2018; Wang et al., 2019). All the *pif* genes are conserved in baculoviruses and some of them are also found in nudiviruses, hytrosavirues, polydnavirus and other invertebrate viruses (Abd-Alla et al., 2008; Burke et al., 2013; Garcia-Maruniak et al., 2008; Jehle et al., 2006; Wang et al., 2017), suggesting a possibly conserved entry process exploited by these invertebrate DNA viruses.

ODV-E66 is one of the major ODV specific envelope proteins (Braunagel et al., 2003; Hong et al., 1994; Hou et al., 2013; Wang et al., 2010b) that is conserved in all lepidopteran baculoviruses (Jehle et al., 2006). Deletion of *odv-e66* resulted in a significant decrease in the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) oral infectivity, while it does not impair BV replication (Xiang et al., 2011b). Consistent with the result of AcMNPV, deletion of ODV-E66 from Bombyx mori nucleopolyhedrovirus (BmNPV) did not affect BV infectivity in infected cells (Ono et al., 2012). ODV-E66 of AcMNPV and BmNPV have been identified as substrate-speicfic endo-chondroitin lyases (Sugiura et al., 2011, 2013), and crytal structure of AcMNPV ODV-E66 showed the protein has a similar 3D structure to that of bacterial polysaccharide lyase 8 (PL8) family enzymes (Kawaguchi et al., 2013). *In vitro* biochemistry analyses showed that BmNPV ODV-E66 could digest chondroiton sulfates (CSs) from the peritrophic membrane (PM) of the silkworm, suggesting a potential role of ODV-E66 in destroying the PM of the host's midgut to facilitate ODV

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infection (Sugiura et al., 2011, 2013). However, this hypothesis lacks *in vivo* evidence.

A previous proteomic study identified ODV-E66 as the major ODV envelope protein of Helicoverpa armigera nucleopolyhedrovirus (HearNPV), an extensively used biological control agent against cotton bollworm in China (Hou et al., 2013; Sun, 2015). In this study, we investigated the function of ODV-E66 during HearNPV infection. The results showed that deletion of ODV-E66 had a more significant effect on oral infection of HearNPV in 4th instar larvae compared to in 3rd instar larvae. The oral infectivity of *odv-e66* deleted HearNPV could be rescued by calcofluor white, an agent that destroys the PM, suggesting that the PM is one of the targets for ODV-E66. Moreover, we found that *odv-e66* deletion could alleviate the damage to the PM caused by HearNPV infection, supporting the chondroitin lyase activity of ODV-E66 is functional *in vivo*.

2. Materials and methods

2.1. Insect cells, virus and infection

The cell line HzAM1 (McIntosh and Ignoffo, 1983) was maintained in Grace's insect medium supplemented with 10% fetal bovine serum (Gibco-BRL) at 27 °C. Cotton bollworm *Helicoverpa armigera (H. armigera)* larvae were reared on artificial diet at 28 °C. An *in vivo* cloned strain of HearNPV (HearNPV G4) was used as the parental virus (Chen et al., 2001; Sun et al., 1998). The bacmid HaBacHZ8 (Wang et al., 2003) and bacmid-derived control virus vHaBac-*egfp-ph* (Song et al., 2008), previously generated, were used in this study.

2.2. Transcriptional analysis of HearNPV odv-e66 gene

HzAM1 cells (3×10^5) were infected with HearNPV G4 strain at a multiplicity of infection (MOI) of 5. Cells were collected at different times post-infection (p.i.) and the total RNA was extracted with Trizol reagent (Invitrogen). 3′RACE experiment was performed using 3′-full RACE core set (TAKAR) according to the manufacturer's instructions. Total RNA (1 μg) from each time point was transcribed by M-MLV reverse transcriptase and an oligo$(dT)_{15}$ adapter primer to synthesize the first cDNA strand. A PCR reaction was carried out on the cDNA template with an anchor primer (5′-CTGATCTAGAGGTACCGGATCC-3′) and an *odv-e66*-specific forward primer (5′-CAACACCAATACGATGAC ACTAG-3) and the products were analyzed on 2% agarose gels.

2.3. Construction of recombinant viruses

The *odv-e66* in the bacmid HaBacHZ8 was replaced with a gene cassette containing an enhanced green florescent protein (*egfp*) gene driven by the *Drosophila hsp70* promoter and chloramphenicol resistance (cm^r) gene as follows. Briefly, 542 bp sequence upstream of the *odv-e66* gene was amplified by PCR with primers HA96UF (5′-GGGGA TATCTTAAATGTATTCGCGTATAATACTT-3′; EcoR V site underlined) and HA96UR (5′-GGGTCTAGATTGGACGCCGACCAGTTG -3′; Xba I site underlined). A 448 bp sequence downstream of the *e66* gene was obtained with the primers HA96DF (5′-GGGGGTACCCAACATTGACGGA CTACGA -3′; Kpn I site underlined) and HA96DR (5′-GGGCTCGAGTT GTATTAATGAAACGCTTTGA -3′; Xho I site underlined) using HearNPV DNA as a template. Using the pKS-*egfp-*cm^r plasmid (Wang et al., 2008), the upstream homologous fragment was cloned upstream of the *egfp-*cm^r cassette while the downstream homologous fragment was inserted downstream of the cassette. The resulting plasmid was designated pKS-dele*66*. The linear fragments for homologous recombination were generated from pKS-dele*66* by Kpn I/Xba I digestion and used to transform *E. coli* DH10B containing HaBacHZ8 and pKD46. The ODV-*e66*-deleted bacmid was generated by homologous recombination in *E. coli*, and screened by kanamycin and chloramphenicol resistance as described previously (Hou et al., 2002). The correct bacmid clone was authenticated by PCR analyses and designated HaBac^{e66-KO}. A pair of primers, HA96 P–F (5′-TATGGATCCCTAATGTACGTATGGAATGCTG-3′; BamH I site underlined) and HA96 P-R (5′- TATCTCGAGATGATGT TGACCGTAATTGCTGTA-3′; Xho I site underlined) were designed to amplify the complete sequence of *e66* containing its putative promoter and the terminal codon from HearNPV G4. The PCR product was cloned into the transfer vector pFB-DUAL-*ph* (Song et al., 2008) and named pFB-DUAL-*ph-e66*. Transpositions were performed on the Tn7 attachment site of HaBac^{e66-KO} using pFB-DUAL-*ph* and pFB-DUAL-*ph-e66* as the donor plasmids. The generated bacmids were designated HaBac$^{e66-KO\text{-}ph}$ and HaBac$^{e66\text{-}REP\text{-}ph}$, respectively.

2.4. Transfection and infection assays

Transfections were performed with 1 μg recombinant bacmids (HaBac$^{e66-KO\text{-}ph}$, HaBac$^{e66\text{-}REP\text{-}ph}$ and HaBac-*egfp-ph*) and 15 μl Lipofectin according to the Bac-to-Bac Expression Systems manual (Invitrogen) and previously described (Wang et al., 2010a). For the infection assay, at 6 days post-transfection (p.t.), 1 ml supernatant from the transfections was centrifuged at 3000 g for 10 min to remove cell debris and the supernatants were used to infect HzAM1 cells. Cells were monitored by fluorescence microscopy at 96 h post transfection (h p.t.) or post-infection (h p.i.).

To detect of ODV-E66 expression in cells infected with recombinant viruses, HzAM1 cells were infected with vHaBa c$^{e66-KO\text{-}ph}$, vHaBac$^{e66\text{-}REP\text{-}ph}$, vHaBac-*egfp-ph* and HearNPV G4 and collected at 72 h p.i. Cellular proteins were separated on 12% SDS-PAGE and Western blot was performed as described previously by using polyclonal antibody (pAb) against ODV-E66 (Hou et al., 2013).

2.5. One-step virus growth curves

HzAM1 cells were infected with vHaBac$^{e66-KO\text{-}ph}$, vHaBac$^{e66-REP\text{-}ph}$ and vHaBac-*egfp-ph* at an MOI of 5. Supernatants were collected at different time points (12, 24, 48, 72 and 96 h p.i.) and BV titres were determined by $TCID_{50}$ (50% tissue culture infective dose) end-point dilution assay with green fluorescence as a marker of infection. The growth curves were generated by arithmetic mean data of triplicates and titres of the viruses were statistically analyzed using one-way ANOVA.

2.6. Larval bioassays

OBs from vHaBac$^{e66-KO\text{-}ph}$ and vHaBac$^{e66-REP\text{-}ph}$ were harvested from infected *H. armigera* larvae and purified as previously described (Deng et al., 2007). The 3rd and 4th instars *H. armigera* larvae were starved prior to the droplet bioassay as described previously (Sun et al., 2004). The 50% lethal concentration (LC_{50}) was determined by feeding the 3rd and 4th instar larvae with a set of serial dilutions of each virus. The assays were done in triplicates with forty-eight insects used for each dose. The LC_{50} and potency ratio data were analyzed according to previously described (Li et al., 2015).

To investigate whether damage of the PM could enhance the infectivity of *e66*-deletion viruses, newly molted 4th-instar *H. armigera* arvae were treated with calcofluor white as described previously (Song et al., 2008). A cohort of 48 larvae was used for each treatment, and performed twice. A concentration of 3×10^4 OBs/ml (approximately equal to the LC_{50} of vHaBac$^{e66-REP\text{-}ph}$) of vHaBac$^{e66-KO\text{-}ph}$ and vHaBac$^{e66-REP\text{-}ph}$ with or without calcofluor (1%) were used to infect early 4th-instar *H. armigera* larvae using the droplet method. The data were subjected to chi-square test analysis.

2.7. Chondroitin lyase activity of ODV-E66

Two truncations of HearNPV E66 were used for chondroitin lyase activity, including the HearNPV ODV-E66 (23–672 aa) lacking the N

terminal sorting motif (SM) (Hong et al., 1997) and the HearNPV ODV-E66 (44–672 aa) corresponding to the AcMNPV ODV-E66 (70–704 aa) which has chondroitin lyase activity (Sugiura et al., 2011). Briefly, the two truncated fragments were amplified by using forward primers HA96 (23–672)-F (5′-GCGGGATCCACAAACGACAATATATTGCC-3′) and HA96 (44–672)-F (5′-GCGGGATCCGATGATTTAAAAATATTCG AAC-3′), the reverse primer for both truncations was HA96-R (5′-GCG GAGCTCAAATTTAAACTGATTAGCGTTG-3′). The PCR products were cloned into expression plasmid pET-28a with an N terminal His-tag for purification, expressed in soluble form in BL21 *E. coli* cells, and purified by nickel column. Chondroitin lyase activity was measured as reported (Hong et al., 2002). The reaction mixture containing 200 μg substrate chondroitin sulfate (CS) (Sigma) and 1 μg of each purified truncated proteins in 250 μl of 50 mM phosphate buffered saline (PBS, pH 7.0) was placed in the warmed sample cell of a UV spectrophotometer and the absorbance at 232 nm was measured at 3-min intervals over 30 min. The catalyzed reaction was carried out at 37 °C and the experiment was repeated three times.

2.8. Transmission electron microscopy (TEM) analysis

HzAM1 cells (1×10^6) were infected with vHaBac$^{e66-\text{KO-}ph}$ and vHaBac$^{e66-\text{REP-}ph}$ at an MOI of 5. Infected cells were collected at 72 h p.i. and washed twice with PBS. Occlusion bodies (OBs) were isolated and purified from infected *H. armigera* larvae (Deng et al., 2007). All samples were processed for ultrastructural analyses as previously described (van Lent et al., 1990) and examined by transmission electron microscopy (Deng et al., 2007).

2.9. Scanning electron microscopy (SEM) analysis of PM

3rd instar *H. armigera* larvae were fed two different doses (3×10^5 and 3×10^7 OBs/ml) of OBs from vHaBa c$^{e66-\text{KO-}ph}$ and HearNPV by the droplet method (Sun et al., 2004). At 4 h p.i., the larvae were dissected according to (Nielsen-Leroux and Charles, 1992) with slight modification. Briefly, the larvae were chilled on ice, submersed in ice-cold PBS (pH 7.4) and then fixed onto wax plate. A small lateral initiating incision was made near the anal proleg, and the integument was subsequently cut longitudinally and spread apart, exposing the gut. After the whole gut was pulled out, a transection was immediately made to separate foregut and midgut. The PM was stripped from the midgut, washed in PBS and then fixed with 2.5% glutaraldehyde. The fixed PM was washed with PBS, dehydrated with a gradient alcohol series (30%, 50%, 70%, 80%, 90%, 95%, 100%), washed with isoamyl acetate, and subsequently subjected to critical point drying using HITACHI HCP-2 (Japan). The samples were sputter-coated with gold and observed under SEM (Shang et al., 2017).

3. Results

3.1. Transcription and protein expression of HearNPV odv-e66

A baculoviral late transcription initiation motif, ATAAG, is located 20 nt upstream of the translational start codon ATG, suggesting HearNPV *odv-e66* may be expressed at the late phase of HearNPV infection. By using 3′RACE transcriptional analysis, the *odv-e66* transcript was detected from 18 to 72 h p.i. (Fig. 1A). Western blot analysis showed that a pAb anti-ODV-E66 antiserum reacted with a protein band running at an expected size of 76 kDa expressed from 24 to 96 h p.i. (Fig. 1B). Taken together, our data confirmed that *odv-e66* is a late gene of HearNPV.

3.2. Construction of odv-e66-deleted and repaired recombinant HearNPV viruses

An *odv-e66*-deleted bacmid HaBac$^{e66-\text{KO}}$ was constructed through homologous recombination as described in materials and methods (Fig. 2A). Positive clones were authenticated by PCR and Hind III digestion (data not shown). Then, by using Bac-to-Bac system, HearNPV *polyhedrin* (*ph*) gene alone was introduced into HaBac$^{e66-\text{KO}}$ to generate HaBac$^{e66-\text{KO-}ph}$, or *e66* and *ph* genes were simultaneously inserted into HaBac$^{e66-\text{KO}}$ to generate HaBac$^{e66-\text{REP-}ph}$ (Fig. 2B). Both recombinant bacmids were confirmed to be correct by PCR analysis (data not shown).

3.3. HearNPV ODV-E66 is not required for BV production

HzAM1 cells were transfected with bacmids HaBac$^{e66-\text{KO-}ph}$, HaBac$^{e66-\text{REP-}ph}$ and HaBac-*egfp-ph* to generate recombinant viruses. Expression of *egfp* was monitored by fluorescence microscopy. No obvious differences in *egfp* expression were observed at 96 h p.t. or 96 h p.i. among all the three viruses (Fig. 2C). Western blot analysis was performed to confirm the deletion and repair of *odv-e66*. As expected, ODV-E66 was detected in cells infected with the vHaBac$^{e66-\text{REP-}ph}$, vHaBac-*egfp-ph* or HearNPV at 72 h p.i., but not in the cells infected with vHaBac$^{e66-\text{KO-}ph}$ (Fig. 2D). To assess the impact of the deletion on BV production, HzAM1 cells were infected with vHaBac$^{e66-\text{KO-}ph}$ or vHaBac$^{e66-\text{REP-}ph}$ with vHaBac-*egfp-ph* as a positive control at an MOI of 5. One-step growth assays were performed by $TCID_{50}$, and the results showed that the vHaBac$^{e66-\text{KO-}ph}$ had similar growth kinetics and titres as the repaired virus vHaBac$^{e66-\text{REP-}ph}$ or control virus vHaBac-*egfp-ph* (Fig. 2E).

3.4. Deletion of ODV-E66 did not affect ODV occlusion into polyhedra

Transmission electron microscopy was performed to investigate the impact, if any, of *odv-e66* on ODV morphogenesis. Cells infected with vHaBac$^{e66-\text{KO-}ph}$ and vHaBac$^{e66-\text{REP-}ph}$ were harvested at 72 h p.i. and processed for EM observations. Both vHaBac$^{e66-\text{KO-}ph}$ and vHaBac$^{e66-\text{REP-}ph}$ infected cells exhibited typical signs of baculovirus infection such as enlarged nuclei, a remarkable electron-dense virogenic stroma (VS) and presence of rod-shaped nucleocapsids (Fig. 3A and B). ODV occlusion into OBs in vHaBac$^{e66-\text{KO-}ph}$ infected cells (Fig. 3A) was similar to those in vHaBac$^{e66-\text{REP-}ph}$ infected cells (Fig. 3B). OBs purified from infected larvae were also observed by EM and the results further confirmed that the deletion of HearNPV *odv-e66* did not have an obvious influence the ODV occlusion into OBs (Fig. 3C and D).

3.5. Deletion of ODV-E66 decreases the oral infectivity of HearNPV

To determine the effect of *odv-e66* deletion on oral infectivity of HearNPV, bioassays were initially performed by the droplet method on 3rd instar *H. armigera* larvae. The results showed that the LC_{50} value of vHaBac$^{e66-\text{KO-}ph}$ is 2.6 times higher than that of the repaired virus vHaBac$^{e66-\text{REP-}ph}$ (Table 1). When the bioassay experiment was conducted with 4th instar larvae, the difference in LC_{50} value between the two recombinant viruses became more significant, with the LC_{50} value of vHaBac$^{e66-\text{KO-}ph}$ was 26 fold higher than that of the repaired virus vHaBac$^{e66-\text{REP-}ph}$, 10 fold more than in 3rd instar larvae (Table 1).

3.6. Destruction of the PM rescues the oral infectivity of odv-e66 deleted HearNPV

Since ODV-E66 has a chondroitinase activity and was proposed to play an important role in destroying the host PM during ODV entry (Sugiura et al., 2011, 2013; Xiang et al., 2011b) calcofluor white, an agent known to destroy the PM, was used in the larval bioassay. Treatment of 1% calcofluor white increased the mortality of vHaBac$^{e66-\text{KO-}ph}$ to almost an equivalent level of vHaBac$^{e66-\text{REP-}ph}$ (Table 2). The result showed that destruction of the PM efficiently rescued the oral infectivity of vHaBac$^{e66-\text{KO-}ph}$, implying that the PM may be the

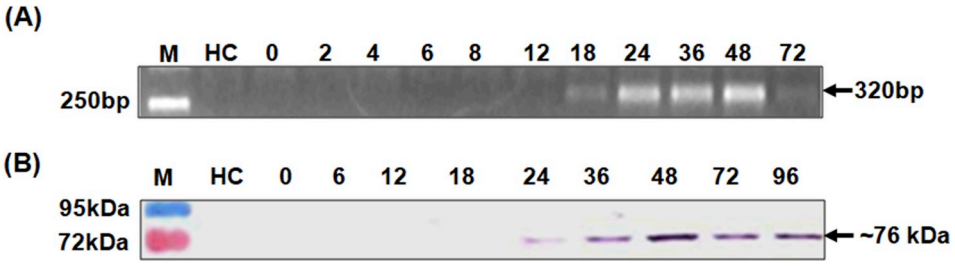

Fig. 1. Temporal analysis of transcription, translation and virion localization of ODV-E66. (A) Transcriptional pattern by 3′RACE analyses. RT-PCR analysis of *odv-e66* transcription in HearNPV G4 infected HzAM1 cells. Numbers above each lane indicated the time (h p.i.) when total RNA was isolated. (B) Expression time course of ODV-E66. Cellular proteins were harvested from HearNPV-G4 infected cells at the indicating h p.i., and separated on 10% SDS-PAGE. An anti ODV-E66 pAb was used in Western blots.

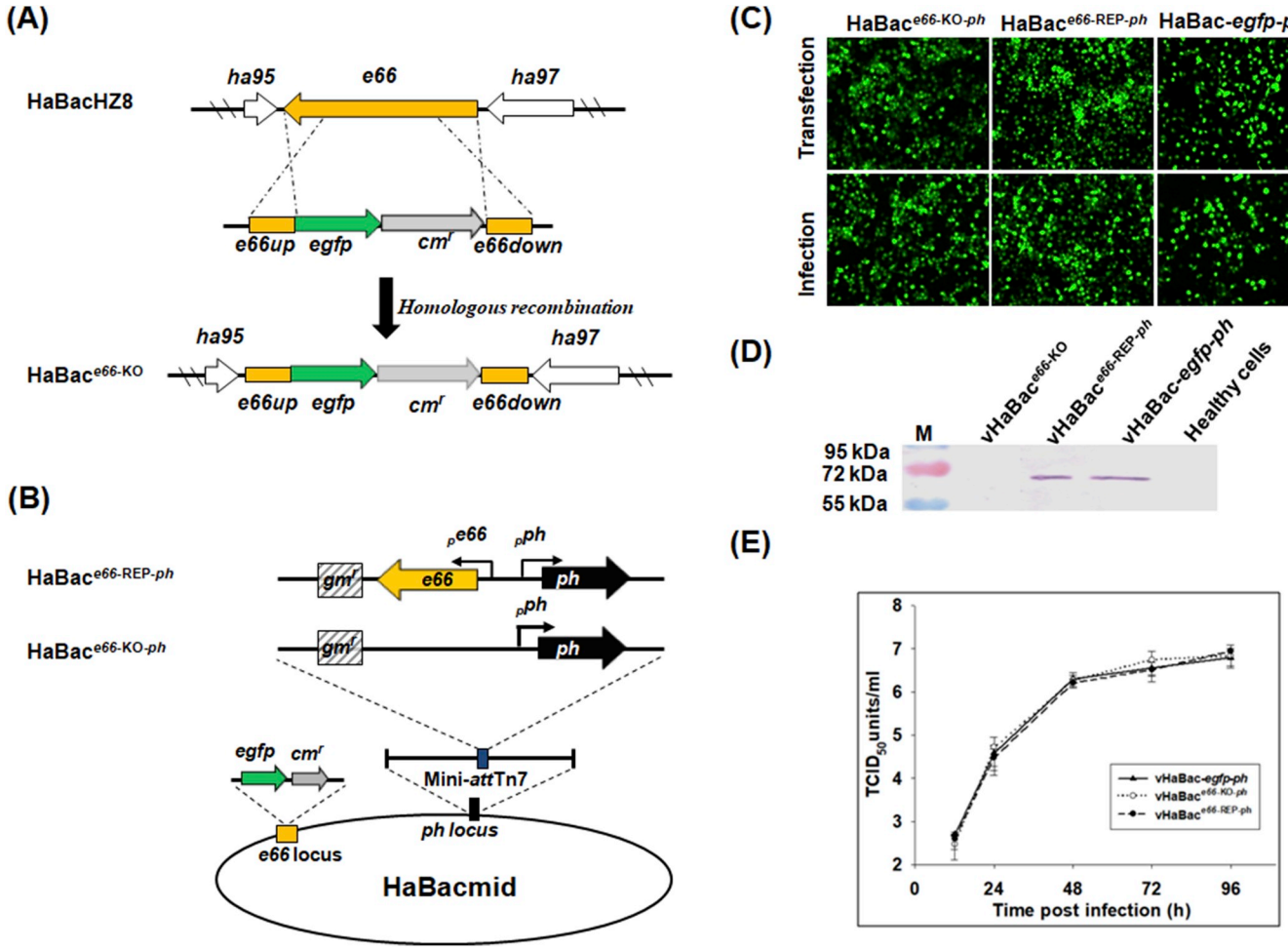

Fig. 2. Construction and characterization of recombinant bacmids. (A) Generation of HaBac$^{e66\text{-}KO}$ by replacing entire *e66* gene with two gene cassettes (*cm*^{r} and *gfp*) using homogenous recombination method. (B) Generation of HaBac$^{e66-KO\text{-}ph}$ and HaBac$^{e66-REP\text{-}ph}$ from bHaBac$^{e66-KO\text{-}ph}$ through transposition by using the donor plasmids pFB-DUAL-*ph* and pFB96-*ph*, respectively. (C) Transfection-infection analysis. HzAM1 cells were transfected with bacmids HaBac$^{e66-KO\text{-}ph}$, HaBac$^{e66-REP\text{-}ph}$ or HaBac-*egfp-ph*. At 6 d p.t., the transfection supernatants were used to infect healthy HzAM1 cells. Fluorescence images were taken at 96 h p.t. or p.i. (D) Detection of *odv-e66* expression in recombinant viruses infected cells. Cells were infected with vHaBac$^{e66-KO\text{-}ph}$, vHaBac$^{e66-REP\text{-}ph}$, vHaBac-*egfp-ph*, and health cells were used as negative control. Cellular proteins were harvested at 72 h p.i. and separated on 10% SDS-PAGE. Anti-ODV-E66 pAb was used in Western blots. M: protein molecular weight marker. (E) One-step growth curves of recombinants' BVs. HzAM1 cells were infected with vHaBac$^{e66-KO\text{-}ph}$, vHaBac$^{e66-REP\text{-}ph}$ and vHaBac-*egfp-ph* at an MOI of 5. BV $TCID_{50}$ titres were determined at different time points (12, 24, 48, 72 and 96 h p.i.). Growth curves were generated by arithmetic mean data of three infections.

target of ODV-E66.

3.7. HearNPV ODV-E66 contains chondroitin lyase activity

The chondroitin lyase activity of AcMNPV ODV-E66 has been characterized previously (Sugiura et al., 2011). We therefore investigated whether HearNPV ODV-E66 also contains chondroitin lyase activity. According to sequence alignment with AcMNPV ODV-E66, two truncated form of HearNPV ODV-E66 (23–672) and HearNPV ODV-E66 (44–672) (Fig. 4A) were cloned and both were expressed in *E. coli* system in soluble forms (data not shown). The chondroitin lyase activity assay showed that both truncated proteins, could degrade the substrate CS and yield unsaturated glucuronic acid (GlcUA) residues that have absorption at 232 nm (Fig. 4B). The shorter version, HearNPV ODV-E66 (44–672) showed higher chondroitin lyase activity than ODV-E66 (23–672) (Fig. 4B), which is similar to the result of AcMNPV ODV-E66 enzyme activity test (Sugiura et al., 2011).

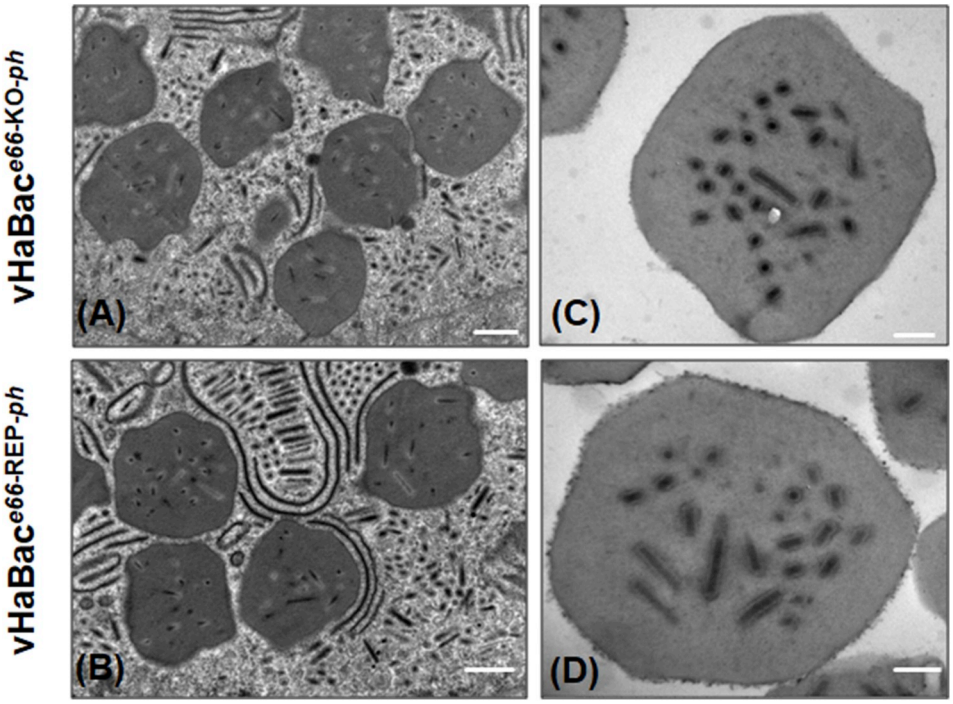

Fig. 3. Virion morphogenesis by TEM of *e66*-deletion. HzAM1 cells were infected with (A) vHaBac$^{e66-\text{KO-}ph}$, or (B) vHaBac$^{e66-\text{REP-}ph}$ at an MOI of 5. OBs were purified from vHaBac$^{e66-\text{KO-}ph}$ (C) or vHaBac$^{e66-\text{REP-}ph}$ (D) infected larvae. At 96 h p.i, infected cells and purified OBs were subjected to TEM examination. Bars, 500 nm (A and B) and 200 nm (C and D).

3.8. HearNPV ODV-E66 is involved in PM degradation of larval midgut

To determine whether ODV-E66 participates in the degradation of the PM in the larval midgut, the 3rd instar *H. armigera* larvae were fed with two different doses of OBs from vHaBac$^{e66-\text{KO-}ph}$ and HearNPV. The PM was stripped from the infected larvae after 4 h p.i. and observed under SEM. The PM of mock-infected larvae had a rather smooth and dense surface structure, even in the administration of high virus dosage (Fig. 5, left panel). For vHaBac$^{e66-\text{KO-}ph}$ infected larvae, the PM had a slightly rough surface compared with that of the mock-infected larvae (Fig. 5, middle panel). In contrast, the PM of larvae infected with the wild type (WT)T virus HearNPV G4 had a surface with obvious fibrous structures (red arrows) and many small pores (white arrows) compared with that of vHaBac$^{e66-\text{KO-}ph}$ and mock-infected groups (Fig. 5, right panel). Our speculation is that during the WT virus infection, ODV-E66 may digest the glycans of the PM structural proteins, leading to the further degradation of the proteins by the insect midgut endogenous proteases (Wang and Granados, 1997, 2001), and the exposure of the underlying fibrous structure (might be chitin fibers, red arrows). The results suggested that ODV-E66 is involved in PM degradation of larvae midgut.

4. Discussion

ODV-E66 is a major envelope protein of baculovirus ODV that plays a role in oral infection process with a yet unknown mechanism. In this study, we constructed *odv-e66*-deleted and repaired recombinant HearNPVs and showed that ODV-E66 was irrelevant to BV replication in cultured cells, but its deletion had a profound effect on HearNPV oral infectivity. Further experiments suggested ODV-E66 was involved in PM degradation.

During oral infection, ODV must first pass through the PM before they gain access to midgut cells. The PM is a dense extracellular matrix composed of chitin, glycoproteins and proteoglycans, which protects midgut cells against ingested toxins or pathogens. The PM is recognized to serve as a barrier against baculovirus infection (Wang and Hu, 2019). For example, destruction of the PM by Calcofluor white significantly increased larval susceptibility to virus infection in different baculovirus-host systems (Song et al., 2008; Wang and Granados, 2000). Analysis of the PM morphology of resistant- and non-resistant larvae

Table 2
Effects of calcofluor white on the oral infectivity of HaBac$^{e66-\text{KO-}ph}$ and HaBac$^{e66-\text{REP-}ph}$ in 4th instar *H. armigera* larvae.

Virus	Concentration (OBs/ml)	Mortality(dead/total)	
		Test 1	Test 2
vHaBac$^{e66-\text{KO-}ph}$	4×10^4	7/48[a]	9/48[a]
vHaBac$^{e66-\text{KO-}ph}$ + calcofluor white	4×10^4	39/48	41/48
vHaBac$^{e66-\text{REP-}ph}$	4×10^4	22/48[a]	24/48[a]
vHaBac$^{e66-\text{REP-}ph}$ + calcofluor white	4×10^4	42/48	43/48

[a] $P < 0.001$.

Table 1
LC_{50} of vHaBac$^{e66-\text{KO-}ph}$ and vHaBac$^{e66-\text{REP-}ph}$ in the 3rd and 4th instar *H. armigera* larvae.

Virus	Instar	LC_{50} (95% CL) (10^4 × OBs/ml)	Slope (95% CL)	Potency ratio (95% CL)[a]
vHaBac$^{e66-\text{KO-}ph}$	3rd	0.94(0.67,1.31)	0.74 (0.59, 0.90)	
vHaBac$^{e66-\text{REP-}ph}$	3rd	0.36 (0.29, 0.47)	1.09 (0.92, 1.26)	2.563 (1.323, 5.828)
vHaBac$^{e66-\text{KO-}ph}$	4th	109.87 (78.00, 160.52)	0.60 (0.50, 0.71)	
vHaBac$^{e66-\text{REP-}ph}$	4th	3.79 (2.89, 5.02)	0.85 (0.70, 1.00)	26.305 (11.635, 72.831)

LC ratios with confidence levels (CL) that include 1.0 indicate that the LC_{50} values of the viruses compared are not significantly different from each another ($P > 0.05$).

[a] Potency ratio was calculated by dividing the LC_{50} value of the v HaBac$^{e66-\text{KO-}ph}$ by that of vHaBac$^{e66-\text{REP-}ph}$.

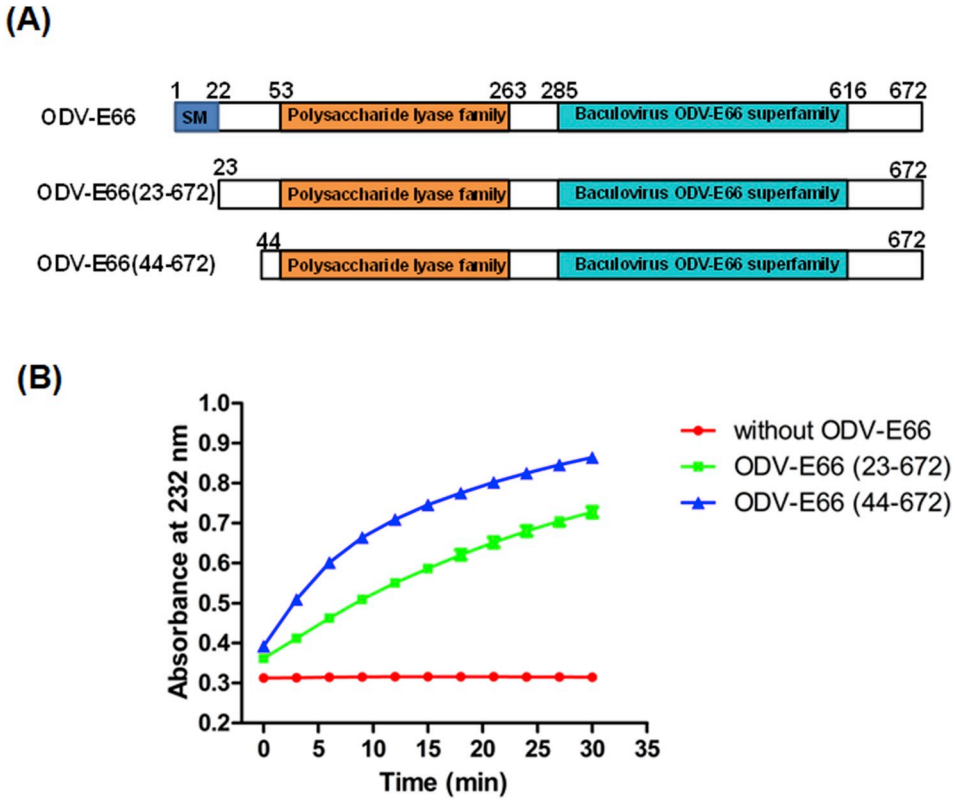

Fig. 4. Chondroitin lyase activity assay of ODV-E66. (A) Linear diagrams of the predicted domains of ODV-E66. A signal sequence (residues 1–22) is predicted at the N terminal of ODV-E66 and the conserved region (residues 53–263) is predicted as the polysaccharide lyase family domain. The region (residues 285–616) is the baculovirus E66 superfamily sequence. Two truncated proteins used in activity assays as shown. (B) Chondroitin lyase activity assay with chondroitin sulfate (CS) as substrate. The catalyzed reaction was carried out at 37 °C and absorbance at 232 nm was measured. The error bars represent SD of triplicate experiments.

implicated a role of the PM in the resistance of *A. gemmatalis* larvae against baculovirus infection (Levy et al., 2011). Accordingly, baculoviruses have developed their own approaches to overcome the barrier posed by the PM, as evidenced by specific biochemical (degradation of PM proteins) and structural changes (more fragile) in the PM of infected larvae compared with those of untreated controls (Derksen and Granados, 1988). Enhancin, a virus encoded metalloproteinase, was known to alter the PM structure by degradation of a major PM protective protein mucin, thus enhances virus infectivity (Wang and Granados, 1997). However, only a few alpha- and betabaculoviruses encode this protein (van Oers and Vlak, 2007). ODV-E66 is conserved in alpha- and betabaculoviruses. Recently, ODV-E66 has been identified to be a novel chondroitin lyase, and since CS is enriched in the PM, it has been suggested that ODV-E66 may promote oral infection by degrading the CSs in the PM. To confirm this hypothesis, we used calcofluor to destroy PM of *H. armigera* larvae, and the results showed that the oral infectivity of vHaBac$^{e66-\mathrm{KO}\text{-}ph}$ was rescued to almost the equivalent level of the repaired virus, suggesting that the PM is the target of HearNPV ODV-E66 (Table 2). Next, we demonstrated that HearNPV ODV-E66 had the chondroitin lyase activity and digest chemically synthesized CS *in vitro* (Fig. 4), which is similar to the function of ODV-E66 from AcMNPV and BmNPV (Sugiura et al., 2011, 2013).

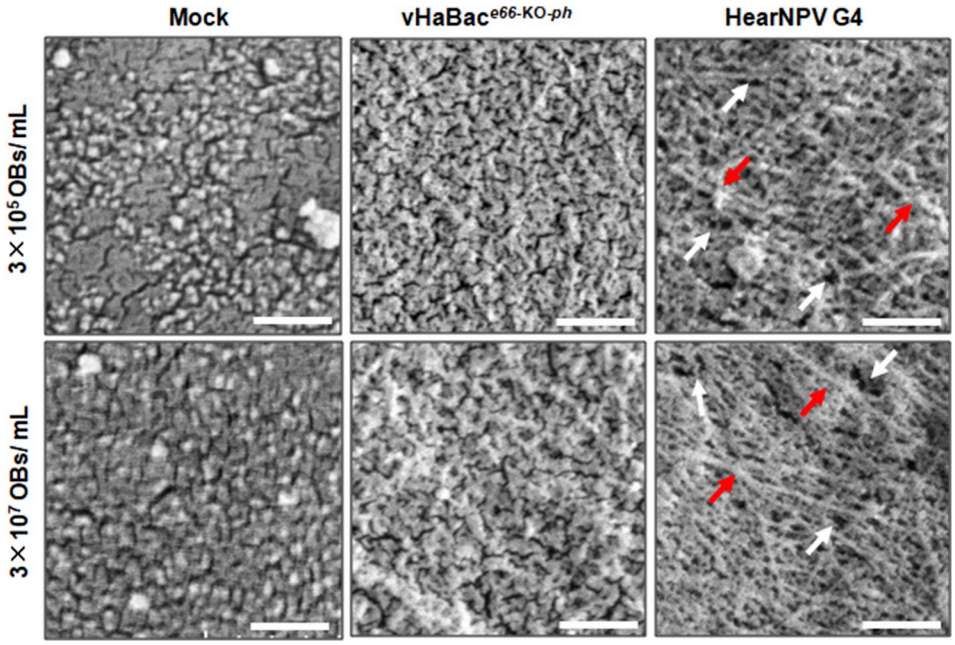

Fig. 5. SEM analysis of PM. The 3rd instar of *H. armigera* larvae were fed different dose of OBs from vHaBac$^{e66-\mathrm{KO}\text{-}ph}$ and the WT virus (HearNPV G4). PM was separated from the larvae at 4 h p.i. and viewed by SEM to observe the surface morphology. The red and white arrows indicate the fibrous structures and small pores, respectively. Bars, 250 nm.

Furthermore, SEM observations showed that PM structure was complete when the larvae infected with *odv-e66*-deleted HearNPV, while it was severely disrupted when the larvae infected with wild-type HearNPV (Fig. 5). Our results, together with the previous finding of *in vitro* cleavage of CS extracted from *Bombyx mori* PM by BmNPV ODV-E66 (Sugiura et al., 2013), highlighted a role of ODV-E66 in the degradation of PM, allowing virus to gain access to midgut epithelium cells for primary infection. This may explain the need for ODV-E66 for proper oral infectivity of HearNPV in the higher instar larvae than the lower instar ones (Table 1), since the PM becomes thicker and denser during insects grow up, which is more difficult for virus to pass through.

Deletion of *odv-e66* in AcMNPV resulted in a severe decrease in oral infectivity in 3rd instar *Plutella xylostella* larvae, with 1000-fold increase in 50% lethal dose compared to that of the control virus (Xiang et al., 2011b). However, deletion of HearNPV *odv-e66* led to only a modest impact on oral infectivity in 3rd instar *H. armigera* larvae, with LC_{50} value increased by only 2.6 fold (Table 1). This indicates that the function of ODV-E66 may not be identical in different baculoviruses or different host susceptibilities to different baculoviruses. Interestingly, a 26-fold increase in LC_{50} value was observed when the 4th instar *H. armigera* larvae were administrated *per os* with *odv-e66*-deleted HearNPV (Table 1). These results confirmed that ODV-E66 plays a crucial role in *per os* infection of HearNPV, especially for that in higher instar larvae. In addition, AcMNPV has a wide host range, it is not known whether the chondroitin lyase activity of AcMNPV ODV-E66 become much stronger during evolution to facilitate expansion of host range. Further investigation would be done to compare the chondroitin lyase activity of ODV-E66s of HearNPV and AcMNPV.

It is also found that the chondroitin lyase activity of the longer truncation HearNPV ODV-E66 (23-672aa) is lower than that of HearNPV ODV-E66 (44-672aa), similar to the phenomena found in AcMNPV ODV-E66 (Sugiura et al., 2011). This indicates that ODV-E66 may be subject to cleavage to activate its chondroitin lyase activity. Furthermore, it is not known whether the activation of ODV-E66 occurs in midgut and which host factor is involved in the process. Apart from the function in degradation of PM, other unknown functions might be unveiled, since ODV-E66 is also found in polydnavirus which do not need to pass through PM to establish infection (Burke et al., 2013).

In conclusion, we suggest that HearNPV ODV-E66 was involved in ODV-mediated primary infection, and it is likely to degrade the PM to facilitate efficient primary infection. Besides ODV-E66, some other viral proteins, such as enhancin and GP37 (fusolin-like protein) (Liu et al., 2019) also target at the PM. Actually, we observed that the PM of $HaBac^{e66-KO\text{-}ph}$ infected larvae had a less smooth surface structure compared with that of the mock-infected larvae (Fig. 5), implying the involvement of other viral factors in the PM degradation in HearNPV. Different from the enhancin, which selectively degraded the mucin component from the PM, ODV-E66 would target CSs from PM. It should be further investigated whether ODV-E66 could degrade PM *in vitro* and rescue the oral infectivity of *odv-e66* deleted HearNPV. If possible, it will be helpful to promote the use of ODV-E66 as a possible additive of baculoviral insecticides in future.

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