Bot. Bull. Acad. Sin. (2005) **46:** 11-20

Improved plant protective efficacy of a baculovirus using an early promoter to drive insect-specific neurotoxin expression

Shu-Jen TUAN[1, 2], Roger F. HOU[3], Suey-Sheng KAO[1], Chi-Fen LEE[1], and Yu-Chan CHAO[2,*]

[1]*Biopesticide Department, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Wufeng, Taichung 413, Taiwan, ROC*

[2]*Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 115, Taiwan, ROC*

[3]*Department of Entomology, National Chung Hsing University, Taichung 402, Taiwan, ROC*

(Received April 5, 2004; Accepted September 30, 2004)

Abstract. Genetically improved *Autographa californica* (Speyer) multiple nucleopolyhedrovirus (AcMNPV), the type species of the genus Nucleopolyhedrovirus, was constructed with an insect-selective toxin gene ($LqhIT_2$) derived from the venom of the Israeli yellow scorpion, *Leiurus quinquestriatus* (Ehrenberg). $LqhIT_2$ was expressed by two distinct and temporally regulated promoters. These two promoters were the early promoter *p-PCm* (a *pu*-enhanced minimal *CMV* promoter) and the very late *p10* promoter, resulting in recombinant viruses named $vAPcmIT_2$ and $vAP10IT_2$, respectively. Western blot and bioassay analysis showed that the toxin gene under the control of the early *p-PCm* promoter was expressed 24 h earlier than under the very late *p10* promoter control, and $vAPcmIT_2$ killed faster than $vAP10IT_2$. The EC_{50} (effective concentration, EC) values of wild type AcMNPV, $vAP10IT_2$, and $vAPcmIT_2$ to the 3rd-instar larvae of *Trichoplusia ni* (Hübner) were 1.00, 0.19, and 0.17 polyhedral inclusion bodies (PIBs)/mm^3, respectively. Compared to the very late promoter, the early promoter could shorten the effective time (ET_{50}) by 14~17 h. Furthermore, for the 4th-instar larvae, compared to the wild-type virus and vAP10G, a 19~23% and 30~33% reduction in ET_{50} were found using $vAP10IT_2$ and $vAPcmIT_2$, respectively. Field trials of these two viruses showed an effective paralysis of the infected larvae which then stopped feeding, resulting in a decrease in the leaf area eaten compared to those larvae infected with wild-type virus and another control virus vAP10G, the GFP-expression recombinant AcMNPV. Scorpion toxin driven by the early *p-PCm* promoter shortened the time-to-paralysis of insect larvae, and thus provided an economical pest control advantage over that driven by the very late *p10* promoter.

Keywords: AcMNPV; *Autographa californica* multicapsid nucleopolyhedrovirus; Baculovirus; Cabbage; Depressant neurotoxin; *Leiurus quinquestriatus hebraeus*; $LqhIT_2$; Plant protection; Recombinant virus; Scorpion toxin; *Trichoplusia ni*.

Introduction

Since chemical pesticides frequently pose environmental and health risks, carefully selected biological control agents, like predators, parasitoids, and pathogens, have become attractive alternatives for the suppression of insect pests in the field. Baculoviruses (Baculoviridae) are arthropod-specific pathogens that have served as microbial biopesticides for control of many lepidopteran pests for several decades (Entwistle and Evans, 1985). These pathogens are characterized by large, circular double-stranded DNA genomes and rod-shaped virions that are enveloped within polyhedrin to form polyhedra (Tinsley and Kelly, 1985; Whitt and Manning, 1988). While baculoviruses have the potential for insect control, infected lepidopteran larvae still cause considerable crop damage due to continued feeding for several days after the initial infection, resulting in reduced efficacy in the field (Bonning and Hammock, 1996; Tuan et al., 1997; 1998).

*Corresponding author. Phone: 886-02-2788-2697; E-mail: mbycchao@imb.sinica.edu.tw

A number of studies have established that the efficacy of baculovirus for insect pest control is greatly improved by the insertion of foreign genes into the viral genome. These include genes encoding the *Manduca sexta* (Linnaeus) diuretic hormone (Maeda, 1989), the *Heliothis virescens* (Fabricius) juvenile hormone esterase (Hammock et al., 1990), the *Bacillus thuringiensis* (Berliner) δ-endotoxin (Martens et al., 1990; 1995), mite or spider toxins (Tomalski and Miller, 1991; Hughes et al., 1997; Priknod'ko et al., 1998), proteases (Harrison and Bonning, 2001), and the scorpion insect-specific neurotoxins (Stewart et al., 1991; Maeda et al., 1991; McCutchen et al., 1991; Fuxa et al., 1998; Gershburg et al., 1998; van Beek et al., 2003). All these studies reveal the significant contribution of exogenous gene products to the efficacy of baculovirus in pest control.

The $LqhIT_2$ depressant insect toxin, derived from the Israeli yellow scorpion *Leiurus quinquestriatus hebraeus* (Ehrenberg), is a polypeptide of 61 residues (Zilberberg et al., 1991). Injection of $LqhIT_2$ toxin into *Sarcophaga falculata* (Pandelle) blowfly larvae induced symptoms typical of the transient excitatory effect that preceeds the onset of prolonged flaccidity. The larvae are immobilized and

paralyzed and become completely flaccid (Zlotkin et al., 1993; Gershburg et al., 1998; Harrison and Bonning, 2000). The depressant toxin's mode of action involves depolarization of the axonal membrane, blockage of the evoked action potential, and changes in the amplitude and the kinetics of the sodium current. Flaccid properties of the host insect are attributed to the opening of sodium channels and inability to inactivate them (Benkhalifa et al., 1997). The higher efficacy of the virus expressing the depressant toxin $LqhIT_2$ over the excitatory toxin $LqhIT_1$ suggests that pharmacokinetic factors and/or promoter efficiency may play a role during infection of insect pest larvae by recombinant baculoviruses (Gershburg et al., 1998).

When the scorpion toxins were tested using recombinant baculoviruses, the insecticidal activity of the very late *p10* promoter was higher than that of the early *p35* promoter (Gershburg et al., 1998). These data suggest that promoter efficiency may play a role in the infection of insect pest larvae by recombinant baculoviruses since an early promoter should exert an effect earlier than the very late promoter. If the early promoter is strong enough, it should show more lethality in the recombinant virus. Previously, the human cytomegalovirus minimal (*CMVm*) promoter, ligated *in cis* with a newly discovered activator, the polyhedrin upstream (*pu*) sequence, and the early *p-PCm* promoter (*pu* plus *CMVm*) was shown to result in high level expression of foreign genes. In these experiments, although less luciferase was expressed by the early *p-PCm* promoter rather than by the very late *p10* promoter, the former expressed earlier with better total luciferase activity (Wu et al., 2000; Lo et al., 2002).

The *Autographa californica* nucleopolyhedrovirus (AcMNPV) is the type species of the Nucleopolyhedrovirus (Volkman, 1995). Its broad insect infectivity spectrum of more than 30 species lepidopteran also made this virus a more acceptable microbial insecticide (Granados and Williams, 1986). The pathogenicity of AcMNPV against nine important agricultural pests was studied previously (Tuan et al., 1997). Recombinant AcMNPV expressing insect-specific toxins under different temporally regulated promoters showed various efficacies. The immediate early *ie1* promoter expressed AaIT earlier than the very late *p10* promoter and significantly reduced the feeding activity of infected larvae (Jarvis et al., 1996). The time required for paralysis or death was promoter-dependent, and the late 6.9K DNA binding protein gene promoter was generally more effective than P_{synXIV}, *HSP70*, and *DA26* promoters for expression of Tox34 (Lu et al., 1996). The hybrid late and very late promoter were able to drive expression of a tox34 gene and showed superior reductions in kill times compared to the very late promoter only (Tomalski and Miller, 1992). The *hr5/ie1* promoter lead to a faster response than the very late *p10* promoter for expression of either $LqhIT_2$ or AaIT (van Beek et al., 2003). In order to further improve the efficacy of recombinant AcMNPV for earlier insect pest control, a newly developed early promoter *p-PCm* (Lo et al., 2002) was used to drive a scorpion depressant toxin gene, $LqhIT_2$. Our results demonstrate that the scorpion toxin is expressed earlier by the early *p-PCm* promoter than the very late *p10* promoter and that insecticidal efficacy of recombinant AcMNPV expressing $LqhIT_2$ was significantly improved compared to wild-type AcMNPV.

Materials and Methods

Cell Cultures and Viruses

The cell line *Spodoptera frugiperda* IPLB-Sf21AE (Sf21, Vaughn et al., 1977) was maintained at 26°C in a modified TNM-FH medium that contained 8% fetal bovine serum (Lee et al., 1998; Lin et al., 1999). The GFP-expression recombinant virus, vAP10G (Chao et al., 1996) and the C6 strain of wild-type AcMNPV were propagated in the Sf21 cell line. The vAP10G was here used as control because it was constructed by the cotransfection with commercial linearized genomic DNA of AcMNPV, and we used the same material and methodology to construct both neurotoxin-expressing recombinant viruses $vAP10IT_2$ and $vAPcmIT_2$. At 5~7 days post infection (d p.i.), the infected cells were pelleted and resuspended in 0.5% SDS solution for 10 min, and the number of polyhedra were counted by a hemocytometer and stored at 4°C in sterile H_2O.

Insects

The larvae of cabbage loopers, *Trichoplusia ni* (Hübner), were collected from Shi-hu or Wu-feng County in Taiwan and reared on an artificial diet (Tuan et al., 1997). These insects were reared at 25±1°C with 70±5% relative humidity (RH) and 14:10 h (L:D) photoperiod. All tested insects were the Shi-hu strain 4thor Wu-feng strain 3rd instar larvae after three generations of rearing. The mass-rearing artificial diet contained 0.1% sorbic acid and p-methyl-benzoate for anti-bacteria and anti-fungi purposes. Pupae and eggs were washed for 10-15 min in 0.5% NaClO or 4% formalin solution, after which they were rinsed for 15 min with running water followed by air-drying. During the course of experiments, all precautions to ensure against contamination with microorganisms (equipment autoclaving, surface wiping with 70% ethanol, instrument flaming, formalin fumigating for all rearing apparatus, and the use of disposable containers and instruments) were applied. The insects in preliminary work and in all experiments were observed daily and by smears under light or phase-contrast microscopy to determine if they were pathogens in origin.

Construction of Plasmids and Recombinant Virus

We excised a complete $LqhIT_2$-coding sequence (included Bombyxin signal sequence and $LqhIT_2$ mature peptide sequence, 250 bp in length) by digestion with *Bgl*II and *Eco*RI (PharMingen) from $pBmLqhIT_2$ (provided by Maeda's Lab., Maeda et al., 1991; Gershburg et al., 1998). The sequence then was inserted into a polyhedrin gene containing transfer vector pAcUW21 between a blunt-ended *Bgl*II site and the *Eco*RI site. The polyhedrin gene was useful for later experiments in forming polyhedral inclusion bodies for insect infection by feeding. The scor-

pion depressant toxin gene was expressed under the control of the very late *p10* promoter of AcMNPV, and named pAP10IT$_2$. For the early expression of scorpion depressant toxin gene, the very late *p10* promoter of plasmid pAP10IT$_2$ was replaced with an early *p-PCm* promoter, which contains a *CMVm* promoter that is activated *in cis* by a upstream *pu* element already situated in the plasmid (Lo et al., 2002) to generate pAPcmIT$_2$ (Figure 1). The *CMVm* promoter was excised from the vector pTRE (Clontech) between *Xba*I and *Bam*HI sites. The 3' end of *CMVm* promoter fragment was ligated into the *Bgl*II cloning site through the *Bam*HI site. The 5' end of the *CMVm* promoter fragment was ligated into *Xba*I site with the aid of an *Xba*I-*Xho*I (5'-3') adaptor, resulting in the *Xba*I-*Xho*I-*CMVm*-*Bam*HI (*Bgl*II) linker. Recombinant viruses, that expressed scorpion depressant toxin, were constructed by the cotransfection of transfer vectors pAP10IT$_2$ or pAPcmIT$_2$ separately with linearized genomic DNA of AcMNPV (vAcRP23.LacZ, PharMingen), using Lipofectin (Life Technologies). Polyhedra-positive recombinant

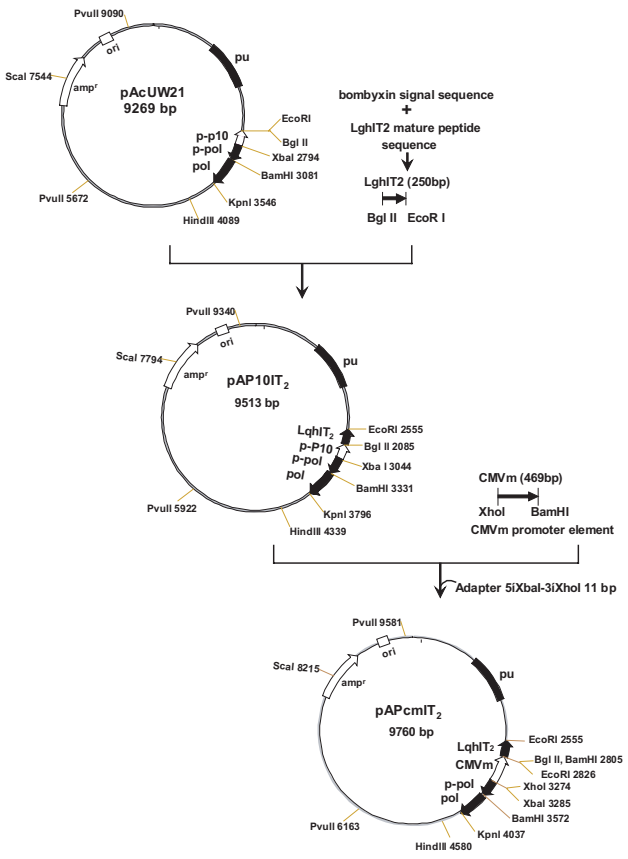

Figure 1. Construction of the transfer vectors pAp10IT$_2$ and pAPcmIT$_2$. The LqhIT$_2$ gene was excised from the transfer vector pBmLqhIT$_2$ by *Bgl* II and *Eco*R I digestion, and inserted into pAcUW21. The scorpion toxin gene was cloned juxtaposed to the *p10* promoter yielding the vector pAp10IT$_2$. The vector pAp10IT$_2$ was modified by replacing the *p10* promoter with the *CMVm* promoter using the restriction sites of *Xho* I, *Bam*H I, *Xba* I and *Bgl* II, and an *Xba* I-*Xho* I linker, resulting in a new plasmid pAPcmIT$_2$.

viruses, vAP10IT$_2$ and vAPcmIT$_2$ were isolated by four rounds of end-point dilution. They were verified by restriction fragment length analysis and then confirmed by PCR analyses. The sequences of these two primers were primers *polh*: 5' CCG ATG TAA ACG ATG GGC TT 3', and *EcoIT* 5' GAA TTC TTA TCC ACA GGT ATT CGT 3'.

Western Analysis

Isolated recombinant viruses were further confirmed and the timing of toxin expression was analyzed. AcMNPV-mediated expression of LqhIT$_2$ under the control of the very late *p10* and early *p-PCm* promoters in insect cells was analyzed by Western blotting. The polyclonal antibody was from rabbits immunized by a sub-dermal multi-injection of synthetic peptide mixed with adjuvant (amino acid sequence: H-DGYIKRRDGCDDKTWK-NH$_2$, conjugated with Keyhole Limpet Hemocyanin as a carrier protein). SF 21 cells in 6-well microplates were inoculated with vAP10IT$_2$, vAPcmIT$_2$, and vAP10G recombinant viruses or with wild-type AcMNPV at a multiplicity of infection (moi) = 1. Infected cells were collected at 4, 8, 12, 18, 24, 36, 48, 72 and 96 h post infection (h p.i.), and were centrifuged at 1000 *g* for 5 min, after which the resulting supernatants were collected. The cell pellets were resuspended in double-distilled H$_2$O treated with 0.5% SDS, then vortexed for 5 min and washed twice. Cell extracts and medium samples were mixed with equal volumes of sample buffer and analyzed by SDS-PAGE in 4% stacking gel and 15% resolving gel. Gels were electroblotted onto polyvinylidene fluoride membranes (PVDF, Millipore) and blocked with Tris-buffer saline with 0.1% Tween-20 containing 3% skim milk at 4°C overnight. Subsequently, the membrane was incubated with polyclonal rabbit anti-LqhIT$_2$ antibody for 1 h at room temperature followed by horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Finally, the HRP spots on the membrane were detected by an enhanced chemiluminscence kit (ECL, Amersham Life Science) following the protocol provided by the manufacturer.

Lethal Dose and Time Measurements

Each candidate recombinant virus was screened by a bioassay of activity against the host larvae. The pathogenicity of scorpion toxin containing recombinant viruses (vAP10IT$_2$ and vAPcmIT$_2$) and wild-type AcMNPV were compared. The vAP10G, a recombinant virus containing a GFP coding region driven by the very late *p10* promoter, was also used as another control. The median lethal concentration (LC$_{50}$) and median lethal time (LT$_{50}$) were determined on the 3rd- and 4th-instar larvae of *T. ni*. Molting larvae at the same age were selected and enclosed individually in a 30-well plate overnight, and the next morning the newly molted larvae were starved for 8 h to synchronize larval growth. These larvae were bioassayed with a diet-contaminated method (Tuan et al., 1997) and maintained at 25±1°C, 70±5% RH, and under a 14:10 h photoperiod. Tested larvae were fed with a set of five serial 10-fold dilutions of each virus stock (from 780 to 0.078 polyhedral

inclusion bodies, PIBs/mm^3) on a small plug of artificial diet. Sixty larvae in three replicates were inoculated in each treatment. For lethal time or effective time determining assay, 4th-instar larvae were treated with 78 and 7.8 PIBs/mm^3, and 3rd-instar larvae were treated with 78 and 0.78 PIBs/mm^3 (to obtain ~90-100%, and <75% mortality within seven days). Larvae that consumed the diet were then fed another plug diet without virus and examined two or three times daily for a response. Tested larvae were scored as responders if they were paralyzed or dead. In doubtful cases, the larva was touched slightly with a paint brush five times and scored as responder only if it failed to move within 30 s. All data were recorded until the 10th d p.i., corrected with Abbott's formula, and analyzed using probit analysis (Finney, 1971) to determine the LC_{50} (concentration of PIBs/mm^3 to kill 50% of the test larvae), EC_{50} (concentration of PIBs/mm^3 necessary to resulting in 50% of the test larvae to paralyze or cease feeding), ET_{50} (mean time at which 50% of test larvae cease to respond to a stimulus), ET_{90} (time at which 90% of test larvae cease to respond to a stimulus), and LT_{50} (mean time at which 50% of test larvae are killed) values. All of the bioassays were performed at least three times with different virus batches. The resulting values for each virus were averaged and analyzed by one-way ANOVA. The means were separated by the Fisher's Least Significant Difference Test (Steel and Torrie, 1980)

Field Trial

Newly molted 4th-instar *T. ni* larvae were starved for 16 h, and then transferred to potted cabbage with 12~14 leaves. Thirty larvae on each pot were confined in a fine-mesh nylon cage. The cabbages were treated individually with sterile water (as control), recombinant viruses with ($vAP10IT_2$ and $vAPcmIT_2$) or without (vAP10G) scorpion toxin gene, and wild-type AcMNPV, that were applied at 10^7 PIBs/ml using a handheld sprayer. Tween 20 (0.05%) was added to all viral suspensions and control. Three potted cabbages were tested for each treatment. The insects were checked twice daily to record signs and symptoms of disease and calculate mortality. The dead larvae were collected and stored at 4°C, then checked by microscopy at a magnification of 400X. Six days after application, all larvae, living and dead, were collected from potted cabbages. The surviving larvae were weighed, reared individually on artificial diet at 24±1°C, and checked twice daily for death or pupation. Larvae failing to respond to gentle stimulation by the paint brush within 30 s were scored as dead. Mean leaf area eaten per cabbage (sum of 12 leaves) was determined in infested control and virus-treated cabbage pots at the 6th day after virus application. All leaves were removed from the plant and Xeroxed. Each intact leaf-image was cut out and scanned with a leaf area meter (Model Li-3100 Area Meter, Li-Cor, USA). The holes in each leaf were then trimmed off carefully and scanned again. The differences between two scans were recorded as the area consumed for that leaf, and these differences in all leaves for each plant were summed up. All field trials were performed at least three times with different virus batches. Leaf areas eaten by tested larvae for each treatment group were compared by one-way ANOVA followed by Fisher's Least Significant Difference Test (Steel and Torrie, 1980).

Results

Construction and Expression of $LqhIT_2$ by Recombinant AcMNPV

Transfer vectors that contained the AcMNPV polyhedrin gene and the scorpion toxin gene $LqhIT_2$ under the control of the very late *p10* or the early *p-PCm* promoters (Lo et al., 2002) were constructed (Figure 1). The production of 6 kD $LqhIT_2$ toxin proteins by individual isolates was subsequently examined. Western analysis showed that $LqhIT_2$ was first detectable in $vAPcmIT_2$-infected cells at 12 h p.i. Expression of $LqhIT_2$ peaked at 36 h p.i. in $vAPcmIT_2$-infected cells and subsequently declined by 48 h p.i. The scorpion toxin expressed by $vAP10IT_2$ was first clearly detectable at 36 h p.i., reached a plateau around 48 ~ 72 h p.i., and declined to 96 h p.i. (Figure 2). Based on size (6 kDa) and its specific expres-

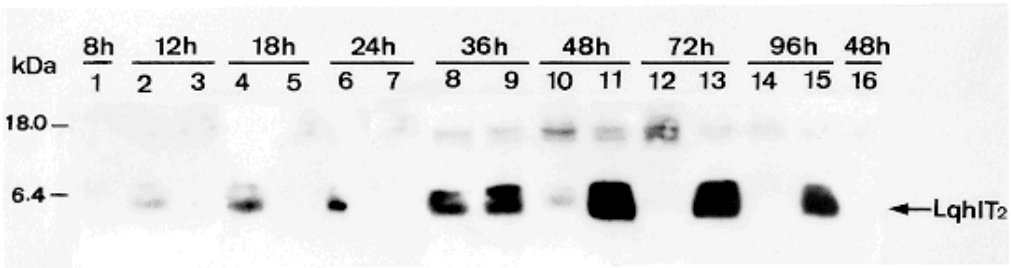

Figure 2. Time course expression of $LqhIT_2$ in insect cells. Sf21 cells were infected with $vAPcmIT_2$, $vAP10IT_2$ and AcMNPV at moi = 1. Immunoblot analysis of scorpion toxin expression was assayed at various times p.i. Cell extracts were prepared at the indicated times (h), separated by 15% SDS-PAGE, and immunoblotted with anti-$LqhIT_2$ polyclonal antibody. Lanes 1, 2, 4, 6, 8, 10, 12, and 14 were cell extracts from $vAPcmIT_2$-infected Sf21 cells. Lanes 3, 5, 7, 9, 11, 13, and 15 were cell extracts from $vAP10IT_2$-infected Sf21 cells, and lane 16 was cell extracts from AcMNPV-infected Sf21 cells at 48 h post infection. Kaleidoscope pre-stained standards (Bio-Rad, catalog 161-0324) were bovine serum albumin 85 kDa, carbonic anhydrase 41.8 kDa, soybean trypsin inhibitor 31.8 kDa, lysozyme 18 kDa, and aprotinin 6.4 kDa.

sion in infected cells (vs wild-type AcMNPV-infected cells), we feel confident that $LqhIT_2$ is recognized by the antibody used in this experiment (Figure 2, lane 16). Even though the detectable level of $LqhIT_2$ toxin in the $vAPcmIT_2$-infected cells was not as abundant as in $vAP10IT_2$-infected cells after 48 h p.i., the efficacy of insect killing was faster and higher for the former than for the latter viruses. This might be due to the better secretion of the toxin protein by the *p-PCm* promoter early in the viral infection. Since the vast volume of medium is 50 × greater than that of the cells, we tried and failed to probe the $LqhIT_2$ toxin from the medium of the cells infected with recombinant viruses by Western blotting.

Dose- and Time-Mortality Responses of Infected Insect

Bioassays were carried out to measure the insecticidal efficacy of the recombinant viruses against *T. ni*. The larvae infected with the scorpion toxin-containing recombinant viruses showed a prolonged flaccid paralysis, and they were effectively immobilized, rather than swollen and liquefied as with the wild-type AcMNPV and vAP10G. The LC_{50} values of wild-type AcMNPV, vAP10G, $vAP10IT_2$, and $vAPcmIT_2$ to the early 4th-instar *T. ni* larvae were 1.43, 2.76, 1.40, and 1.36 PIBs/mm^3, respectively, and differences were not significant (Table 1). Although toxin-expressing recombinant viruses killed insect larvae much more quickly than non-toxin-expressing viruses, the host LC_{50} values of their 4th-instar larvae were not significantly different. However for the 3rd-instar larvae, the EC_{50} values of wild-type AcMNPV, $vAP10IT_2$, and $vAPcmIT_2$ were 1.00, 0.19, and 0.17 PIBs/mm^3, respectively (Table 2). This is a more than fivefold difference in virulence between the recombinants and wild-type AcMNPV. In addition, the ET_{50} values against the 3rd-instar larvae of *T. ni* at a concentration of 78 PIBs/mm^3 were 80, and 66 h, respectively for $vAP10IT_2$, and $vAPcmIT_2$. For either lower or higher concentrations as 0.78 or 78 PIBs/mm^3, $vAPcmIT_2$ resulted 18% (17 h) or 12% (14 h) reductions, respectively, in effective time compared to $vAP10IT_2$ (Table 2). The time of paralysis of the 4th instar *T. ni* fed with the recombinant baculoviruses (measured by immobility of the larvae) was determined. In general, the effective time (ET_{50}) was 9~12 h earlier than the lethal time (LT_{50}). Insects infected with $vAPcmIT_2$ and $vAP10IT_2$ provided significantly lower ET_{50} values, 83.2 and 87.1 h, respectively, and they were 19~41 h earlier than those of larvae infected with wild type AcMNPV and vAP10G. The ET_{90} values also demonstrated that toxin-expressing recombinants resulted in 36~61 h ear-

Table 1. LC_{50} comparison of *Trichoplusia ni* 4th-instar larvae infected with recombinant or wild-type baculoviruses.[1), 2)]

Virus	LC_{50} (PIBs/mm^3)		
	Mean	95% Confident limits	
		Lower	Upper
AcMNPV	1.43a	0.91	2.07
vAP10G	2.76a	1.75	4.08
$vAP10IT_2$	1.40a	0.74	2.25
$vAPcmIT_2$	1.36a	0.71	2.20

[1)]Polyhedral inclusion bodies (PIBs)/mm^3 of contaminated diet. LC_{50} value was calculated from the cumulative mortalities up to 10 days post infection. 180 tested larvae were assayed in each treatment.

[2)]LC_{50} values were calculated using probit analysis. Means followed by the same letter were not significantly different by the analysis of variance at $P<0.05$. F value = 2.11<F (3, 8, 0.05); thus, a hypothesis of no difference among treatments is not rejected.

Table 2. EC_{50} and ET_{50} comparisons of *Trichoplusia ni* 3rd-instar larvae infected with recombinant or wild type baculoviruses.[1), 2), 3), 4)]

Virus	Time and dose to paralysis and/or death response		
	EC_{50} (PIBs/mm^3)[2)]	ET_{50} (h)[3)]	ET_{50} (h)[4)]
	Mean±SD	Mean±SD	Mean±SD
AcMNPV	1.00 ± 0.47b	—	—
$vAP10IT_2$	0.19 ± 0.08a	141.7 ± 5.0b	80.4 ± 5.4b
$vAPcmIT_2$	0.17 ± 0.07a	124.7 ± 5.1a	66.0 ± 4.7a

[1)]Median effective concentration (EC_{50}) and median effective time (ET_{50}) values were calculated using probit analysis with 95% confident limits. Means within a column labeled with different letters are significantly different at $P<0.05$, Least Significant Difference. SD, standard deviation.

[2)]Polyhedral inclusion bodies (PIBs)/mm^3 of contaminated diet. EC_{50} value was calculated from the cumulative mortalities and paralysis until 10 days post inoculation. F value= 8.7>F (2, 6, 0.05).

[3)]Time in hours since larvae were placed on the contaminant diet. The concentration given is 0.78 PIBs/mm^3. F value = 44.9>F (2, 6, 0.05).

[4)]The concentration given is 78 PIBs/mm^3. F value = 44.5>F (2, 6, 0.05).

Table 3. Lethal and effective time comparisons by paralysis/death responses of *Trichoplusia ni* 4th-instar larvae infected with recombinant or wild-type baculoviruses.[1), 2), 3), 4)]

Virus	Time to paralysis and/or death response[2)]			
	LT_{50} (h)[3)]	LT_{50} (h)[4)]	ET_{50} (h)[4)]	ET_{90} (h)[4)]
AcMNPV	132.7b	112.2c	107.6b	136.5b
vAP10G	173.3c	130.4d	124.3c	159.7c
$vAP10IT_2$	115.7a	98.6b	87.1a	98.9a
$vAPcmIT_2$	100.0a	92.9a	83.2a	100.7a

[1)]Time in hours from when larvae were placed on the contaminant diet.

[2)]Median lethal time (LT_{50}), median effective time (ET_{50}), and effective time to paralyze or kill 90% of tested larvae (ET_{90}) values were calculated using probit analysis with 95% confidence limits. Means within a column labeled with different letters are significantly different at $P<0.05$, Least Significant Difference.

[3)]The inoculate concentration is 7.8 PIBs/mm^3. F value= 42.9>F (3, 8, 0.05).

[4)]The inoculate concentration is 78 PIBs/mm^3. F values of LT_{50}, ET_{50}, and ET_{90} were 43.6, 24.0, and 41.0, respectively, and all >F (3, 8, 0.05).

lier response times than the non-recombinant viruses. The LT_{50} values against the 4th-instar *T. ni* at a high concentration of 78 PIBs/mm^3 were 112.2, 130.4, 98.6, and 92.9 h, for infection by AcMNPV, vAp10G, $vAP10IT_2$, and $vAPcmIT_2$, respectively (Table 3). The LT_{50} of these viruses was also significantly different at 7.8 as well as at 78 PIBs/mm^3. A 20-73% reduction in LT_{50} or effective time (ET_{50}) was found using $vAPcmIT_2$ compared to wild-type AcMNPV (Table 3). The baculoviruses expressing the depressant toxin showed improved insecticidal efficacy (faster paralysis) compared to the toxin-negative viruses (Figure 3). At 60 to 84 h p.i. of the 4th instar larvae, 10~20% higher mortality resulted from $vAPcmIT_2$ compared to $vAP10IT_2$. At 96 h p.i., more than 85% of tested insects were killed by either $vAPcmIT_2$ or $vAP10IT_2$ while only 10% of the larvae were killed by either vAP10G or wild-type AcMNPV at the same incubation time. It took 156 h for vAP10G and wild type AcMNPV to kill 85-98% of the insect larvae (Figure 3).

Field Experiments

In the field, both toxin-expressing recombinant viruses killed the 4th-instar larvae of *T. ni* faster than the parental or the GFP-expression recombinant viruses. Toxin expression by the recombinant virus induced larval paralysis in the field, resulting in many larvae ceasing to feed and falling off the plants before death. Over 20% of larvae treated with $vAPcmIT_2$ were found paralyzed/dead on the soil, compared to none in wild-type treatments at the 3rd day post application (p.a.) treated at 10^7 PIBs/ml. On the 6th day p.a., more than 80% of larvae infected with $vAPcmIT_2$ and $vAP10IT_2$ fell off the plants before death while most larvae infected with vAp10G and wild-type AcMNPV kept eating and grew into 5th-instar larvae. The mortality and average body weight of larvae on the 6th day p.a. were significantly different between toxin-expressing recombinant viruses and non-toxin viruses. By 6 days p.a., the mortalities of larvae infected with wild-type AcMNPV, vAp10G, $vAP10IT_2$, and $vAPcmIT_2$ were 96.7, 67.4, 96.7, and 95.1%, respectively (Table 4).

Virus treatment significantly reduced insect damage compared to the untreated controls, but $vAPcmIT_2$ and $vAP10IT_2$ showed no significant difference. Larvae fed toxin-expressing recombinants ate less than larvae treated with wild-type AcMNPV, resulting in a 22% reduction of body weight (Table 4). Untreated larvae caused 842 cm^2 loss in leaf area while the $vAPcmIT_2$ and $vAP10IT_2$-treated plots exhibited a dramatic reduction in cabbage plant damage, with up to 64% less leaf area consumed compared

Table 4. Comparison of mortality and body weight of *Trichoplusia ni* 4th-instar larvae infected with recombinant and wild-type AcMNPV.[1), 2), 3), 4), 5)]

Treatment (10^7 PIBs/ml)	Mortality (%)		Average body weight[3)] (mg)	
	6th d p.a.[3)]	Final[4)]	Mean[5)]	SD
AcMNPV	36.7^b	96.7^a	107.3^b	13.6
vAP10G	16.7^c	67.4^b	134.7^c	8.6
$vAP10IT_2$	84.3^a	96.7^a	84.2^a	3.2
$vAPcmIT_2$	80.2^a	95.1^a	83.7^a	4.6
Control	2.2^d	10.0^c	146.2^c	11.8

[1)]Means labeled with different letters are significantly different at $P<0.05$, Least Significant Difference.

[2)]All viral suspensions used were 10^7 polyhedral inclusion bodies (PIBs)/ml. Adjuvant was dissolved in 2,000-fold diluted Triton®.

[3)]Data were calculated at the 6th day post application (d p.a.). F value= 292.9>F (4, 10, 0.05).

[4)]Final mortality was recorded up to pupation of tested insects. F value= 376.9>F (4, 10, 0.05).

[5)]Average body weight was recorded at the 6th day post application (d p.a.). F value= 32.9>F (4, 10, 0.05).

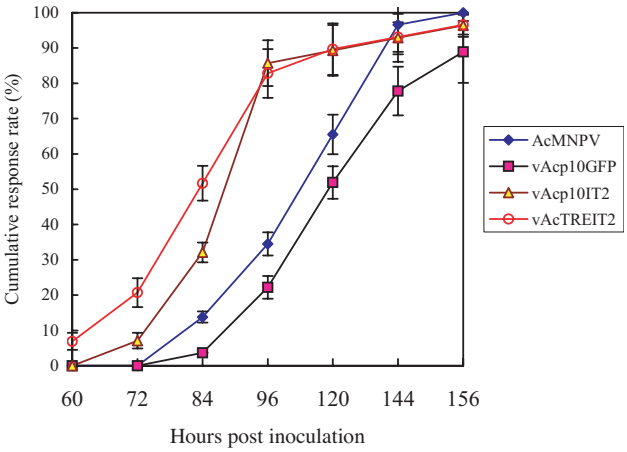

Figure 3. Cumulative percentage of *T. ni* 4th-instar larvae that had ceased to respond to stimuli after inoculation with recombinant or wild type AcMNPV at a concentration of 78 polyhedral inclusion bodies (PIBs)/mm^3.

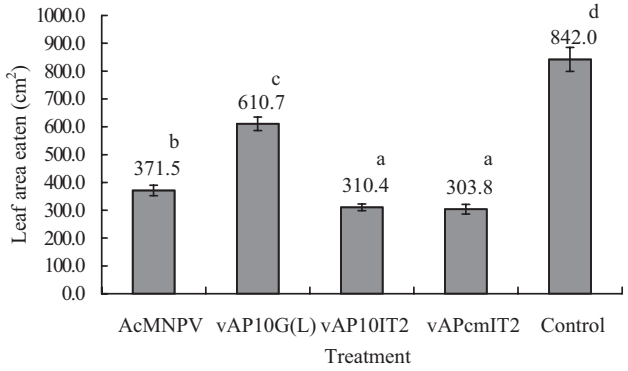

Figure 4. Comparison of leaf area eaten by *T. ni* 4th-instar larvae infected with recombinant and wild-type AcMNPV at the 6th day post application. All viral suspensions were adjusted to a concentration of 10^7 polyhedral inclusion bodies (PIBs)/ml with 2,000 fold-diluted Triton®. Means labeled with different letters are significantly different at the 5% level analyzed at P <0.05, Least Significant Difference. F value = 102.6>F (4, 10, 0.05).

to control (Figure 4). Toxin-expressing recombinant-treated plots had significantly less crop damage, about 16~18% less than those treated with the wild-type AcMNPV, which resulted in about 50% less loss compared to vAp10G. Most insects infected with vAPcmIT$_2$ and vAP10IT$_2$ fell onto the ground while other insects infected with wild-type AcMNPV and vAp10G usually remained on the plant after death, where they liquefied and released of large quantities of viruses (Figure 5).

Discussion

The scorpion toxin-expressing recombinant viruses always killed host larvae more quickly than the wild-type AcMNPV regardless of larval size, and the maximal efficacy would occur in the early stages of pest development (Smits and Vlak, 1988; McCutchen et al., 1991; Hoover et al., 1995; Ignoffo and Garcia, 1996; Hughes et al., 1997; Ignoffo and Garcia, 1997; Gershburg et al., 1998; Milks et al., 1998; Harrison and Bonning, 2001). To speed up the time required to kill or paralyze pest insects, however depends upon the action of the toxin and on the species and instars of the tested insects. The timing and efficacy of the promoter used to drive the toxin gene is also very important (Lu et al., 1996; Prikhod'ko et al., 1998; Harrison and Bonning, 2000). The appropriate choice and combination of different signal sequences or promoters could maximize the efficacy of insect-selective toxin-expressing recombinant viruses (van Beek et al., 2003). Furthermore, the speed of kill of toxin-expressing recombinant could be further reduced by coproducing synergistic toxins (Prikhod'ko et al., 1998; Regev et al., 2003).

The very late *p10* promoter is a popular choice for driving a high-level of foreign gene expression in baculoviruses. Although it is slightly weaker than the polyhedrin promoter, it activates foreign gene expression

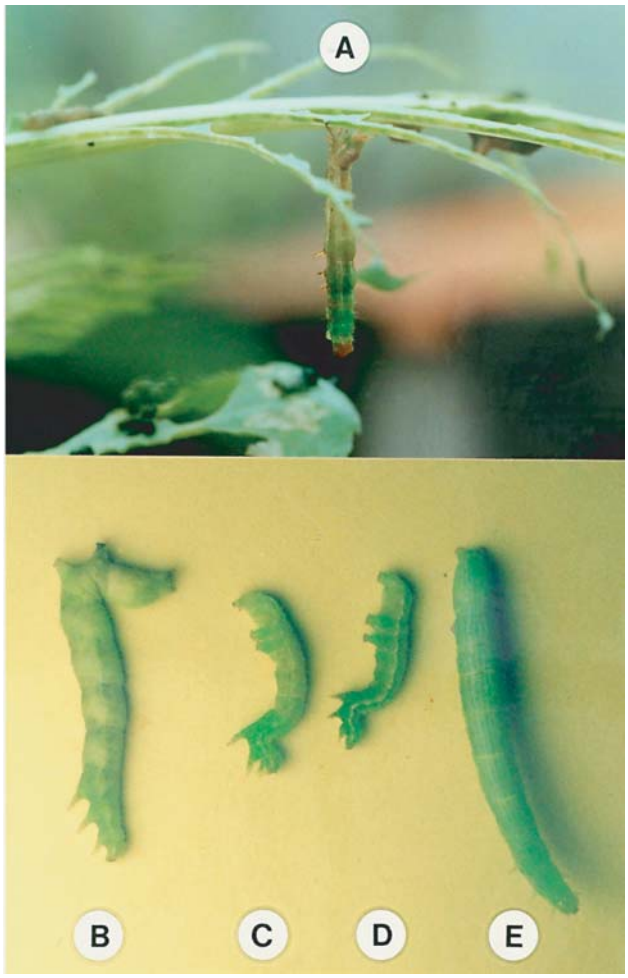

Figure 5. Treatment of *T. ni* larvae with wild-type or recombinant AcMNPV. Figures showed the infection of larvae with different viruses. A: wild-type AcMNPV; B: vAP10G; C: vAP10IT2; D: vAPcmIT2; E: uninfected larvae as a control.

earlier (Roelvink et al., 1992). In the experiment on toxin protein expression by two distinct, temporally regulated viral promoters for pest control using recombinant baculoviruses, Gershburg et al. (1998) revealed advantages for the use of the very late *p10* promoter over the early *p3*5 promoter. In a further experiment, we showed that the early *p-PCm* promoter could be better than the very late *p10* promoter for controlling insect pests. This is probably due to an early and sufficient level of $LqhIT_2$ scorpion depressant toxin protein expression by the former promoter resulting in an earlier cessation of feeding.

Bioassays on the 2nd-instar larvae of *Heliothis virescens* (Fabricius) with recombinant virus expressing AaIT, an excitatory neurotoxin from *Androctonus australis hector* (Ewing) controlled by the very late *p10* promoter, demonstrated a significant 30% decrease in the time to kill (LT_{50} 88 hrs) compared to wild-type AcMNPV (LT_{50} 125 hrs) (McCutchen et al., 1991). Compared with AaIT, the depressant neurotoxin $LqhIT_2$ causes qualitatively different neurophysiological effects and different symptoms when injected into blowfly larvae (Zlotkin et al., 1993). Expression of $LqhIT_2$ reduced survival times of *Heliothis zea* (Boddie) and *H. virescens* to a significantly greater extent than expression of AaIT, and recombinant viruses expressing toxin from the late *p6.9* promoter killed larvae faster than a recombinant virus utilizing the very late *p10* promoter (Harrison and Bonning, 2000). Furthermore, better results could be achieved most recently by the combined expression of the excitatory toxin, $LqhIT_1$ and the depressant toxin, $LqhIT_2$ than if each had been applied alone using recombinant viruses (Regev et al., 2003).

In our studies, we demonstrated that the early *p-PCm* promoter provides for earlier and relatively effective expression. At 12 h p.i., expression of $LqhIT_2$ by the early *p-PCm* promoter was evident, and expression by the very late *p10* promoter can only be observed beginning by 36 h p.i. Although the very late *p10* promoter expressed significantly more proteins at the peak of toxin expression, the expression of the early *p-PCm* promoter was sufficient, so that early expression proved to enhance the control of pest insects. Furthermore, although the total amount of the engineered luciferase protein produced by the early *p-PCm* promoter in our previous study was less than that produced by the very late *p10* promoter, significantly less protein degradation and higher total luciferase enzymatic activities were both found by using the early *p-PCm* promoter (Wu et al., 2000; Lo et al., 2002). The better quality of the protein produced by the early *p-PCm* promoter was further reflected in bioassay tests. Both the effective time and the lethal concentration (EC_{50} in Table 2) data demonstrated that the control efficacy of $vAPcmIT_2$ was superior to that of $vAP10IT_2$ against middle instars of *T. ni*. We also found more significant differences between ET_{50} and EC_{50} occurred in the 3rd -instar *T. ni* larvae than in the 4th-instar (Table 2 and data not shown). Larvae always became less susceptible to baculoviruses with age for ten- to thousand-fold differences of susceptibility between younger and elder larvae (Smite and Vlak, 1988; Tuan et al., 1994; 1995; 1999). Therefore, these experiments suggested that viruses, whether recombinants or wild type, should be applied at early stages of pest development to maximize the bioinsecticidal activity and plant protection efficacy.

All the recombinant viruses used in our studies were derived from linear commercial viral DNA rather than the genome of wild-type baculovirus. As the bioassay data showed, the highest LC_{50} for the larvae (Table 1), the highest rate of leaf area eaten by the treated larvae (Figure 4), and the lowest level of larval mortality with the highest body weight (Table 4) were all performed by the vAP10G. The efficacy of vAP10G was not only worse than our toxin-gene containing viruses, it also significantly underperformed the wild type virus. This suggests that if a field-collected wild type virus were used for the insertion of $LqhIT_2$ in later experiments, significant better control of pest insects would be achieved using the same *p-PCm* promoter.

Acknowledgment. We thank Mr. D. Chamberlin for careful reading and editing of the manuscript. This work was supported by grants from Academia Sinica, AS93-AB-IMB-01, and from National Science Council, Taiwan, ROC, NSC 90-2313-B-001-014.

Literature Cited

Benkhalifa, R., M. Stankiewicz, B. Lapied, M. Turkov, N. Zilberberg, M. Gurevitz, and M. Pelhate. 1997. Refined electrophysiological analysis suggests that a depressant toxin is a sodium channel opener rather than a blocker. Life Sci. **61:** 819-830.

Bonning, B.C. and B.D. Hammock. 1996. Development of recombinant baculoviruses for insect control. Annu. Rev. Entomol. **41:** 191-210.

Chao, Y.C., S.L. Chen, and C.F. Li. 1996. Fluorescent insects by infection. Nature **380:** 396-397.

Entwistle, P.F. and H.F. Evans. 1985. Viral control. *In* G.A. Kerkut and L.I. Gilbert (eds.), Comprehensive Insect Physiology Biochemistry and Pharmacology. Pergamon Press, New York, Vol. 7, pp. 347-412.

Finney, D.J. 1971. Probit analysis. 3rd ed. Cambridge Univ. Press. London, 333 pp.

Fuxa, J.A., J.R. Fuxa, and A.R. Richter. 1998. Host-insect survival time and disintegration in relation to population density and dispersion of recombinant and wild-type nucleopolyhedroviruses. Biol. Control **12:** 143-150.

Gershburg, E., D. Stockholm, O. Froy, S. Rashi, M. Gurevitz, and N. Chejanovsky. 1998. Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins. FEBS Letters **422:** 132-136.

Granados, R. and K. Williams. 1986. In vivo infection and replication of baculoviruses. *In* R.R. Granados and B.A. Federici (eds.), The Biology of Baculoviruses, Vol. 1. CRC Press, Boca Raton, Florida, pp. 89-108.

Hammock, B.D., B.C. Bonning, R.D. Possee, T.N. Hanzlik, and S. Maeda. 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature **344:** 458-461.

Harrison, R.L. and B.C. Bonning. 2000. Use of scorpion neuro-

toxins to improve the insecticidal activity of *Rachiplusia ou* multicapsid nucleopolyhedrovirus. Biol. Control **17:** 191-201.

Harrison, R.L. and B.C. Bonning. 2001. Use of proteases to improve the insecticidal activity of baculoviruses. Biol. Control **20:** 199-209.

Hoover, K., C.M. Schultz, S.S. Lane, B.C. Bonning, S.S. Duffey, B.F. McCutchen, and B.D. Hammock. 1995. Reduction in damage to cotton plants by a recombinant baculovirus that knocks moribund larvae of *Heliothis virescens* off the plant. Biol. Control **5:** 419-426.

Hughes, P.R., H.A. Wood, J.P. Breen, S.F. Simpson, A.J. Duggan, and J.A. Dybas. 1997. Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. J. Invertebr. Pathol. **69:** 112-118.

Ignoffo, C.M. and C. Garcia. 1996. Rate of larval lysis and yield and activity of inclusion bodies harvested from *Trichoplusia ni* larvae fed a wild or recombinant strain of the nuclear polyhedrosis virus of *Autographa californica*. J. Invertebr. Pathol. **68:** 196-198.

Ignoffo, C.M. and C. Garcia. 1997. Effect of viral concentration and body weight on mortality of larvae of *Trichoplusia ni* (Lepidoptera: Noctuidae) exposed to wild-type or recombinant strains of the nuclear polyhedrosis virus of *Autographa callifornia* (Lepidoptera: Noctuidae). Biol. Control **26:** 955-960.

Jarvis, D.L., L.M. Reilly, K. Hoover, C. Schulz, B.D. Hammock, L. Guarino. 1996. Construction and characterization of immediate early baculovirus pesticides. Biol. Control **7:** 228-235.

Lee, J.C., H.H. Chen, and Y.C. Chao. 1998. Persistent baculovirus infection results from deletion of the apoptotic suppressor gene *p35*. J. Virol. **72:** 9157-9165.

Lin, J.L., J.C. Lee, M.L. Li, and Y.C. Chao. 1999. Persistent Hz-1 virus infection in insect cells: evidence for viral DNA insertion in host chromosomes and viral infection in a latent status. J. Virol. **73:** 128-139.

Lo, H.R., C.C. Chou, T.Y. Wu, J.P.Y. Yuen, and Y.C. Chao. 2002. Novel baculovirus DNA elements strongly stimulate activities of exogenous and endogenous promoters. J. Biol. Chem. **277:** 5256-5264.

Lu, A., S. Seshagiri, and L.K. Miller. 1996. Signal sequence and promoter effects on the efficacy of toxin-expressing baculoviruses as biopesticides. Biol. Control **7:** 320-332.

Maeda, S. 1989. Increased insecticidal effect by a recombinant baculovirus carring a synthetic diuretic hormone. Biochem. Biophys. Res. Commun. **165:** 1177-1183.

Maeda, S., S.L. Volrath, T.N. Hanzlik, S.A. Harper, K. Majima, D.W. Maddox, B.D. Hammock, and E. Fowler. 1991. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. Virology **184:** 777-780.

Martens, J.W.M., G. Honee, D. Zuidema, J.W.M. van Lent, B. Visser, and J.M. Vlak. 1990. Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirusin insect cells. Appl. Environ. Microbiol. **56:** 2764-2770.

Martens, J.W.M., M. Knoester, F. Weijts, S.J.A. Groffen, Z. Hu, D. Bosch, and J.M. Vlak. 1995. Characterization of baculovirus insecticides expressing tailored Bacillus thuringiensis CryIA(b) crystal proteins. J. Invertebr. Pathol. **66:** 249-257.

McCutchen, B.F., P.V. Choudary, R. Crenshaw, D. Maddox, S. C. Kamita, N. Palekar, S. Volrath, E. Fowler, B.D. Hammock, and S. Maeda. 1991. Development of a recombinant baculovirus expressing and insect-selective neurotoxin: potential for pest control. Biotechnology **9:** 848-852.

Milks, M.L., I. Burnstyn, and J.H. Myers. 1998. Influence of larval age on the lethal and sublethal effects of the nucleopolyhedrovirus of *Trichoplusia ni* in the cabbage looper. Biol. Control **12:** 119-126.

Prikhod'ko, G.G., H.J.R. Popham, T.J. Felcetto, D.A. Ostlind, V.A. Warren, M.M. Smith, V.M. Garsky, J.W. Warmke, C.J. Cohen, and L.K. Miller. 1998. Effects of simultaneous expression of two sodium channel toxin genes on the properties of baculoviruses as biopesticides. Biol. Control **12:** 66-78.

Regev, A., H. Rivkin, B. Inceoglu, E. Gershburg, B.D. Hammock, M. Gurevitz , and N. Chejanovsky. 2003. Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively. FEBS Letter **537:** 106-110.

Roelvink, P.W., M.M.M. Van Meer, C.A.D. De Kort, R.D. Possee, B.D. Hammock, and J.M. Vlak. 1992. Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and *p10* genes. J. Gen. Virol. **73:** 1481-1489.

Smits, P.H. and J.M. Vlak. 1988. Biological activity of *Spodoptera exigua* nuclear polyhedrosis virus against *S. exigua* larvae. J. Invertebr. Pathol. **51:** 107-114.

Steel, R.G.D. and J.H. Torrie. 1980. "Principles and Procedures of Statistics: A Biometrical Approach" 2nd ed., McGraw-Hill, New York.

Stewart, L.M.D., M. Hirst, M.L. Ferber, A.T. Merryweather, P.J. Cayley, and R.D. Possee. 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature **352:** 85-88.

Tinsley, T.W. and D.C. Kelly. 1985. Taxonomy and nomenclature of insect pathogenic viruses. *In* K. Maramorosch and K.E. Sherman (eds.), Viral Insecticides for Biological Control. Academic Press, London, pp. 3-26.

Tomalski, M.D., and L.K. Miller. 1991. Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. Nature **352:** 82-85.

Tomalski, M.D., and L.K. Miller. 1992. Expression of a paralytic neurotoxin gene to improve insect baculoviruses as pesticides. BioTechnology **10:** 545-549.

Tuan, S.J., S.S. Kao, and D.J. Cheng. 1994. Histopathology and pathogenicity of *Spodoptera exigua* nuclear polyhedrosis virus isolated in Taiwan. Chinese J. Entomol. **14:** 33-45.

Tuan, S.J., S.S. Kao, D.J. Cheng, Roger F. Hou, and Y.C. Chao. 1999. Comparison of the characterization and pathogenesis of three lepidopteran nucleopolyhedroviruses (HearNPV, SpeiNPV and SpltNPV) isolated from Taiwan. Chinese J. Entomol. **19:** 167-186.

Tuan, S.J., S.S. Kao, U.L. Leu, and D.J. Cheng. 1995. Pathogenicity and propagation of *Spodoptera litura* nuclear polyhedrosis virus isolated in Taiwan. Chinese J. Entomol. **15:** 19-33.

Tuan, S.J., S.S. Kao, Y.C. Chao, and R.F. Hou. 1997. Investigation of pathogenicity of AcMNPV to nine lepidopteran pests in Taiwan. Chinese J. Entomol. **17:** 209-225.

Tuan, S.J., W.L. Chen, and S.S. Kao. 1998. *In vivo* mass pro-

duction and control efficacy of *Spodoptera litura* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. Chinese J. Entomol. **18:** 101-116.

van Beek, N., A. Lu, J. Presnail, D. Davis, C. Greenamoyer, K. Joraski, L. Moore, M. Pierson, R. Herrmann, L. Flexner, J. Foster, A. Van, J. Wong, D. Jarvis, G. Hollingshaus, and B. McCutchen. 2003. Effect of signal sequence and promoter on the speed of action of a genetically modified *Autographa californica* nucleopolyhedrovirus expressing the scorpion toxin LqhIT$_2$. Biol. Control **27:** 53-64.

Vaughn, J.L., R.H. Goodwin, G.J. Tompkins, and P. McCawley. 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro **13:** 213-217.

Volkman, L.E. 1995. Virus taxonomy: the classification and nomenclature of viruses. *In* F.A. Murphy, C.M. Fauquet, D. H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G..P. Martelli, M. A. Mayo, and M.D. Summers (eds.), The Sixth Report of the ICTV. Springer-Verlag Wien, Inc., New York, pp. 104-113.

Whitt, M.A. and J.S. Manning. 1988. A phosphorylated 34-kda protein and a subpopulation of polyhedrin are thiol-linked to the carbohydrate layer surrounding a baculovirus occlusion body. Virology **163:** 33-42.

Wu, T.Y., D.G. Lin, S.L. Chen, C.Y. Chen, and Y.C. Chao. 2000. Expression of highly controllable genes in insect cells using a modified tetracycline-regulated gene expression system. J. Biotechnol. **80:** 75-83

Zilberberg, N, E. Zlotkin, and M. Gurevitz. 1991. The cDNA sequence of a depressant insect selective neurotoxin from the scorpion *Buthotus judaicus*. Toxicon **29:** 1155-1158.

Zlotkin, E., M. Gurevitz, E. Fowler, and M.E. Adams. 1993. Depressant insect selective neurotoxins from scorpion venom: chemistry, action, and gene cloning. Arch. Insect Biochem. Physiol. **22:** 55-73.

利用早期啟動子表現昆蟲特異性神經毒以提升重組桿狀病毒對植物保護之效力

段淑人[1, 2]　侯豐男[3]　高穗生[1]　李至芬[1]　趙裕展[2]

[1] 行政院農委會農業藥物毒物試驗所生物藥劑系

[2] 中央研究院分子生物研究所

[3] 國立中興大學昆蟲學系

將一段以色列黃蠍之鎮定神經毒（LqhIT$_2$）基因，分別插入早期起動子（*p-PCm*）及非常晚期起動子（*p10*）之下游，構築成含蠍毒之加州苜蓿夜蛾核多角體病毒重組病毒，分別命名為 vAPcmIT$_2$ 及 vAP10IT$_2$。vAPcmIT$_2$ 在細胞感染後 12 小時，即可偵測到微量之蠍毒蛋白，較 vAP10IT$_2$ 表現蠍毒之時效提早約 24小時。重組病毒感染寄主後，於蟲體內表現蠍神經毒，造成幼蟲神經麻痺停止取食，全身癱軟無力致死。野生型加州苜蓿夜蛾核多角體病毒、vAP10IT$_2$ 及 vAPcmIT$_2$ 對擬尺蠖三齡幼蟲之半致死濃度（EC$_{50}$）分別為 1.00、0.19 及 0.17 PIBs/mm^3；早期及晚期表現之含蠍毒重組病毒之半有效時間（EC$_{50}$）亦有顯著性之差異，前者可提早 14～17 小時。而對擬尺蠖四齡幼蟲之而言，vAP10IT$_2$ 及 vAPcmIT$_2$ 可分別較野生型病毒縮短 19% 及 23% 之半有效時間; 若和 vAP10G 相比，更是縮短了 30% 及 33% 之半有效時間，可顯著提早發揮防蟲效果。在田間甘藍盆栽試驗，含蠍毒重組病毒處理組亦較螢光重組病毒及野生型病毒顯著減少葉片受害面積；含蠍毒重組病毒處理組在擬尺蠖幼蟲死亡率、存活率及幼蟲平均體重方面亦較未含蠍毒病毒組具顯著性差異。故含蠍毒重組病毒之殺蟲時效顯著優於野生病毒，而早期表現起動子表現神經毒亦較晚期表現者之殺蟲效果為佳，可達到更好的植物保護效果，減少甘藍葉片受害率。

關鍵詞：加州苜蓿夜蛾核多角體病毒；桿狀病毒；甘藍；鎮定神經毒；以色列黃蠍；植物保護；重組病毒；蠍毒；擬尺蠖。