Functional studies of per os infectivity factor 3 of Helicoverpa armigera nucleopolyhedrovirus

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PIF3 is one of the six conserved per os infectivity factors (PIFs) of baculoviruses. In this study, PIF3 of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) was analysed by infectivity bioassays using a series of recombinant viruses harbouring various PIF3 truncation/substitution mutants. The results demonstrated that the N-terminal region (L26–Y45) and C-terminal region (T160–Q199) are essential for HearNPV oral infectivity. In the C-terminal T160–Q199 region, there are three conserved cysteines (C162, C164 and C185). Our results showed that substitutions of C162 or C164, predicted to be involved in disulfide-bond formation, led to a severe decrease in HearNPV per os infectivity. Mutation of C185, predicted not to be involved in disulfide-bond formation, did not affect the per os infectivity. The data suggest that disulfide bonds are important for PIF3 conformation and function. Immunofluorescence assays showed that none of the mutations affected the subcellular localization of PIF3 to the nuclear ring zone region of infected cells. Western blot results showed that all mutants except C162G and C185G failed to incorporate PIF3 into occlusion-derived viruses, which resulted in impaired oral infectivity of the latter. The data provide insights for future study of PIF3 function.

Successful infection of the natural larval host by a baculovirus is largely dependent on the function of a number of proteins known as per os infectivity factors (PIFs), and a deletion of any one PIF abrogates infectivity. So far, the precise mechanisms of the per os infection remain largely unknown. Occlusion-derived viruses (ODVs) attach to midgut-cell receptor(s) and enter by direct fusion of the viral envelope with the host-cell membrane (Horton & Burand, 1993). To date, six genes encoding PIFs have been identified as essential for ODV infectivity, including p74 (Kuzio et al., 1989; Yao et al., 2004), pif1 (Kikhno et al., 2002), pif2 (Pijlman et al., 2003; Fang et al., 2006), pif3 (Ohkawa et al., 2005; Li et al., 2007), pif4 (Fang et al., 2009) and pif5 (odv-c56) (Harrison et al., 2010; Sparks et al., 2011). Absence of any of these will lead to severe impairment or complete loss of oral infection. All of the pif genes are conserved among all sequenced baculoviruses and nudiviruses, whilst some pif genes are also found in salivary-gland hypertrophy viruses (SGHVs) and white spot syndrome virus (Wang et al., 2011), indicating a highly conserved entry mechanism for these invertebrate viruses. A detailed understanding of the functions of the PIFs will undoubtedly be helpful to illustrate this conserved virus-entry process.

Existing data show that P74, PIF1, PIF2, PIF3 and PIF5 are all ODV-specific proteins and are located on the ODV envelope (Faulkner et al., 1997; Kikhno et al., 2002; Song et al., 2008; Harrison et al., 2010). PIF4, unlike the other PIFs, was detected in the envelope fractions of both budded virus (BV) and ODV (Fang et al., 2009). PIF1, PIF2 and PIF3 form a protein complex in ODVs, and P74 is associated loosely with the protein complex (Peng et al., 2010). P74 has been confirmed to bind ODVs with midgut columnar cell microvilli (Haas-Stapleton et al., 2004). PIF1 and PIF2 are also involved in initial binding steps; however, there is no evidence to show that PIF3 participates in this process, suggesting that PIF3 might mediate another essential event during early oral infection (Okawa et al., 2005).

Li et al. (2007) have shown that the N-terminal hydrophobic sequence of PIF3 of Autographa californiae multiple nucleopolyhedrovirus (AcMNPV) acts as a nuclear-localization signal (NLS) and is essential for establishing primary infection in midgut cells. Alignment of the amino acid sequence of the PIF3 proteins demonstrates a hydrophobic transmembrane (TM) domain at the N terminus, followed by a region containing charged amino acids, and the C terminus of PIF3 is more conserved than the N terminus.
In this study, PIF3 of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) was analysed by constructing a series of recombinant viruses harbouring various PIF3 truncation/substitution mutants and subsequent bioassays. The results demonstrated that the N-terminal region (L26–Y45) and C-terminal region (T160–Q199) of PIF3 are essential for oral infectivity. It was also found that mutations of C162 and C164, predicted to be involved in disulfide-bond formation, led to a severe decrease in HearNPV per os infectivity. Mutation of C185, which is not expected to be involved in disulfide-bond formation, did not affect the per os infectivity. The results indicate that disulfide bonds appear to be important for the functional conformation of PIF3.

**RESULTS**

**Bioinformatic analyses of PIF3**

Sequence comparisons of PIF3 homologues are summarized in Fig. 1(a). The N terminus of PIF3 proteins is less conserved than the C terminus. The N-terminal region, a hydrophobic TM domain is conserved in all PIF3 proteins, followed by positively charged amino acids in most proteins. There are two glycines (G108 and G146, indicated in the protein of HearNPV) and seven cysteine residues (C70, C103, C124, C143, C162, C164 and C185) that are highly conserved in baculoviruses, nudiviruses and SGHVs (Fig. 1a), suggesting important roles in PIF3 conformation and function. In HearNPV PIF3, there are 12 cysteines that may be implicated in disulfide-bond formation. The PredictProtein server (http://www.predictprotein.org/) revealed that 11 of the 12 cysteines are very likely to be involved in disulfide-bond formation, whereas the last cysteine residue, C185, was predicted to be unlikely to participate in disulfide-bond formation (Fig. 1b).

**Construction of recombinant HearNPVs with truncated or mutated pif3 genes**

In order to determine the functional domains of PIF3, a series of truncation or substitution mutants of PIF3 was constructed (Fig. 2). These included the N-terminal (pif3Δ26–45 and pif3Δ26–65) and C-terminal (pif3Δ180–199 and pif3Δ160–199) truncation mutants, the C162G, C164G and C185G substitution mutants, and a double mutant (C162G/C164G). Recombinant bacmids containing various pif3 truncations and cysteine mutations were constructed as described in Methods. The accuracy of all bacmids was confirmed by PCR (data not shown). Recombinant HearNPVs were produced after transfection of each recombinant bacmid DNA into HzAM1 cells. Progeny was identified by the presence of green fluorescence and polyhedra in cells infected by the recombinant HearNPVs, and the BV titres of the recombinants were similar to those of the parental virus (data not shown).

To detect the expression of PIF3, Western blot analyses were conducted on HzAM1 cells infected with the parental and recombinant viruses. Fig. 3(a) shows that anti-PIF3 antiserum can detect the expected 22.4 kDa PIF3 band in HearNPV-infected cells, but not in vHaBac5pif3-ph-infected cells or healthy cells. Truncated forms of PIF3 were also detected in cells infected with vHaBac-pif3Δ26–45-ph (~20.0 kDa), vHaBac-pif3Δ26–65-ph (~18.0 kDa), vHaBac-pif3Δ160–199-ph (~18.0 kDa) or vHaBac-pif3Δ180–199-ph (~20.0 kDa) (Fig. 3b). A band close to the molecular mass of native PIF3 was detected in cell samples infected with vHaBac-pif3ΔC162G-ph, vHaBac-pif3ΔC164G-ph, vHaBac-pif3ΔC162/164G-ph or vHaBac-pif3ΔC185G. The data showed that mutated PIF3 proteins were expressed by the recombinant viruses.

**The N-terminal (aa 26–45) and C-terminal (aa 160–199) regions are essential for the PIF3-mediated oral infection process**

It was shown previously that the N-terminal 21 aa of AcMNPV PIF3 act as an NLS and are essential for establishing primary infection in midgut cells (Li et al., 2007). In this study, we investigated further the function of the N-terminal region after the NLS (L26–Y45, L26–Q65), as well as part of the C-terminal region (T160–Q199, R180–Q199), by constructing deletion mutants. All of the truncation mutants were analysed by oral bioassays. Preliminary feeding assays were carried out using a very high concentration [10⁸ occlusion bodies (OBs) ml⁻¹] of each virus and the results showed that vHaBac-pif3Δ26–45-ph, vHaBac-pif3Δ26–65-ph and vHaBac-pif3Δ160–199-ph were non-infectious to H. armigera larvae by oral ingestion, and the oral infectivity of vHaBac-pif3Δ180–199-ph was reduced in comparison to that of the control virus vHaBac-pif3R-ph; the latter led to 100% mortality of H. armigera larvae (Table 1). Further bioassay showed that the LC₅₀ value of vHaBac-pif3Δ180–199-ph dropped by six orders of magnitude in comparison to that of the control virus (Table 2; Fig. 4). Our results demonstrated that the N-terminal region (L26–Y45, L26–Q65) and C-terminal region (T160–Q199) are essential for the function of PIF3, whilst the C-terminal region (R180–Q199) plays an important role in PIF3 function.

**Cysteine residues 162 and 164 are critical for oral infectivity**

Bioinformatic analysis showed that there are three highly conserved cysteine residues within the C-terminal T160–Q199 region, including C162, C164 and C185 (Fig. 1). Recombinant viruses with site mutations to each of the above cysteine residues were generated and tested for oral infectivity. Preliminary feeding assays showed that oral infectivity of vHaBac-pif3ΔC162G-ph was reduced, in each mutant, and obliterated totally in the double mutant vHaBac-pif3ΔC162/164G-ph. However, the oral infectivities of vHaBac-pif3ΔC164G-ph and vHaBac-pif3ΔC185G-ph seem to be comparable with that of the control virus vHaBac-pif3R-ph (Table 1).
Further, the LC50 values of vHaBac-pif3C162G-ph, vHaBac-pif3C164G-ph and vHaBac-pif3C185G-ph were determined and compared with that of the control, vHaBac-pif3R-ph. The data presented in Table 2 show that the LC50 values of vHaBac-pif3C162G-ph and vHaBac-pif3C164G-ph were respectively 7.61 × 10^9 and 3.15 × 10^7 OBs ml⁻¹, which, in comparison to vHaBac-pif3R-ph, were reduced by about 2 × 10⁶- and 1 × 10⁴-fold, respectively (Table 2). Statistical analyses indicated that the LC50 values of vHaBac-pif3C162G-ph and vHaBac-pif3C164G-ph were significantly different from that of vHaBac-pif3R-ph (P < 0.05; Table 2; Fig. 4). The LC50 value of vHaBac-pif3C185G-ph was similar to that of the control virus vHaBac-pif3R-ph, with no statistically significant difference (Table 2). The data

![Fig. 1. Computational analyses of PIF3. (a) Sequence alignment of PIF3 proteins in baculoviruses and their putative homologues in nudiviruses and SGHVs. Conserved cysteine and glycine residues are shown on a black background. GenBank accession numbers for PIF3 proteins and virus names are as follows: HearNPV, NP_075167; AcMNPV, NP_054145; Neodiprion lecontii NPV (NeloNPV), YP_025266; Culex nigripalpus NPV (CuniNPV), NP_203350; Cydia pomonella granulovirus (CpGV), NP_148819; Gryllus bimaculatus NV (GbNV), YP_001111270; Heliothis zea NV-1 (HzNV), NP_690507; Oryctes rhinoceros NV (OrNV), YP_002321418; Glossina pallidipes SGHV (GpSGHV), YP_001687024; Musca domestica SGHV (MdSGHV), YP_001883434. (b) Prediction of the disulfide-bonding state of cysteines in HearNPV PIF3 via the PredictProtein server (http://www.predictprotein.org/). AA, Amino acid sequence; DB_state, predicted disulfide-bonding state (1, disulfide-bonded; 0, no disulfide bond); DB_conf, confidence of disulfide-bonding state prediction (0, low to 9, high). Boxes indicate cysteine residues mutated in this study.](image-url)
Fig. 2. Schematic representation of recombinant HearNPVs with various truncations/mutations in the pif3 gene. The names of the truncations/mutations of pif3 genes are indicated on the left. Polh, Polyhedrin; pPolh, polyhedrin promoter; pP10, p10 promoter; pPif3, estimated promoter region of HearNPV pif3; TM, the sequences encoding the amino acids of the TM domain.

indicate that C162 and C164 are important for PIF3 function, whereas C185 is dispensable for the PIF3-mediated oral infection process. The severely decreased oral infectivity of vHaBac-pif3C162G-ph and vHaBac-pif3C164G-ph is in agreement with the bioassay result of vHaBac-pif3A160–199-ph; the latter completely lost its oral infectivity.

The truncations or mutations appear to have no impact on the subcellular localization of PIF3

The subcellular localization of the PIF3 proteins of the mutants were detected by immunofluorescence assays using samples collected at 48 h post-infection (p.i.). Similar to the PIF3 cellular distribution of the control virus vHaBac-pif3R-ph, all of the truncated or cysteine-mutated PIF3 proteins were localized at the ring zone region of the infected nucleus (Fig. 5). Therefore, the N-terminal (aa 26–45) and C-terminal (aa 160–199) deletions and C162G, C164G and C162/164G mutations did not affect the cellular trafficking and localization of HearNPV PIF3 under the experimental conditions.

Most mutants showed defective incorporation of PIF3 proteins into the recombinant ODVs

Western blot analyses were performed to test the incorporation of PIF3 into the recombinant ODVs. As Fig. 6 shows, anti-PIF3 antiserum detected the expected band for PIF3 in the ODVs of the control virus vHaBac-pif3R-ph, as well as in those of vHaBac-pif3C162G-ph, which has a similar LC50 value to the control virus. A similar but weaker band was also detected in the ODVs of vHaBac-pif3C164G-ph, indicating that C162G PIF3 was able to be incorporated into ODVs, although the bioassay showed that the oral infectivity of the virus was severely impaired. PIF3 was not detected in the ODV samples of any of the other mutants (Fig. 6). For vHaBac-pif3C162/164G-ph, vHaBac-pif3A26–45-ph, vHaBac-pif3A26–65-ph and vHaBac-pif3A160–199-ph, lack of PIF3 incorporation could explain the complete loss of their oral infectivity. For VHaBac-pif3C164G-ph and vHaBac-pif3A160–199-ph, which still possessed slight oral infectivities, a possible explanation could be that tiny amounts of PIF3 that were not detectable by Western blot were incorporated into ODVs.

DISCUSSION

PIF3 is one of the six conserved PIFs and a component of the PIF1, PIF2, PIF3 and P74 complex (Peng et al., 2010). Unlike P74, PIF1 and PIF2, which have been shown to initiate binding of ODVs to midgut epithelial cells, PIF3 appears not to be involved in the binding and fusion processes (Haas-Stapleton et al., 2004; Ohkawa et al., 2005). A study of PIF3 structure in relation to function should help in the elucidation of the function of this protein.

PIF3 is located at the envelope of HearNPV ODVs (Song et al., 2008). Current knowledge of ODV envelope protein traffic is that, after the proteins are synthesized, they are integrated into the membrane of the endoplasmic reticulum and then targeted to the inner nuclear membrane, then transit into intranuclear membrane vesicles and finally form the ODV envelope (Braunagel & Summers, 2007). The inner nuclear membrane-sorting motif (INM-SM) was first identified in the N-terminal 33 aa of AcMNPV ODV-E66 (Braunagel et al., 2004) and has been predicted in many ODV envelope proteins (Braunagel et al., 2009). Study of the INM-SM of AcMNPV ODV-E66 revealed that it contains two functional domains: a hydrophobic domain of approximately 18 aa and a positively charged amino acid within 4–8 aa from the end of the hydrophobic sequence (Braunagel et al., 2004). HearNPV PIF3 contains a putative INM-SM in that aa 3–23 form a hydrophobic TM domain, and there are three positively charged lysines located at aa 25, 31 and 32. Previously, it was shown that the N-terminal 21 aa hydrophobic domain of AcMNPV PIF3 serves as an NLS and is essential for oral infection (Li et al., 2007). In this study, we analysed the N-terminal region downstream of the hydrophobic TM domain further. Bioassay results showed that vHaBac-pif3A26–45-ph and vHaBac-pif3A26–65-ph had lost oral infectivity completely (Table 1), suggesting that both L26–Y45 and L26–Q65 are essential for PIF3 function. As L26–Y45 is a part of L26–Q65, it remains unknown whether the H46–Q65 region is
essential for PIF3 function. Immunofluorescence assays demonstrated that, although the regions were predicted to be part of the INM-SM of PIF3, the deletions did not affect the trafficking of PIF3 to the ring zone region of the infected nucleus (Fig. 5). This suggested that the charged amino acids K31 and K32 are not important for the inner nuclear membrane targeting of HearNPV PIF3. As the remaining N terminus still contains a positively charged K25, we predict that the N-terminal 25 aa may be a sufficient INM-SM. Western blot analysis showed that the deletion of the N-terminal regions resulted in defective incorporation of PIF3 proteins into ODVs, which explains the loss of oral infectivity of the recombinants.

The C-terminal region of PIF3 is more conserved than the N-terminal region. When the last 20 aa, R180–Q199, were deleted from PIF3, the LC50 value of the recombinant virus increased by six orders of magnitude compared with that of control virus (Table 2), indicating that this region, although not essential, is important for oral infection. Further truncation of T160–Q199 resulted in complete loss of oral infectivity (Table 1). Western blot analysis showed that deletion of the C-terminal region (T160–Q199 or R180–Q199) led to defective incorporation of PIF3 into ODVs (Fig. 6) and, as a consequence, impairment of oral infectivity.

Within the C-terminal region, three cysteines, C162, C164 and C185, are conserved in all sequenced PIF3 proteins and were subjected to further analysis. Individual substitution showed that the LC50 values of vHaBac-pif3C162G-ph and vHaBac-pif3C164G-ph were reduced by 2×10^6- and 1×10^4-fold, respectively, in comparison with that of vHaBac-pif3R-ph, suggesting that both C162 and C164 are critical to PIF3 function. Bioassay results showed that the LC50 value of vHaBac-pif3C185G was similar to that of vHaBac-pif3R-ph. Therefore, substitution of C185 did not affect the oral infectivity of the virus.

Among cysteine mutations, C162G and C185G did not have an obvious impact on PIF3 incorporation (Fig. 6); in contrast, the mutants with C164G and C162G/C164G could not be incorporated into ODVs correctly, suggesting a pivotal role of C164 for PIF3 incorporation. Therefore, except for C162G, the lost or reduced oral infectivity of recombinant viruses could be explained by the deficient packaging of truncated/mutated PIF3 proteins into the recombinant ODVs. For C162G, although the mutated PIF3 proteins were incorporated into ODVs, the bioassay data suggest that PIF3 function was severely damaged. Therefore, although C162 may not be involved in PIF3 incorporation, it plays an important role in PIF3 function.

### Table 1. Infectivity of recombinant viruses to third-instar H. armigera larvae

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mortality</th>
<th>Test 1</th>
<th>Test 2</th>
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<tbody>
<tr>
<td>vHaBac-pif3R-ph</td>
<td></td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>vHaBac-pif3K326–45-ph</td>
<td>2*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>vHaBac- pif3K326–45-ph</td>
<td>1*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>vHaBac-pif3A160–199-ph</td>
<td>12</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>vHaBac-pif3C162G-ph</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>vHaBac-pif3C164G-ph</td>
<td>48</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>vHaBac-pif3C162/164G-ph</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>vHaBac-pif3C185G-ph</td>
<td>48</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td></td>
<td>0</td>
<td>1*</td>
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*Denotes that no OBs were observed in the dead larvae. OB concentration, 1×10^8 OBs ml^−1.

![Western blot analyses of the expression of truncated/mutated PIF3 proteins in infected HzAM1 cells. (a) Truncated PIF3 proteins in infected cells. (b) Cysteine-mutated PIF3 proteins in infected cells. The marker was a prestained protein molecular mass marker.](https://www.microbiologyresearch.org/content/journal/jgv/93/4/378/Fig3.jpg)

![Western blot analyses of the expression of truncated/mutated PIF3 proteins in infected HzAM1 cells. (a) Truncated PIF3 proteins in infected cells. (b) Cysteine-mutated PIF3 proteins in infected cells. The marker was a prestained protein molecular mass marker.](https://www.microbiologyresearch.org/content/journal/jgv/93/4/378/Fig3.jpg)
As C162 and C164 are predicted to be involved in disulfide-bond formation (Fig. 1b), it is logical to suggest that disulfide bonds play an important role in PIF3 function. Disulfide bonds generally play an important role in the folding and stability of proteins. It is interesting to note that most PIFs contain multiple conserved cysteines. Alignments of the PIF homologues from baculoviruses, nudiviruses and SGHVs showed that P74, PIF1, PIF2 and PIF5 contain six, 12, eight and four conserved cysteines, respectively (data not shown). PIF4 is an exception, with only two conserved cysteines. It has been shown that PIF1, PIF2, PIF3 and P74 form a protein complex in ODVs (Peng et al., 2010). The putative disulfide bonds may stabilize PIFs and/or the PIF complex to protect them from digestive enzymes in the larval midgut. Once in the alkaline, reducing environment of the midgut, they may be reversibly reduced and cause conformational change to the PIFs/PIF complex to give them functional structure. Future investigation of the structures of the PIFs will help us to understand mechanisms of oral infection.

**Table 2. Median lethal concentrations (LC$_{50}$) and regression slopes of the concentration–mortality relationship of recombinant viruses**

LC$_{50}$ values were calculated by probit analysis with PASW Statistics (version 18.0) and are reported with 95% confidence limits (CL). For each set of treatments, values with different footnote symbols (*, †) are significantly different at $P<0.05$. For heterogeneity, goodness of fit to the linear regression line was checked by $\chi^2$ test. If $P<0.05$, a heterogeneity factor was used to calculate the 95% CLs; otherwise, no heterogeneity factor was used. Relative potency ratios were calculated by dividing the LC$_{50}$ value of vHaBac-pif3R-ph by those of the recombinant viruses. Significance of difference was based on whether the 95% CL of the potency ratio included the value 1.0 (Robertson & Preisler, 1992).

<table>
<thead>
<tr>
<th>Virus</th>
<th>LC$_{50}$ (95% CL) (PIBs ml$^{-1}$)</th>
<th>Slope ± SEM</th>
<th>Heterogeneity</th>
<th>Potency ratio (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vHaBac-pif3R-ph</td>
<td>3.22 (2.55–3.97) $\times 10^3$*</td>
<td>1.232 ± 0.079</td>
<td>16.812/28</td>
<td>0.952 –</td>
</tr>
<tr>
<td>vHaBac-pif3A180–199-ph</td>
<td>3.27 (2.00–7.37) $\times 10^3$†</td>
<td>1.234 ± 0.121</td>
<td>12.038/13</td>
<td>0.525 $9.51 \times 10^{-7}$ (4.51 $\times 10^{-8}$, 1.12 $\times 10^{-5}$)</td>
</tr>
<tr>
<td>vHaBac-pif3C162G-ph</td>
<td>7.61 (3.15–3.59) $\times 10^3$†</td>
<td>0.787 ± 0.123</td>
<td>3.904/10</td>
<td>0.952 $9.92 \times 10^{-7}$ (3.62 $\times 10^{-8}$, 1.37 $\times 10^{-5}$)</td>
</tr>
<tr>
<td>vHaBac-pif3C164G-ph</td>
<td>3.15 (2.36–4.23) $\times 10^3$‡</td>
<td>0.971 ± 0.061</td>
<td>15.849/13</td>
<td>0.257 $9.35 \times 10^{-7}$ (1.68 $\times 10^{-5}$, 3.98 $\times 10^{-4}$)</td>
</tr>
<tr>
<td>vHaBac-pif3C185G-ph</td>
<td>3.50 (2.86–4.22) $\times 10^3$‡</td>
<td>1.407 ± 0.092</td>
<td>8.838/28</td>
<td>0.990 0.99 (0.76, 1.31)</td>
</tr>
</tbody>
</table>

Fig. 4. Relationship between logarithms of different concentrations of recombinant virus and mortality. Forty-eight third-instar *H. armigera* larvae were infected with different concentrations of each recombinant virus by the droplet method. Larval death was monitored daily and the final mortality for each virus concentration was calculated. Each data point represents the mean value from three separate infections; error bars indicate SD.
METHODS

Insect cells, insects and virus. The Helicoverpa zea cell line HzAM1, originally isolated from H. zea pupal ovarian tissue (McIntosh & Ignoffo, 1983), was maintained at 28°C in Grace’s medium (Gibco-BRL) supplemented with 10% (v/v) FBS. Anti-PIF3 polyclonal antiserum was produced in our laboratory as reported previously (Song et al., 2008). HearNPV strain G4, the genome of which has been sequenced (GenBank accession no. AF271059; Chen et al., 2001), was used as the parental virus and propagated in H. armigera larvae reared on an artificial diet at 27°C. HaBacHZ8 is an infectious bacmid of HearNPV constructed previously in our laboratory (Wang et al., 2003). The pif3-repaired control virus vHaBac-pif3R-ph was constructed previously in our laboratory (Song et al., 2008).

Computational analysis. Disulfide-bonding state prediction of HearNPV PIF3 was performed using the PredictProtein server (http://www.predictprotein.org/). Alignment of PIF3 homologues was performed by using the CLUSTAL W program in MegAlign (DNASTAR) and displayed by using the GeneDoc program.

Construction and identification of HearNPV bacmids containing truncated or mutated pif3. The pif3 ORF, including its putative promoter region, was amplified by PCR from HearNPV G4 genome DNA using primers pif3F (XhoI site) and pif3R (KpnI site). To generate two N-terminal truncation mutants, pif3Δ26–45 and pif3Δ26–65, the coding sequence of the hydrophobic TM domain of pif3 (pif31–25) was firstly amplified by PCR with primers pif3F and pif3TM25R (SphI site) and inserted into the XhoI and SphI sites of a transfer vector pFB-DUAL-ph (Song et al., 2008). pif3Δ36–199 and pif3Δ36–199 were amplified by pif3Δ36–45F (SphI) and pif3R, and pif3Δ36–45F (SphI) and pif3R. These two fragments were inserted into the transfer vector, respectively generating pFB-DUAL-pif3Δ26–45-ph and

Fig. 5. Immunofluorescence microscopy to visualize the subcellular localization of PIF3 mutants. HzAM1 cells were infected with each recombinant virus. At 48 h p.i., the cells were fixed and probed with the first antibody (anti-PIF3 antiserum), followed by incubation with the secondary antibody (rhodamine-conjugated anti-rabbit IgG). Cells were then stained with Hoechst and observed under a confocal microscope. Bars, 10 μm.

Fig. 6. Western blot analysis of PIF3 proteins in recombinant ODVs. ODVs were purified from OBs of each recombinant virus and Western blot analyses were performed using antibodies against PIF3 and VP39. The marker was a prestained protein molecular mass marker.
pFB-DUAL-pif3Δ26-65-ph. Two C-terminal truncation mutants, pif3Δ180-199 and pif3Δ160-199, were amplified with pif3F and pif3Δ180-199R (KpnI site) and pif3F and pif3Δ160-199R (KpnI site), respectively. The cysteine-substitution mutants pif3Δ162G, pif3Δ162G-ph and pif3Δ185G were constructed by using overlap-extension PCR as described previously, with mutagenic primers (Ho et al., 1989). The mutated pif3 genes were inserted into the Xhol and KpnI sites of pFB-DUAL-ph, generating transfer vectors pFB-DUAL-pif3Δ180-199-ph, pFB-DUAL-pif3Δ160-199-ph, pFB-DUAL-pif3Δ162G-ph, pFB-DUAL-pif3Δ162G-ph and pFB-DUAL-pif3Δ185G-ph. All primers are listed in Supplementary Table S1, available in JGV Online. All genes in the final transfer vectors were amplified with Pyrobest DNA polymerase (TaKaRa) and cloned into the pGEM-T Easy vector (Promega) for sequencing.

After confirming accuracy of the constructs by sequencing, the above transfer vectors were transposed to the pif3-deletion bacmid (HaBac-Ampf3), which contains an egfp gene at the pif3 location (Song et al., 2008), according to the Bac-to-Bac manual (Invitrogen), generating recombinant bacmids HaBac-pif3Δ26-45-ph, HaBac-pif3Δ26-65-ph, HaBac-pif3Δ180-199-ph, HaBac-pif3Δ160-199-ph, HaBac-pif3Δ162G-ph, HaBac-pif3Δ162G-ph, HaBac-pif3Δ185G-ph and HaBac-pif3Δ185G-ph. The recombinant bacmids were selected by gentamicin resistance and identified by PCR using M13 primers.

Transfection and infection assays. HA2M1 cells (5 × 10^5) were seeded into 35 mm tissue-culture dishes. 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, centrifuged briefly at 1000 g for 5 min to remove cell debris and then used to infect fresh HA2M1 cells. The titres of the recombinant BVs were determined by an end-point dilution assay (King & Possee, 1992).

Bioassay. About 10 μl BVs (10^6 TCID50 units ml^-1) per larva was injected into the haemocoel of late third-instar H. armigera larvae to generate OBs for bioassays. The OBs were harvested and purified from infected larvae as described by Sun et al. (1998). To test whether the recombinant viruses had lost their oral infectivity, experiments were carried out by droplet feeding of 10^5 OBs ml^-1 of each virus to early third-instar H. armigera larvae (Hughes et al., 1986). 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. The experiments were done in duplicate.

For the recombinant viruses that kept oral infectivity (vHaBac-pif3Δ180-199-ph, vHaBac-pif3Δ162G-ph, vHaBac-pif3Δ162G-ph and vHaBac-pif3Δ185G-ph), bioassays were conducted by exposing larvae to different virus concentrations: 1 × 10^3, 3 × 10^3, 1 × 10^4, 3 × 10^4, 1 × 10^5, 3 × 10^5, 1 × 10^6, 3 × 10^6 and 1 × 10^7 OBs ml^-1 (an additional concentration of 3 × 10^6 OBs ml^-1 was used for vHaBac-pif3Δ180-199-ph). vHaBac-pif3Δ185G-ph was used as the control. Forty-eight larvae were used for each concentration of each virus, and the experiments were repeated twice. Probit analysis was used to calculate LC50 values, 95% confidence limits and regression slopes. Data from three replicates were pooled to calculate the final LC50 values, as long as there was no significant difference between the LC50 values and regression slopes of the three replicates. LC50 values between each pair of recombinant viruses were compared by the lethal dose ratio method of Robertson & Preisler (1992).

Western blot analyses of recombinant viruses. Western blot analyses were used to identify the expression of PIF3 mutants in the recombinant BV-infected cells, as well as the incorporation of PIF3 into the recombinant ODVs. ODVs were released from OBs of recombinant viruses by DAS buffer (0.1 M Na2CO3, 0.5 M NaCl, 0.01 M EDTA, pH 10.9), followed by centrifugation at 18,000 g for 30 min at 4 °C. The sedimented ODVs and HA2M1 cells infected with each recombinant virus were used for Western blot analyses. Protein samples were separated by SDS-PAGE (12% polyacrylamide) and transferred onto a Hybond-N nitrocellulose membrane (Amersham Biosciences). A polyclonal anti-HearNPV PIF3 antisera (Song et al., 2008) was used as the primary antibody, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Jackson ImmunoResearch) was used as the secondary antibody. The signal was detected by using a BCIP/NBT kit (Sino-America).

Immunofluorescence confocal microscopy. HA2M1 cells were infected with individual recombinant viruses at an m.o.i. of 3. Cell samples were collected at 48 h p.i. The cells were washed twice in PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized with 0.15% Triton X-100 in PBS for 10 min. After being washed a further three times with PBS, cells were blocked for 30 min in blocking buffer (5% BSA in PBS) and incubated with anti-PIF3 antisera (1:500 dilution) overnight at 4 °C. Then, the cells were rinsed three times in PBS for 10 min each, followed by incubation with rhodamine-conjugated anti-rabbit IgG for 2 h at room temperature. Finally, cells were stained with Hoechst 33258 (Beyotime) and examined under a laser-scanning confocal microscope (PerkinElmer UltraView VOX) for fluorescence.

ACKNOWLEDGEMENTS

The work is supported by the 973 project (2009CB118903), an NSFC grant (31130058), a PSA project from the Ministry of Science and Technology of China and the Royal Academy of Sciences of the Netherlands (2008DFB30220) and a Chinese Academy of Sciences project (KSCX2-EW-G-16). The authors would like to thank Dr Xiulan Sun for statistical analysis, Ms Yanfang Zhang for cell culture and Dr Basil M. Arif for scientific editing of the manuscript.

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