Protein–protein interactions of the baculovirus *per os* infectivity factors (PIFs) in the PIF complex

Qin Zheng, Yunwang Shen, Xiangshuo Kon, Jianjia Zhang, Min Feng and Xiaofeng Wu*

**Abstract**

After ingestion of occlusion bodies, the occlusion-derived viruses (ODVs) of the baculoviruses establish the first round of infection within the larval host midgut cells. Several ODV envelope proteins, called *per os* infectivity factors (PIFs), have been shown to be essential for oral infection. Eight PIFs have been identified to date, including P74, PIFs 1–6 and Ac110. At least six PIFs, P74, PIFs 1–4 and PIF6, together with three other ODV-specific proteins, Ac5, P95 (Ac83) and Ac108, have been reported to form a complex on the ODV surface. In this study, in order to understand the interactions of these PIFs, the direct protein–protein interactions of the nine components of the *Autographa californica* multiple nucleopolyhedrovirus PIF complex were investigated using yeast two-hybrid (Y2H) screening combined with bimolecular fluorescence complementation (BiFC) assay. Six direct interactions, comprising PIF1–PIF2, PIF1–PIF3, PIF1–PIF4, PIF1–P95, PIF2–PIF3 and PIF3–PIF4, were identified in the Y2H analysis, and these results were further verified by BiFC. For P74, PIF6, Ac5 and Ac108, no direct interaction was identified. P95 (Ac83) was identified to interact with PIF1, and further Y2H analysis of the truncation and deletion mutants showed that the predicted P95 chitin-binding domain and amino acids 100–200 of PIF1 were responsible for P95 interaction with PIF1. Furthermore, a summary of the protein–protein interactions of PIFs reported so far, comprising 10 reciprocal interactions and two self-interactions, is presented, which will facilitate our understanding of the characteristics of the PIF complex.

**INTRODUCTION**

Baculoviruses are a group of insect-specific viruses with enveloped nucleocapsids containing double-stranded DNA [1]. The family *Baculoviridae* comprises the four genera *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* [2]. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) has been well characterized as the model of baculoviruses. Two different virus phenotypes, called budded virus (BV) and occlusion-derived virus (ODV), are produced during the baculovirus infection cycle. Although BV and ODV contain the same genome, they differ in the composition of their viral envelope. ODV obtains its envelope from intranuclear membrane vesicles [3], while the BV envelope is acquired as it buds through the modified plasma membrane [4]. Moreover, the two phenotypes play different roles in the virus cycle [4, 5]. BVs, which bud out of the infected cells, mediate the cell-to-cell transmission of the virus, while ODVs ensure the spread of the virus between hosts [6]. ODVs are occluded within protein crystals called occlusion bodies (OBs), which are responsible for the protection of the virus in the environment [7]. Once OBs that contaminate the diet of susceptible larvae are ingested, they are dissolved by the alkaline environment (pH 8.7–11) of the midgut lumen and release the ODVs [5]. The ODVs then pass through the peritrophic membrane (PM) and infect the midgut cells [8–11], thereby initiating the infection.

Eight ODV envelope proteins, P74 (PIF0) [12, 13], Ac119 (PIF1) [14], Ac22 (PIF2) [15], Ac115 (PIF3) [16], Ac96 (PIF4) [17], Ac148 (PIF5) [18], Ac68 (PIF6) [19] and Ac110 [20], have been designated as *per os* infectivity factors (PIFs) due to their essential role in oral infectivity. Notably, oral infectivity is prevented when any one of these PIF genes is deleted, whereas cell-to-cell infection through BVs is maintained.

PIFs are implicated in ODV binding and fusion with midgut cell membranes, but their precise functions are still unknown. While Ac5 has been shown to form a complex on the ODV surface. In this study, in order to understand the interactions of these PIFs, the direct protein–protein interactions of the nine components of the *Autographa californica* multiple nucleopolyhedrovirus PIF complex were investigated using yeast two-hybrid (Y2H) screening combined with bimolecular fluorescence complementation (BiFC) assay. Six direct interactions, comprising PIF1–PIF2, PIF1–PIF3, PIF1–PIF4, PIF1–P95, PIF2–PIF3 and PIF3–PIF4, were identified in the Y2H analysis, and these results were further verified by BiFC. For P74, PIF6, Ac5 and Ac108, no direct interaction was identified. P95 (Ac83) was identified to interact with PIF1, and further Y2H analysis of the truncation and deletion mutants showed that the predicted P95 chitin-binding domain and amino acids 100–200 of PIF1 were responsible for P95 interaction with PIF1. Furthermore, a summary of the protein–protein interactions of PIFs reported so far, comprising 10 reciprocal interactions and two self-interactions, is presented, which will facilitate our understanding of the characteristics of the PIF complex.
unknown. For instance, P74, the first PIF identified [12], was reported to be essential for ODV binding to the host midgut epithelial cells [21, 22] and it interacts with a host protein with a molecular mass of approximately 35 kDa in the brush border membrane vesicles [23, 24]. PIF1 and PIF2 have also been identified as ODV envelope attachment proteins in *in vivo* fluorescence dequenching assays [16], but live imaging of ODV entry into midgut cells captured using confocal fluorescence microscopy revealed that they were more likely to function after ODV binding to midgut cells [22]. Interestingly, communoprecipitation (CoIP) combined with proteomic analysis indicated that nine proteins, P74, PIF1, PIF2, PIF3, PIF4, Ac5, PIF6, P95 and Ac108, formed a complex with a molecular mass of approximately 480 kDa [25]. This PIF complex has a highly stable core complex (170 kDa) consisting of PIF1, PIF2, PIF3 and PIF4 on the envelope of ODV, with which the other proteins appear to interact loosely [26].

Monitoring protein–protein interactions and macromolecular complex formation in living cells is extremely useful for understanding the dynamics and mechanism of oral infectivity. Currently, several methods are available for studying protein interactions in living cells, such as the yeast two-hybrid (Y2H) system, fluorescence resonance energy transfer (FRET) and the bimolecular fluorescence complementation (BiFC) assay [27]. In the Y2H system, two proteins are fused with the DNA-binding domain (BD) and activation domain (AD), respectively, which are brought into proximity to activate transcription of four independent reporter genes as the interaction is present between these two proteins. The BiFC assay is a tool for evaluating protein–protein interactions in living cells. The BiFC assay relies on the reconstruction of a fluorescent protein from two non-fluorescent fragments when they are brought into close proximity. Y2H and BiFC have been proven to be versatile and simple non-invasive technologies for studying protein interactions in living cells. Compared with other technologies *in vitro*, such as CoIP and pull-down assays, the Y2H and BiFC methods are more suitable for the detection of specific interactions and can be performed under conditions that are closer to the natural states of proteins.

In this study, the genes encoding these nine proteins, i.e. P74, PIF1, PIF2, PIF3, PIF4, PIF6, Ac5, Ac108 and P95 (Ac83), were expressed for interaction analysis. Six interactions of proteins that participate in the PIF complex were identified. Furthermore, we showed that the predicted P95 chitin binding domain (CBD) was crucial for P95 association with the PIF complex. The results from this study provide further evidence as to how the PIF complex is organized and how its components interact.

**RESULTS**

**Y2H analyses of protein–protein interactions in the PIF complex**

Nine ODV envelope proteins, most of which have been proven to be PIFs, except Ac5 and Ac108, were identified as components of the PIF complex by immunoprecipitations combined with proteomic analysis [25]. They are encoded by highly conserved genes that are present in nearly all baculovirus genomes, with the exception of Ac5, which is only present in alphabaculoviruses. To analyse the protein–protein interactions, coding sequences of these proteins were amplified from the AcMNPV genome. As shown in Fig. 1, with the exception of Ac5, transmembrane (TM) domains were found in eight of these proteins by prediction using the TMHMM server v. 2.0 (www.cbs.dtu.dk/services/TMHMM/). For the Y2H system, since TM domains may prevent access of proteins to the yeast nucleus where reporter genes are activated, ORFs lacking the TM domain were amplified and characterized in this study. Specifically, for proteins containing only one predicted TM domain, i.e. PIF1, PIF2, PIF3, PIF4, PIF6, Ac108 and P95, truncated ORFs without a TM domain were constructed. For P74, the TM domains in the hydrophobic C-termini were removed by truncation. Finally, all 9 fragments were cloned into the pGBK7T and pGADT7 vectors, resulting in 18 constructs.

The combination of the pGBK7T and pGADT7 constructs used and the results of the interactions are shown in Table 1. In general, a total of six interactions were identified. The interactions of PIF1–PIF2, PIF1–PIF3, PIF1–PIF4, PIF2–PIF3 and PIF3–PIF4 showed reciprocal interactions, whereas the interaction of PIF1–P95 could only be detected in one direction. Among these interactions, PIF1–PIF3, PIF2–PIF3 and PIF1–P95 were strong interactions, while the others appeared to be less robust and resulted in intermediate growth. Remarkably, most of the interactions were identified among PIF1, PIF2, PIF3 and PIF4, corresponding to previous results that indicated that they formed a stable complex [25]. According to our results, we assumed that this stable complex is mainly assembled through the strong interactions of PIF1 and PIF2, PIF1 and PIF3, PIF1 and PIF4, PIF2 and PIF3, and PIF3 and PIF4. Direct interactions were also identified among other components of the PIF

![Fig. 1. Schematic of the predicted TM domains located on the ODV envelope surface. The orientation of the proteins in the PIF complex was predicted by the TMHMM server v. 2.0 (www.cbs.dtu.dk/services/TMHMM/). The N-termini or C-termini of the proteins are indicated as ‘N’ or ‘C’. The ORFs corresponding to the orientation outside the membrane were amplified for Y2H analysis.](image-url)
complex. For example, the results showed that P95 could interact directly with PIF1, while no interaction was found for P74, Ac5, Ac108 or PIF6. The self-interactions of these nine proteins were also tested, but no self-association was identified in the Y2H assay.

**BiFC verification of the protein–protein interactions in the PIF complex**

Given that removal of the TM domain might influence the secondary and tertiary protein structure and result in a negative Y2H interaction, BiFC studies were conducted to verify the results from Y2H. The N-terminal and C-terminal fragments of red fluorescent protein (RFP) were fused to the N- and C-termini of ORFs of the P74, PIF1, PIF2, PIF3, PIF4, PIF6, Ac5, Ac108 and P95, generating a set of four fusion proteins for each virus-encoded protein (Fig. 2). Subsequently, combinations of two pairs of nRFP and cRFP terminal fusions of these nine proteins were co-transfected into Sf9 cells, and the reconstituted RFP signal was monitored by fluorescence microscopy. To exclude the possibility that low transfection efficiencies resulted in false negatives, all of the Sf9 cells without RFP signal were further verified for the expression of the constructs by Western blot analysis. Because gp64 has a propensity for trimerization, it was used in this investigation as a positive control for interacting proteins. Consistent with the results of Y2H analysis, no self-association of individual proteins was identified in the BiFC assay.

**The predicted P95 CBD and amino acids (aa) 100–200 of PIF1 are responsible for P95 interaction with PIF1**

P95 (ac83) was recently identified as being involved in per os infectivity, but no BVs were produced by a virus with Ac83 knocked out when transfected into Sf9 cells, although viral DNA replication was unaffected [29]. Fig. 4 shows the location of a typical type II CBD (aa 224–282) in P95, indicating that it may play a role in ODV binding to the PM, which is a chitin-rich structure [29]. P95 is a core gene, and all predicted P95 homologues contain a putative C2HC-type zinc finger (aa 148–197) with the consensus sequence C-X₅-C-X₉-C (where X is any amino acid) and a TM domain at the N-terminus (aa 5–27). In addition, a proline-rich region (aa 671–698) is present in P95 homologues [29]. We found that P95 was associated with PIF1 by Y2H screening (Table 1). To determine which domains of P95 and PIF1 are responsible for the interaction, a series of truncations and deletion mutants were created (Fig. 4). It has previously been shown that the PIF complex seems to have a structural order with a highly stable complex consisting of PIF1, PIF2, PIF3 and PIF4, which can be further separated into two formats, with PIF1, PIF2 and PIF3 forming a stable core and PIF4 interacting strongly with this stable core. Hence, if the pif4 gene is deleted, PIF1, PIF2 and PIF3 can still form a stable subcomplex, but deletion of pif1, pif2 or pif3 leads to complete disruption of the complex [25]. From these results, it was clear that the pairwise interactions among PIF1, PIF2 and PIF3 contributed to the formation of the subcomplex, while PIF4 associated closely with this subcomplex by interacting with PIF1 and PIF3. No red fluorescence was detected in the other combinations, such as combinations of P74, Ac5, Ac108 and PIF6, indicating that no interaction or very weak interactions existed among these proteins. Consistent with the results of Y2H analysis, no self-association of individual proteins was identified in the BiFC assay.
Y2H assays. The results presented in Table 3 showed that fragments of P95 containing aa 28–300, aa 28–500, aa 28–700, aa 141–839 and aa 201–839 could interact with PIF1, but not fragments containing aa 28–150, aa 28–200, aa 301–839, aa 501–839 and aa 701–839. Among these interactions, PIF1–P95 aa 28–700 and PIF1–P95 aa 141–839 were strong interactions, and PIF1–P95 aa 28–500 and PIF1–P95 aa 201–839 were intermediate interactions, while aa 28–300 generated a weak interaction. These results indicated that truncation of aa 200–300 of P95, where the predicted CBD (aa 224–282) was located, abrogated its ability to interact with PIF1, suggesting that the CBD is essential for interaction. The CBD was then deleted, and the interaction between the mutant and PIF1 was tested. Consistent with the truncation assay, deletion of CBD could efficiently prevent interaction between P95 and PIF1 (Table 3), highlighting the importance of this region for interaction. For PIF1, fragments containing aa 25–200, aa 25–300, aa 25–400 and aa 101–527 were observed to interact with P95, but not the other truncations (Table 3). The interactions of P95–PIF1 aa 25–200 and P95–PIF1 aa 101–527 were intermediate, while P95–PIF1 aa 25–200 and P95–PIF1 aa 25–300 generated weak interactions (Table 3). This indicated that the aa 100–200 of PIF1 seemed to be essential for interaction, and this region was deleted for further analysis. As shown in Table 3, the deletion of aa 100–200 of PIF1 abrogated its interaction with P95.

Table 2. Summary of the interactions of the components in the PIF complex, as detected by BiFC assay

<table>
<thead>
<tr>
<th>AcMNPV ORF</th>
<th>PIZn–nRFP/PIZc–nRFP</th>
<th>Red fluorescence of cells co-expressing constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIZn–cRFP/PIZc–cRFP</td>
<td>P95</td>
</tr>
<tr>
<td>Ac138</td>
<td>P74</td>
<td>–</td>
</tr>
<tr>
<td>Ac119</td>
<td>PIF1</td>
<td>+</td>
</tr>
<tr>
<td>Ac22</td>
<td>PIF2</td>
<td>–</td>
</tr>
<tr>
<td>Ac115</td>
<td>PIF3</td>
<td>–</td>
</tr>
<tr>
<td>Ac96</td>
<td>PIF4</td>
<td>–</td>
</tr>
<tr>
<td>Ac5</td>
<td>Ac5</td>
<td>–</td>
</tr>
<tr>
<td>Ac88</td>
<td>PIF6</td>
<td>–</td>
</tr>
<tr>
<td>Ac108</td>
<td>Ac108</td>
<td>–</td>
</tr>
<tr>
<td>Ac83</td>
<td>P95</td>
<td>–</td>
</tr>
</tbody>
</table>

Red fluorescence was detected by co-expression of binary constructs of N- and C-terminal RFP fusion proteins (e.g. P74–nRFP and PIF4–cRFP) in all possible pairwise combinations in Sf9 cells. False positives were excluded by co-expression of either an N- or a C-terminal fusion of the target protein with a counterpart of a non-fused fragment of RFP in all possible combinations. The presence (+) and absence (–) of red fluorescence are indicated.
ability to interact with P95. Therefore, these data suggested that aa 100–200 of PIF1 are responsible for the interaction between PIF1 and P95.

**DISCUSSION**

In this paper Y2H and BiFC assays were used in combination to study the protein–protein interactions of the PIF complex that is essential for initiating primary infections of baculoviruses. Six direct interactions, including PIF1–PIF2, PIF1–PIF3, PIF1–PIF4, PIF2–PIF3, PIF3–PIF4 and PIF1–P95, were identified. For P74, PIF6, Ac5 and Ac108, no direct interaction was found, although they have been shown to be associated with the PIF complex [25]. Interactions among the components of ODV of *Helicoverpa armigera* nucleopolyhedrovirus were previously detected by Peng et al. [30], and 9 self-interactions and 13 reciprocal interactions for a total of 22
interactions were identified among 39 ODV structural proteins. Furthermore, the 68 interactions with 45 viral proteins and 5 host proteins reported in baculoviruses were summarized in that study [30]. Remarkably, five PIFs, comprising P74, PIF1, PIF2, PIF3 and ODV-E56 (PIF5), and seven PIF–PIF interactions, including five reciprocal interactions, PIF1–ODV-E56, P74–ODV-E56, PIF2–PIF3, PIF2–ODV-E56 and PIF3–ODV-E56, and two self-interactions, ODV-E56–ODV-E56 and PIF3–PIF3, were reported [30]. Specifically, HA98 (PIF3) was reported to have a self-interaction [30], but this was not the case for its homologue Ac115 (PIF3) in our study. Sequence alignment of HA98 and Ac115 showed that 37 % of the amino acids are identical between these two proteins (Fig. S2). It needs to be further verified whether the low homology led to the different interaction results for HA98 and Ac115. On the other hand, it is also possible that Ac115 has a self-interaction, but this could not be identified in our study. Based on our results and those of Peng et al. [30], a model revealing how PIFs interact with each other is proposed (Fig. 5). Ten reciprocal interactions and two self-interactions are presented. The 10 binary interactions comprise PIF1–PIF2, PIF1–PIF3, PIF1–PIF4, PIF1–P95, PIF2–PIF3, PIF3–PIF4, ODV-E56–PIF1, ODV-E56–PIF2, ODV-E56–PIF3 and ODV-E56–P74, while the two self-interactions comprise PIF3–PIF3 and ODV-E56–ODV-E56. Other PIFs might also be involved in the formation of the PIF complex, but how they are involved with each other will require additional studies.

Clearly, ODV entry into midgut epithelial cells requires multiple steps. Firstly, the OBs ingested by the larva must be dissolved, and the virions released into the midgut lumen. The second step is the passing through the larval PM to allow the virions to reach the brush-border cells of the midgut. The last step is the binding and entry of the virions into the midgut epithelium cells, followed by migration of the nucleocapsids to the nuclei, liberation of the genome, replication and the production of progeny viruses, which will be released to infect other cells [14]. PIFs appear to be the ODV envelope proteins that facilitate the binding and entry of virions. Previous studies have focused on the role of single PIF proteins in this process [21, 22, 31], while neglecting their interaction with other proteins. Our studies and those of Peng et al. [25, 26] indicated that virion entry is a complex process involving a number of viral proteins. The organization of the PIF complex on the ODV surface suggests that several PIFs may function in combination, but little is known about this complex. Furthermore, previous studies have shown that the complex formed by the PIF1, PIF2, PIF3 and PIF4 proteins is so stable that it can withstand 2 % SDS/5 % β-mercaptoethanol with heating at 50 °C for 5 min [26]. We speculate that the formation of this stable complex is important for efficient viral entry, similar to aggregates of

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**Fig. 4.** Schematic of the P95 and PIF1 mutants. C-terminal and N-terminal truncations, as well as internal deletions were performed for P95 (a) and PIF1 (b). Dotted lines represent deletions, and solid lines represent fragments retained. PR, proline-rich region.
gp120 and gp41 in human immunodeficiency virus type 1 (HIV-1). For HIV-1, envelope proteins clustering into a single focus may facilitate the alteration of the virus surface in preparation for productive entry [32]. Similarly, baculovirus gp64 also appears to form a postfusion trimer, with the hydrophobic patch at the tip acting as both a receptor binding site and a fusion peptide [28].

As an ODV attachment protein, P74 binds to a specific receptor on primary target cells within the midgut [23, 24]. Moreover, the inability of P74-null occlusion bodies to show oral infectivity can be rescued by a mixed infection with the purified P74 protein expressed in Sf9 cells [24]. Previous studies have shown that P74 is also associated with the PIF complex [30]. However, we did not detect any interaction of P74 with other proteins in the complex. This led us to speculate that it might bind at the interacting surface of two other proteins that could not be detected in our experiment. Interestingly, P74 was also reported to be efficiently cleaved by an OB endogenous alkaline protease and a host trypsin during ODV release, and this proteolytic cleavage could be part of a mechanism for activation of P74 [33]. We suggest that these two sequential cleavage events may alter the spatial structure of P74, thus increasing its affinity to other PIFs, but this needs to be verified.

Interference with the chitin component of the PM often leads to a defect in the integrity and normal function of the PM [34]. Disruption of the PM is required for virions to pass from the midgut lumen to the midgut epithelial cells. A previous study showed that damage to the PM utilizing calcofluor could not rescue the defects of the oral infectivity of the p74, pif1, pif2 and pif3-deletion viruses, indicating that the PM is not likely to be the functional target of these PIFs [31].

Nevertheless, gp37 of Spodoptera litura multicapsid nucleopolyhedrovirus has a predicted CBD and was found to have an affinity for chitin [35]. In our analysis of the protein–protein interactions of the PIF complex, we found that P95 could interact with PIF1. We further confirmed that the predicted CBD of P95 and amino acids 100–200 of PIF1 are required for the interaction between PIF1 and P95. Considering that PIF1 was the only component that could interact with P95 in the

### Table 3. Interactions of mutants of P95 and PIF1 in yeast cells

<table>
<thead>
<tr>
<th>pGBK T7</th>
<th>pGAD T7</th>
<th>Growth of colonies on:</th>
<th>pGBK T7</th>
<th>pGAD T7</th>
<th>Growth of colonies on:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DDO</td>
<td>QDO</td>
<td></td>
<td>DDO</td>
</tr>
<tr>
<td>PIF1</td>
<td>Empty*</td>
<td>+++</td>
<td>–</td>
<td>Empty</td>
<td>Empty†</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 28–150</td>
<td>+++</td>
<td>–</td>
<td>P53</td>
<td>T‡</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 28–200</td>
<td>+++</td>
<td>–</td>
<td>Empty*</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 28–300</td>
<td>+++</td>
<td>+</td>
<td>aa 25–100</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 28–400</td>
<td>+++</td>
<td>+</td>
<td>aa 25–200</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 28–300</td>
<td>+++</td>
<td>+</td>
<td>aa 25–300</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 141–389</td>
<td>+++</td>
<td>++</td>
<td>aa 25–400</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 201–389</td>
<td>+++</td>
<td>++</td>
<td>aa 101–527</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 301–389</td>
<td>+++</td>
<td>–</td>
<td>aa 201–527</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 501–389</td>
<td>+++</td>
<td>–</td>
<td>aa 301–527</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>aa 701–389</td>
<td>+++</td>
<td>–</td>
<td>aa 401–527</td>
<td>P95</td>
</tr>
<tr>
<td>PIF1</td>
<td>ΔCBD</td>
<td>+++</td>
<td>–</td>
<td>Δ100–200</td>
<td>P95</td>
</tr>
</tbody>
</table>

DDO, Leu- and Trp-deficient medium; QDO, Ade-, Leu-, Trp- and His-deficient medium.

–, No growth; +, weakly positive growth; ++, intermediate growth; +++ , strong growth.

*Autotransactivation.
†Negative control.
‡T indicates SV40 large T antigen and along with p53 is a positive control.

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Fig. 5. Summary of the protein–protein interactions in the PIF complex. The proteins were classified into two groups: PIFs (purple circles) and non-PIFs (blue circles). The interactions identified in this study are indicated by solid lines, and the interactions reported by Peng et al. [30] are indicated by dashed lines.
PIF complex, we suggest that the deletion of the CBD might dissociate P95 from the PIF complex and reduce the oral infectivity of the virus. This would be consistent with the previous results showing that deletion of the P95 CBD severely attenuated the oral infectivity of AcMNPV [29].

In conclusion, the cumulative data from this and prior studies indicate that some PIFs form a complex on the ODV envelope surface through interaction with each other. Our study elucidated the character of the protein interactions in this complex as detected by both Y2H and BiFC assays. Further studies employing biochemical and molecular biological approaches are needed to define the molecular mechanisms of this multimeric complex in mediating baculovirus entry into its host cells.

**METHODS**

**Cell culture**

*Spodoptera frugiperda* Sf9 cells were grown in TC100-insect medium (Applichem) supplemented with 10 % (v/v) fFBS (Sigma) at 27 °C.

**Y2H assays among the components of the PIF complex**

As shown in Fig. 1, truncated ORFs without a TM domain of P74, PIF1, PIF2, PIF3, PIF4, Ac5, PIF6, P95 and Ac108 were PCR-amplified from the AcMNPV genome and cloned into the pGBK17 and pGADT7 vectors using the primers listed in Table S2. Competent yeast cells were transformed with 100 ng of the desired plasmids, followed by heat shock in Tris/EDTA/lithium acetate/polyethylene glycol/DMSO solution according to the manufacturer’s protocol (see Clontech’s ‘Yeastmaker Yeast Transformation System 2 User Manual’ for the transformation procedures). The transformants were screened for growth on minimal synthetic defined (SD) base medium plates lacking tryptophan or leucine. Yeast two-hybrid analysis was performed by placing colonies in 500 µl 2 × concentration of YPDA and incubating with shaking at 200 r.p.m. at 30 °C for 24 h according to the manufacturer’s protocol (see Clontech’s ‘Matchmaker Gold Yeast Two-Hybrid System User Manual’ for the hybrid procedures). Hybrids were selected on SD plates lacking leucine and tryptophan (SD/-Leu/-Trp) to ensure the presence of both bait and prey vectors, and SD plates lacking leucine, tryptophan, adenine and histidine (SD/-Ad/-His/-Leu/-Trp) to identify the interaction. Each pair of combinations was repeated twice.

**BiFC assay among the components of the PIF complex**

As shown in Fig. 2, binary vectors PIZc–nRFP, PIZc–cRFP, PIZn–nRFP and PIZn–cRFP were constructed by inserting two fragments of mCherry into pIZ/V5-His vector, respectively [27]. The ORFs of gp64, P74, PIF1, PIF2, PIF3, PIF4, PIF6, Ac5, Ac108 and P95 were amplified by PCR. The sequence-specific primers are listed in Table S3. The resulting PCR products were inserted into pIZc–nRFP, PIZc–cRFP, PIZn–nRFP and PIZn–cRFP. The orientation of each insert was confirmed by sequencing. Co-transfections of vectors into Sf9 cells were performed using Lipofectamine 293 *in vitro* transfection reagent (Signagen Laboratories). Fluorescence signals from the transfected cells were examined using a fluorescence microscope at 3 days post-transfection.

**Western blot analysis**

Cells co-transfected with the different constructs were harvested at 3 days post-transfection by centrifugation at 2388 g for 5 min. The cells were resuspended in lysis buffer (10 mM Tris/HCl, pH 8.0, 130 mM NaCl) with Protease Inhibitor Cocktail tablets (Sangon), after which Laemmli buffer [125 mM Tris-HCl, 2 % sodium dodecyl sulfate, 5 % β-mercaptoethanol, 10 % glycerol, 0.001 % bromophenol blue, (pH 6.8)] was added. After denaturation for 10 min at 95 °C, the samples were analysed by 12 % SDS-PAGE. Proteins were transferred to PVDF membranes (Whatman) by wet transfer. After blocking in 2.5 % fat-free milk in TBS buffer (137 mM NaCl, 20 mM Tris/HCl, pH 7.6) overnight at 4 °C, the membranes were incubated with different primary antibodies. The dilutions of antibodies against P74 and PIFs1–3 were as previously described [26]; other proteins were detected with anti-His mouse monoclonal antibody (Abcam) at a concentration of 100 ng IgG ml⁻¹. Subsequently, the membranes were incubated with goat anti-mouse or goat anti-rabbit antibodies conjugated with horseradish peroxidase (1:5000; Sigma). Chemiluminescence was observed using the ECL-Plus Western blotting detection system (Amersham Biosciences).

**Analysis of PIF1 interaction with P95 by C- and N-terminal truncations, as well as internal deletions**

To generate the P95 and PIF1 truncations, PCR products were generated using the pGBK17-P95 and pGBK7-PIF1 constructs as the templates. For generation of the CBD deletion construct, overlap extension PCR was used. Firstly, the sequences upstream and downstream of the CBD were amplified using the primers P95-F/-P95-CBD-R and P95-CBD-F/P95-R. The PCR products of the upstream and downstream regions were used as the templates to amplify the CBD deletion fragment of P95 using the primers P95-F/-P95-R. The PCR products were cloned into the pGADT7 vector. The same method was used for generation of the aa 100–200 deletion construct of PIF1. The primers used to amplify the P95 and PIF1 mutants are listed in Table S4. The clones were verified by enzyme digestion and sequencing, and then transformed into competent yeast cells. The vectors pGBK17-p53 that which encode a fusion of the murine p53 protein (72-390aa) and the GAL4 DNA-BD (aa 1-147), as well as pGADT7-T, which carries a fusion of the SV40 large protein (72-390aa) and the GAL4 DNA-BD (aa 1-147), as well as pGADT7-T, which carries a fusion of the SV40 large T antigen (aa 87-708) and the GAL4 DNA-BD (aa 768–881), were used as positive control plasmids. Both of these were gifts from Dr Lijian Luo (College of Animal Sciences, Zhejiang University). Y2H analysis was performed as described above (see the Y2H assays among the components of the PIF complex for the Y2H procedures).

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Conflicts of interest
We declare that there are no conflicts of interest for our study.

Ethical statement
All the experiments involving animals were conducted in strict accordance with the Institutional Animal Care and Use Committee of Zhejiang University.

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