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Host-range expansion of *Spodoptera exigua* multiple nucleopolyhedrovirus to *Agrotis segetum* larvae when the midgut is bypassed

Agata K. Jakubowska,[1,2,3] Dwight E. Lynn,[2]† Salvador Herrero,[1] Just M. Vlak[2] and Monique M. van Oers[2]

Correspondence
Monique M. van Oers
monique.vanoers@wur.nl

[1]Department of Genetics, University of Valencia, Dr Moliner 50, 46100 Burjassot, Spain

[2]Laboratory of Virology, Wageningen University, PO Box 629, 6700 AP Wageningen, The Netherlands

[3]Department of Biological Control and Quarantine, Institute of Plant Protection, Miczurina 20, 60-318 Poznan, Poland

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Given the high similarity in genome content and organization between *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) and *Agrotis segetum* nucleopolyhedrovirus (AgseNPV), as well as the high percentages of similarity found between their 30 core genes, the specificity of these NPVs was analysed for the respective insect hosts, *S. exigua* and *A. segetum*. The LD_{50} for AgseNPV in second-instar *A. segetum* larvae was 83 occlusion bodies per larva and the LT_{50} was 8.1 days. AgseNPV was orally infectious for *S. exigua*, but the LD_{50} was 10 000-fold higher than for SeMNPV. SeMNPV was not infectious for *A. segetum* larvae when administered orally, but an infection was established by injection into the haemocoel. Bypassing midgut entry by intrahaemocoelic inoculation suggested that the midgut is the major barrier in *A. segetum* larvae for infection by SeMNPV. Delayed-early genes of SeMNPV are expressed in the midgut of *A. segetum* larvae after oral infections, indicating that the virus is able to enter midgut epithelial cells and that it proceeds through the first phases of the infection process. The possible mechanisms of *A. segetum* resistance to SeMNPV in *per os* infections are discussed.

INTRODUCTION

Baculoviruses are large, double-stranded DNA viruses that infect invertebrates, primarily insects. They are a promising alternative to chemical pesticides for control of insect pests because they are able to kill insect larvae within a few days. Baculoviruses are safe because they are restricted to arthropods and are non-pathogenic to vertebrates and plants (Burges *et al.*, 1980). Some baculoviruses, including the prototype *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Mamestra brassicae* multicapsid nucleopolyhedrovirus, have broad host ranges and can cause mortality in larvae of an array of insect species belonging to different families. Other baculoviruses have a host range restricted to a few or even one insect species, such as *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) (Federici, 1997; Goulson, 2003). High host specificity is advantageous from a safety perspective, but can be problematic for commercialization of baculoviruses as bioinsecticides. Production of a baculovirus with a wide host range is economically more attractive than production of a very specific baculovirus able to control only one or a few closely related insect species.

Virus host range is determined by the ability of a virus to enter cells, replicate and produce infectious progeny in particular species. Baculovirus infection initiates by oral ingestion of occlusion bodies (OBs) by an insect larva. In the alkaline environment of the insect midgut, OBs dissolve and release occlusion-derived virions (ODVs), which are responsible for initiating a primary infection (Granados & Lawler, 1981). The ODV envelope binds to and fuses with the membrane of columnar cells of the midgut epithelium (Granados, 1978). The ability of the virus to enter these cells is mediated by *per os* infectivity factors (PIFs) (reviewed by Slack & Arif, 2007). Once the virus has entered the midgut cells, replicated and budded through the basal lamina of the midgut cells, it must successfully enter cells of other tissues, replicate and spread the infection within the insect body to produce appropriate amounts of progeny virus to infect other insects. A second viral phenotype, budded virus (BV), is responsible for the movement of the virus from the basal lamina of the midgut

†Present address: INSell Consulting, 247 Lynch Road, Newcastle, ME 04553, USA.

A supplementary table showing sequences of the specific primer sets used in this study is available with the online version of this paper.

cells into the haemocoel or trachea (Granados & Lawler, 1981; Washburn *et al.*, 1995) and for further spreading the disease in insect tissues to cause a systemic infection. BVs enter secondary target cells by absorptive endocytosis (Hefferon *et al.*, 1999; Lung *et al.*, 2002).

Baculovirus host specificity may be determined at multiple levels and depends on many events, due to the complex infection cycle of these viruses. The first barrier for virus infection is the peritrophic membrane (PM) lining the insect midgut. Baculoviruses use the enzymic activity of chitinases and enhancins to disrupt PMs during invasion of insect midgut cells (Hegedus *et al.*, 2009; Peng *et al.*, 1999; Wang & Granados, 2001). Sloughing of the PM and midgut cells constitutes an important lepidopteran defence against baculovirus infection (Haas-Stapleton *et al.*, 2003; Shapiro & Argauer, 1997; Wang & Granados, 2000). Once the virus passes the PM, it attaches to microvilli of columnar epithelial cells. The nucleocapsids (NCs) enter the cytoplasm by direct membrane fusion (Granados & Lawler, 1981; Horton & Burand, 1993). The NCs are transported to the nucleus in a cytoskeleton-dependent manner and enter the nucleus through nuclear pores (Ohkawa *et al.*, 2002), after which transcription and replication can occur.

Host-range data are often lacking when a novel virus species is described. In general, baculoviruses are said to have a narrow host range, although in-depth studies on the specificity of these viruses are very limited. Moreover, verification that the progeny virions produced belong to the same virus species as those used for the cross-infections does not always occur (Cory, 2003). In fact, several studies have shown that the progeny virus obtained may have resulted from contamination of the virus preparation or the induction of a latent virus already present in the insect (Bourner & Cory, 2004; Cory *et al.*, 2000; Doyle *et al.*, 1990; Takatsuka *et al.*, 2007). Hence, it is important to determine the identity of the progeny virus in cross-infection studies.

The complete genome sequences of *Agrotis segetum* nucleopolyhedrovirus (AgseNPV) and SeMNPV have been determined (IJkel *et al.*, 1999; Jakubowska *et al.*, 2006). These viruses show $<10\,\%$ difference in genome size (147 129 and 135 611 bp, respectively) and a striking collinearity of the genes shared between these viruses. In addition, a high level of sequence similarity between common genes is observed. This prompts the following questions: (i) is SeMNPV infectious for *A. segetum* larvae? Conversely, (ii) is AgseNPV able to infect *S. exigua* larvae orally? SeMNPV is highly specific for *S. exigua* larvae and the ability to infect other insect species has not been demonstrated (Gelernter & Federici, 1986). AgseNPV has a wider host range including *Agrotis ipsilon*, *Agrotis exclamationis*, *Agrotis puta*, *Noctua comes*, *Peridroma saucia*, *Xestia sexstrigata* and *Xestia xanthographa* (Bourner & Cory, 2004). However, the virus used to determine the host range of AgseNPV is different from the sequenced Polish AgseNPV isolate (Jakubowska *et al.*, 2006) used in the present work and may represent a different virus species (Jakubowska *et al.*, 2005).

In the current study, the infectivity of AgseNPV and SeMNPV for the insects *A. segetum* and *S. exigua* was examined. The results show that, despite the high level of genome similarity and collinearity between AgseNPV and SeMNPV, the latter cannot infect *A. segetum* larvae by the oral route, but it can establish a systemic infection when the midgut barrier is bypassed.

RESULTS

Per os infectivity of SeMNPV and AgseNPV against *S. exigua* and *A. segetum* larvae

In the first experiment, the cross-infectivity of SeMNPV and AgseNPV for *A. segetum* and *S. exigua* larvae was explored. Larvae of both species were fed high doses of suspended OBs. AgseNPV fed at the titre of 10^8 OBs ml^{-1} caused a fatal infection in 30 % of the second-instar (L2) *S. exigua* larvae. OBs were present in various tissues, including the fat body of infected and dead insects (data not shown). The identity of the virus recovered from *S. exigua* larvae was determined by restriction enzyme (REN) analysis of DNA extracted from OBs isolated from the AgseNPV-infected larvae. The *Pst*I REN pattern of OB DNA from cross-infected *S. exigua* larvae was identical to that of the input virus (Fig. 1a) and therefore activation of

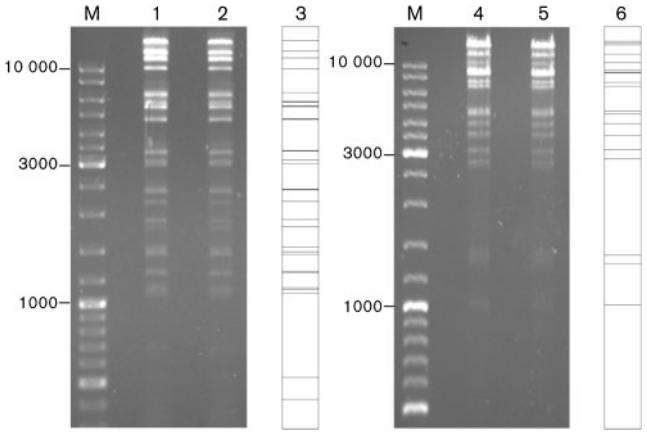

Fig. 1. REN analysis. Lanes: 1, *Pst*I DNA REN profile for AgseNPV OBs used for infecting *S. exigua* larvae; 2, *Pst*I DNA REN profile for OBs resulting from *S. exigua* infection with AgseNPV; 3, schematic of the *Pst*I REN profile of AgseNPV; 4, *Pst*I digestion of SeMNPV DNA derived from BVs used for injecting *A. segetum* larvae; 5, *Pst*I DNA REN profile for OBs resulting from injecting *A. segetum* larvae with SeMNPV; 6, diagram of *Pst*I REN profiles of SeMNPV. M, DNA molecular marker pUC19 DNA digested with *Msp*I (Fermentas) with sizes in bp. Schematics of REN profiles were generated from the genomic sequences with NEBcutter v. 2.0 (Vincze *et al.*, 2003).

a latent virus or contamination with other NPVs in the original isolate was excluded.

A bioassay was conducted to compare the infectivity of AgseNPV for both *A. segetum* and *S. exigua*. Five doses of AgseNPV were applied to diet plugs and offered to larvae. A higher dose range of AgseNPV was used for *S. exigua*, as the experiment described above had shown that these larvae are less susceptible to oral infection by AgseNPV than *A. segetum*. The LD_{50} values obtained for AgseNPV in L2 larvae were 83 OBs for *A. segetum* compared with 8.3×10^5 OBs for *S. exigua* (Table 1); hence, these species showed a 10 000-fold difference in susceptibility to AgseNPV.

Oral inoculation of *A. segetum with* SeMNPV did not lead to fatal infection. None of the newly moulted *A. segetum* L2 larvae fed with 10^8 SeMNPV OBs ml^{-1} died from virus infection. Microscopic inspection showed that no OBs were present in the tissues of these larvae.

Intrahaemocoelic (IH) inoculation of *A. segetum* larvae with SeMNPV BVs

Given the observation that SeMNPV is not orally infectious for *A. segetum* larvae, IH injections were performed with SeMNPV BVs in L3 *A. segetum* larvae. More than 70 % of the larvae injected with SeMNPV BVs at a dose of 10^6 $TCID_{50}$ units per larva died from virus infection, resulting in the production of large numbers of OBs. The DNA of the input and the recovered virus showed identical REN patterns (Fig. 1b), confirming that SeMNPV had replicated in *A. segetum* larvae and produced OBs. This result suggests that the midgut of *A. segetum* larvae is the main barrier for successful infection with SeMNPV.

Detection of SeMNPV- and AgseNPV-specific transcripts in cross-infected larvae

Quantitative RT-PCR (qRT-PCR) was performed with total RNA isolated at different times post-infection (p.i.) from *A. segetum* larvae infected *per os* (L2) or injected IH (L3) with SeMNPV alone or with a mixture of SeMNPV and AgseNPV. No early (*DNApol*) or very late (*p10*) viral transcripts were detected at any time after *per os* infection with SeMNPV alone. The larvae continued to grow regularly and finally pupated (not shown). In larvae infected *per os* with a mixture of AgseNPV and SeMNPV, AgseNPV early (*ie-1*) and very late (*p10*) transcripts were first detected at 12 and 48 h p.i., respectively (Fig. 2a, b), but again, no SeMNPV transcripts were found. The prevalence of AgseNPV *ie-1* transcripts increased by approximately 20 times between 24 and 96 h p.i. The levels of AgseNPV *p10* transcripts increased strongly between 72 and 96 h p.i., but a large variation was seen between individual larvae (Fig. 2b).

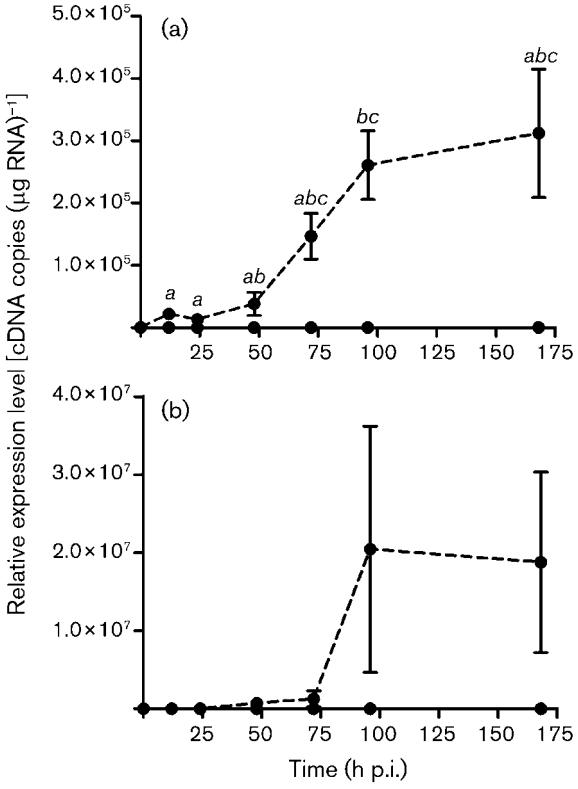

Fig. 2. Viral early gene expression in *A. segetum* larvae inoculated orally with SeMNPV OBs in a mixture with AgseNPV OBs. Total RNA from infected larvae was analysed by qRT-PCR using primer sets specific for (a) the SeMNPV *DNApol* gene (solid line) and the AgseNPV *ie-1* gene (dashed line) and (b) the *p10* genes of both viruses (solid line, SeMNPV; dashed line, AgseNPV). Target-gene copy numbers were calculated based on SeMNPV and AgseNPV genome molecular mass and standard curves. Data show means ± SD (n=3). Values with different letters (*a*, *b*, *c*) are significantly different ($P \leqslant 0.05$).

As the midgut is the first tissue affected in oral NPV infections, isolated *A. segetum* midguts were analysed for the expression of SeMNPV genes in single and mixed infections. Low expression levels of the SeMNPV *DNApol* gene were detected in one of five midgut samples collected

Table 1. LD_{50} values of AgseNPV infection in *A. segetum* and *S. exigua* larvae

Insect species	LD_{50} value	95 % confidence interval	Regression line
A. segetum	8.3×10^1	2.2×10^1–3.2×10^2	$y=0.4x+1.2$
S. exigua	8.3×10^5	3.7×10^5–1.9×10^6	$y=0.7x+0.9$

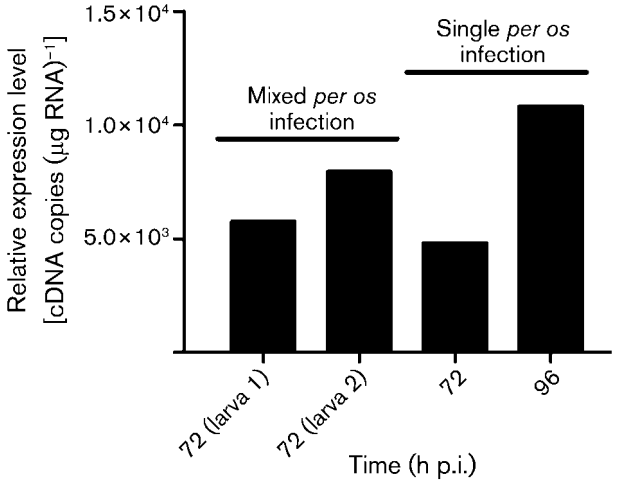

Fig. 3. Viral early gene (*DNApol*) expression in the midguts of *A. segetum* larvae inoculated orally with SeMNPV OBs alone and with a mixture of SeMNPV and AgseNPV OBs. Midguts were isolated from infected larvae at different times p.i. Total midgut RNA was analysed by qRT-PCR using primers specific for SeMNPV *DNApol*. Target-gene copy numbers were calculated based on SeMNPV genome molecular mass and standard curves.

from L4 larvae infected with SeMNPV at 72 h p.i. [4.8×10^3 cDNA copies (μg RNA)$^{-1}$] and at 96 h p.i. [1.1×10^4 cDNA copies (μg RNA)$^{-1}$]. Two of five midguts collected at 72 h p.i. from mixed-infected larvae showed SeMNPV *DNApol* transcripts [5.8×10^3 and 7.9×10^3 cDNA copies (μg RNA)$^{-1}$] (Fig. 3). None of these samples showed expression of the SeMNPV *p10* gene.

After IH inoculation of *A. segetum* L3 larvae with SeMNPV, early gene transcripts of this virus (*DNApol*) were first detected at 48 h p.i. and the levels of these transcripts increased by 3.9 times between 72 and 96 h p.i. (Fig. 4a). Very late SeMNPV transcripts (*p10*) appeared also at 48 h p.i. and their level increased (by 50 times) between 72 and 96 h p.i. (Fig. 4b).

In mixed IH inoculations with AgseNPV and SeMNPV BVs, early SeMNPV transcripts (*DNApol*) were detected at 24 h p.i. – earlier than with SeMNPV alone – and between 24 and 48 h p.i. they increased by approximately 75 times (Fig. 4a). Between 48 and 96 h p.i., levels of SeMNPV early transcripts decreased strongly (25 times), and at 168 h p.i. they were no longer detectable. SeMNPV *p10* transcripts were detected in mixed IH inoculations at 48 h p.i. and were maintained at the same level throughout the recording time.

AgseNPV early transcripts (*ie-1*) were detected as early as 12 h p.i. in mixed IH inoculations and their level continued to increase until 168 h p.i. (Fig. 4c). The transcript number increased significantly (6.9 times) between 48 and 96 h p.i. AgseNPV very late transcripts (*p10*) were detected already at 24 h p.i. and increased by 2.6 times between 48 and 72 h p.i. (Fig. 4d). These data suggest that SeMNPV gene expression in mixed infections is enhanced at early times p.i., but suppressed at later times p.i.

AgseNPV early (*ie-1*) and very late (*p10*) gene expression in larvae infected *per os* was almost identical in level and timing to that in larvae infected by IH inoculation (Fig. 5a, b).

DISCUSSION

Host-specificity studies of two closely related NPVs, SeMNPV and AgseNPV, in *S. exigua* and *A. segetum* revealed that, despite a high level of sequence similarity and a remarkably similar genome organization and gene content (Jakubowska *et al.*, 2006), these viruses differ significantly in their capacity to cross-infect the other insect species *per os*. Whilst AgseNPV caused lethal infections in *S. exigua* larvae when administered orally, SeMNPV was able to kill *A. segetum* larvae only when injected into the haemocoel (Table 1). Although *S. exigua* larvae were susceptible to oral infection with AgseNPV, they are, based on LD_{50} values, less susceptible to AgseNPV by four orders of magnitude than *A. segetum*. Hence, *S. exigua* appears to be a semi-permissive host for AgseNPV.

Monitoring of the SeMNPV infection process in *A. segetum* larvae by following the level of early and late viral transcripts by qRT-PCR revealed that the main barrier for infection in this virus/host system is the midgut. After oral infection of *A. segetum* larvae with SeMNPV, no viral transcripts were detected when RNA was isolated from the total larval body; however, in a few isolated midguts, we detected early SeMNPV transcripts. *p10* transcripts were never found. Thus, the entry of SeMNPV into midgut epithelial cells of *A. segetum* is possible. The presence of delayed-early transcripts (*DNApol*) and the lack of late viral transcripts (*p10*) suggest that the infection is blocked at the transition from early to late transcription. The fact that we only found these early transcripts in a small number of isolated midguts may indicate that the efficiency of entry is low or that the infected cells are rapidly sloughed off. The results also suggest that SeMNPV may be able to replicate in cells of more insect species than thought previously, once this midgut barrier is passed.

Successful baculovirus infection depends on many factors and may be blocked at any point in the infection cycle. Many virus–host systems have been studied to shed light on insect susceptibility to baculovirus infections and apparently many mechanisms exist that lead to a block in infection. Studies with AcMNPV carrying a *lacZ* reporter gene in non-permissive *Heliothis zea* and fully permissive *Heliothis virescens* larvae showed that immune responses may also define baculovirus host range (Washburn *et al.*, 1995). For AcMNPV in *Spodoptera frugiperda* larvae, the main barriers for fatal infection were the inability to infect midgut epithelial cells efficiently and the loss of infected

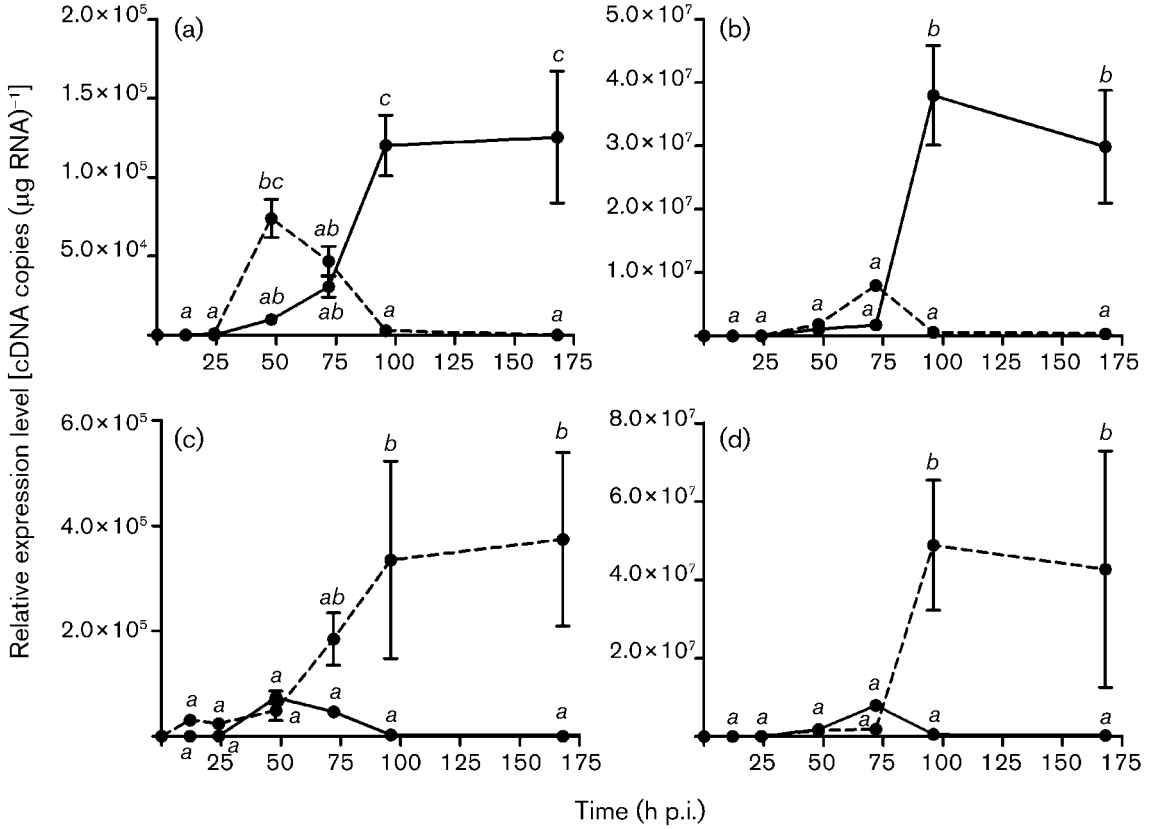

Fig. 4. Viral early and late gene expression in *A. segetum* larvae infected with SeMNPV alone or in a mixture with AgseNPV by IH inoculation. (a) SeMNPV *DNApol* expression (solid line, single infection; dashed line, mixed infection); (b) SeMNPV *p10* expression (solid line, single infection; dashed line, mixed infection); (c) SeMNPV *DNApol* expression (solid line) and AgseNPV *ie-1* expression (dashed line) in mixed IH inoculations; (d) SeMNPV *p10* expression (solid line) and AgseNPV *p10* expression (dashed line) in mixed IH inoculations. Total larval RNA was analysed by qRT-PCR using primers specific for the SeMNPV *DNApol* and *p10* genes and the AgseNPV *ie-1* and *p10* genes. Target-gene copy numbers were calculated based on SeMNPV and AgseNPV genome molecular mass and standard curves. Data show means ± SD (n=3). Values with different letters (*a*, *b*, *c*) are significantly different ($P \leqslant 0.05$).

tracheal cells (Haas-Stapleton *et al.*, 2003). AcMNPV in *S. frugiperda* larvae shows the same pattern of infection as SeMNPV in *A. segetum*: *S. frugiperda* is extremely resistant to oral infection with AcMNPV, but is highly susceptible to systemic infection by IH inoculation with BVs. These studies suggested that the ability of ODVs to bind to epithelial midgut cells plays a crucial role in the ability to infect insect larvae orally. The binding of ODVs to midgut cells is mediated by three PIFs (P74, PIF-1 and PIF-2), associated with the ODV envelope (Haas-Stapleton *et al.*, 2004; Ohkawa *et al.*, 2005; Song *et al.*, 2008). In line with this, an AcMNPV p74 mutant could only infect *Trichoplusia ni* and *H. virescens* larvae (Faulkner *et al.*, 1997; Haas-Stapleton *et al.*, 2004) when injected IH. So far, the detailed role of PIFs in ODVs midgut entry remains enigmatic, but a reason for the resistance to oral infections could be inefficient binding to and fusion with primary target midgut cells. The level of amino acid similarity between SeMNPV and AgseNPV PIFs ranges from 74 to 88 %. Sequence similarity, however, seems not to be very informative in this case, as the similarity between PIFs of SeMNPV, which does not infect *A. segetum* larvae orally, and *A. ipsilon* nucleopolyhedrovirus, which infects *A. segetum* larvae, is at the same level, between 74 and 89 %. As SeMNPV can enter *A. segetum* midgut cells, the PIFs are in this case clearly not as specific as thought originally (Song *et al.*, 2008).

A salient observation was that AgseNPV clearly out-competed SeMNPV in a single passage after mixed IH inoculations (Figs 2 and 3). AgseNPV probably possesses some features in comparison to SeMNPV that enable a faster or more efficient infection process, leading to a higher mortality and greater yield of progeny virus. Alternatively, AgseNPV may activate insect responses that result in the clearance of SeMNPV. The competition between viruses may happen at different stages in the infection process, including release of BVs from midgut

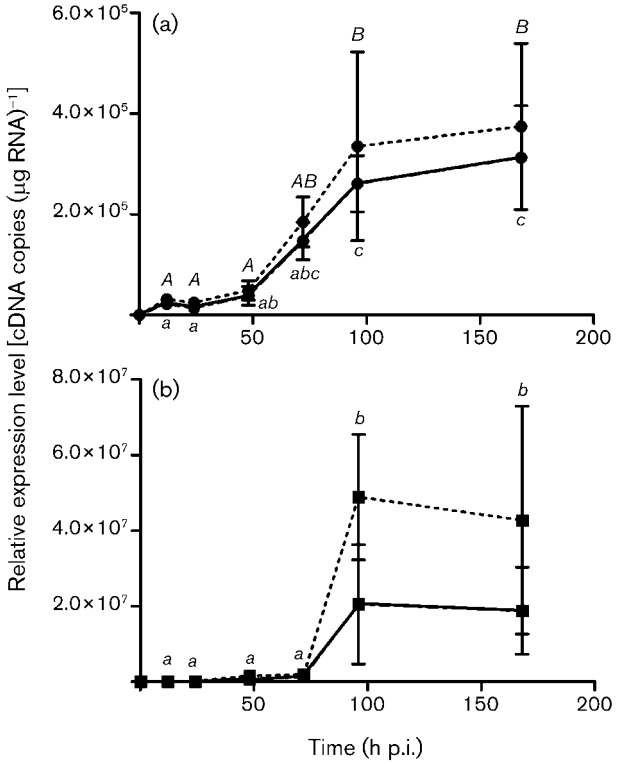

Fig. 5. AgseNPV early and late gene expression in *A. segetum* larvae. *A. segetum* larvae were infected *per os* (dashed lines) or by IH inoculation (solid lines) with a mixture of SeMNPV and AgseNPV. AgseNPV *ie-1* (a) and *p10* (b) expression was analysed in infected and injected larvae. Total larval RNA was analysed by qRT-PCR using primers specific for the AgseNPV *ie-1* and *p10* genes. Target-gene copy numbers were calculated based on SeMNPV and AgseNPV genome molecular mass and standard curves. Data show means ± SD (n=3). Values with different letters (*a*, *b*, *c*; *A*, *B*, *C*) are significantly different ($P \leqslant 0.05$). *Per os* and IH inoculation were analysed separately.

cells, entry into secondary target cells, efficacy of replication and transcription and assembly of a new generation of BVs and OBs. We also find of interest the observation that, at 48 and 72 h p.i., the abundance of both early and late SeMNPV transcripts was higher in mixed infections than in single infections with SeMNPV. This suggests that AgseNPV assists in the replication of SeMNPV in *A. segetum* larvae in the first few days after co-injection. At 96 h p.i., the situation changed dramatically in favour of AgseNPV transcripts, indicating that AgseNPV, despite serving as a helper for SeMNPV in the beginning, finally takes over completely. These data are in agreement with co-infection studies in various *Spodoptera* species with SeMNPV, *S. frugiperda* multiple nucleopolyhedrovirus and *S. littoralis* multiple nucleopolyhedrovirus (Simón *et al.*, 2004), with the difference that, when *S. frugiperda* and *S. littoralis* larvae were co-infected with SeMNPV and their natural viruses, all classes of SeMNPV transcripts were found in both *per os* infections and IH inoculation. In our study, SeMNPV transcripts were not detected in oral infections, except for early transcripts in a few midgut samples. Simón *et al.* (2004) also reported that SeMNPV transcript levels increased in *S. frugiperda* and *S. littoralis* in co-infections with respect to SeMNPV infection alone, similar to what was seen here in *A. segetum*. However, in REN analysis, they detected only the homologous virus in co-infected larvae and concluded that REN was not sufficiently sensitive to detect the heterologous virus. The lack of SeMNPV detection in REN analysis might also have been due to the time point of OB collection. OBs were probably collected from dead larvae, when the homologous virus might have already outcompeted the heterologous one, as in the current study. SeMNPV infection in cultured *S. frugiperda* cells is improved by co-infection with AcMNPV (Yanase *et al.*, 1998) and those authors suggested that AcMNPV may provide transcripts or their products that are used by SeMNPV for replication. Also, in our study, the replication of SeMNPV may increase at the early stages of infection in co-infected insects compared with insects inoculated IH with SeMNPV alone, due to the presence of AgseNPV gene products.

A. segetum and *S. exigua* and their respective viruses now represent an attractive model for studying the baculovirus infection process and to reveal the barriers in specificity and the identification of host-range factors. It is clear that, in the case of SeMNPV, this barrier is beyond initial entry and the involvement of PIFs. Furthermore, high genomic collinearity and gene sequence similarity make the SeMNPV/AgseNPV system highly suitable to study baculovirus adaptation, evolution and speciation.

METHODS

Insects and viruses *A. segetum* and *S. exigua* larvae were reared on an artificial diet at 25 °C, 70 % humidity and a 16 : 8 h photoperiod as described previously (Hinks & Byers, 1976; Smits *et al.*, 1986). AgseNPV used in this study was isolated in 1975 from *A. segetum* larvae collected in cabbage crops in Poland (Jakubowska *et al.*, 2005). The virus was freshly amplified in L2 larvae of a current laboratory culture of *A. segetum*. The SeMNPV isolate used in this study for oral infections and IH injections was SeMNPV-US1 (Gelernter & Federici, 1986). Both virus stocks are non-cloned, field isolates.

AgseNPV infections in *S. exigua* and *A. segetum* larvae. To determine the oral infectivity of AgseNPV for *S. exigua*, L2 larvae were infected by the droplet-feeding method (Hunter-Fujita *et al.*, 1998) with a virus suspension of 10^8 AgseNPV OBs ml^{-1}. The consumed volume was 1–2 µl. Phenol red was added to monitor the ingestion of the suspension. Larvae that ingested the virus were provided with a fresh diet and reared individually until death or pupation. Mortality was checked daily until pupation.

For determining the median lethal dose (LD_{50}) of AgseNPV for both *S. exigua* and *A. segutum*, L2 larvae of both species were infected individually by feeding with diet discs contaminated with five different doses of AgseNPV, ranging from 10^1 to 10^5 OBs per larva for *A. segetum* and from 10^3 to 10^8 OBs per larva for *S. exigua*.

Control larvae were given diet plugs with water. Larvae were given a fresh diet after they consumed the entire disc. Thirty larvae were used per treatment and the same number of larvae served as a mock-infected group. The bioassay was conducted twice and the LD_{50} values for both viruses were calculated by probit analysis (Finney, 1952).

SeMNPV in *A. segetum* larvae. To determine the oral infectivity of SeMNPV for *A. segetum* larvae, L2 larvae were infected by the droplet-feeding method as described above for *S. exigua* larvae. A concentration of 10^8 SeMNPV OBs ml^{-1} was used. According to the fact that no mortality was observed after oral administration of SeMNPV to *A. segetum* larvae, infectivity of SeMNPV by IH inoculation was analysed. *A. segetum* L3 larvae were injected IH with 10 µl SeMNPV BV-containing haemolymph mixed with phenol red solution to control the injections. To this aim, BV-containing haemolymph was collected at 6 days p.i. from L4 *S. exigua* larvae infected orally with SeMNPV at a dose of 10^3 OBs per larva by cutting the prolegs. The haemolymph was mixed with an equal volume of Grace's medium (Gibco, Invitrogen Inc.), centrifuged at 1000 ***g*** to remove haemocytes and filtered through a 0.45 µm non-pyrogenic filter. Virus titre was determined in an end-point dilution assay (Vlak, 1979) in *Se*301 cells (Hara *et al.*, 1993) and a virus stock with a titre of 10^8 $TCID_{50}$ units ml^{-1} was used for IH inoculation. Injected larvae were reared individually until death or pupation and mortality was recorded daily.

Infections for qRT-PCR. To determine the possible barriers in *A. segetum* larvae for oral infection by SeMNPV, the infection process was monitored by measuring the presence and accumulation of viral transcripts using qRT-PCR. For comparison, the development of the infection was also monitored in *A. segetum* larvae infected with SeMNPV by IH inoculation.

For monitoring oral infections by qRT-PCR, L2 *A. segetum* larvae were infected by droplet feeding with 10^8 OBs SeMNPV ml^{-1}. The consumed volume was 1–2 µl. Additionally, a group of *A. segetum* larvae was infected with a mixture of 10^8 OBs SeMNPV ml^{-1} and 10^3 OBs AgseNPV ml^{-1} to test complementation of SeMNPV by AgseNPV. Three to five larvae were collected at 0, 12, 24, 48, 72, 96 and 168 h p.i. for total RNA isolation. The experiment was performed in triplicate and the data were analysed by one-way ANOVA and Tukey test for multiple pair analysis. Additionally, at 24, 72 and 96 h p.i., midguts and haemolymph were collected from five larvae individually at each time point for total RNA isolation.

For monitoring IH inoculation, L3 *A. segetum* larvae were injected with 10 µl SeMNPV BVs (10^8 $TCID_{50}$ ml^{-1}) and with a mixture of BVs consisting of 10^8 $TCID_{50}$ SeMNPV ml^{-1} and 10^6 $TCID_{50}$ AgseNPV ml^{-1} (100 : 1). SeMNPV-containing haemolymph was prepared as described above. AgseNPV-containing haemolymph was collected and prepared, as described above for *S. exigua* larvae, from *A. segetum* larvae infected with AgseNPV at a dose of 10^3 OBs per larva. The virus titre was determined in AiE1611T cells (Harrison & Lynn, 2008). Injected larvae were reared individually and three to five larvae were collected per treatment at 0, 12, 24, 48, 72, 96 and 168 h p.i. for RNA isolation. The experiment was performed three times and the data were analysed as described above.

DNA extraction and REN analysis. DNA was extracted from infected larvae that died from virus infections. OBs were purified as described by Muñoz *et al.* (1997). Virions were released from OBs by incubation in 0.1 M Na_2CO_3 for 15 min at 37 °C. DNA was extracted according to the method described by Reed *et al.* (2003). After phenol/chloroform extraction, DNA was dialysed for 48 h against 1 mM Tris/HCl, 0.1 mM EDTA (pH 8.0). For REN analysis, 1.0–1.5 µg DNA was incubated with 10 U enzyme for 3.5 h at 37 °C. Reactions were stopped at 65 °C for 10 min. The resulting DNA fragments were separated by electrophoresis in 0.7 % agarose gels containing 40 mM Tris/acetate, 1 mM EDTA (pH 8.0). After electrophoresis, the gels were stained with an ethidium bromide solution (0.5 µg ml^{-1}) for 30 min and analysed under UV light.

RNA extraction from insect larvae and detection of transcripts by qRT-PCR. Total RNA was extracted from insect larvae by using TRIpure isolation reagent (Roche Diagnostics) according to the manufacturer's protocol. The concentration and integrity of RNA samples were determined spectrophotometrically at 260 nm, as well as by agarose gel electrophoresis.

qRT-PCR was employed to determine the presence or absence of SeMNPV and AgseNPV transcripts in cross-infectivity studies. One microgram of RNA was subjected to DNase I (Invitrogen) treatment for 15 min at room temperature in a volume of 10 µl. DNase I activity was inhibited by incubation in 2.5 mM EDTA at 65 °C for 10 min. cDNA was synthesized by using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The absence of contaminating DNA was verified by performing PCR without a reverse transcription step with all sets of primers. Five microlitres of cDNA at 1 : 5 or 1 : 10 dilutions was used for qRT-PCR. All reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in a total reaction volume of 25 µl. Forward and reverse primers were added to a final concentration of 300 pM. All primers used were designed on the basis of the reported genome sequences of SeMNPV (IJkel *et al.*, 1999) and AgseNPV (Jakubowska *et al.*, 2006) and are listed in Supplementary Table S1 (available in JGV Online). Non-template controls were analysed for each set of primers in order to verify the absence of non-specific background signals. RNA isolated from mock-infected larvae served as a negative control. All reactions were performed in duplicate. Dilutions (10^{-1}–10^{-8}) of purified SeMNPV and AgseNPV DNA were used for standard curves. This DNA was quantified with a BioPhotometer (Eppendorf) and the number of target gene copies was calculated based on the DNA concentration and the molecular mass.

PCRs were performed in an ABI PRISM 7000 (Applied Biosystems). The program used for all primer sets was 15 min at 95 °C, followed by 50 cycles of 94 °C for 15 s and 60 °C for 30 s. The fluorescence signal was acquired at 60 °C. At the end of the PCRs, dissociation curves were obtained by monitoring fluorescence data while heating DNA samples slowly from 60 to 90 °C at intervals of 1 °C. Primer sets were selected based on the presence of a single melting peak, a measure for specific amplification. Moreover, cross-reactions of the SeMNPV- and AgseNPV-specific primer sets were excluded by performing reactions with both AgseNPV and SeMNPV DNA as templates.

qRT-PCR data were visualized and analysed by using the 7000 System Sequence Detection software, version 1.2.3 (Applied Biosystems). Relative quantification of gene copies was performed by applying C_t values from each reaction based on the appropriate standard curves. Note that standard curves were prepared with genomic DNA and thus the calculated number of gene copies in cDNA cannot be considered as an absolute value, as reverse transcription efficiency is not included in the calculations.

Early and late gene-specific primer sets were used to determine how far the infection proceeds. Initially, three sets of primers were designed for the detection of AgseNPV and SeMNPV transcripts, specific for the immediate-early gene *ie-1*, the early DNA polymerase gene (*DNApol*) or the very late *p10* gene. IE-1 is the principal transregulator of early gene expression, DNA polymerase is essential for baculovirus DNA replication and P10 is involved in releasing OBs from cell nuclei in the very late phase of virus infection. Due to the high similarities in the sequence of the AgseNPV and SeMNPV primer sets, the AgseNPV *DNApol* primers also amplified the

SeMNPV *DNApol* gene, and the primer set designed to amplify the SeMNPV *ie-1* gene also amplified the AgseNPV *ie-1* gene. These two primer sets have therefore been discarded and only primer sets that amplified one virus template specifically were used in the study. Primer sets amplifying *ie-1* and *p10* were used for detection of AgseNPV-specific transcripts and, for detection of SeMNPV transcripts, primer sets for *DNApol* and *p10* were used.

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