Prevalence of latent virus in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) and its activation by a heterologous virus

Lucien N'Guessan KOUASSI,¹ Katsuo TSUDA,^{1,*} Chie GOTO,² Shigeyuki MUKAWA,²
Yositaka SAKAMAKI,¹ Kanetosi KUSIGEMATI¹ and Masayuki NAKAMURA¹

¹ Laboratory of Plant Pathology and Entomology, Faculty of Agriculture, Kagoshima University; Kagoshima 890–0065, Japan

² Insect Pest Management Research Team, National Agricultural Research Center; Tsukuba, Ibaraki 305–8666, Japan

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Abstract

Nested PCR amplification of a *Spodoptera litura* nucleopolyhedrovirus (SpltNPV) *lef-8* gene fragment was performed on egg masses and larvae of *S. litura*, and resulted in detection of a latent SpltNPV in 20.0% of “healthy” laboratory stock samples, and in 22.6% of samples collected at different periods and in remote places in Kagoshima Prefecture. The PCR product sequences showed 99% similarity to the published *lef-8* sequence of SpltNPV, confirming that the amplification products were derived from SpltNPV. Further, cross-inoculation of laboratory stock *S. litura* larvae with *Mythimna separata* NPV (MyseNPV) activated the latent virus, which provoked a change in the virulence of MyseNPV samples collected from cadavers of *S. litura*. Our data suggest that caution should be taken when using *S. litura* as a host to produce heterologous NPVs.

Key words: *Spodoptera litura*; latent virus; activation; nested PCR; virulence variation

INTRODUCTION

Due to their pronounced host specificity and high virulence to susceptible insect hosts, nucleopolyhedroviruses (NPVs) of the *Baculoviridae* are being developed as biocontrol agents for lepidopteran pest insects. Before application as biopesticides, however, a range of experimental studies including identification, host range determination, and efficacy evaluation are necessary. Currently, the cheapest way to produce NPVs for such studies is by propagation through host insects, and although this is usually accomplished in the homologous host species there are occasional exceptions. For example, in Japan, the production of viruses for experimental use has been carried out in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) larvae because this insect is permissive to some viruses (Okada, 1977; Tsuda et al., 2005), is easy to rear, and is large enough to yield substantial amounts of virus for assays. However, the production of viruses in a heterologous host entails the risk that activation of a latent virus might lead to a

false interpretation of bioassay results. Latency describes the state of a virus that does not produce obvious signs of infection, including sublethal effects, but can be vertically transmitted between host generations (Hale and Margham, 1988). Concerning insects, latent virus has intrigued researchers for many years, but only with recent molecular techniques has it become possible to demonstrate with accuracy the presence of insect latent virus and understand its mechanism (reviewed by Podgwaite and Mazzone, 1986; Il'inykh and Ul'yanova, 2005).

Triggers or stress factors such as extreme temperatures, crowding, high humidity, and unsuitable diet can convert a latent to an overt infection (Fuxa et al., 1999). Although true cross-infection may occur during a cross-inoculation (El-Salamouny et al., 2003; Jakubowska et al., 2005), numerous reports have also shown that cross-inoculation testing with heterologous baculovirus isolates is the best stressor for production of a different virus progeny (i.e., the host's own virus) (Longworth and Cunningham, 1968; Maleki-Milani, 1978; Jurkovičová,

* To whom correspondence should be addressed at: E-mail: ktsuda@agri.kagoshima-u.ac.jp
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1979; McKinley et al., 1981; Cory et al., 2000). While latent viruses have been studied in several insects, nothing is known about their occurrence in *S. litura*, although it is an important pest species in Japan.

In this study, we address two issues: the first is to determine whether *S. litura* harbors latent NPV, and to evaluate the prevalence of latent infection in field insects and laboratory stock. The second is to assess the effects of latent virus when heterologous NPVs are propagated in laboratory stock of *S. litura*.

MATERIALS AND METHODS

Insects. Two types of insects were used in this study. The first type consisted of laboratory stocks of *S. litura* and *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) that were collected from fields in Kagoshima Prefecture in 1997 and 2003, respectively. Both species were reared continuously in the laboratory at ca. 25°C on artificial diet (Okada, 1977), prepared by a modified process in which dried maize leaves and soybean were used instead of dried pinto beans.

The second type were larvae and egg masses of *S. litura* collected in September, November, and December 2007 from fields in three geographical locations of Kagoshima Prefecture (Kagoshima City, Ibusuki City, Kanoya City). Egg masses, or larvae from the same egg mass, were divided into two batches: one was reared until the next generation according to the procedure described above, and the other was stored at -20°C for DNA extraction. This type represented our field-collected insects.

Viruses. The *M. separata* NPV Geihoku isolate (MyseNPV G) was discovered in Geihoku, Hiroshima Prefecture, in 1968 (Okada, 1977). MyseNPV G was propagated in larvae of *M. separata*, and the collected virus was then propagated through *S. litura*. The viruses collected from cadavers of *M. separata* and *S. litura* were named MyseNPV G (M) and MyseNPV G (S), respectively. Occlusion bodies (OBs) of these two types of viruses were purified by glycerol density gradient centrifugation (Hunter-Fujita et al., 1998), and then suspended in distilled water. OBs in the suspension were counted with a Thoma hemocytometer and stored at 4°C until use.

Extraction of DNA from insects, nested PCR amplification, and sequencing. To avoid contamination by exogenous virus or DNA, all plasticware used in this study (microtubes, homogenizers, tips, petri dishes) was disposable and sterilized before the experiments.

One egg mass or larvae from the same egg mass were considered as one sample. Laboratory stock and field-collected *S. litura* samples were ground in DNA extraction buffer (0.15 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.0), incubated at 65°C for 20 min, and centrifuged at 16,000×*g* for 10 min. DNA in the supernatant was extracted by phenol-chloroform, collected by ethanol precipitation, suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and stored at 4°C. The DNA was then used in a nested PCR reaction.

First, about 500 ng of DNA was used to amplify a 241-bp fragment of the *late expression factor-8* (*lef-8*) gene of *S. litura* NPV (SpltNPV) (GenBank Accession No. AY706581) using the primers SINPVlef-8F3 (5'-CGACGTTTTCGTGCAAAC-3') and SINPVlef-8R3 (5'-GTCGACGATCCGAGCTTT-3'), whose specificity was maximized by selecting a region which showed low sequence similarity with MyseNPV G (AB308407). The PCR amplification was performed in a reaction mixture of 10 µl using the following conditions: initial denaturation of 3 min at 94°C; 30 cycles of 20 s at 94°C, 20 s at 60°C, 45 s at 72°C; and final extension of 5 min at 72°C. The PCR products (8 µl) were separated in a 1% agarose gel along with a 100 bp ladder (Takara Bio, Japan), and the remaining 2 µl was added to a PCR mixture to amplify a nested 217-bp product of the same gene, using the conditions described above and the low-similarity-region primers SINPVlef-8Fnes (5'-ACGAAAGCATCTAGTGCG-3') and SINPVlef-8Rnes (5'-CCGAGCTTTGATTTGAGC-3'). The PCR products were then separated in an agarose gel as described above.

For DNA sequencing, fragments amplified by nested PCR were ligated into pT7Blue T-Vector (Novagen, Germany) and cloned in *Epicurian coli* XL1-Blue (Stratagene, USA). Sequencing was carried out with a Big DyeTM terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) using T7 and M13 M4 primers on an ABI Prism 3100 Genetic Analyzer apparatus (Applied Biosystems). Analysis of sequencing data

was carried out using GENETYX-MAC Network version 13.0.3 (GENETYX Corp., Japan).

Viral DNA extraction and analysis. The purified OBs isolated from dead larvae were incubated in 0.02 M NaOH for 10 min to release virions, and centrifuged at $13,000\times g$ for 30 min. Virions were disrupted by suspending the pellet in TE buffer containing 1% SDS and 200 $\mu\text{g/ml}$ proteinase K, and incubating the mixture at 55°C for 3 h. DNA was extracted as described above, and either digested with *EcoRI* (Takara Bio) for 4 h or used in a PCR reaction with the SINPVlef-8F3/SINPVlef-8R3 primer pair as described above. For the other reaction, amplification of the partial *lef-8* gene was performed according to the method of Lange et al. (2004), using degenerate primer pairs (prL8-1B, prL8-2; Jehle et al., 2006), with minor modifications. Amplification reactions were performed under touchdown PCR cycling conditions: step 1: 94°C denaturing for 3 min; step 2: 94°C for 20 s, 55°C for 20 s (ramping 3°C per 3 cycles), 72°C for 45 s (step 2 cycled 15 times); step 3: a further 20 cycles as above but without ramping, at a fixed annealing temperature of 60°C, followed by a final elongation step of 72°C for 5 min. PCR products were then sequenced and analyzed as described above.

Bioassays. Bioassays were conducted in two ways. In the first, third-instar *S. litura* and *M. separata* were challenged with both MyseNPV G (M) and MyseNPV G (S). Virus preparations were serially diluted from 10^9 to 10^2 OBs/ml, and 10- μl aliquots of four to eight virus concentrations were spread on small pieces of artificial diet in glass test tubes. Twenty-five newly molted larvae were allowed to feed individually on the contaminated diet for 48 h, and then transferred to virus-free diet. As controls, larvae were fed on artificial diet without virus suspension. All treatments were incubated at 25°C in a 14-h light and 10-h dark photoperiod. Mortality due to infection was monitored daily until death or pupation. Bioassays with MyseNPV G (M) were replicated three times in both species, and those with MyseNPV G (S) were replicated 4 times and twice in *M. separata* and *S. litura*, respectively. The median lethal concentration (LC_{50}) with the respective 95% confidence limits (CLs) was estimated for both viruses in both species by probit analyses (Finney, 1978) using the program PriProbit 1.63 (Sakuma, 1998). The ratio of LC_{50} s

between the two hosts for MyseNPV G (M) and MyseNPV G (S) was calculated by the method of Robertson and Preisler (1992).

In the second bioassay method, MyseNPV G (M) was used to infect 115 third-instar larvae of *S. litura*. Dead larvae were homogenized individually with 500 μl of distilled water. Each homogenate was inoculated into 5 third-instar *M. separata* and/or *S. litura* larvae. Mortality was recorded, and the presence of OBs in tissue smears of dead larvae was confirmed by phase contrast microscopy at $\times 400$ magnification.

RESULTS

PCR amplification and sequencing of SpltNPV *lef-8* gene fragment from *S. litura*

PCR amplification of the SpltNPV *lef-8* gene fragment was performed on egg masses and larvae of *S. litura* to detect SpltNPV in “healthy” *S. litura*. Although no product was visible in the first PCR reaction, nested PCR of several samples yielded a 217-bp fragment (Fig. 1). The PCR product was detected in egg masses and larvae of insects from laboratory stock. It was also found in field-collected samples, at all places and periods of collection (Table 1). The product was detected in 20.0% and 22.6% of laboratory stock and field-collected samples, respectively. All offspring derived from the insect batches not used for DNA extraction survived without any apparent symptoms of viral infection.

To make certain that the PCR product was not generated by *S. litura* DNA but by SpltNPV, se-

Fig. 1. Detection of SpltNPV *lef-8* 217-bp fragment by nested PCR. Lanes 1 and 2, larvae and eggs of laboratory stock *S. litura*, respectively; lanes 3 and 4, larvae and eggs of Kagoshima field-collected *S. litura*, respectively; lanes 5 and 6, larvae and eggs of Ibusuki field-collected *S. litura*, respectively; lane 7, larvae of Kanoya field-collected *S. litura*; lane 8, PCR positive control (SpltNPV DNA); lane M, 100 bp ladder size marker.

quencing of the *lef-8* nested PCR product of one sample each from laboratory stock (GenBank Accession No. AB451186) and field-collected insects (from Kanoya City) (GenBank Accession No. AB451185) was performed. BLAST searching showed 99% and 90% similarity with the published

lef-8 sequences of SpltNPV (AY706581) and SpliNPV (AY706585), respectively, confirming that the amplification product was derived from SpltNPV (Fig. 2).

To ascertain whether any naturally occurring SpltNPV on the surface of field insects might interfere with our results, five samples that proved negative for SpltNPV by nested PCR were mixed with DNA extraction buffer containing 10^2 OBs of SpltNPV. DNA extraction and PCR amplification were carried out as usual. No viral DNA was detected, proving that any virus present on the surface of insect material was not amplified and hence did not interfere with detection assays (data not shown).

Pathogenicity of MyseNPV types

Table 2 shows the relative pathogenicity of MyseNPV G (M) and MyseNPV G (S) against third-instar larvae of *M. separata* and *S. litura*. According to the 95% CLs of the LC_{50} values, while *M.*

Table 1. Detection by nested PCR of *S. litura* latent virus in *S. litura* collected at different periods and in separate localities in Kagoshima Prefecture

Period (2007)	Geographic location			Total
	Kagoshima	Ibusuki	Kanoya	
September	8/21	2/13	3/9	13/43
November	5/9	6/37	1/56	12/102
December	2/5	2/10	14/30	18/42
Total	15/35	10/60	18/95	43/190

x/y: number of samples (larvae and eggs) in which SpltNPV gene was detected/number tested.

Field	CGAAAGCATCTAGTGC	CGCATCGTGTACGAGTTTAAACGCAAACGGTTTCCCGTCGAGATC
Laboratory	CGAAAGCATCTAGTGC	CGCATCGTGTACGAGTTTAAACGCAAACGGTTTCCCGTCGAGATC
SpltNPV	CGAAAGCATCTAGTGC	CGCATCGTGTACGAGTTTAAACGCAAACGGTTTCCCGTCGAGATC
SpliNPV	CGAAAGCATCTAGT	ACGCATCGTGTACGAGTTTAAACGCAAACGGTTTCCAGTCGAGATC
Field	AAACTGTTTAAAGATAT	TCTATTGTCAATCATCACGAAGGCATGATATGTATACGCAAA
Laboratory	AAACTGTTTAAAGATAT	TCTATTGTCAATCATCACGAAGGCATGATATGTATACGCAAA
SpltNPV	AAACTGTTTAAAGATAT	TCTATTGTCAATCATCACGAAGGCATGATATGTATACGCAAA
SpliNPV	AAACTGTCCAAAAGTAT	TCTATTGTCAACCATCACGAAGGCATGATATGTATACGCAAG
Field	CATATTCTAATAAACG	ACAAAGTTAACATTTTCGGCTCTGCTCACTCCGTACGAGTACCAC
Laboratory	CATATTCTAATAAACG	ACAAAGTTAACATTTTCGGCTCTGCTCACTCCGTACGAGTACCAC
SpltNPV	CATATTCTAATAAACG	ACAAAGTTAACATTTTCGGCTCTGCTCACTCCGTACGAGTACCAC
SpliNPV	CAGGTTCCGAATAAA	TGAAAAGTAAACATTTTCGGCTCTGCTCACTCCGTACGAGTACCAC
Field	AACGGCGAATCTATT	GTTGCTCAAATCAAAGCTCGGA
Laboratory	AACGGCGAATCTATT	GTTGCTCAAATCAAAGCTCGGA
SpltNPV	AACGGCGAATCTATT	GTTGCTCAAATCAAAGCTCGGA
SpliNPV	AACGTCGAATCTAT	CATTGGCCAGATCGGAGCCCGGA

Fig. 2. Alignment of amplified SpltNPV *lef-8* 217-bp sequence from DNA extracted from laboratory stock (Laboratory) and field-collected (Field) *S. litura* with the partial nucleotide sequences of *S. litura* NPV (SpltNPV) and *S. littoralis* NPV (SpliNPV) in GenBank. Underlined letters indicate nucleotides which differ from the SpltNPV sequence.

Table 2. Dose mortality response of third instar larvae of *Spodoptera litura* and *Mythimna separata* following inoculation with MyseNPV G (M) and MyseNPV G (S)

Virus	Host	LC_{50} (95% CL) [$\times 10^6$]	Slope (SE)	χ^2 ^a	df	Ratio (95% CL) ^b
MyseNPV G (M)	<i>M. separata</i>	0.7 (0.2–2.3)	0.81 (0.11)	11.81*	4	—
	<i>S. litura</i>	31.8 (21.7–46.5)	1.08 (0.09)	1.83	3	46.1 (19.4–109.8)
MyseNPV G (S)	<i>M. separata</i>	3.8 (2.5–5.9)	0.85 (0.07)	5.89	4	—
	<i>S. litura</i>	4.6 (2.9–7.3)	1.12 (0.12)	5.93	4	1.2 (0.6–2.3)

^a Pearson Chi-square for the homogeneity test. Values followed by asterisk are significant ($p < 0.05$).

^b Ratio of LC_{50} s with respect to the virus/host combination.

Fig. 3. Examination of MyseNPV samples for the presence of SpltNPV by PCR amplification of a SpltNPV *lef-8* 241-bp fragment. Lane 1, MyseNPV G (S) DNA; lane 2, MyseNPV G (M) DNA; lane 3, DNA from occlusion bodies from a mixture of homogenates of 114 individual *S. litura* larvae that died after inoculation with MyseNPV G (M); lane 4, PCR positive control (SpltNPV DNA). Lane M, 100 bp ladder size marker.

separata was more susceptible than *S. litura* to MyseNPV G (M), the two species were almost equally susceptible to MyseNPV G (S). According to the parallelism test, probit lines for MyseNPV G (M) were not parallel ($\chi^2=8.53$, $df=1$, $p<0.05$), and this was also the case for MyseNPV G (S) ($\chi^2=4.27$, $df=1$, $p<0.05$). Ratios of LC_{50} s between the two hosts for MyseNPV G (M) and MyseNPV G (S) were 46.1 and 1.2, respectively.

Examination of MyseNPV G

The purity of each type of MyseNPV G was checked using PCR. SpltNPV was detected in MyseNPV G (S) but not in MyseNPV G (M) (Fig. 3).

Bioassays: examination of progeny virus

Larvae of *S. litura* and *M. separata* were inoculated with homogenate prepared from individual cadavers of *S. litura* infected with MyseNPV G (M) in order to verify whether the mortalities were caused by the inoculated MyseNPV G or the latent virus of *S. litura*. Among 115 homogenates made from individual dead larvae of *S. litura*, 114 caused mortality when inoculated into *M. separata*, but one failed to kill *M. separata* although it provoked death in larvae of *S. litura*. The *EcoRI* profile of the viral DNA obtained from this unique sample was similar to that of SpltNPV described by Takatsuka et al. (2003) (Fig. 4, lane 4). Sequence analy-

Fig. 4. *EcoRI* profiles of viral DNA extracted from: lane 1, MyseNPV G; lanes 2 and 3, occlusion bodies from individual *S. litura* that had died after inoculation with MyseNPV G and that caused death in *M. separata*; lane 4, occlusion bodies from individual *S. litura* that had died after inoculation of MyseNPV G and that failed to kill *M. separata*. Lane M, λ -HindIII digest.

sis of the partial *lef-8* gene of DNA from the unique sample (GenBank Accession No. AB451187) amplified by degenerate primers showed 100, 98, 97, and 59.1% identity with the *lef-8* sequences of SpltNPV-A17-3 (AY706580), SpltNPV-S37 (AY706581), SpltNPV-G2 (AF325155), and MyseNPV G (AB30847), respectively, confirming that the virus isolated from the unique sample is SpltNPV, a species different from MyseNPV G.

However, although two samples randomly chosen from the other 114 presented *EcoRI* profiles resembling that of the inoculum MyseNPV G (Fig. 4, lanes 2 and 3), a PCR reaction performed with DNA extracted from OBs of a mixture of the 114 samples revealed the presence of SpltNPV (Fig. 3, lane 3).

DISCUSSION

Spodoptera litura is permissive for several NPVs. To explore the possibility of using this insect as an alternative host to produce OBs of heterologous NPVs, our study has focused on a latent virus harbored by *S. litura* which may affect some properties of virus samples collected after propagation in larvae of the insect, particularly their viru-

lence, a very important parameter for the success of NPVs as bioinsecticides.

Latency, defined as the ability of a virus to survive in a host or host generations without causing recognizable symptoms, has been demonstrated in several lepidopteran insects (Podgwaite and Mazzone, 1986; Il'inykh and Ul'yanova, 2005). Thanks to the use of molecular techniques applied mainly to the *polyhedrin* gene (*polh*) (Cameron and Possee, 1989; Hughes et al., 1997), evidence for the presence of latent NPV infections of insects has been achieved. The combination of *polh* and *lef-8* and *-9*, two other highly conserved baculovirus genes, is a very suitable gene set to identify lepidopteran-specific NPVs (de Moreas and Maruniak, 1997; Herniou et al., 2003; Lange et al., 2004; Jehle et al., 2006). The present study could also provide information on a latent virus in *S. litura* by amplifying target SpltNPV DNA sequences. Based on detection of viral DNA by nested PCR amplification and sequencing of a *lef-8* fragment, we showed that 20% of eggs and larvae of a healthy laboratory stock of *S. litura*, reared continuously for more than 10 years, proved positive for the virus, indicating that SpltNPV exists in a latent state in *S. litura*. This result is supported by samples collected from field that showed an infection rate of 22.6% although no death was recorded in offspring derived from these collections. The virus was detected in samples collected from different geographical locations of Kagoshima Prefecture (Kagoshima City, Ibusuki City and Kanoya City), at different periods. This occurrence of the virus at various places and times permitted us to confirm our results. Our findings are similar to those of Eastwell et al. (1999), who detected latent virus in laboratory cultures, and in 23% of larvae of codling moth populations from British Columbia, using the PCR reaction. Application of PCR permitted Burden et al. (2003) to demonstrate a higher rate (50 to 100%) of latent infection in natural cabbage moth populations in England, and, similar to our results, the virus was present in insects from geographically remote regions. Moreover, they demonstrated that the viral infection rate of these insects when reared in the laboratory (under conditions without external viral contamination) usually did not decrease over the five generations during which they were observed. The percentages found in our study might reflect a similarity of infection

rates between laboratory stocks and field-collected *S. litura*, as shown for the cabbage moth, although this will need to be monitored over a longer period. Overall, we conclude that this is the first report of a latent NPV infecting *S. litura*.

The activation of latent virus by heterologous baculoviruses has been discussed over the last 100 years (see Podgwaite and Mazzone, 1986, for a review). For instance, Longworth and Cunningham (1968) demonstrated by morphological and serological analysis that latent virus had been activated in both species when larvae of *Aglaia urticae* and *Porthetria (Lymantria) dispar* were each fed the NPV of the other species. More recently, Cooper et al. (2003) demonstrated, by analyzing DNA profiles of viruses resulting from cross-inoculation of the forest tent caterpillar *Malacosoma disstria* with the western tent caterpillar *M. californicum pluviale* NPV (McpINPV), that 88% of these infections were caused by *M. disstria* NPV (MadiNPV) rather than McpINPV. Our study indicated that a similar phenomenon can occur as a result of propagation of MyseNPV through *S. litura*. Although no SpltNPV genes were detected in the MyseNPV G inoculum, the presence of SpltNPV genes in the OBs of the virus sample obtained after passage through *S. litura* larvae suggested that activation of SpltNPV by MyseNPV G had occurred in *S. litura*. Our hypothesis was confirmed by sequence analysis and by the *EcoRI* profile similarity between SpltNPV isolates and that of OBs collected from a *S. litura* larva that died after inoculation with MyseNPV G.

Moreover, the virulence of the heterologous virus MyseNPV G propagated through *S. litura* (MyseNPV G (S)) was different from that of the same virus propagated through the homologous host *M. separata* (MyseNPV G (M)). The virus became more infectious to *S. litura* and less infectious to *M. separata* when propagation occurred through this insect, as indicated by the ratios of LC_{50} s between the two hosts for MyseNPV G (M) and MyseNPV G (S), which were 46.1 and 1.2, respectively. The non-pathogenicity to *M. separata* of the unique sample obtained after inoculation of *S. litura* with MyseNPV G suggested that activation of a latent virus was responsible for the change in virulence of the MyseNPV G sample following propagation through *S. litura*. This phenomenon might be the origin of the variability in LC_{50} s in

the results of Tsuda et al. (2005), Okada (1977), and ours (unpublished data). The LC₅₀s of MyseNPV G recorded by Tsuda et al. (2005) and Okada (1977), who propagated MyseNPV G through *S. litura*, were about 10⁴ OBs/ml, while the LC₅₀ of MyseNPV G propagated through *M. separata* was about 10⁷ OBs/ml against second-instar *S. litura* (unpublished data). This variability is not surprising, since SpltNPV has been shown to be ~1,000-fold more pathogenic than MyseNPV G against *S. litura* (unpublished data).

The use of *S. litura* as an alternative host to produce heterologous NPVs should therefore be reconsidered; at least, it is important to confirm (preferably by nested PCR) the virus-free nature of any *S. litura*, above all in eggs, that are to be used for heterologous NPV production.

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