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Functional analysis of the baculovirus *per os* infectivity factors 3 and 9 by imaging the interaction between fluorescently labelled virions and isolated midgut cells

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Abstract

Baculovirus occlusion derived viruses (ODVs) contain ten known *per os* infectivity factors (PIFs). These PIFs are crucial for midgut infection of insect larvae and form, with the exception of PIF5, an ODV entry complex. Previously, R18 dequenching assays have shown that PIF3 is dispensable for binding and fusion with midgut epithelial cells. Oral infection nevertheless fails in absence of PIF3. PIF9 has not been analysed in much depth yet. Here, the biological role of these two PIFs in midgut infection was examined by monitoring the fate of fluorescently labelled ODVs when incubated with isolated midgut cells from *Spodoptera exigua* larvae. Confocal microscopy showed that in absence of either PIF3 or PIF9, the ODVs bound to the brush borders, but the nucleocapsids failed to enter the cells. Finally, we discuss how the results obtained for PIF3 with dequenching assays and confocal microscopy can be explained by a two-phase fusion process.

Full text

The *Baculoviridae* are a family of arthropod-specific viruses with large circular DNA genomes (1). Insect larvae become orally infected when food is consumed that is contaminated with viral occlusion bodies (OBs). Infectious occlusion derived viruses (ODVs) are embedded in the protein matrix of these OBs and are released in the alkaline environment of the insect's midgut lumen. The first step in the infection process is binding and fusion of the ODV-envelope with the plasma membrane of the microvilli of midgut epithelial cells (2, 3). After primary infection of the midgut epithelium, the virus spreads systemically to other tissues by means of a different virus phenotype, the budded viruses (BVs).

Infection of the midgut epithelium cells by ODVs requires at least ten different ODV-envelope proteins: the *per os* infectivity factors or PIFs (4-16). Deletion of any of the *pif*-genes abolishes the oral infectivity of the ODVs without affecting their formation or occlusion into OBs. The production and infectivity of BVs remain unaffected by *pif*-gene deletions. All PIFs, except PIF5, participate in the formation of the ODV entry complex (14, 16-19). The initial steps of midgut infection have previously been studied by fluorescence dequenching assays with R18-loaded ODVs and brush border membrane vesicles (7, 20, 21). These studies showed that PIF0, 1 and 2 were important for the binding of ODVs to the vesicles, while PIF3 was dispensable for ODV binding and fusion (7, 20, 21). These findings would indirectly imply that the binding and fusion of the ODV envelope with the cellular plasma membrane do not require the presence of the entry complex, as the entry complex fails to form in absence of PIF3 (18), a concept that is not easy to understand. To gain more insight into the biological function of PIF3 we visualized the interaction of ODVs with the brush border of isolated midgut cells of *S. exigua* larvae using confocal microscopy imaging, as previously described (22). We also examined the role of the recently discovered PIF9 in this analysis. Like PIF3, PIF9 is also needed for entry complex formation (15).

Generation of fluorescent ODVs with an inactivated pif3 or pif9 gene

For this study, we produced fluorescently labelled ODVs of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) lacking either PIF3 or PIF9. The *pif3* gene of the bacmid bMON14272 derived from the E2 strain (23, 24) was deleted by insertion of the chloramphenicol acetyl transferase (*cat*) resistance gene via λ-red mediated homologous recombination (25). To this aim, a *cat*-amplicon, with arms homologous to sequences flanking the *pif3* region to be deleted, was produced by PCR with Phusion polymerase (Thermo Fischer). We used the pCR-TOPO-loxLE-cat-loxRE plasmid (kindly provided by L. Galibert, at the time

at Généthon, France) as template for the PCR in combination with the previously described primers (26). *E. coli* MW003 cells (MW001 (27) with the *bla* gene removed), carrying the AcMNPV bacmid were transformed with the *cat*-amplicon. The expression of the λ RED recombinase (25) was activated in these bacterial cells by heat induction at 42°C for 10 min. The transformed bacteria were incubated on LB-agar plates in the presence of chloramphenicol (34 µg/ml) and kanamycin (50 µg/ml) for up to two days at 32°C. The insertion of the *cat*-gene in the *pif3*-ORF was confirmed by PCR (**Supplementary Figure 1**) with primers that annealed outside the recombined region (5'-CAGTTGTAAACGCGTCTGTAC-3'and 5'-CATGTTGAACTTTTGGCAAATACTTATTC-3'). The *pif3* deletion bacmid was electroporated into DH10ß *E. coli* cells that contained the transposase helper plasmid pMON7124 (24). These resulting cells were used for site-directed transposition using the pFastBac-*polh-egfp:vp39* plasmid (22) with the aim to restore the polyhedrin (*polh*) gene to allow OB formation and to introduce a *gfp:vp39* fusion construct under control of the *p10* promoter to label the nucleocapsids (**Fig. 1,** del-*pif3*). The *pif9* (*ac108)* mutant bacmid previously described (15) was transposed in a similar way to obtain mut-*pif9* (**Fig. 1**). BV-stocks were generated by transfection of Sf21 cells with the bacmids, using Expres2TR transfection reagent (Expres2ion Biotechnologies). High titre BV stocks were obtained by an additional infection round in Sf21 cells. OBs were produced by infection of TnH5 cells with BVs at an MOI of 2 and isolated as described before (15). The fluorescent "wild type" (Wt) virus used as control in this study has been described before (22). Western blot analysis confirmed the absence of PIF3 in the envelope of *pif3*-deletion ODVs, while all the tested viruses were shown to incorporate the eGFP:VP39 fusion protein into their virions (**Fig. 2**).

Infectivity assays in S. exigua larvae

First the infectivity of the BVs of the fluorescent *pif3* and *pif9* mutant viruses was determined by injection of 1 µl BV-stock (3,5 x 10^7 $TCID_{50}$ / ml) into the hemocoel of L4 *Spodoptera exigua* larvae, using an Humapen Luxura insulin pen. When the larvae were injected with Wt or one of the mutant BVs stocks, fluorescence was observed in at least 11 out of 12 larvae for each virus at 3 days post infection (dpi) (**Fig. 3a**), and almost all larvae finally died of the infection (**Table 1**), indicating that the Wt and *pif* mutant viruses replicated efficiently in the larvae when bypassing the midgut. The oral infectivity of the del-*pif3* and mut-*pif9* OBs was determined in separate bioassays, in which the del-*pif3* was tested in duplicate (as others already tested *pif3* deletion mutants extensively) and the *pif9* mutant in triplicate. When L3 instars were orally inoculated with OB stocks (3 x 10^8 ml^{-1}) using droplet feeding, only the larvae that were fed with the Wt OBs showed fluorescence (**Fig. 3b**) and significant mortality (**Table 1**). Hence,

the fluorescently labelled del-*pif3* and mut-*pif9* viruses have the same phenotype as previously described for the non-fluorescent variants (15, 26).

Interaction between ODVs and the brush borders of midgut epithelial cells

To visualize the interaction between (mutant) ODVs and the epithelial cell brush border, midguts of twenty *S. exigua* L5 instar larvae were dissected by cutting of the larva's head and posterior abdominal segment, after which the midgut was pulled out and the peritrophic membrane was removed. The midguts were collected in Grace's medium, supplemented with 50 µg/ml gentamycin, rinsed twice with this medium and further incubated in Graces medium, containing 2 mg/ml collagenase from *Clostridium histolyticum* (Sigma, blend type H), for 90 min. at 27°C under vigorous shaking at 850 rpm. The cells were separated from the remaining midgut tissues by filtering through a 200 µm meshwork of nylon, fixed at the bottom of a microtube. The primary midgut cells were collected and pelleted by centrifugation at 200 **g** for 10 min, and subsequently washed three times with Grace's medium. In parallel, the ODVs were isolated in a slightly modified way from what has been described before (19), in which ODVs were released from larva-derived OBs by EDTA-containing DAS-buffer and were further purified by ultracentrifugation in a sucrose gradient. For our experiments, the ODVs were released from ~3.0 x10^8 cell culture-derived OBs by a 10 min incubation in EDTA-free DAS-buffer (0.1 M Na_2CO_3, 166 mM NaCl; pH 10.5) and subsequent disruption of the OB membrane by two sonication bursts.

This alkaline ODV suspension was further used to suspend the isolated midgut cells after the last washing step and the resulting cell-virus suspension was incubated on ice for 15 min to allow the ODVs to bind to the brush borders prior to imaging. The cell-virus suspension (50-100 µl) was transferred to a circular compartment on the object holder, which was created by placing a silicone spacer between the object- and cover glass. The midgut cells were examined for (bound) ODVs in the brush border area by detection of GFP fluorescence by confocal microscopy (Zeiss LSM-510), using a 63x oil objective lens and excitation with an Argon laser. The ODV-midgut cell interaction was then analysed by construction of a z-stack consisting of 15 – 20 optical slices of 600-700 nm each in a time lapse experiment with a duration of 20 min. It turned out to be crucial to add limited amounts of ODVs to be able to clearly discriminate individual ODVs over time. The images were analysed by using Zen 2.3 lite (blue edition) software.

First, more than twenty Wt ODVs were followed over time in their interaction with eight separate midgut cells to validate the experimental conditions (**Table 2**). These ODVs were

detected in the brush border for 2 to 6 minutes before they disappeared from the cell surface (**Fig. 4a**). About half of the ODVs disappeared within 2-4 minutes after the start of the time lapse experiment. As the ODVs under study could not be found in the surrounding focal planes, it was concluded that the nucleocapsids had entered the midgut cells. Apparently, the fluorescent signal disappeared by decomposition of the grouped nucleocapsids of the multiple capsid ODVs upon cell entry to allow transport of individual nucleocapsids through the microvilli on their way towards the nucleus, as was observed before in electron microscopy studies (2, 3, 28). It should be noted that it took longer for the Wt ODVs to enter the cells under the present experimental conditions than previously described (22). This might be explained by the usage of ODVs that were released from cell culture-derived OBs (C-ODVs) instead of OBs from infected larvae (L-ODVs), as used in the previous study. Infection with C-ODVs might initially be hampered by remnant polyhedrin protein that sticks to the virus envelope, but will be degraded by co-occluded host proteases in L-ODV preparations (29-31). However, as we now know that the remaining PIF-proteins in *pif* deletion mutants are highly sensitive to degradation by such co-occluded proteases during OB-dissolution (19), we decided to use C-ODVs for our experiments. The remnant polyhedrin was probably degraded by digestive enzymes of the primary midgut cell (32) when the virus and cell suspensions were mixed.

The experiment was repeated with mutant ODVs, for which seven ODVs of the *pif3* deletion mutant and fourteen of the *pif9* mutant were analysed, each on four distinct midgut cells (**Table 2**). The ODVs of both mutants were detected at the cell surface until the end of the measurements, even after continuous excitation with the laser for 20 minutes of the eGFP:VP39 fusion protein (**Fig. 4b**). Hence, the disappearance of wild type ODVs could not be explained by fading of the fluorescent signal by continuous excitation of the eGFP:VP39 fusion protein with the laser. These observations show that in absence of PIF3 or PIF9 the ODVs are still able to bind to the brush borders of midgut epithelial cells (**Fig. 4b**), but the nucleocapsids fail to enter the cell.

PIF3 and 9 might be required to overcome an intermediate hemifusion state during midgut infection.

Two different phenotypes regarding binding and fusion have been observed before with R18 dequenching assays using brush border vesicles with various mutant ODVs. It had been found that PIF0, 1 and 2 are important for ODV binding, while PIF3 is dispensable for binding and fusion (7, 20, 21). The ODVs of the *pif3* deletion mutant nevertheless fail to establish an midgut infection. Our observations show that in absence of PIF3, the ODVs bind to the brush border

(**Fig. 4b**), just as indicated before by the R18 dequenching assays (7). Whether the ODV-envelope of this mutant also fused with the brush border plasma membrane could not be confirmed with the currently used assay. Our observations nevertheless show that the nucleocapsids stayed together and failed to enter the cell. This indicates that PIF3 is indeed required for the translocation of the nucleocapsids into the host cell as proposed by others (7). However, it has been observed with mutants of other viruses that R18-dequenching also takes place after hemifusion of the lipid membranes, in which only the outer leaflets of the bilayers fuse (33, 34). It could therefore be reasoned that in absence of PIF3, only the outer leaflets fuse and that this PIF is required to overcome this hemifusion intermediate state (**Fig. 5**). This might explain why the R18-dequenching assays with the *pif3* deletion mutant suggested that membrane fusion took place, while the infection of the midgut epithelium failed.

ODVs that lacked PIF9 also bound to the epithelial cell brush borders. Whether the binding of these mutant ODVs is indeed not affected quantitatively is not known, as the live imaging assay is biased for ODVs that bind to the brush borders; i.e. non-interacting ODVs were not analysed in the time lapse experiments. However, as we regularly encountered bound ODVs, it is reasoned that binding is not significantly affected by the absence of PIF9. Based on our assay we cannot say for sure whether PIF9 is involved in fusion, but our observations nevertheless show that midgut cell entry of the nucleocapsids failed in absence of PIF9, as shown previously for PIF1 and 2 (22) and now also for PIF3.

Despite the fact that we know that both mutants are unable to form the ODV entry complex (15, 18), the mutant ODVs were nevertheless found to be able to bind to the brush border of the isolated midgut cells. Formation of the entry complex is apparently not important for the ODV binding properties as long as PIF0, 1 and 2 are present in the ODV envelope. It was also observed for both *pif 3* and *pif 9* mutants that the entry of the nucleocapsids was abolished. It could be concluded from these observations that formation of the entry complex is crucial for early stages of midgut infection to complete the membrane fusion process at the epithelial cell brush border and/or for the translocation of the nucleocapsids into the cytosol.

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fluorescent mut-*pif9* ODVs, and Wendy van Elst and Priyanka Thaoroijam for their assistance with midgut cell isolation and assay optimization.

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297 **Table 1:** Mortality of *S. exigua* larvae after injection with BVs or oral inoculation with OBs

Injection with BVs	**3.5 x 10^7 $TCID_{50}$ / ml**		
mock	0/12		
del-*pif3-egfp:vp39*	11/12		
mut-*pif9-egfp:vp39*	11/12		
Wt-*egfp:vp39*	12/12		
Oral infectivity 3.0 x 10^8 OBs / ml	**Exp. 1**		**Exp. 2**
mock	0/14		0/12
del-*pif3-egfp:vp39*	0/13		0/11
Wt-*egfp:vp39*	12/13		11/11
Oral infectivity 3.0 x 10^8 OBs / ml	**Exp. 1**	**Exp.2**	**Exp. 3**
mock	0/12	0/14	0/12
mut-*pif9-egfp:vp39*	0/15	0/12	0/12
Wt-*egfp:vp39*	12/15	12/13	10/12

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301 **Table 2:** Overview of the observations of fluorescent ODVs interacting with the brush border of primary

302 midgut cells.

Virus	**Nr. of observations**	**Nr. of observed cells**	**Average observation time before disappearance (min.)**
Wild type	21	8	4.5 ± 2
del-*pif3*	7	4	-
mut-*pif9*	14	4	-

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Figures with legends

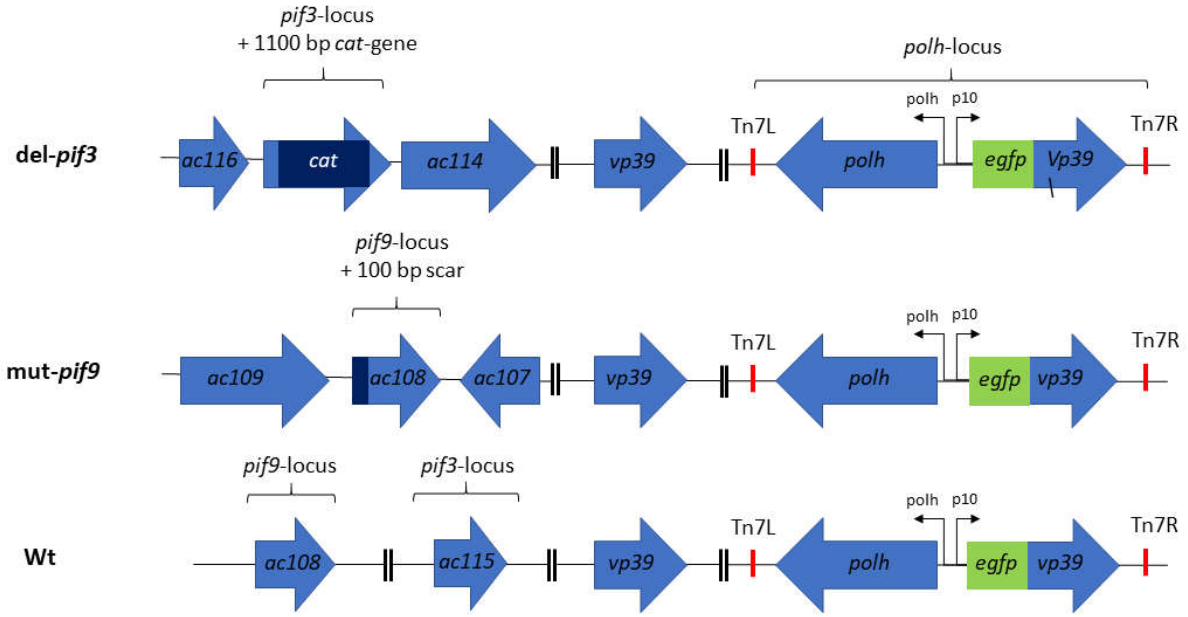


Fig. 1. Overview of the constructed bacmids. The *pif3* deletion and *pif9* mutant bacmids (the latter is described in Boogaard *et al.,* 2019) were transposed with a pFastbac-*polh-egfp:vp39* vector to enable the mutant viruses to produce OBs and fluorescently labelled nucleocapsids. The fluorescent wild type virus was previously described in (22) and was used in this study as wild type control.

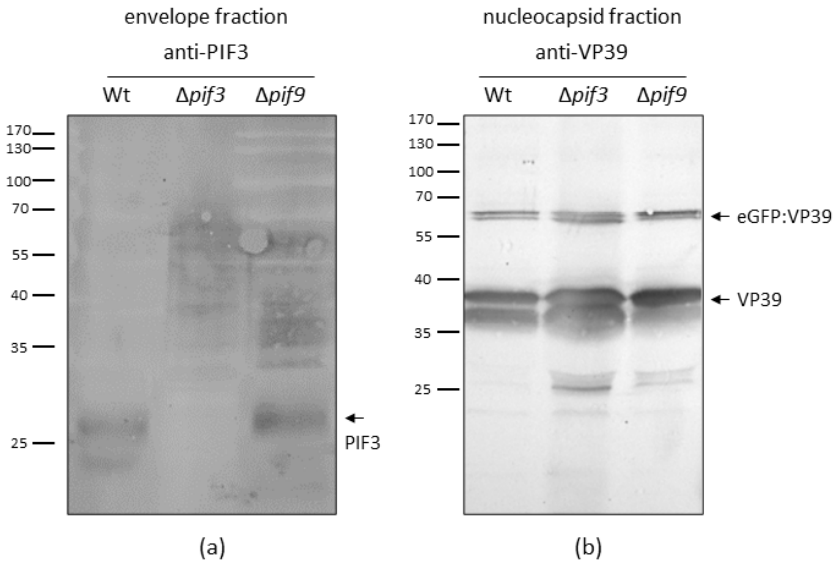


Fig. 2. Western blot analysis of the ODV-envelope and nucleocapsid fractions of wild type (Wt), *pif3* deletion mutant and *pif9* mutant viruses. The envelope fractions were analysed with antiserum against PIF3 (a) and the nucleocapsid fractions with VP39 antiserum (b).

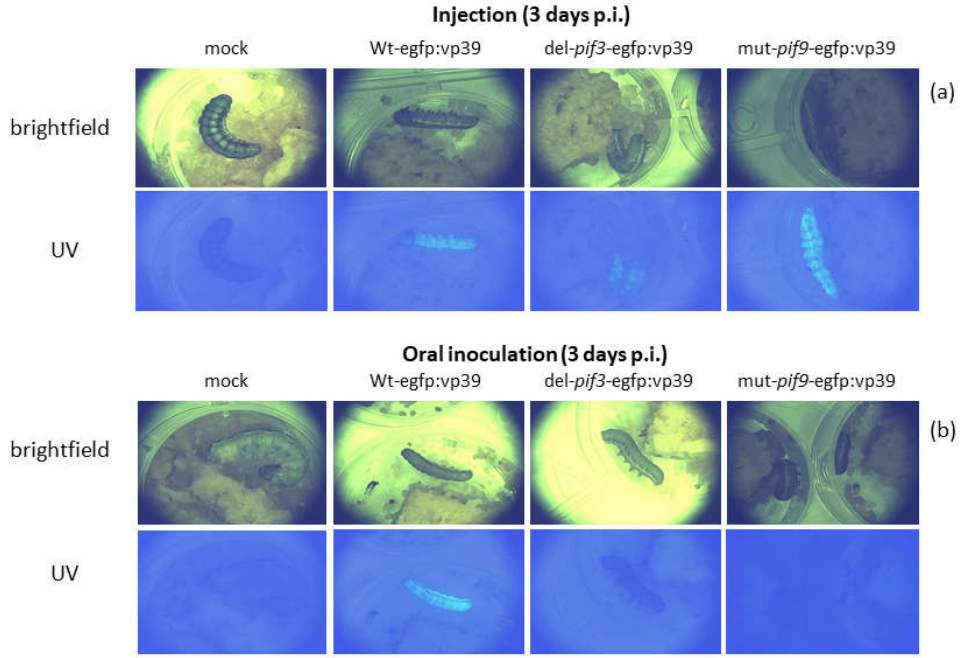


Fig. 3. Detection of viral infection. The larvae were examined under UV-light to observe green fluorescence after injection with BVs (a) or oral inoculation with OBs (b). Green fluorescence is indicative of GFP:VP39 production and hence for viral replication.

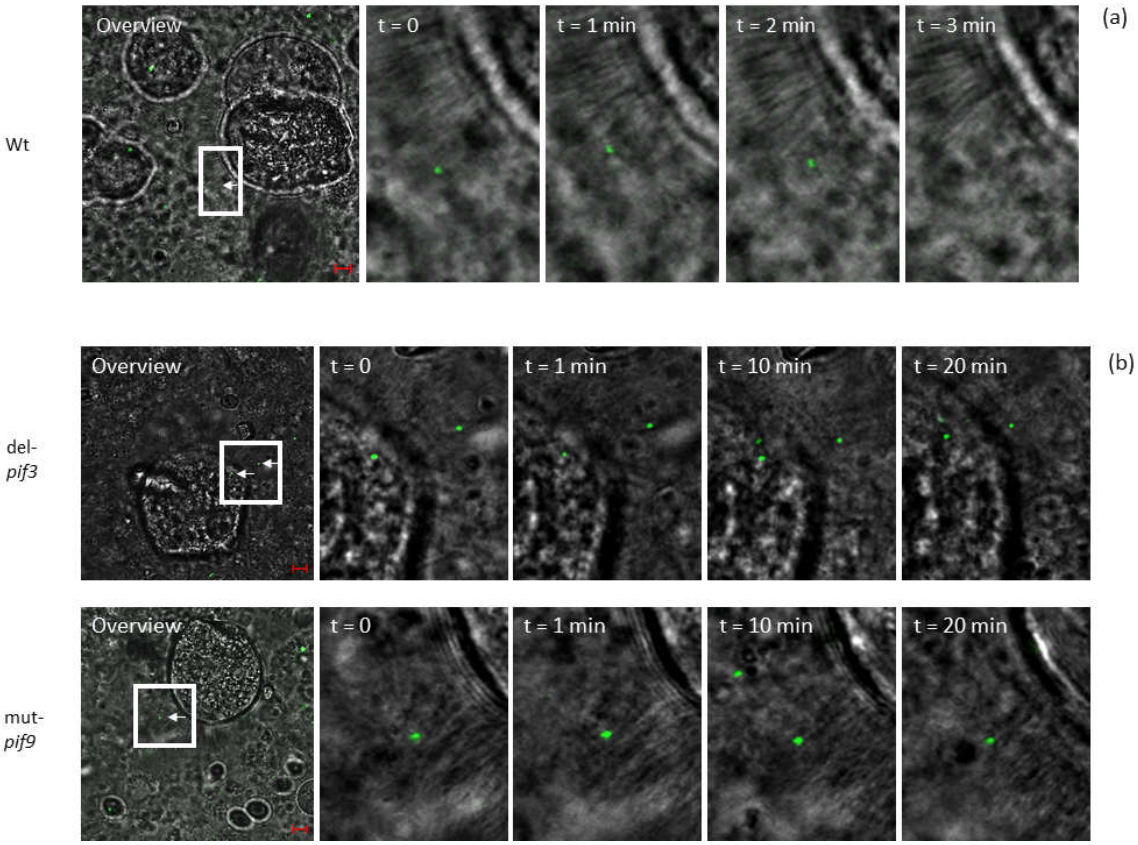
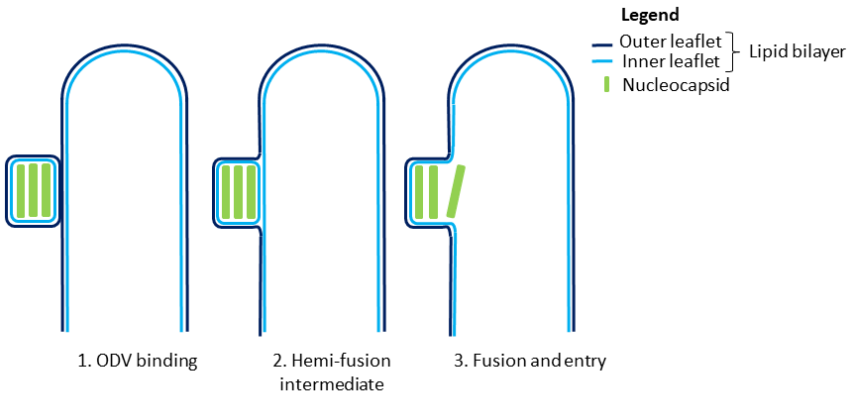


Fig. 4. Time lapse analysis of single fluorescently labelled ODVs on the brush border of a primary midgut cell. Cells were incubated with ODVs from the wilt type (Wt) virus (a), the *pif3* deletion mutant (b) or the pif9 mutant (c) and these ODVs were followed by confocal microscopy in their interaction with the brush border of a primary midgut cell. The red scale bars correspond with 5 µm. The Wt ODV shown in (a) disappeared within 3 minutes after the start of the observation.



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335 **Fig. 5.** Model for the membrane fusion event during primary infection of the midgut
336 epithelium. In the first step (1) the ODV binds to the microvilli membrane. The fusion of the
337 outer and inner leaflets of the lipid bilayers are considered as separate events. First the outer
338 leaflets fuse leading to the formation of a hemifusion intermediate (step 2), followed by the
339 fusion of the inner leaflets and the release of the nucleocapsids into the cytosol (step 3). It is
340 hypothesized that in absence of PIF3, membrane fusion is stuck in the hemifusion state, and
341 hence, entry of the nucleocapsids into the host cell will be prevented.

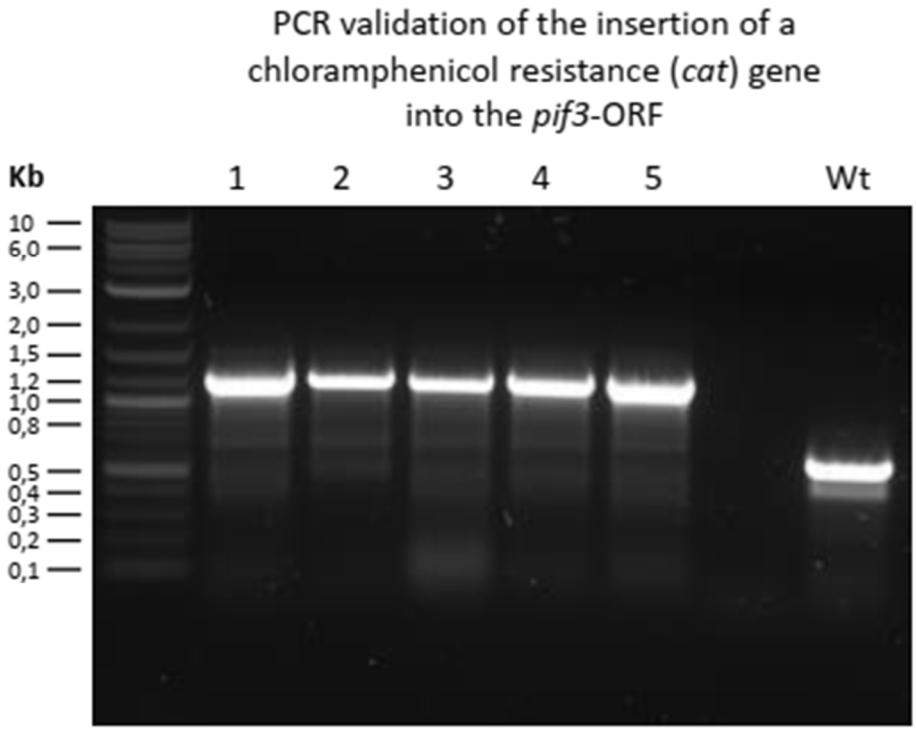

Fig. S1: Confirmation of the insertion of the chloramphenicol acetyl transferase (*cat*) resistance gene into the *pif3-l*ocus by PCR. Insertion of the *cat*-gene resulted in the formation of an amplicon of 1500 bp (clone 1-5), while without insertion a 500 bp amplicon was produced (Wt). Clone 1 was used for further studies.

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