In vivo characterization of a group II nucleopolyhedrovirus isolated from Mamestra brassicae (Lepidoptera: Noctuidae) in Japan

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A Japanese isolate of Mamestra brassicae nucleopolyhedrovirus (MabrNPV) was identified phylogenetically as a group II nucleopolyhedrovirus (NPV) that is related closely to other NPVs isolated from Mamestra spp. based on nucleotide sequence data of its polh, egt and lef-3 genes. The multiplication of MabrNPV in M. brassicae larvae was characterized following inoculation at various doses and in combination with the fluorescent brightener Tinopal by measuring temporal changes in the concentrations of its viral DNA using real-time quantitative PCR. The growth curves of budded-virus replication were analysed by fitting the data of viral DNA concentration in the host haemolymph to a modified Gompertz model. When fifth-instar larvae were inoculated with an LD\textsubscript{95} equivalent dose of MabrNPV and Tinopal, the time lag between the onset of primary and secondary infection was estimated to be 25 h. Another 65 h was required to reach a plateau titre equivalent to a level of 10\textsuperscript{8} virions ml\textsuperscript{-1} in the haemolymph. All larvae died during the sixth instar following this inoculation regime. In contrast, following inoculation with a 1000-fold higher dose of MabrNPV and Tinopal, the time lag between the onset of primary and secondary infection was only 20 h. Subsequently, the same plateau titre was reached after a further 20 h. Following this inoculation regime, most larvae died during the fifth instar. Quantification of viral DNA by real-time quantitative PCR and application of the Gompertz model are valuable for the characterization of baculovirus replication in vivo.

INTRODUCTION

Baculoviruses, including nucleopolyhedroviruses (NPVs) and granuloviruses, are large, dsDNA viruses that are specific for invertebrate hosts, mainly lepidopteran larvae. Baculoviruses have evolved a biphasic life cycle during which they produce genetically identical progeny occlusion-derived virus (ODV) and budded virus (BV). ODVs are found in occlusion bodies and initiate primary infection in midgut cells upon ingestion by a susceptible host. BVs released by the midgut cells initiate secondary infections in other cells throughout the host.

Lepidopteran NPVs can be divided phylogenetically into two groups, I and II (Zanotto et al., 1993; Herniou et al., 2001). The infection process of NPVs, including in vivo and in vitro multiplication, has been characterized mainly using the group I NPVs, such as Autographa californica NPV (AcMNPV), Orgyia pseudotsugata NPV (OpMNPV) and Bombyx mori NPV (BmNPV) (e.g. Williams & Faulkner, 1997; Bonning, 2005). BVs of the group I NPVs contain a glycoprotein, GP64, an envelope-fusion protein required for the spread of infection to other cells and for efficient budding from the cell surface (Monsma et al., 1996; Oomens & Blissard, 1999). In contrast, BVs of group II NPVs lack GP64, but possess in its place the F protein (Lung et al., 2003). Thus, it is possible that functional differences between these fusion proteins may lead to differences between the multiplication strategies of the group I and group II NPVs in the host haemocoel. Several studies of the multiplication of the group II NPVs, such as Lymantria dispar NPV (LdMNPV; Riegel & Slavicek, 1997), Helicoverpa zea NPV (HzSNPV; Granados et al., 1981), Mamestra brassicae NPV (MabrNPV; Kondo et al., 1995), Spodoptera exigua NPV (SeMNPV; Hara et al., 1994) and Spodoptera frugiperda NPV (Knudson & Tinsley, 1974), have been performed in insect cell lines. These studies, however, are limited in comparison with those of group I NPVs. In terms of in vivo studies, there are several reports on the lethal effect and the survival time of the host larvae using recombinants of LdMNPV (Slavicek et al., 1999), SeMNPV (Dai et al., 2000) and Helicoverpa armigera NPV (Sun et al., 2002, 2004) and on the infection process of HzSNPV (Washburn et al., 2001, 2003), but only a few reports on the multiplication of other group II NPVs (Evans et al., 1981).

The cabbage armyworm, M. brassicae L., is an important insect pest of vegetables and ornamental plants in Europe and Asia. Several NPVs have been isolated from M. brassicae over wide regions of Europe and Japan (Aruga et al., 1981;...
Okada, 1977; Vlak & Gröner, 1980; Brown et al., 1981). These viruses, named MabrNPV, have been considered useful biological-control agents for *M. brassicae* (Akutsu, 1972; Brown et al., 1981; Evans & Allaway, 1983). There are several reports of the identification and characterization of MabrNPV strains isolated in Europe (Kelly & Brown, 1980; Vlak & Gröner, 1980; Erlandson, 1990; Clarke et al., 1996; Rovesti et al., 2000), as well as NPVs isolated from *Mamestra configurata*, a species related closely to *M. brassicae*, in North America (Erlandson, 1990; Li et al., 2002, 2005). However, Japanese strains of MabrNPV are poorly characterized, especially at the molecular level. For the practical use of NPVs as a control agent of noctuid pests, it is necessary to identify and characterize the virus strain in advance. The objective of our study was the phylogenetic and phenotypic characterization of a MabrNPV isolate derived from a diseased larva of *M. brassicae* found in Tokyo, Japan.

In order to gain a better understanding of the multiplication of MabrNPV in vivo, we measured the increase in MabrNPV DNAs over time in the haemolymph of *M. brassicae* by using real-time quantitative PCR (RTQ-PCR). RTQ-PCR has been utilized as a rapid and simple method for the detection of baculovirus titres in *vitro* (Rosinski et al., 2002; Lo & Chao, 2004; Vanarsdall et al., 2005) and we confirmed the effectiveness of this method for *in vivo* studies. As the baculovirus virion is generally composed of a single nucleocapsid (Williams & Faulkner, 1997), we assumed that there was a direct correlation between the amount of viral genomic DNA and the number of BVs. Moreover, the ability of stibene-derived fluorescent brightener to enhance the infection of MabrNPV was tested. In this report, we elucidated the multiplication of MabrNPV in the host haemolymph with two factors of virus growth: the time taken from primary infection to secondary infection and the trend of BV increase following low and extremely high inoculation doses of ODVs.

**METHODS**

**Insect, virus and fluorescent brightener.** *M. brassicae* was collected in the field in Tsukuba, Japan, and maintained continuously on an artificial diet, Insecta LFS (Nihon Nosan-Kogyo Co., Ltd), in our laboratory for more than 10 years. All experiments were conducted at 25 °C under a 16 h light/8 h dark photoperiod.

MabrNPV T strain (Aruga et al., 1960; Akutsu, 1972) was obtained from Dr Kisaku Akutsu at the Tokyo Metropolitan Agricultural Experiment Station. MabrNPV T was propagated in larvae of *M. brassicae* and purified basically as described previously (Kunimi & Yamada, 1990) but using 10 mM Tris/HCl (pH 7.5) instead of PBS. The concentration of polyhedral inclusion bodies (PIBs) in the stock suspension was determined by using a Thoma haemocytometer under a phase-contrast microscope. PIBs were stored at 4 °C until use. Although no submolar bands were observed in the original MabrNPV obtained from Dr Akutsu by restriction-endonuclease analysis, an isolate named T5 was newly isolated from MabrNPV T by using conventional methods of low-dose inoculation (Smith & Crook, 1988) for five passages in larvae of *M. brassicae* and used for DNA sequencing.

The ability of the fluorescent brightener Tinopal (Fluorescent brightener 28, Tinopal UNPA-GX; Sigma) to enhance the infectivity of MabrNPV T was tested at a final concentration of 1 % (w/w).

**Identification and sequencing of the polh, egt and lef-3 genes of MabrNPV T.** DNA was extracted from PIBs of MabrNPV T5 basically as described by Goto et al. (1992). Genomic DNA from MabrNPV T5 was digested with HindIII or EcoRV and ligated into pBluescript II KS (+) and transformed into *Escherichia coli* JM109 competent cells. DNA sequencing was conducted by using an ABI PRISM BigDye Terminator v1.1 Cycle Sequencing kit and a capillary sequencer ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The individual viral DNA fragments were sequenced by using universal or M13 primers. For the amplification and sequencing of the internal regions of the polyhedrin (polh) or ecysstoid UDP-glucosyltransferase (egt) genes, specific or degenerate primers were synthesized as follows: PFS002 (5′-GGIICGGYAAAYCAAC-3′), PER001 (5′-GGIRCTCGGGCRAAYTCYTT-3′) and P1172R (5′-CGGTTCAGAAGTGCACAA-3′) for polh, and EGT + 98, EGT + 1363, EGT + 1089D and EGT + 5127D (Clarke et al., 1996 with slight modifications); Nakai et al., 2002) for egt.

Homologous sequences were carried out by using the updated DDBJ/EMBL/GenBank database via the NCBI website and the BLAST algorithm (Altschul et al., 1990). Nucleotide sequences of the NPVs used in this comparison were as follows: AcMNPV (GenBank accession no. L22858), BmNPV (GenBank no. L33180), *Choristoneura fumiferana* NPV (*CfMNPV*; GenBank no. AFS12031), *Epiphysis postvittana* NPV (*EppNPV*; GenBank no. AY104326), OpMPNV (GenBank no. U75930), *Adoxophyus heteronematus* NPV (*AhnDPNV*; GenBank no. AP006270), HzSNPV (GenBank no. AF334030), LdMNPV (GenBank no. AF081810), *Mamestra configurata* NPV strain 90/2 (MacoNPV A; GenBank no. AF67808), *M. configurata* NPV B (MacoNPV B; GenBank no. AJ126275), SmMNPV (GenBank no. AF196823), *Spodoptera litura* NPV (GenBank no. AF325155), and *M. brassicae* NPV strain Oxford (MabrNPV Oxford; GenBank no. M20927 for polh and U41999 for egt). Pairwise and multiple sequence alignments were performed by using CLUSTAL W (Thompson et al., 1994) implemented in the BioEdit program, version 7.0.1 (Hall, 1999). Neighbour-joining (NJ) distance, maximum-parsimony (MP) and minimum-evolution (ME) analyses (1000 bootstrap replicates) were inferred from the amino acid sequence alignments by using MEGA version 2.1 (Nei & Kumar, 2000). Introduced gaps were treated as missing data and MP trees were analysed by a max-mini branch-and-bound search.

**Droplet-feeding bioassay.** Larvae of *M. brassicae* were inoculated by a modified droplet-feeding method (Kunimi & Fuxa, 1996). Fourth-instar larvae at a molting stage (i.e. head capsule slippage) were selected and transferred to 0–6 ml plastic tubes without food. Within 24 h of molting, each fifth-instar larva was inoculated with 3–2 μl of a solution containing 10 % (w/v) sucrose and 1 % (w/v) red food colouring (Ogura Shokuhin kakou) in 10 mM Tris/HCl (pH 7.5), as well as PIBs and 1 % (w/v) Tinopal as appropriate. Larvae that completely ingested the droplet were transferred to 24 ml plastic cups and reared individually on a virus-free diet. The concentrations of MabrNPV used in the experiments were 10^5, 10^6, 10^5, 10^6, 10^6, 10^5, 10^5, 10^6, 10^6 and 10^9 PIBs ml⁻¹ without Tinopal, and 10^5, 10^5, 10^5, 10^5 and 10^5 PIBs ml⁻¹ with Tinopal. Experiments were replicated three times with 28–36 larvae per treatment. Larvae were observed daily for mortality until 21 days after inoculation. Tissue smears were prepared from dead larvae and examined for the presence of PIBs by using a phase-contrast microscope.

In order to determine the lethal times following inoculation at an LD₉₅ equivalent dose (i.e. 1·1×10⁵ PIBs per larva without Tinopal and 5·8×10⁵ PIBs per larva with Tinopal) or at a 1000-fold LD₉₅ dose (i.e. 5·8×10⁶ PIBs per larva with Tinopal), 47–48 larvae per treatment were used and checked at 12 h intervals after inoculation. Replicate inoculations were performed with 23–24 larvae per treatment in order to determine larval body weight at 120 h post-inoculation (p.i.).
Collection of haemolymph. Newly moulted fifth-instar larvae were inoculated with MabrNPV alone or MabrNPV and 1% (w/v) Tinopal at an LD$_{95}$ equivalent dose or at a 1000-fold LD$_{95}$ dose with 1% (w/v) Tinopal as described above. Cell-free haemolymph was prepared basically as described by Granados & Lawler (1981). Haemolymph was collected from individual larvae at 4 h intervals from 12 to 80 h p.i. and at 90, 100, 110 and 120 h p.i. by snipping off a larval proleg. Ten microlitres of haemolymph was mixed with 190 µl PBS in a microfuge tube and centrifuged at 2000 g for 10 min to pellet cells. Subsequently, 100 µl supernatant (equivalent to 5 µl haemolymph) was used for DNA extraction for RTQ-PCR. The supernatant was added to 500 µl lysis buffer [0-6% (w/v) SDS, 12 mM Tris/HCl (pH 8.0), 12 mM EDTA] incubated overnight at room temperature and stored at $-20^\circ$C until DNA extraction. At each time point, haemolymph was collected from six larvae inoculated with MabrNPV alone and from three larvae inoculated with MabrNPV and Tinopal.

DNA preparation for RTQ-PCR. Viral DNAs were extracted from BVs in the haemolymph of infected insects as described by Schlink & Reski (2002) with some modifications. Specifically, 3 µl of a 4 mg ml$^{-1}$ solution of RNase A (>100 U ml$^{-1}$) was added to the haemolymph in lysis buffer and incubated at 37°C for 30 min. After cooling to room temperature, 200 µl 4 M ammonium acetate was added and the precipitated proteins were pelleted by centrifugation at 15000 g for 4 min. In order to precipitate the viral DNAs, 2-propanol (600 µl) and glycogen (1 µl of a 20 mg ml$^{-1}$ solution) were added to the supernatant. The DNAs were pelleted by centrifugation (15000 g for 3 min), washed with 70% ethanol, dried, redissolved in 20 µl sterilized Milli-Q water (Millipore) and stored at $-20^\circ$C until use.

Quantification of MabrNPV viral DNA by RTQ-PCR. RTQ-PCR was performed by using a DNA Engine Opticon 2 System (MJ Research). The reaction mixture (10 µl) contained 4 µl DNA, 5 µl DyNAme Master Mix with SYBR Green (Finnzymes) and 0.5 µM each MabrNPV polyhedrin primer. The MabrNPV primers (forward primer, 5’-AACATGTGAAGCCGCCAGACCA-3’; reverse primer, 5’-AGAGCATGTTGAGCCAGGAA-3’) were designed on the basis of the polh gene of MabrNPV T5 to amplify a 195 bp product. All of the RTQ-PCRs were carried out under the same conditions: 95°C for 10 min, followed by 38 cycles of 95°C for 10 s and 64°C for 50 s. Known dilutions of viral DNAs purified as described above from MabrNPV PIBs were used as internal standards for each RTQ-PCR. Agarose-gel electrophoresis and thermal denaturation (melting-curve analysis) were performed to confirm specific replica formation.

Data analysis. Probit analysis (Finney, 1978) by using the computer program SPSS, version 11.5.1 (SPSS Inc.), was used to analyse mortality data. A log-normal distribution was assumed for the data of the lethal time. JMP software, version 5.0.1 (SAS Institute, Inc.), was used to analyse the significance of differences among survival times by using the parametric survival analysis and to make pairwise comparisons by using the log-rank test of the Kaplan–Meier method with a Bonferroni adjustment (Sokal & Rohlf, 1981). The significance of differences in larval body weights was determined using a Kruskal–Wallis test of the JMP software suite. Pairwise comparisons of larval body weights were also determined using a Wilcoxon test with a Bonferroni adjustment (Sokal & Rohlf, 1981). The amount of viral DNA in the haemolymph of infected hosts was used to estimate the concentration of BVs by a modified Gompertz model (Zwietering et al., 1990):

$$\log_{10} \text{[viral DNA copies (µl haemolymph)]}^{-1} + 1 = A \exp\left( - \exp\left( \frac{\mu_{o}/c}{A} (L - \lambda) + 1 \right) \right)$$

where $A$ is the common logarithm of the maximum BV concentration in the haemolymph, $\mu_{o}$ is the maximum rate of increase and $\lambda$ is the intercept of the growth curve with the $x$ axis. This intercept indicates the starting point of viral increase, namely the duration of the time lag between the onset of primary and secondary infection.

One copy of viral genomic DNA was calculated to be $1 \times 10^{-7}$ ng, assuming that the size of the dsDNA genome of MabrNPV is 155 kbp. In this study, the RTQ-PCR detection limit was regarded as a measurement below 10 copies per reaction, i.e. 10 copies (µl haemolymph)$^{-1}$. Values below the detection limit were regarded as zero in order to calculate the parameters of the Gompertz model. The equation parameters were estimated with the JMP software suite.

RESULTS

Phylogenetic information from the polh, egt and lef-3 genes

Preliminary shotgun sequencing generated approximately 20.5 kbp of nucleotide sequence data (data not shown), including 1874 bp of the lef-3 region (GenBank no. AB198075). Two additional regions of the MabrNPV T5 genome containing the polh and egt genes, consisting of 1900 bp (GenBank no. AB198073) and 2073 bp (GenBank no. AB198074), respectively, were also sequenced specifically. In order to elucidate the genetic relationships between MabrNPV T5 and other lepidopteran NPVs, phylogenetic analyses were performed by using the deduced amino acid sequences of polh, egt and lef-3. A typical phylogenetic tree for the egt gene is shown in Fig. 1. All of the trees demonstrated that MabrNPV T5 was a member of the group II NPVs and was related closely to NPVs isolated from Mamestra spp.

Alignment of the coding sequences of polh (741 bp), egt (1551 bp) and lef-3 (1182 bp) of MabrNPV T5 showed 99% identities to the corresponding genes of MacoNPV B and MabrNPV Oxford (with the exception of the lef-3 gene of MabrNPV Oxford, which was not available for comparison). The deduced amino acid sequence of MabrNPV T5 polh showed 100 and 99% identity to the polyhedrin sequences of MacoNPV B and MabrNPV Oxford, respectively. The deduced amino acid sequence of MabrNPV T5 egt showed 99 and 95% identity to the EGT proteins of MacoNPV B and MabrNPV Oxford, respectively. The alignment of nucleotide and deduced amino acid sequences of the egt genes of these three NPVs revealed that MabrNPV Oxford had a start codon at a position 36 nt upstream from those of the other two NPVs (Fig. 2a, b). Additionally, two point mutations in the middle of the ORF resulted in two frame-shift mutations (Fig. 2c, d). The deduced amino acid sequence of MabrNPV T5 lef-3 showed 98% identity to the corresponding sequence of MacoNPV B.

Effect of Tinopal on the virulence of MabrNPV

The addition of Tinopal to the inoculum greatly reduced the LD$_{95}$ values of MabrNPV from $9 \times 10^{5}$ to $5 \times 10^{6}$ PIBs per larva, from $6 \times 10^{4}$ to $9 \times 10^{5}$ PIBs per larva and from $1 \times 10^{6}$ to $9 \times 10^{6}$ PIBs per larva, respectively, in each trial. The LD$_{95}$ values were also reduced from $9 \times 10^{5}$ to $3 \times 10^{4}$ PIBs per larva, from $2 \times 10^{5}$ to $7 \times 10^{5}$ PIBs.
per larva and from $4.1 \times 10^5$ to $6.0 \times 10^4$ PIBs per larva, respectively (Table 1). In all of the trials, the slopes of the probit mortality lines became steeper with the addition of Tinopal to the inoculum. In the second and third trials, the addition of Tinopal resulted in significantly steeper slopes in comparison with inoculation of MabrNPV alone (parallelism test: $x^2 = 9.50, P = 0.002$; and $x^2 = 19.35, P < 0.001$, respectively). The addition of Tinopal did not cause any significant change in the slope of the probit mortality line in the first trial (parallelism test: $x^2 = 1.83, P = 0.176$).

The survival time and effect of MabrNPV infection on larval development were determined following inoculation with an LD$_{95}$ equivalent dose of MabrNPV alone (‘NPV alone’) or MabrNPV in combination with Tinopal (‘NPV + Tinopal’). In addition, the survival time was determined following inoculation with a dose that was 1000-fold higher than the LD$_{95}$ dose of MabrNPV in combination with Tinopal (‘high NPV + Tinopal’). The median survival times of larvae inoculated with ‘NPV alone’, ‘NPV + Tinopal’ and ‘high NPV + Tinopal’ were estimated to be 7.0, 8.0 and 6.5 days, respectively. Following the ‘NPV alone’ treatment, larval death occurred in a wide range of 4.5–8.5 days after inoculation. Following the ‘NPV + Tinopal’ and ‘high NPV + Tinopal’ treatments, larval death was observed from 7.5 to 9.0 days and from 6.0 to 7.5 days after inoculation, respectively. By the parametric survival analysis, the progression rates to death differed significantly among all of the treatments (likelihood-ratio test: d.f. = 2, $x^2 = 140.9, P < 0.01$). Following the ‘NPV alone’ treatment, 42.6 % of larvae died.

![Fig. 1. NJ distance tree based on deduced amino acid sequences of the egt gene. Numbers above or below the nodes indicate bootstrap values obtained for MP, NJ distance and ME analyses, respectively. NCBI Protein database accession numbers are shown in parentheses.](image-url)

![Fig. 2. Comparison of egt genes of NPVs isolated from Mamestra spp. Alignment of amino acid (a, c) and the corresponding nucleotide (b, d) sequences of the variable region. Dots indicate sequences where amino acids or bases are identical to those of MabrNPV T5 and dashes indicate gaps in the alignment. The numbers above the sequence indicate the length from the predicted start codon of MabrNPV T5. Methionine and the corresponding codon are highlighted in shaded boxes (a, b). The boxed amino acids correspond to the boxed nucleotide sequences (c, d) and the gaps indicated by arrowheads cause a shift of the reading frame.](image-url)
Table 1. Log-dose-probit parameters for MabrNPV with or without Tinopal against *M. brassicae* fifth-instar larvae

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope ± SE</th>
<th>Intercept ± SEM</th>
<th>No. PIBs per larva</th>
<th>χ² (d.f.)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>LD₉₀</td>
<td>95% CL</td>
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<td>Trial 1</td>
<td></td>
<td></td>
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<tr>
<td>MabrNPV alone</td>
<td>1.63 ± 0.17</td>
<td>-3.10 ± 0.87</td>
<td>9.1 × 10⁴</td>
<td>6.6 × 10³–1.3 × 10⁵</td>
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<td>MabrNPV + Tinopal</td>
<td>2.03 ± 0.24</td>
<td>3.48 ± 0.21</td>
<td>5.6 × 10⁵</td>
<td>2.8 × 10⁴–1.2 × 10⁵</td>
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<td>Trial 2</td>
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<tr>
<td>MabrNPV alone</td>
<td>1.72 ± 0.11</td>
<td>0.08 ± 0.52</td>
<td>6.6 × 10⁴</td>
<td>2.7 × 10⁴–1.7 × 10⁵</td>
</tr>
<tr>
<td>MabrNPV + Tinopal</td>
<td>1.77 ± 0.22</td>
<td>3.28 ± 0.26</td>
<td>9.3 × 10⁵</td>
<td>2.0 × 10⁴–3.3 × 10⁵</td>
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<tr>
<td>Trial 3</td>
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<tr>
<td>MabrNPV alone</td>
<td>1.14 ± 0.12</td>
<td>0.27 ± 0.49</td>
<td>1.5 × 10⁵</td>
<td>5.2 × 10⁴–4.7 × 10⁵</td>
</tr>
<tr>
<td>MabrNPV + Tinopal</td>
<td>2.04 ± 0.24</td>
<td>3.03 ± 0.25</td>
<td>9.3 × 10⁵</td>
<td>3.1 × 10⁴–3.5 × 10⁵</td>
</tr>
</tbody>
</table>

during the fifth instar, whereas the remainder died during the sixth instar. Following the ‘NPV + Tinopal’ treatment, all of the larvae died during the sixth instar, and 95.8% of larvae died during the fifth instar following the ‘high NPV + Tinopal’ treatment.

The body weights of larvae inoculated with MabrNPV alone or a combination of MabrNPV and Tinopal are shown in Fig. 3. The median body weight of control larvae (i.e. no NPV or Tinopal treatment) was 873 mg. The median body weight of larvae treated with Tinopal was 879 mg, indicating that Tinopal was not toxic to larvae. The body weights of larvae inoculated with virus were significantly lower than those of the control or Tinopal-treated larvae. The median body weight of ‘NPV alone’, ‘NPV + Tinopal’ and ‘high NPV + Tinopal’-treated larvae was 442, 611 and 180 mg, respectively. The body weight of larvae treated with ‘NPV alone’ was significantly lower than that with the ‘NPV + Tinopal’ treatment and significantly higher than that with the ‘high NPV + Tinopal’ treatment.

**Multiplication of MabrNPV in the larval haemolymph**

In order to analyse the *in vivo* multiplication of MabrNPV, the amount of viral DNA in the haemolymph of infected larvae was quantified by RT-Q-PCR and these values were fitted to a modified Gompertz model in order to generate virus-growth curves (Fig. 4). Three parameters \(A, \mu_m\) and \(\lambda\); the units of these parameters are \(\log_{10}(\text{copies \, \mu l}^{-1})\), \(\log_{10}(\text{copies \, \mu l}^{-1})\) h⁻¹, and h, respectively) that determine the shape of the curves were estimated in order to fit the model (Table 2). Following the ‘NPV alone’ treatment, larvae were divided into ‘sixth-instar type’ and ‘fifth-instar type’ at 52 h p.i. and subsequent times, as the larval stage could be distinguished on the basis of head capsule slippage (Fig. 4a). Ecdysis of ‘sixth-instar type’ larvae treated with ‘NPV alone’ and ‘NPV + Tinopal’ was observed at 68 and 72 h p.i. The parameters of the \(A, \mu_m\) and \(\lambda\) values for the ‘NPV alone’ treatment were estimated separately for the ‘sixth-instar type’ and ‘fifth-instar type’ larvae after 52 h p.i., whereas they were determined together before 48 h p.i. Estimates of the \(A, \mu_m\) and \(\lambda\) values for the ‘sixth-instar type’ larvae were 6.14, 0.137 and 15.3, respectively, and 6.69, 0.161 and 17.6, respectively, for the ‘fifth-instar type’ larvae. The BV growth curve following the ‘NPV + Tinopal’ treatment is shown in Fig. 4(b), and estimates of \(A, \mu_m\) and \(\lambda\) were 6.12, 0.145 and 26.5, respectively. The BV growth curve following the ‘high NPV + Tinopal’ treatment is shown in Fig. 4(c), and estimates of \(A, \mu_m\) and \(\lambda\) were 6.35, 0.441 and 18.7, respectively.

**DISCUSSION**

Analysis of the *polh*, *egt* and *lef-3* genes suggested strongly that MabrNPV T is a group II NPV and that it is related closely to other NPVs isolated from *Mamestra* spp., especially MacoNPV B and MabrNPV Oxford. We also confirmed similarity among these NPVs with an analysis of

![Fig. 3. Box plot of body weight of *M. brassicae* larvae at 120 h after inoculation of MabrNPV with or without Tinopal. The horizontal line in the box indicates the median value of body weight with each treatment. The upper and lower hinges of the box indicate the 75th and 25th percentiles of the data-set, respectively. The ends of the vertical lines indicate the minimum and maximum data values. Treatments with different letters are significantly different (Kruskal–Wallis test: d.f. = 4, \(\chi^2 = 99.17\), \(P < 0.05\)).](image-url)
the partial lef-8 and lef-9 genes, using the method described by Lange et al. (2004) and Jehle et al. (2006). The phylogenetic analysis of amino acid sequences of the egt gene demonstrated clearly that MabrNPV T was related more closely to MacoNPV B than to MabrNPV Oxford, although their nucleotide sequences showed 99% identity. We found that differences in the amino acid sequences of EGT of MabrNPV Oxford and the other two NPVs resulted from

**Table 2. Growth parameters of MabrNPV viral DNA in M. brassicae larval haemolymph fitted to the modified Gompertz model**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Instar type*</th>
<th>Parameter of the model†</th>
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<tr>
<td></td>
<td></td>
<td>A</td>
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<td></td>
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<td>Value</td>
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<tr>
<td>NPV alone‡</td>
<td>Sixth§</td>
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<td></td>
<td>Fifth‖</td>
<td>6·69</td>
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<tr>
<td>NPV + Tinopal‡</td>
<td>Sixth§</td>
<td>6·12</td>
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<tr>
<td>High NPV + Tinopal§</td>
<td>Fifth‖</td>
<td>6·35</td>
</tr>
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*Larval-instar type was divided into fifth or sixth from head capsule slippage before moulting.
†A, \( \log_{10} \) (maximum concentration of viral DNA in the host haemolymph); \( \mu_m \), maximum rate of viral increase; \( \lambda \), time lag between the onset of primary and secondary infection.
‡Inoculation with an LD\(_{95}\) equivalent dose.
§The growth curve was estimated with the measurements collected from fifth-instar moulting stage and sixth-instar larva at 52 h p.i. and after, and with those taken together before 48 h p.i.
‖The growth curve was estimated with the measurements collected from fifth instars at 52 h p.i. and after, and with those taken together before 48 h p.i.
§§Inoculation with a 1000-fold LD\(_{95}\) equivalent dose.

**Fig. 4.** Temporal changes in concentration of viral DNA of MabrNPV in the cell-free haemolymph of *M. brassicae* larvae after inoculation. Fifth-instar larvae were inoculated with MabrNPV at an LD\(_{95}\) equivalent dose (a), MabrNPV at an LD\(_{95}\) equivalent dose with Tinopal (b), or MabrNPV at a 1000-fold LD\(_{95}\) equivalent dose with Tinopal (c). ○, Data from larvae in fifth instar before moulting stage; ●, data from larvae in fifth moulting stage or sixth instar. Solid lines indicate the fitting curve estimated from the modified Gompertz model. Dotted lines indicate the 95% CL of the regression curves. For the ‘NPV alone’ treatment, the growth curves were estimated from ‘sixth-instar type’ [a(i)] and ‘fifth-instar type’ [a(ii)] data, respectively.
point mutations within the *egt* gene of MabrNPV Oxford. These results and our data from shotgun sequencing suggested strongly that MabrNPV T is a genetic variant of MacoNPV B and possibly of MabrNPV Oxford. It is interesting that MabrNPV T isolated from Japan appeared to be related closely to MacoNPV B and MabrNPV Oxford, which were isolated from Canada (Li *et al.*, 2002) and Europe (Doyle *et al.*, 1990), respectively.

We demonstrated that the susceptibility of *M. brassicae* to MabrNPV was increased dramatically when Tinopal was added to the inoculum. The LD\(_{95}\) values of the three trials varied in a relatively broad range from 4.1 \(\times\) 10\(^5\) to 2.0 \(\times\) 10\(^6\) (4-9-fold) following inoculation with MabrNPV alone, and in a relatively narrow range from 3.7 \(\times\) 10\(^1\) to 7.9 \(\times\) 10\(^1\) (2-1-fold) following inoculation with MabrNPV and Tinopal. These results suggested that the LD\(_{95}\) values of MabrNPV are considerably stable, especially with Tinopal. When larvae were inoculated with MabrNPV alone, the slope of the probit mortality line was flat, suggesting that the larvae were quite variable in their susceptibility to MabrNPV. The differences in weight gain and multiple larval stages (fifth and sixth instar) during which the larvae died were consistent with this variability. In contrast, the steep slope of the probit mortality line following inoculation with MabrNPV and Tinopal suggested that the addition of Tinopal diminished the variability in larval susceptibility. Previous studies (Wang & Granados, 2000; Mukawa *et al.*, 2003; Okuno *et al.*, 2003) have reported that the ingestion of a fluorescent brightener inhibits the formation of the peritrophic membrane, which serves as a barrier to pathogenic micro-organisms of the host midgut. The mechanism of viral enhancement by Tinopal may involve alteration of the peritrophic membrane permeability, allowing a large number of ODVs to reach the midgut epithelium cells (Mukawa *et al.*, 2003; Okuno *et al.*, 2003). In addition, Washburn *et al.* (1998) suggested that fluorescent brighteners block the sloughing of infected midgut epithelial cells, which results in decreased larval midgut resistance and increased mortality.

We hypothesize that the addition of Tinopal allows primary infection to initiate with the minimum number of ODVs such that a relatively low virus titre can initiate a systemic infection. Following inoculation with ‘NPV + Tinopal’, larvae survived longer, grew heavier and died during the sixth instar, and the haemolymph concentrations of viral DNAs were relatively low, especially during the early stages of infection. In contrast, following the ‘high NPV + Tinopal’ treatment, the lethal time and weight gain were reduced and death occurred during the fifth instar. The concentration of viral DNAs also increased rapidly and reached a plateau within a short period of time. Following inoculation at an LD\(_{95}\) equivalent dose without Tinopal, the concentration of viral DNAs varied widely and could be divided into two groups corresponding to ‘fifth-instar type’ and ‘sixth-instar type’ larvae. These results suggested that the level of viral multiplication in haemolymph is related directly to survival time, weight gain and the stage of death. Therefore, we concluded that Tinopal overcomes the midgut defence against the virus and that the primary infection was initiated uniformly with small numbers in the case of ‘NPV + Tinopal’ inoculation or with large numbers of ODVs in the case of ‘high NPV + Tinopal’ inoculation. The data from the ‘NPV alone’ treatment suggested that the primary infection was initiated with a variable number of ODVs.

In order to estimate the rate of virus multiplication *in vivo*, we fitted the data of the concentration of MabrNPV genomic DNA in the haemolymph to the modified Gompertz model. Two reports have adopted the Gompertz model to study the multiplication of NPVs (van Beek *et al.*, 2000; Ishii *et al.*, 2002), but a detailed explanation of the biological significance of the three Gompertz parameters (\(A, \mu_l\) and \(\lambda\)) that determine the shape of the curve has not been discussed. In our study, the \(\lambda\) parameter, a measure of time lag between the onset of primary and secondary infection, was estimated to be 26.5 and 18.7 h p.i. [with 95 % confidence limits (CL) of 23.2–29.6 and 17.3–20.1 h p.i., respectively] following inoculation with ‘NPV + Tinopal’ or ‘high NPV + Tinopal’, respectively. These results suggested that the secondary infection following inoculation with a minimum number of ODVs started at 23–30 h p.i. and that this period was shortened by over 3 h when infection was initiated with large numbers of ODVs, whilst those of ‘NPV alone’ treatment were estimated with a broad 95 % CL. In the larval tissue of *Trichoplusia ni* infected with AcMNPV, newly synthesized nucleocapsids can be detected in the cell cytoplasm by electron microscopy as early as 12 h p.i. and BVs are released into the haemocoel by 16–24 h p.i. (Granados & Lawler, 1981). The infection of AcMNPV progresses from the midgut to tracheae of larvae of *T. ni* at 12–16 h p.i. (Engelhard *et al.*, 1994; Washburn *et al.*, 1995, 2003). These findings indicate that the onset of AcMNPV secondary infection occurs at 12–16 h after virus exposure. Our results suggest that the shift from primary infection to secondary infection takes longer in *M. brassicae* infected with MabrNPV than in *T. ni* infected with AcMNPV.

Granados & Lawler (1981) reported that the concentration of AcMNPV in the haemolymph of *T. ni* larvae reached 10\(^9\) p.f.u. ml\(^{-1}\) at 24 h p.i. In the haemolymph of *Heliothis virescens*, the BV titre of AcMNPV has been detected at a level of 10\(^8\)–10\(^9\) p.f.u. ml\(^{-1}\) at 48–72 h after oral infection (Trudeau *et al.*, 2001; Kamita *et al.*, 2003). However, there is little information about the final BV concentrations for other baculoviruses *in vivo*. In this study, the maximum concentration of BV in the host haemolymph, which is represented by the A parameter of the Gompertz model, was estimated at approximately 10\(^8\) virions ul\(^{-1}\) following all of the treatments. However, the 95 % CL of the A parameter of ‘sixth-instar type’ larvae treated with ‘NPV alone’ and ‘NPV + Tinopal’ were estimated to be 5.53–6.82 and 5.76–6.53, respectively. These estimates suggested that the maximum BV concentration is occasionally <10\(^8\) virions ul\(^{-1}\). The A parameter of ‘fifth-instar type’ larvae treated
with ‘NPV alone’ and ‘high NPV + Tinopal’ was found to be higher, sometimes reaching a BV concentration of $10^7$ virions $\mu l^{-1}$.

As shown in Fig. 4, the concentration of BV reached a plateau at 60–80 h p.i. for the ‘NPV alone’ treatment. The plateau titre appeared to occur later ( > 90 h p.i.) in the ‘NPV + Tinopal’-treated larvae and sooner (40 h p.i.) in the ‘high NPV + Tinopal’-treated larvae. The time required to reach the plateau titre is related to the $\mu_m$ parameter, the slope of the virus-growth curve during exponential growth. The values of the $\mu_m$ parameter following inoculation with ‘NPV + Tinopal’ and ‘high NPV + Tinopal’ were estimated to be 0.15 and 0.44, respectively. The slope of the Gompertz formula reflects not only the rate of increase per virion, but also the volume of larval haemolymph. In fact, the median body weight of larvae following ‘NPV + Tinopal’ treatment was 3.4-fold higher than that of larvae following ‘high NPV + Tinopal’ treatment. This suppression of larval development following ‘high NPV + Tinopal’ treatment seems to be related to the inhibition of larval moult and a high rate of viral increase following the early appearance of virus in the haemolymph. Under the same conditions as our experiment, Agui & Hiruma (1982) reported that the ecdysteroid titre in larvae of M. brassicae peaked at 30 h before larval ecdysis to the sixth instar. This period of time corresponds with 38–42 h p.i. in our experiments. At this time, the BV concentration had almost reached a plateau ($10^6$ virions $\mu l^{-1}$) following the ‘high NPV + Tinopal’ treatment. On the other hand, the BV concentration following ‘NPV + Tinopal’ treatment was below $10^5$ virions $\mu l^{-1}$ at the same time. In the former case, larvae stayed in the fifth instar, possibly because the concentration of EGT, a viral protein that inactivates the host ecdysteroid (Cory et al., 2001), was high enough to impede ecdysis, whilst in the latter case larvae moulted to the sixth instar. These results suggest that virus concentration correlates with the expression rate of the viral genes such as egt and regulation of host development.

From our results, we concluded that MabrNPV T is a member of the group II NPVs. We found that when infection initiates from a minimum number of ODVs, it took approximately 25 h before initiation of secondary infection and another 65 h before the maximal concentration of virus ($10^9$ virions $ml^{-1}$) in the host haemocoel was reached. When the initial virus dose was increased, the primary and secondary infection cycles were accelerated. To improve our understanding of the virulence of baculoviruses in vivo, our future work will study the differences in virus multiplication between group I and group II NPVs and granuloviruses in a common permissive host.

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