Midgut-based resistance to oral infection by a nucleopolyhedrovirus in the laboratory-selected strain of the smaller tea tortrix, *Adoxophyes honmai* (Lepidoptera: Tortricidae)

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**Abstract**

A strain of *Adoxophyes honmai* resistant to *Adoxophyes honmai* nucleopolyhedrovirus (AdhoNPV) was established from a field-collected colony by repeated selection. Fifth-instar larvae of this resistant strain (R-strain) had over 66,666-fold greater resistance in terms of 50% lethal concentration values to oral infection of AdhoNPV than non-selected strain larvae (susceptible for AdhoNPV; S2-strain). In this study, the mechanism of resistance to AdhoNPV was determined in R-strain larvae. An assessment of viral genome replication in AdhoNPV-infected S2- and R-strain larvae by quantitative PCR showed no viral genome replication occurring in R-strain larvae. Transcription of AdhoNPV ie-1, vp39 and polyhedrin genes was also not detected in R-strain midgut cells. Besides, a fluorescent brightener had no effect on AdhoNPV infection in either S2- or R-strain. However, binding and fusion of occlusion-derived virus with R-strain were significantly lower than those of S2-strain. These findings suggest that R-strain *Adoxophyes honmai* larvae possess a midgut-based resistance to oral infection by AdhoNPV in which midgut epithelial cells are infected less efficiently.

**INTRODUCTION**

A nucleopolyhedrovirus (NPV) is a circular double-stranded DNA virus belonging to the family Baculoviridae, which consists of the genera *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* [1]. Baculoviruses are used as biological control agents to control agricultural pests instead of chemical pesticides because viruses of this family exclusively infect insects and do not cause disease in vertebrates or non-target insect taxa. The process of baculovirus infection starts when host larvae ingest viral occlusion bodies (OBs) [2]. OBs are dissolved in the midgut juice to release occlusion-derived viruses (ODVs). ODVs pass the host chitin–protein matrix (known as the peritrophic membrane (PM)), which lines the midgut and directly binds to midgut epithelial cells. Fusion of the ODV envelope with midgut epithelial cell plasma membrane leads to the entry of viral nucleocapsids into the cytosol. Nucleocapsids migrate to the nucleus, where viral DNA is uncoated, viral genes are expressed and the viral genome is replicated. Progeny viruses are produced, some of which bud to haemocoel as the budded virus virion phenotype, which spreads infection to other tissues in the host.

Resistance of lepidopteran insects to baculoviruses has been reported in some laboratory-selected populations [3–8]. Some field populations of *Cydia pomonella* in Europe also have acquired resistance against *Cydia pomonella* granulovirus (CpGV), which is used as a biological control agent [9–11]. The mode of inheritance of CpGV resistance was different among different resistance populations [12, 13], and the resistance mechanism is still not clear.

The smaller tea tortrix, *Adoxophyes honmai* (Tortricidae, Lepidoptera), is a major pest of tea plants in Japan. This pest has acquired resistance to some chemical pesticides and also to sex pheromone compounds used for mating disruption [14, 15]. In our laboratory, to characterize resistance to a potential baculovirus control agent, a resistant strain (R-strain) of *Adoxophyes honmai* against *Adoxophyes honmai*...
nucleopolyhedrovirus (AdhoNPV) was established with repeated selection by administration of AdhoNPV OB doses corresponding to 60–70% lethal concentration (LC_{60}–LC_{70}). This viral-selected strain has resistance both to oral inoculation of OBs and to haemocoelic injection of budded viruses (M. Nakai, K. Takahashi, K. Iwata, K. Tanaka, J. Kunimi, unpublished). In this study, we focused on the cause of resistance against oral infection of AdhoNPV observed in our R-strain *Adoxophyes honmai* colony to reveal mechanisms blocking the viral infection. We identified resistance mechanisms that impacted midgut primary infection.

**RESULTS**

**Dose–response assay**

Bioassays were performed with AdhoNPV OB for fifth-instar *Adoxophyes honmai* larvae of S2-strain (non-selected and susceptible) and R-strain (selected and resistant) to check their susceptibility for oral infection of AdhoNPV. The mortalities of S2-strain were 8.0–98.0% for inoculum concentrations (10^4–10^7 OBs mℓ^-1) (Table 1). The 50% lethal concentration (LC_{50}) value of S2-strain was 1.5×10^8 OBs mℓ^-1 (Table S1, available in the online Supplementary Material). On the other hand, in R-strain, no larval mortality was measured even with OB concentrations ranging from 10^7 to 10^{10} OBs mℓ^-1 (Table 1). Therefore, the LC_{50} of R-strain was estimated to be more than 10^{10} OBs mℓ^-1 in this study. In addition, resistance ratio (LC_{50} of R-strain divided by LC_{50} of S2-strain) was calculated to be over 66,666 (Table S1).

**Viral replication by time course**

To evaluate viral replication in S2- and R-strain larvae, the viral genome copies in 1 ng total DNA isolated from whole larval homogenates of larvae inoculated with 10^8 OBs mℓ^-1 of AdhoNPV were measured by quantitative PCR (qPCR). On day 0 post-inoculation (dpi), there was no significant difference between the viral genome copies in S2-strain and those in R-strain (Wilcoxon test; *H*=3.15, df=1, *P*=0.076). From 1 dpi onward, the viral genome copies increased with time in S2-strain larvae, and they were significantly higher than R-strain viral genome copies at each time point (1 dpi: *H*=6.82, df=1, *P*<0.05; 2 dpi: *H*=3.94, df=1, *P*<0.05; 3 dpi: *H*=6.82, df=1, *P*<0.05; 4 dpi: *H*=6.82, df=1, *P*<0.05; 5 dpi: *H*=6.82, df=1, *P*<0.05) (Fig. 1). In R-strain larvae, there was no increase of the viral genome copies (Fig. 1), and all inoculated larvae of R-strain pupated at 6 or 7 dpi (data not shown).

**Expression of viral genes in midgut epithelial cells**

Expression of *ie-1*, *vp39* and *polyhedrin* was assessed by reverse transcription PCR (RT-PCR) in midgut epithelial cells of S2- and R-strain larvae inoculated with 10^8 OBs mℓ^-1 of AdhoNPV. The *ie-1* gene is transcribed very soon after virus entry [16], while *vp39* and *polyhedrin* are transcribed during the late phase, after viral DNA replication begins [17]. All three viral genes were detected at 24, 48 and 72 h post-inoculation in the midgut epithelial cells of S2-strain larvae (Fig. 2a). On the other hand, expression of these three viral genes was not detected in the midgut epithelial cells of R-strain larvae at any time points (Fig. 2b). Transcripts of the housekeeping gene *beta-actin* were detected, confirming that RNA extraction and cDNA synthesis were successful. The same results were obtained with three replicates of the experiment.

**Effect of PM on host susceptibility to AdhoNPV**

Since viral gene expression was not detected in midgut epithelial cells in R-strain larvae, the hypothesis that the PM of R-strain larvae was blocking the viral entry into midgut epithelial cells was tested. To elucidate the influence of the PM

**Table 1. Mean mortality of Adoxophyes honmai larvae inoculated with AdhoNPV at different concentrations**

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<tr>
<th>Conc (OBs mL^-1)</th>
<th>Mortality±SE (%)</th>
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<tr>
<td></td>
<td>S2-strain R-strain</td>
</tr>
<tr>
<td>10^4</td>
<td>8.0±0.10 –</td>
</tr>
<tr>
<td>10^5</td>
<td>14.3±2.86 –</td>
</tr>
<tr>
<td>10^6</td>
<td>44.5±5.24 –</td>
</tr>
<tr>
<td>10^7</td>
<td>69.9±0.83 –</td>
</tr>
<tr>
<td>10^8</td>
<td>83.5±1.31 –</td>
</tr>
<tr>
<td>10^9</td>
<td>94.1±3.09 0.0±0.00</td>
</tr>
<tr>
<td>10^{10}</td>
<td>98.0±0.98 0.0±0.00</td>
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![](https://i.imgur.com/3QX2Q.png)
on primary infection by AdhoNPV, the PM of larvae was removed by administration of virus with a fluorescent brightener, Tinopal UNPA-GX. Fifth-instar larvae of the S2-strain were fed with 1.5 × 10^6 OBs ml^{-1} of AdhoNPV, with or without 1 % Tinopal UNPA-GX. Both the treatments showed 55.8±4.76 and 53.9±1.67 % (mean±SE) mortalities in three replications, and there was no significant difference among them (t-test; t = −0.33, df=4, P =0.76) (Table 2). When the experiment was repeated with R-strain larvae, no mortality was observed in the presence or absence of Tinopal UNPA-GX (Table 2). To confirm the destruction of PMs by 1 % Tinopal UNPA-GX, PMs with food contents from larvae of S2-strain and R-strain inoculated with 1 % Tinopal UNPA-GX were examined. To study the morphology of PMs in larvae fed on 1 % Tinopal UNPA-GX, PMs of S2- and R-strain larvae of *Adoxophyes honmai* were disrupted at 30–60 min post-inoculation, and gut compartments were run out in removing midgut epithelial cells (Fig. S1). Larvae fed with distilled water as negative control contained intact PMs with food contents in both strains (Fig. S1). To confirm the viral-enhancement activity of 1 % Tinopal UNPA-GX, increased mortality and disrupted PMs were observed in *Mythimna separata* (Noctuidae, Lepidoptera) larvae administered with Autographa californica multiple nucleopolyhedrovirus (AcMNPV) and 1 % Tinopal UNPA-GX (Table S2).

**Fluorescence-dequenching assay**

To monitor the viral attachment to midgut epithelial cells of R-strain, fluorescence-dequenching assay was conducted. ODVs bound and fused to midgut epithelial cells of *Adoxophyes honmai* with progressing of time course and increasing of the viral amount were investigated by using the laboratory strain of *Adoxophyes honmai*, which is susceptible for AdhoNPV infection. These experiments indicated that there was no significant difference of the amount of octadecyl rhodamine B chloride (R18)-labelled ODV binding or fusion with midgut epithelial cells during 30–120 min post-inoculation (Fig. 3a), and the amount of bound or fused ODVs got higher with increasing viral concentration and saturated at the inoculation of around 0.9 µg µl^{-1} of R18-labelled ODVs (Fig. 3b).

Fifth-instar larvae of S2- and R-strains were inoculated with 1.1 µg R18-labelled ODVs, which caused 100 % mortality among S2-strain larvae, but no mortality among R-strain larvae (Fig. 4a). Amounts of R18-labelled ODVs equal to 0.19±0.03 µg and 0.08±0.01 µg were bound and fused, respectively, to midgut epithelial cells of S2-strain. On the other hand, 0.04±0.001 µg and 0.007±0.004 µg R18-labelled ODVs were bound and fused, respectively, to midgut epithelial cells of R-strain (Fig. 4b). These amounts of ODVs bound and fused with midgut epithelial cells of R-strain were significantly lower than those of S2-strain (bound: H =27.02, df=1, P<0.0001; fused: H=23.36, df=1, P<0.0001) (Fig. 4b).

**DISCUSSION**

In this study, a pattern of low or no expression of AdhoNPV genes in midgut primary infection and lower attachment of ODV to midgut epithelial cells were observed with R-strain larvae. Data from bioassays conducted with a fluorescent brightener did not support a role for the peritrophic matrix in the midgut-based resistance of the R-strain. The inability of AdhoNPV to enter R-strain midgut cells accounts for the complete lack of viral replication in R-strain larvae and represents a novel mode of resistance observed for an otherwise permissive host against a baculovirus. Based on these
mechanisms, fifth-instar larvae of R-strain showed over 66666-fold greater resistance than larvae of S2-strain.

This is the first study to our knowledge monitoring the ODV attachment with different strains of one permissive host against a baculovirus by fluorescence-dequenching assay. This assay is based on the self-dequenching character of the fluorescent probe, R18, which is used to detect cell-membrane fusion [18]. Horton and Burand [19] indicated that the primary infection of baculovirus was caused by the direct binding and membrane fusion of ODVs with midgut epithelial cells by using the fluorescent dye R18 in Lymantria dispar NPV and brush border membrane vesicles of the host larvae. This fluorescence-dequenching assay was also used to detect ODV attachment to midgut epithelial cells in vivo using the virus–host pair of AcMNPV and a noctuid insect [20]. The procedure has been used for the study of the functions of *per os* infectivity factors in AcMNPV [20–22]. According to time course and saturation of ODV binding and fusion experiments in this study, the fluorescence-dequenching assay is adaptable to detect the viral attachment of AdhoNPV ODVs with *Adoxophyes honmai* larvae. Consequently, experimental conditions for the following experiment were determined as: *Adoxophyes honmai* larvae of S2- and R-strain were inoculated with a saturating dose (1.1 µg) of labelled ODVs and dissected at 30 min post-inoculation.

Saturation of bound or fused ODVs indicates that the receptor(s) of AdhoNPV ODV is located on midgut epithelial cells of *Adoxophyes honmai* larvae (Fig. 3b), even though receptor(s) of baculoviruses has not been identified to date. Binding and fusion of ODVs to the midgut epithelial cells of R-strain larvae were lower than those of S2-strain larvae (Fig. 4b). This observation suggests that the resistance to AdhoNPV in R-strain larvae may be due to a missing or mutated epithelial cell receptor(s). The absence of viral binding to the midgut caused resistance to AcMNPV in non-permissive host, *Spodoptera frugiperda* larvae [23]. Recombinant viruses lacking *per os* infectivity factors (p74, pif1 or pif2) showed lower binding and fusion with host midgut epithelial cells in fluorescence-dequenching assays, correlating with lower infectivity from oral inoculation [20, 21]. Thus, ODV binding and fusion with midgut epithelial

<table>
<thead>
<tr>
<th>Strain</th>
<th>Conc (OBs ml⁻¹)</th>
<th>Tinopal</th>
<th>Mortality±se (%)</th>
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<tr>
<td>S2</td>
<td>1.5×10⁹</td>
<td>+</td>
<td>55.8±4.76*</td>
</tr>
<tr>
<td>R</td>
<td>1.0×10⁹</td>
<td>+</td>
<td>53.9±1.67*</td>
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*, Administration with Tinopal UNPA-GX; –, without Tinopal UNPA-GX as negative control.

*No significant difference between AdhoNPV with Tinopal UNPA-GX as negative control.

Fig. 3. Fluorescence-dequenching assay in *Adoxophyes honmai* larvae. (a) Time course of binding (solid bars) and fusion (blank bars) of ODVs to midgut epithelial cells in laboratory strain of *Adoxophyes honmai* inoculated with 0.5 µg R18-labelled ODV. Midgut epithelial cells were collected at each time point. Different letters (A, bound; a, fused) indicate significant difference by nonparametric comparisons for all pairs using Steel-Dwass test (P<0.05). Error bars indicate standard errors (six or seven larvae examined for each time point). (b) Saturation of bound (solid circles) and fused (blank circles) ODVs with midgut epithelial cells in laboratory strain of *Adoxophyes honmai* inoculated with 0.009–0.9 µg µl⁻¹ of R18-labelled ODV. Midgut epithelial cells were collected at 30–60 min post-inoculation. Error bars indicate standard errors (six or seven larvae examined for each concentration).
cells are essential to establish the midgut primary infection of baculoviruses, and binding and fusion are likely influenced by specific host–virus interactions. It is necessary to identify receptors in the midgut epithelium of Adoxophyes honmai larvae to prove this hypothesis.

The reduced viral attachment with midgut epithelial cells of R-strain may not be sufficient to explain the complete refractoriness of R-strain larvae to AdhoNPV because the amount of ODVs bound and fused with midgut epithelial cells of R-strain was not zero. It is possible that some viral nucleocapsids enter the midgut epithelial cells of R-strain larvae. However, no viral gene expression was detected in midgut epithelial cells of inoculated R-strain larvae. ODVs of AcMNPV can bind and fuse with midgut epithelial cells of a non-permissive host, Anticarsia gemmatalis larvae, but early genes of AcMNPV are not expressed in the midgut [24]. The authors observed cell apoptosis occurring in the midgut epithelial cells of inoculated larvae. Baculoviruses encode anti-apoptosis genes (iap, p35, p45 and apsup) to inhibit the apoptosis of permissive cells [25]. AdhoNPV also encodes some homologues of iap genes [26]. R-strain larvae may have evolved a means to overcome the viral mechanisms for inhibition of apoptosis. There may be other kinds of intracellular defence mechanism that the R-strain has evolved to evade AdhoNPV infection. For example, nucleocapsids of Bombyx mori nucleopolyhedrovirus (BmNPV) are not transmitted to the nuclei of Sf9 cells, which correlates with a lack of infectivity of Sf9 by BmNPV [27]. BM-N cells undergo rRNA degradation and global protein synthesis shutdown when infected by heterologous NPVs [28]. It may be possible that viral gene expression in midgut epithelial cells of R-strain was suppressed by RNA interference. This phenomenon is described for Helicoverpa armigera single NPV and its host, H. armigera [29], even though the innate antiviral effect is not clear. Transgenic silkworm larvae engineered to suppress the ie-1 gene of BmNPV exhibit resistance to BmNPV [30]. A global transcriptome analysis of AdhoNPV-infected R-strain larvae may reveal the expression of RNA interference triggers that target AdhoNPV.

Mechanisms of resistance against baculoviruses in permissive hosts have been studied in Anticarsia gemmatalis, Bombyx mori and C. pomonella. The resistance against Anticarsia gemmatalis multiple nucleopolyhedrovirus in a resistant population of Anticarsia gemmatalis selected in a laboratory was attributed to its thicker PM [31, 32]. The PM acts as a physical barrier in the midgut [33]. The administration of fluorescent brighteners induced destruction of larval PMs and enhanced viral infection [34–36]. This result suggests that PMs of Adoxophyes honmai do not serve as physical barriers against AdhoNPV infection. This does not conflict with the results of bioassays in other tortricid insects. El-Salamony [38] indicates that a fluorescent brightener does not increase the susceptibility of these larvae for their homologous granuloviruses, caused by the lack of physical barrier in the midgut of tortricid

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**Fig. 4.** Mortality and binding or fusion of ODVs to midgut epithelial cells in S2- and R-strain larvae. (a) Percentage of fifth-instar larvae killed by the inoculation of R18-labelled ODV (1.1 µg per larva) 32–35 larvae for each replication; n indicates the total number of larvae examined in three replications. (b) Amounts of labelled ODVs bound or fused with midgut epithelial cells of S2-strain (blank bars) and R-strain (solid bars) of Adoxophyes honmai larvae inoculated with 1.1 µg R18-labelled ODV. Different letters (A, B; a, b) indicate statistically significant differences (bound: H=27.02, df=1, P<0.0001; fused: H=23.36, df=1, P<0.0001). Error bars indicate standard errors.
insects. Therefore, the resistance mechanism of R-strain *Adoxophyes honmai* does not appear to involve the PM.

Some resistant races of *B. mori* against BmNPV highly express antiviral proteins [39]. These proteins (Bmlipase-1 and Bm-serine protease-2) identified from the larval gut juice of *B. mori* reduce the virulence of BmNPV ODV [40, 41]. Besides, transgenic silkworm overexpressing the antiviral lipase exhibits resistance to BmNPV infection [42]. Therefore, R-strain larvae of *Adoxophyes honmai* might express an antiviral factor in midgut that inactivates ODVs, causing midgut-based resistance to oral infection of AdhoNPV. In the future, we should study antiviral proteins in midgut juice of R-strain larvae.

The *C. pomonella* strain CpRR1 exhibits resistance to CpGV that is characterized by infection foci in larval midgut tissue from which there is no expansion or propagation [43]. The authors concluded that the resistance against CpGV in CpRR1 larvae was caused by a block of viral DNA replication. However, the mechanisms of this blockage are still not clear. Similarly, R-strain *Adoxophyes honmai* larvae showed no viral genome replication in this study. This absence of replication is likely attributable to low or no expression of viral genes in the primary infection and lower attachment of ODVs in midgut of R-strain larvae. The possible points of similarity between the mechanisms of virus resistance in R-strain *Adoxophyes honmai* and *C. pomonella* strain CpRR1 merit further investigation. Such studies promise to provide new insight into management of insect resistance to baculovirus products and also host defence mechanisms against baculovirus infection.

**METHODS**

**Insects**

The R-strain of *Adoxophyes honmai* was established by repeated serial inoculation of larvae from a field-collected population from Tukuba, Ibaraki, Japan, in 1998 with AdhoNPV OB suspensions corresponding to LC$_{60}$–LC$_{70}$. The susceptible strain maintained in parallel was lost during rearing, so the susceptible strain (S2-strain) used in this study is derived from a second colony that was developed later from insects collected from the same region as the R-strain. In addition, a laboratory strain of *Adoxophyes honmai* that was susceptible for AdhoNPV was kindly provided by Agro-Kanesho (Saitama, Japan) [44]. These strains of *Adoxophyes honmai* were reared on artificial diets (INSECTA LF or Silkmate 2S; Nosan) at 25°C and with a 16 h light : 8 h dark (16L : 8D) photoperiod.

**Virus**

An isolate of AdhoNPV was isolated from the tea field in Tukuba, Ibaraki, Japan [44], and an in vivo isolated clone [26] derived from this field isolate was used in this study. To produce viral OB stocks, the laboratory strain of *Adoxophyes honmai* was inoculated with AdhoNPV. Then OBs were purified as described previously [44]. The concentration of OB suspension in the distilled water was measured by using a cell counting chamber (Helber bacteria counting chamber; Hawksley) with a phase-contrast microscope. OB suspension was stored at 4°C until it was used.

**Bioassay**

Larval S2- and R-strain *Adoxophyes honmai* were inoculated with AdhoNPV by using a modified droplet feeding method [44]. Newly moulted fifth-instar *Adoxophyes honmai* larvae were fed on a droplet of OB suspension ($10^4$, $10^5$, $10^6$, $10^6.5$ and $10^7$ OBs ml$^{-1}$ for S2-strain; $10^7$, $10^8$, $10^9$ and $10^{10}$ OBs ml$^{-1}$ for R-strain) with 10% (w/v) sucrose and 5% (w/v) red food dye. The same solution without virus was used as a mock inoculum in control experiments. Inoculated larvae were individually reared on artificial diet at 25°C, with 16L:8D photoperiod. Mortalities of each treatment were calculated after all larvae were pupated or were dead. Larvae that did not die from viral infection were excluded from the number of larvae examined. These bioassays were repeated in triplicate for 30–35 larvae per treatment. The dose–response data of S2- and R-strains were analysed by probit analysis by using POLO-PC (Leora Software) [45].

**qPCR**

Newly moulted fifth-instar *Adoxophyes honmai* larvae of S2- and R-strains were inoculated with $10^8$ OBs ml$^{-1}$ of AdhoNPV by the droplet feeding method. Some of the inoculated larvae were immediately frozen at −30°C for use as a 0 dpi sample; other larvae were individually reared and collected daily over the course of 5 days. Total DNA was extracted from whole larvae. Larvae were homogenized in cell lysis solution [10 mM Tris/HCl, 100 mM EDTA, 1% (w/v) SDS, pH 8.0] by using pestles. Homogenates were centrifuged at 6000 g for 5 min at 4°C. Proteinase K (200 µg ml$^{-1}$ final concentration) (Nippon Gene) was added to supernatant and incubated at 50°C for 5 h. To remove contaminating RNA, the sample was treated with RNAse A solution (100 µg ml$^{-1}$ final concentration) (Nippon Gene). Proteins were removed by precipitating with Protein Precipitation Solution (Qiagen). DNA was isopropanol precipitated, pelleted by centrifugation and washed with 70% (v/v) ethanol. Finally, precipitated DNA was dissolved in distilled water. The concentration of each DNA solution was measured by using NanoVue Plus (GE Healthcare). The sample DNAs were diluted to 10 ng µl$^{-1}$ and used for qPCR. The primer pair for qPCR was designed from the genome sequence of *polyhedrin* of AdhoNPV to amplify a region of 112 bp (Table S3). The qPCR was performed by using the StepOnePlus Real-Time PCR System (Life Technologies). The reaction mixture contained 10 ng sample DNA, 30 µM of each primer and 5 µl FastStart Universal SYBR Green Master Mix (ROX; Roche). The qPCR cycle conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. At the end of the cycles, a dissociation curve analysis of the amplified product was performed as follows: 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The C$_t$ value of each sample DNA was measured twice. The quantity of the viral genome (in nanograms) in each sample was calculated from a standard curve generated from 10–$10^{-4}$ ng of DNA.
isolated from AdhoNPV OB. The viral genome copies in 1 ng sample DNA were estimated from the molecular weight of AdhoNPV genome. This experiment was conducted for five larvae at each time point. The differences of the viral genome copies at each time point between S2- and R-strains were analysed by nonparametric comparisons for each pair using Wilcoxon test (IBM 9.0.0 software; SAS Institute).

RT-PCR
Fifth-instar larvae of both strains were inoculated with AdhoNPV OBs (10^6 OBs ml^-1) by the droplet feeding method. Inoculated larvae were dissected to isolate midgut epithelial cells at 0.5, 24, 48 and 72 h post-inoculation. Midgut epithelial cells were carefully removed from the PM in PBS buffer. Total RNA was extracted from larval tissue samples pooled from five larval midguts for each time point using ISOGEN (Nippon Gene), as described in the manufacturer’s protocol. The total RNA samples were treated with RNase-free recombinant DNase I (TaKaRa Bio) to remove the contaminating DNAs. The DNase-I-treated total RNA samples (approximately 100 ng µl^-1) were used as templates for cDNA synthesis using a TaKaRa RNA PCR kit (AMV) ver. 3.0 (TaKaRa Bio). Primers were designed from the genome sequence of ie-1, vp39 and polyhedrin regions of AdhoNPV (Table S3). Primers for amplification of Adoxophyes honmai beta-actin (housekeeping gene) transcripts were used as a control for RNA extraction and cDNA synthesis. The PCR was conducted by using 5 µl TaKaRa EX Taq HS (TaKaRa Bio), and cycle conditions were as follows: 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; 72°C for 5 min. Total DNA from infected larvae was used as a positive control for this PCR. The PCR products were electrophoresed in 1.5 % (w/v) agarose gels, and the gels were stained with ethidium bromide. Then, photographs were taken under UV transillumination (E-BOX-VX/20M; M&S Instruments). This experiment was conducted in triplicate.

Bioassay with a fluorescent brighter, Tinopal UNPA-GX
Newly moulted fifth-instar Adoxophyes honmai larvae of S2- and R-strains were inoculated with AdhoNPV at 1.5×10^5 OBs ml^-1 or 1.0×10^10 OBs ml^-1 mixed with or without 1 % Tinopal UNPA-GX (Fluorescent Brightener 28; Sigma-Aldrich) by the droplet feeding method. These bioassays were repeated in triplicate for 30–35 larvae per treatment. The differences of mortalities between the Tinopal UNPA-GX treatments and negative control were analysed by arcsine transformation and t-test (IBM 9.0.0 software; SAS Institute). To confirm the destruction of PM by 1 % Tinopal UNPA-GX, larvae of S2- and R-strains were fed on 1 % Tinopal UNPA-GX or distilled water and were dissected at 30–60 min post-inoculation. The midgut epithelial cells were carefully removed to examine the PM covering gut contents by stereo microscopy.

Fluorescence-dequenching assay
This assay was conducted as described by Haas-Stapleton et al. [20]. To prepare ODVs, AdhoNPV OBs were solubilized with DAS buffer (0.1 M Na2CO3, 0.1 M NaCl, pH 11.5) for 30 min at 37°C. The solution was neutralized with 1 M Tris/HCl, pH 7.4, and kept for 20 min at room temperature. Then the solubilized OB preparation was centrifuged at 1000 g for 10 min to remove insoluble material. The ODVs in the supernatant were banded on sucrose gradients [30 and 60 % (w/v) sucrose] in Ultra-Clear Centrifuge Tubes (Beckman Coulter) by ultracentrifugation for 30 min at 86 714 g in an SW 41 Ti rotor (Beckman Coulter) at 4°C. The ODV band was collected and diluted with TE buffer (1 mM Tris/HCl, 1 mM EDTA, pH 8.0). ODVs were pelleted by centrifugation at 86 733 g for 30 min in a TLA-55 rotor (Beckman Coulter) at 4°C. The pellet was suspended in TE buffer and stored in the dark at 4°C. For the fluorescence-dequenching assay, ODVs were labelled with a self-quenching fluorescent probe, octadecyl rhodamine B chloride (R18), as described previously [46]. Purified labelled ODV was stored in the dark at 4°C until use. The labelled ODVs were quantified using the Micro BCA Protein Assay kit (Pierce). Fifth-instar larvae of the Adoxophyes honmai laboratory strain were fed with 0.5 µg labelled ODVs by the droplet feeding method and subsequently dissected at 30, 60, 90 and 120 min post-inoculation to monitor the time course of binding and fusion, as described previously [20]. Collected midgut epithelial cells were placed in 200 µl separation buffer (100 mM NaCl, 100 mM EDTA, 100 mM Na2CO3, pH 9.5) and stored at −80°C. They were defrosted, and midgut epithelial cells were suspended in separation buffer by pipetting and removing the basement membrane. The cell suspension (150 µl) was transferred to a 96-well black plate (Greiner Bio-One) and measured for the relative fluorescence units (RFUs) associating with the labelled ODVs fused to each midgut epithelial cell sample using an ARVO MX 1420 Multilabel Counter (PerkinElmer) at excitation/emission wavelengths of 560 nm/583 nm. To quantify the labelled ODVs bound with each midgut epithelial cell sample, Triton X-100 (1 % final concentration) (Sigma-Aldrich) was added to each well. Each cell suspension was allowed to solubilize R18 by incubation overnight in the dark, and then the RFUs associated with the labelled ODVs bound to each midgut epithelial cells were measured. Six or seven larvae were examined for each time point. Measured RFUs were corrected for background fluorescence associated with the midgut epithelial cells of mock larvae in the absence of R18. Additionally, the RFUs of 1 µg inoculum labelled ODVs was determined by incubating it with 1 % (v/v) Triton X-100 in 150 µl separation buffer overnight and then measuring its RFU value. These amounts of ODVs bound or fused with midgut epithelial cells were calculated from RFU µg^-1 of inoculated R18-labelled ODV. The differences between time points were analysed by nonparametric comparisons for all pairs using Steel–Dwass test (IBM 9.0.0 software; SAS Institute). To identify saturating quantities of bound and fused ODVs, the assay was conducted by
inoculating fifth-instar larvae with 0.009–0.9 µg µl⁻¹ of labelled ODVs (six or seven larvae for each concentration). Then RFUs associated with binding or fusion were measured, and the estimated amounts of bound or fused ODVs were calculated from RFU µg⁻¹ of inoculated R18-labelled ODVs. Based on the above preliminary experiments, the time point of dissection and the amount of inoculum ODVs were decided.

Newly moulted fifth-instar Adoxophyes honmial larvae of S2- and R-strains were inoculated with 1.1 µg labelled ODVs by droplet feeding. Then, these inoculated larvae were dissected to isolate midgut epithelial cells at 30 min post-inoculation. RFUs associated with binding or fusion of R18-labelled ODVs were measured, as described above. These amounts of ODVs bound or fused with midgut epithelial cells were calculated from RFU µg⁻¹ of inoculated R18-labelled ODVs. This experiment was conducted with 20 larvae of S2-strain and 19 larvae of R-strain. The differences between ODVs bound or fused with midgut epithelial cells of S2- and R-strains were evaluated by nonparametric comparisons for each pair using Wilcoxon test (MPP 9.0.0 software; SAS Institute).

Funding information
E. H.-S. received a Postdoctoral Fellowship Program (short term) for North American and European Researchers from the Japan Society for the Promotion of Science, PE07024. This work was partially supported by a Grant-in-Aid for Scientific Research (KAKENHI) Scientific Research (B), Japan Society for the Promotion of Science, 16H05052.

Acknowledgements
The authors thank Dr Katsuhiko Ito (Tokyo University of Agriculture and Technology, Japan) for technical assistance and Mr Tomoyuki Miyashita (Tokyo University of Agriculture and Technology) for supporting the preliminary experiment. The authors thank Drs Robert L. Harrison (United States Department of Agriculture Agricultural Research Service, USA) and Taro Ohkawa (University of California, Berkeley, CA, USA) for critical review of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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