Functional studies of per os infectivity factors of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus

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A combined functional investigation on the four per os infectivity factors (PIFs) of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HearNPV) was conducted in this study. HearNPV bacmids with deletions of *p74* (Ha20), *pif1* (Ha111), *pif2* (Ha132) and *pif3* (Ha98) were constructed individually by homologous recombination in *Escherichia coli* cells. Repaired bacmids with respective *pifs* were also constructed. Western blot analyses revealed that all four PIFs were structural components of the envelope of HearNPV occlusion-derived virus (ODV). Electron microscopy showed that deletion of the *pif* did not have any obvious effects on the morphology of the occlusion bodies (OBs). Bioassay analyses indicated that deletion of any of the above *pifs* resulted in loss of oral infectivity of OBs. The mixtures of the four *pif*-deletion mutants also resulted in deficiency of oral infectivity, implying that the four PIFs must be structural components of the same ODV to accomplish their function. Repairing of the respective genes into the *pif*-deletion bacmids could rescue the oral infectivity of the *pif*-deletion viruses. Calcofluor, which can damage the peritrophic membrane (PM), could not rescue the defects of the oral infectivity of the *pif*-deletion viruses, indicating that the PM is not likely to be the functional target of the PIFs.

INTRODUCTION

Baculoviral infection of insect cells produces two kinds of enveloped progeny viruses: budded virus (BV) and occlusion-derived virus (ODV). BVs infect a broad spectrum of cell types and transmit viral infection among the insect tissues within an infected larva, whereas ODVs infect only columnar epithelial cells of the insect midgut and are required for the oral transmission of virus between insect hosts. BVs usually infect cells under slightly acidic conditions of the haemolymph (pH 6.4–6.8) (Chapman, 1998), whereas ODVs need a highly alkaline environment in the pH range 9.2–11 (Federici, 1997).

ODVs are released from the occlusion bodies (OBs) in the alkaline environment of the midgut after the OBs are ingested by insects. The ODVs first pass through the peritrophic membrane (PM), a secreted extracellular matrix that borders the midgut epithelium and subsequently fuse to the midgut epithelial cells, thereby establishing the initial infection (Funk *et al.*, 1997). It was demonstrated that the entry of the *Lymantria dispar* multicaspid nucleopolyhedrovirus (LdMNPV) ODVs into brush-border membrane vesicles occurred in two steps, binding to the cell membrane followed by fusion mediated by ODV attachment and fusion factors (Horton & Burand, 1993).

To date, four genes have been identified as essential for ODV oral infection in several NPVs: *p74* (Kuzio *et al.*, 1989; Yao *et al.*, 2004), *pif1* (Kikhno *et al.*, 2002), *pif2* (Pijlman *et al.*, 2003; Fang *et al.*, 2006) and *pif3* (Ohkawa *et al.*, 2005; Li *et al.*, 2007). In this manuscript, for easy description, *per os* infection factors (PIFs) refer to all four proteins, including *P74*. These *pifs* are all late genes and their expression products are ODV-specific proteins (Slack & Arif, 2007). It has been shown that *P74* and PIF1 are the ODV envelope proteins (Faulkner *et al.*, 1997; Kikhno *et al.*, 2002), while PIF2 and PIF3 are ODV-specific proteins with their detailed locations in ODV fractions unidentified (Fang *et al.*, 2006; Li *et al.*, 2007). Absence of any one of the *pifs* will lead to a block in infection prior to viral gene expression in midgut cells, suggesting that PIFs are involved in early infection events and important for ODV oral infection (Haas-Stapleton *et al.*, 2004; Kuzio *et al.*, 1989; Ohkawa, 1997). The *pifs* are conserved among all sequenced baculoviruses (Wang *et al.*, 2007), suggesting there is a highly conserved entry mechanism for baculoviruses. Elucidation of the function of the PIFs should be helpful to disclose this special entry process. Previous studies indicated that *P74*, PIF1 and PIF2 mediate ODVs binding to the midgut cells during the...
initial infection process (Haas-Stapleton et al., 2004; Ohkawa et al., 2005), but the function of PIF3 and potential other functions of the four PIFs are still unknown. ODVs are released from the OBs in the alkaline environment of the midgut and pass through the PM to form efficacious infection. Obviously, the PM is the first barrier for ODV-initiated primary infection. Whether the PIFs interact with PM is not quite clear.

In this manuscript, the PIFs of the Helicoverpa armigera single nucleocapsid nucleopolyhedrovirus (HearNPV) were studied together. HearNPV is a naturally occurring pathogen of cotton bollworm which was first isolated from diseased larvae in the Hubei province of China and has been widely used for pest control (Zhang, 1994). Although p74 and pif2 of HearNPV had been shown to be essential for oral infection (Yao et al., 2004; Fang et al., 2006), their detailed locations in the ODV fractions have not been studied. In this manuscript, the localizations of all the PIFs in HearNPV virions and their fractions were studied by Western blot and the results showed that they all localized to the ODV envelope. Recombinant HearNPVs with deletions of p74 (Ha20), pif1 (Ha111), pif2 (Ha132) and pif3 (Ha98) were constructed individually. Bioassay results showed that all pifs are indispensable for HearNPV ODV oral infection and that deletion of any one of them led to a block in oral infection, whilst pifs-rescued virus recovered oral infectivity. The mixed infection with OBs of the four individual pif-deletion mutants resulted in deficiency of oral infectivity, but the co-occluded OBs generated by co-infection of four individual pif-deletion BVs could produce efficient oral infection. This suggests that the four PIFs need to be physically integrated into the same ODV to accomplish their function. To test whether PIFs facilitated passage of ODV across the PM, we inoculated pif-deletion occlusion bodies in the presence of Calcofluor. The results show that the addition of 1% Calcofluor failed to enhance the infectivity of pif-deletion occlusion bodies, suggesting that PM is not the functional target of the HearNPV PIFs.

METHODS

Insect cells, insects and virus. The Helicoverpa zea cell line HzAM1, originally isolated from H. zea pupal ovarian tissue (Mclntosh & Ignoffo, 1983), was maintained at 28°C in Grace’s medium (Gibco-BRL) supplemented with 10% fetal bovine serum. HearNPV strain G4, the genome of which has been sequenced (GenBank accession no. AF271059; Chen et al., 2003), was used as the wild-type virus and was propagated in H. armigera, H. zea HzAM1, originally isolated from H. armigera. H. armigera larvae were reared on an artificial diet at 27°C. HaBacH2Z was an infectious bacmid of HearNPV, which was constructed previously in our laboratory (Wang et al., 2003).

Localization of PIFs in ODV fractions and BVs. Western blot analyses were used to localize the PIFs in the virions of HearNPV. To generate anti-PIF antibodies, partial coding sequence of the pifs were amplified from HearNPV G4 genome by specific primers (Supplementary Table S1, available in JGV Online) and fragments containing p74 (1–1380 nt), pif1 (118–1587 nt), pif2 (1–1152 nt) and pif3 (73–600 nt) were cloned into the pET-28a (+) plasmid individually. PIFs as His-tag-fused proteins were expressed in E. coli BL21 cells and purified for rabbit immunizations to generate polyclonal anti-PIFs antiserum.

Construction and identification of pif deletion HearNPV bacmids. To obtain p74 (Ha20), pif1 (Ha111), pif2 (Ha132) and pif3 (Ha98) deletion viruses, a linear homologous recombination method was used (Hou et al., 2002). Briefly, mutants with deletions in p74, pif1, pif2 and pif3 were produced by inserting a cassette containing the enhanced green fluorescent protein gene (egfp) driven by the Drosophila hsp70 promoter and chloramphenicol resistance (Cmr) gene under control of a bacterial promoter into the HaBacH2Z bacmid to replace relevant ORFs Ha20, Ha111, Ha132 and Ha98, respectively. The deletion mutants were constructed as follows: first, the upstream and downstream homologous fragments (about 400–500 bp) of pifs were amplified with specific primers (Supplementary Table S1). A pair of restriction enzyme sites was introduced to homologous fragments of pifs, KpnI/XhoI for upstream fragments and EcoRV/XhoI for downstream fragments. Then the fragments were cloned into the pKS-egfp-Cmr plasmid which was kindly provided by Professor J. M. Valk ( Wageningen University, The Netherlands). The upstream homologous fragment was cloned upstream of the egfp-Cmr cassette while the downstream homologous fragment was inserted downstream of the cassette. The resulting plasmids were designated pKS-delpifs. The linear fragments for homologous recombination were generated from pKS-delpifs by KpnI/XhoI digestion and then transformed into E. coli DH10B containing HaBacH2Z DNA and pKD46. The pif-deletion bacmids were generated by homologous recombination in E. coli and screened by kanamycin and chloramphenicol resistance as described previously (Hou et al., 2002). By using the above methods, the 83–2047 nt of p74, 106–1477 nt of pif1, 23–1130 nt of pif2 and 71–517 nt of pif3 were deleted in HaBacΔp74, HaBacΔpif1, HaBacΔpif2 and HaBacΔpif3, respectively. The deletion bacmids were identified by PCR analyses and HindIII restriction enzyme digestion.

Since the original polyhedrin (ph) gene was disrupted during the construction of the bacmid HaBacH2Z (Wang et al., 2003), ph was reintroduced into HearNPV pif-deletion bacmids. Accordingly, HearNPV ph with putative promoter sequence was amplified by specific primers (Supplementary Table S1) from the HearNPV G4 genome and inserted into the pFastBac-DUAL transfer vector (Invitrogen) under control of the AcMNPV ph promoter, which was referred to as pB-B-DUAL-ph. The recombinant bacmids with deletion of individual pifs and insertion of ph were constructed by using Bac-to-Bac transposition protocol (Pijlman et al., 2002) (Invitrogen) and named HaBacΔp74-ph, HaBacΔpif1-ph,
HaBacApf2-ph and HaBacApf3-ph. The correctness of the ph insertions were verified by PCR.

Construction of pifs-rescued HearNPV bacmids. To construct pif-rescued bacmids, the coding sequence and putative promoter region of p74, pif1, pif2 and pif3 were amplified from the HearNPV G4 genome by specific primers (Supplementary Table S1). The PCR products were first cloned into the pGEM-T Easy vector (Promega). The plasmids were sequenced to confirm the identity of the insertions. p74, pif1, pif2 and pif3 were then inserted into the transfer vector pFB-DUAL-ph under control of the AcMNPV p10 promoter. The transfer vectors were transposed to the respective pif-deletion bacmids according to the Bac-to-Bac transposition protocol. The resulting bacmids with repaired pif and ph were identified by PCR, and named HaBac-p74R-ph, HaBac-pif1R-ph, HaBac-pif2R-ph and HaBac-pif3R-ph, respectively. For control, the egfp–Cm cassette and ph were inserted into the HaBacHZ8 bacmid and the resulting bacmid was named HaBac-egfp-ph.

Transfection and infection assays. HzAM-1 cells were seeded in tissue culture wells with 5 × 10^5 cells per well. Transfection was performed with 0.5 μg bacmid DNA using 12 μl Lipofectin (Invitrogen). At 6 days post-transfection, supernatants containing BVs from transfected cells were harvested by centrifugation and used to infect HzAM-1 cells. The titre of the produced BVs was determined by end-point dilution assay (EPDA).

Western blot analyses of recombinant viruses. HzAM-1 cells were infected individually with vHaBac-egfp-ph, vHaBacApf74-ph, vHaBacApf1-ph, vHaBacApf2-ph, vHaBacApf3-ph, vHaBac-p74R-ph, vHaBac-pif1R-ph, vHaBac-pif2R-ph or vHaBac-pif3R-ph at an m.o.i. of 5. Infected cells were harvested at 96 h p.i. The recombinant ODVs used for Western blot were purified from OBs generated from infected larva. The methods for ODV purification and Western blot analysis were the same as used for PIFs localization.

Electron microscopic observations of cells infected with the pif-deletion viruses. HzAM1 cells were infected individually with vHaBac-egfp-ph, vHaBacApf74-ph, vHaBacApf1-ph, vHaBacApf2-ph, vHaBacApf3-ph or vHaBacApf3-ph at an m.o.i. of 5. Infected cells were fixed at 96 h p.i. and processed for electron microscopic analysis.

Bioassay. The systemic infectivity of BVs was detected by intrahaemocoel injection of BVs into the late third-instar H. armigera larvae. About 10 μl of 10^6 TCID₅₀ units BVs ml⁻¹ was injected into the haemocoel of each larva. Grace’s medium was used as a negative control. The oral infectivity of the recombinant viruses was detected by the droplet method (Hughes et al., 1998) with early third-instar H. armigera larvae. The OBs used for bioassay were harvested and purified from diseased larvae as described by Sun et al. (1998), and 10^6 OBs ml⁻¹ were used for the bioassay.

For bioassay of mixed OBs, equal amounts of OBs of vHaBacApf74-ph, vHaBacApf1-ph, vHaBacApf2-ph and vHaBacApf3-ph with individual concentrations of 10^6 OBs ml⁻¹ were mixed together before being applied to the larvae for droplet bioassay. For bioassay of co-occluded OBs, equal amounts of BVs of vHaBacApf74-ph, vHaBacApf1-ph, vHaBacApf2-ph and vHaBacApf3-ph with an individual titre of 10^6 TCID₅₀ units ml⁻¹ were mixed and injected into the haemocoel of late third-instar larva (10 μl per larva). The co-occluded OBs were harvested and purified from the diseased larvae and 10^6 OBs ml⁻¹ were used for droplet bioassay.

For all the above bioassay experiments, the larvae were kept separately in 24-well plates and monitored daily until all larvae had either pupated or died as a result of virus infection. At least 48 larvae were tested per treatment. All the bioassays were done in duplicates.

To investigate whether damage of PM could enhance the infectivity of pif-deletion viruses, 1 % (w/v) Calcofluor (Wang & Granados, 2000) was used to disturb the PM of H. armigera. Calcofluor (1 %) plus 10⁸ OBs ml⁻¹ recombinant viruses (vHaBacApf74-ph, vHaBacApf1-ph, vHaBacApf2-ph or vHaBacApf3-ph) were used to infect early third-instar H. armigera larvae using the droplet method and the results were compared with the control groups infected only with viruses, but without Calcofluor.

RESULTS

Western blot detection of the four PIFs in HearNPV ODV fractions and BVs

Polyclonal antibodies against HearNPV PIFs (anti-P74, anti-PIF1, anti-PIF2 and anti-PIF3) were generated according to the Methods and used for Western blot analyses. Fig. 1 shows the Western blot results of the PIFs. Using anti-P74, a band with the expected size of P74 (75.6 kDa) was detected in ODV, but not in BV. Further identification showed that P74 located in the envelope but not in the nucleocapsid of ODV (Fig. 1a). Using anti-PIF1, a band with the expected size of PIF1 (58.1 kDa) was identified in the ODV envelope but not in the ODV nucleocapsid or BV (Fig. 1b). Similarly, PIF2 (42.1 kDa) and PIF3 (21.0 kDa) were found specifically in the ODV envelope (Fig. 1c and d). Our Western blot results showed that the four PIFs of HearNPV are all ODV-specific proteins and localized on the ODV envelope. These results are consistent with the report of Slack & Arif (2007). In Fig. 1(d), a very weak band at the expected size of PIF3 is shown in the nucleocapsid fraction of ODV, suggesting that, although PIF3 locates in the ODV envelope, it may interact closely with the nucleocapsid.

Identification of pif-deletion and pif-repaired mutants

The pif-deletion and pif-repaired bacmids were constructed according to the Methods and their structures are shown in Fig. 2(a). The bacmids were confirmed by PCR (data not shown) and HindIII restriction digestion analysis. HindIII restriction digestion profiles of HaBacApf74, HaBacApf1, HaBacApf2 and HaBacApf3 were compared with those of HaBacHZ8 (Fig. 2b). The data showed that, in comparison with the HaBacHZ8 genome, the HaBacApf74 bacmid lost HindIII-C fragment (14.5 kb) with a concomitant appearance of two fragments of 10.2 and 3.6 kb; the HaBacApf1 bacmid lost HindIII-E fragment (11.0 kb) with a concomitant appearance of two fragments of 5.9 and 4.9 kb; the HaBacApf2 lost HindIII-D fragment (12.9 kb) with a concomitant appearance of two fragments of 9.1 and 3.9 kb; and the HaBacApf3 bacmid lost HindIII-A fragment (22.6 kb) with a concomitant appearance of two fragments of 17.6 and 5.8 kb. All the changes were the expected results from the recombination. The results of HindIII restriction digestion showed that the proper deletion mutants were produced.
Similarly, HaBacΔp74-ph, HaBacΔpif1-ph, HaBacΔpif2-ph, HaBacΔpif3-ph, HaBac-p74R-ph, HaBac-pif1R-ph, HaBac-pif2R-ph and HaBac-pif3R-ph bacmids were identified by PCR analyses (data not shown). All the recombinant viruses were infective to HzAM-1 cells, which were characterized by specific green fluorescence and appearance of polyhedra under the microscope.

**Western blot detection of HearNPV PIFs expression in infected HzAM-1 cells and purified ODVs**

Fig. 3(a) showed the Western blot results of extracts of cells infected with pif-deletion and repaired viruses. Using anti-P74, a band with expected size of P74 (75.6 kDa) was detected in the extracts of vHaBac-egfp-ph- and vHaBac-p74R-ph-infected cells, but not in those of mock- and vHaBacΔp74-ph-infected cells. In addition to the 75.6 kDa band, a non-specific band with size of about 65.0 kDa was detected in all the samples, which might be due to the unspecific reactions of anti-P74. Two other proteins with sizes of 36.0 and 38.0 kDa were detected in the vHaBac-egfp-ph- and vHaBac-p74R-ph-infected cells, which might be due to degradation products of P74. Using anti-PIF1, a band with expected size of PIF1 (58.1 kDa) was identified in the extracts of vHaBac-egfp-ph- and vHaBac-pif1R-ph-infected HzAM1 cells, but not in those of the mock- and vHaBacΔpif1-ph-infected cells. Similarly, PIF2 (42.1 kDa) and PIF3 (21.0 kDa) were found specifically expressed in the cells infected with wild-type or repaired viruses, but not in the cells infected with the respective deletion viruses.

Western blot analyses of ODVs of control virus (vHaBac-egfp-ph), deletion viruses (vHaBacΔp74-ph, vHaBacΔpif1-ph, vHaBacΔpif2-ph and vHaBacΔpif3-ph) and repaired
viruses (vHaBac-p74R-ph, vHaBac-pif1R-ph, vHaBac-pif2R-ph and vHaBac-pif3R-ph) were also performed. Results showed that HearNPV PIFs were detected in the ODVs of control and repaired viruses, but not in the ODVs of pif-deletion viruses, confirming that the recombinant viruses were correctly constructed (Fig. 3b).

Electron microscopy of cells infected with the recombinant viruses
To determine if the deletion of PIFs affected the formation of the OBs, the morphology of the OBs of the pif-deletion viruses were observed by electron microscopy. As shown in Fig. 4, the pif-deletion recombinant viruses formed OBs similar to those of the control virus, suggesting that, although the PIFs are ODV-associated proteins, their deletion has little impact on the formation of the OBs.

Fig. 4. TEM images of HzAM-1 cells infected with vHaBac-egfp-ph, vHaBac-p74-ph, vHaBacΔpif1-ph, vHaBacΔpif2-ph or vHaBacΔpif3-ph. Cell samples were collected at 96 h p.i.

pif-deletion viruses lost their oral infectivity, but retained their BV infectivity
To study the infectivity of BV and ODV of the pif-deletion viruses, two kinds of bioassay were carried out. Firstly, supernatants containing BVs of control, pif-deletion and pif-repaired viruses were injected into the haemolymph of late third-instar H. armigera larvae with a titre of 10^6 TCID_{50} units ml^{-1}. The mortalities due to the deleted and repaired viruses were similar to those of the control virus (data not shown). Secondly, OBs of the recombinant viruses isolated from those infected larvae were fed to third-instar H. armigera larvae by the droplet method. The results showed that the pif-deletion mutants were not infectious to H. armigera larvae by oral ingestion, whereas the pif-repaired recombinant viruses were able to fully rescue the oral infectivity (Table 1). Our results confirmed that deletion of each individual pif leads to the lack of oral infectivity, but does not impair infectivity by intrahaemocoelic injection of HearNPV.

Deficiency of oral infectivity could not be rescued by mixed infection of different pif-deletion mutants
In order to find out if the pif-deletion mutants could be rescued by co-infection of other pif-deletion mutants, we conducted bioassays for mixed OBs and co-occluded OBs. For bioassay of mixed OBs, we fed the larvae with a mixture of four deletion mutants (vHaBacΔp74-ph, vHaBacΔpif1-ph, vHaBacΔpif2-ph and vHaBacΔpif3-ph), the concentration of each virus was about 10^8 OBs ml^{-1}. For bioassay of co-occluded OBs, BVs of the four deletion mutants were mixed and injected into the haemocoel of late third-instar H. armigera larvae, the concentration of each virus was about 10^6 TCID_{50} units ml^{-1}. OBs obtained from diseased larvae were used for the bioassay. Results
The concentration of OBs was 3 × 10^6 OBs ml⁻¹ and 1 × 10^8 OBs ml⁻¹ of different vHaBacApifs-ph OBs, and 3 × 10^4 OBs ml⁻¹ vHaBac-egfp-ph OBs plus 1 % Calcofluor as a positive control. The results (Table 2) showed 1 % Calcofluor had increased the mortality of vHaBac-egfp-ph significantly (the mortality was increased from 52 to 95.8 %). However, 1 % Calcofluor had no impact on the mortality of the pif-deletion viruses. Therefore, destroying the PM of the insects did not help OBs of pif-deletion viruses to resume their oral infectivity. Our results suggest that the PM is not the functional target of the PIFs.

**DISCUSSION**

In this paper, we confirmed that P74, PIF1, PIF2 and PIF3 are ODV envelope proteins and indispensable for HearNPV oral infectivity. Deletion of any one of those four genes results in the complete elimination of the oral infectivity of the HearNPV, without changing the infectivity by intrahaemocoelic injection of BVs.

The localization of all four PIFs as ODV envelope proteins (Fig. 1) is consistent with their sequence characters. P74 is a C-terminal transmembrane protein containing two to three transmembrane domains and the hydrophobic C-terminal is responsible for the intranuclear ring zone location and possibly the ODV envelope location of P74 (Slack et al., 2001). The N-termini of PIF1, PIF2 and PIF3 have a hydrophobic domain called sorting motif, which comprises a signature for sorting proteins to the inner nuclear membrane (Braunagel et al., 2004). Although PIFs are ODV envelope-specific proteins, deletion of any one of them seems to have no influence on the formation of the ODV or OBs of HearNPV (Fig. 4). The ODV envelope could be clearly detected in all the pif-deletion mutants (Fig. 4), indicating that pifs are likely to be dispensable for the assembly of the ODV envelope.

As oral infection-related genes pifs are conserved among all sequenced baculovirus genomes. The mechanisms underlying those four proteins, indispensable for ODV oral infection, are not clear. The ODV binding and fusion mechanism is different from that of BV. BVs enter the insect cells through adsorptive endocytosis dependent on endosomal low-pH activation (Volkman & Goldsmith, 1985). The main envelope protein, GP64 of group I (Hefferon et al., 1999) or F protein of group II (Ijkel et al., 2000; Pearson et al., 2002), is responsible for BVs entry (Volkman & Goldsmith, 1985). In contrast to BV, ODV is more specialized. ODVs penetrate the protective PM bordering the midgut epithelium and complete the fusion process at the cellular membrane under an extremely alkaline environment. At least four proteins (P74, PIF1, PIF2 and PIF3) participate in this initial infection process; they act as key factors, exert their function solely or together during primary infection. Our result of the infection of mixed OBs showed that the deficiency of oral infectivity cannot be rescued by the co-existing PIFs in different OBs. The deficiency of oral infectivity, however, could be rescued by co-occluded OBs. Our result is in contrast to a previous report that the deficiency of p74 deletion could be rescued by addition of P74 proteins (Yao

### Table 1. Feeding experiments of recombinant viruses on third instar H. armigera larvae

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. larvae</th>
<th>No. deaths (Test 1/Test 2)</th>
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</thead>
<tbody>
<tr>
<td>HaBac-egfp-ph</td>
<td>48/48</td>
<td>47 + 1*/47</td>
</tr>
<tr>
<td>HaBacAp74-ph</td>
<td>48/48</td>
<td>0/2*</td>
</tr>
<tr>
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<td>48/48</td>
<td>0/0</td>
</tr>
<tr>
<td>HaBacApif2-ph</td>
<td>48/48</td>
<td>2*/1*</td>
</tr>
<tr>
<td>HaBacApif3-ph</td>
<td>48/48</td>
<td>0/0</td>
</tr>
<tr>
<td>HaBac-p74R-ph</td>
<td>48/48</td>
<td>46 + 1*/48</td>
</tr>
<tr>
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<td>48/48</td>
<td>0/0</td>
</tr>
<tr>
<td>HaBac-pif2R-ph</td>
<td>48/48</td>
<td>48/48</td>
</tr>
<tr>
<td>HaBac-pif3R-ph</td>
<td>48/48</td>
<td>48/48</td>
</tr>
<tr>
<td>Mixed OBs</td>
<td>48/48</td>
<td>0/0</td>
</tr>
<tr>
<td>Co-occluded OBs</td>
<td>48/48</td>
<td>48/48</td>
</tr>
<tr>
<td>Control</td>
<td>48/48</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*Denotes no OBs observed in the dead larva. All the concentrations of OBs were 10⁶ OBs ml⁻¹ and all the experiments were performed in duplicates.

showed that mixed OBs did not rescue the deficiency of oral infection; however, co-occluded OBs rescued the deficiency of oral infection (Table 1). As the four PIFs are ODV envelope proteins, our results suggested that the four PIFs need to be co-occluded in the same ODV envelope to accomplish their function.

**PM is not the functional target of the PIFs**

To study whether PM is the functional target of the PIFs, early third-instar H. armigera were infected orally with a mixture of 1 % Calcofluor and 1 × 10⁸ OBs ml⁻¹ of different vHaBacApifs-ph OBs, and 3 × 10⁴ OBs ml⁻¹ vHaBac-egfp-ph OBs plus 1 % Calcofluor as a positive control. The results (Table 2) showed 1 % Calcofluor had increased the mortality of vHaBac-egfp-ph significantly (the mortality was increased from 52 to 95.8 %). However, 1 % Calcofluor had no impact on the mortality of the pif-deletion viruses. Therefore, destroying the PM of the insects did not help OBs of pif-deletion viruses to resume their oral infectivity. Our results suggest that the PM is not the functional target of the PIFs.

### Table 2. Feeding experiments of the recombinant viruses with or without 1 % Calcofluor on third instar H. armigera larvae

<table>
<thead>
<tr>
<th>Virus</th>
<th>Test number</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaBac-egfp-ph</td>
<td>48</td>
<td>25 + 1†</td>
</tr>
<tr>
<td>HaBac-egfp-ph + Calcofluor</td>
<td>48</td>
<td>46</td>
</tr>
<tr>
<td>HaBacAp74-ph</td>
<td>48</td>
<td>0</td>
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<tr>
<td>HaBacΔpif1-ph</td>
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<td>0</td>
</tr>
<tr>
<td>HaBacΔpif2-ph</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>HaBacΔpif3-ph</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>HaBacΔpif3-ph + Calcofluor</td>
<td>48</td>
<td>0</td>
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</tbody>
</table>

*The concentration of OBs was 3 × 10⁴ OBs ml⁻¹ for vHaBac-egfp-ph, and 10⁸ OBs ml⁻¹ for the others.
†Denotes no OBs observed in the dead larva.
et al., 2004). To confirm our results, the individual pif-deletion mutants (10^8 OBs ml^-1) were co-infected with wild-type HearNPV (10^5 OBs ml^-1), at 24, 36, 48 and 72 h p.i., the infected larvae were dissected and observed under the microscope for green florescence. None of the infected larvae showed any florescence, indicating that the deficiency of the pif-deletion mutants can not be rescued by wild-type virus. The difference between our experiments with those of Yao et al. (2004) was that the latter used expressed P74 protein instead of a P74-containing virus. Similarly to our results, Kikhno et al. (2002) also showed that the ODV of SpliNPVΔpif1 could not be rescued by the protein fraction of dissolved OBs from wild-type virus. Our result is accordant with the report of Simón et al. (2005), in which they showed that pif-deletion virus could be rescued by co-oocluded wild-type viruses. Since all the PIFs are ODV envelope proteins and HearNPV is an SNPV, we concluded that the four PIFs need to be co-oocluded in the same ODV envelope to accomplish their function. Calcofluor blocks PM formation by binding to chitin, leading to changes of midgut physiology and loss of protection of the insect midgut from virus infection (Wang & Granados, 2000). Our experiment with Calcofluor suggested that the PM of the insect is not likely to be the functional target of HearNPV PIFs.

Available evidence suggests that ODVs enter apical microvillar cells through direct membrane fusion. Three PIFs, P74, PIF1 and PIF2 have been identified to mediate ODV binding to the midgut cells (Haas-Stapleton et al., 2004; Ohkawa et al. 2005). Some viruses use multiple attachment factors and receptors in parallel or in succession (Smith & Helenius, 2004). Herpes simplex virus (HSV), for example, initially attaches to cells by binding of gC and gB to heparan sulfate proteoglycans. Membrane fusion of HSV is induced by gD after interacting with additional receptors, such as herpes virus entry mediator, nectins or integrins (Spear et al., 2000). Four glycoproteins (gD, gB, gH and gL) are required for cell entry and fusion. The fusion is a sequential process initiated by the interaction of gD with its cellular receptor, then recruitment and activation of gHL and gB for fusion (Subramanian & Geraghty, 2007). So far, it is unknown what P74, PIF1 and PIF2 bind to, and whether the bindings are in parallel or in succession. As ODV fusion was not diminished by the deletion of any one of the PIFs (Haas-Stapleton et al., 2004; Ohkawa et al., 2005), it remains unknown if the fusion is mediated by PIFs and could be complemented by each other. So far, evidence suggests that PIF3 is not responsible for the initial binding and fusion (Ohkawa et al., 2005), the role of PIF3 for oral infection needs to be further investigated. It also remains unknown whether P74, PIF1, PIF2 and PIF3 form a complex. All these are open questions for the mechanism of ODV entry. In the future, experiments would focus on the interaction among HearNPV PIFs and their host counterparts, as well as how these proteins operate together to initiate ODV entry.

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