Full Length Research Paper

Virus efficacy of recombined *Autographa californica* M nucleopolyhedrovirus (Ac*M*NPV) on tea pest *Ectropis obliqua*

Yue-Rong Liang[1], Chen Lin[1], Xun-Lei Wang[1], Jian-Hui Ye[1] and Jian-Liang Lu[2]*

[1]Zhejiang University Tea Research Institute, Hangzhou 310029, PR China.
[2]The State Agriculture Ministry Laboratory of Horticulture Plant Growth, Development and Quality Improvement, Hangzhou 310029, PR China.

Ectropis obliqua is a major tea pest and chitin synthase (CHS) plays a key role in the pest growth and development. A 192 bp conserved domain from *E. obliqua* CHS gene was cloned and it was used to construct recombined *Autographa californica* M nucleopolyhedrovirus (Ac*M*NPV) with double-stranded RNA interference (dsRNAi) method. The recombined Ac*M*NPV virus could propagate in host cells sf9. Injection test showed that the virus efficacy of the recombined Ac*M*NPV on *E. obliqua* larvae was significantly enhanced. It is considered that the CHS dsRNAi mediated by the nuclear polyhedrosis virus will be interesting for development of alternative bio-pesticide to control the tea pest *E. obliqua*.

Key words: Chitin synthase, baculovirus, double-stranded RNA interference, *Ectropis obliqua*.

INTRODUCTION

Chitin, a polymer of N-acetyl-β-D-glucosamine, is a major component of cuticles of the epidermis and trachea, and also the peritrophic matrices lining the gut epithelium in insect (Merzendorfer and Zimoch, 2003). It protects insects from mechanical injuries, toxins and pathogens. Chitin synthase (CHS) catalyzes the key step of chitin biosynthesis pathway and plays an important role in insect growth and development. The CHS genes were cloned in many insects (Tellam et al., 2000, Hogenkamp et al., 2005, Kumar et al., 2008) and could be divided into CHS-A and CHS-B in the gene family (Gagou et al., 2002). The CHS-A was expressed in the epidermis and trachea and the CHS-B in the peritrophic matrices (Tellam et al., 2000; Kumar et al., 2008). Suppressing the expression of CHS gene will block chitin synthesis and inhibit the growth and development of insects. As chitin biosynthesis occurs in insects but not in vertebrates, CHS genes and their expression products in the chitin synthesis pathway are effective potential targets for biological control of insect pests without interfering with the vertebrates (Merzendorfer and Zimoch, 2003).

RNA interference (RNAi) is a specific method to knock out the target gene, which results in the post-transcriptional gene silencing (PTGS) (Fire et al., 1998). The PTGS can be achieved through pathways of sense, antisense, or double-stranded RNA (dsRNA) molecules, among which the dsRNA-mediated genetic interference is the most efficient since it has a stable suppressive action on the expression of protein encoded by the target gene (Kramer & Bentley, 2003). The reaction of dsRNAi is catalyzed by the endoribonucleases which belong to a member of RNase III family (Ketting et al., 2001) and it is mediated by 21- and 22-nucleotide RNAs called small silencing RNAs (Elbashir et al., 2001). The RNA-induced silencing complex (RISC), a kind of multicomponent nuclease complex, could silence the target gene by cleaving and degrading the homologous mRNA (Hammond et al., 2000).

Nuclear polyhedrosis virus (NPV), one of the

*Corresponding author: E-mail: jllu@zju.edu.cn.

Table 1. Primers used in the tests.

Primer number	Direction	Nucleotide sequence	Remarks
CS$_{00}$	Forward	5'-tttgagtacgccatcggccattgg-3'	For cloning and verification of the CHS conserved domain; amplicon, 192 bp
	Reverse	5'-ccatcgatcctccccctggtcgtatcg-3'	
CS$_{01}$	Forward	5'-aaggcgcgcctttgagtacgccatcggccattgg -3'	Embedded with Asc I sites (underlined)
	Reverse	5'-aaatttaaatccatcgatcctccccctggtcgtatcg -3'	Embedded with Swa I site (underlined)
CS$_{02}$	Forward	5'-aatctagatttgagtacgccatcggccattgg -3'	Embedded with Xba I site (underlined)
	Reverse	5'-aaggatccccatcgatcctccccctggtcgtatcg -3'	Embedded with BamH I site (underlined)
INT$_{00}$	Forward	5'-agcgagctagcaaaattccaagat-3'	For verification of recombined bacmid DNA; amplicon, 491bp
	Reverse	5'-tagcatgcaaaaaccctcaataaa-3'	

baculoviruses, is a rod shaped virion consisting of a large circular double stranded DNA with an envelop around. It is an arthropod exclusive virus and has limited host ranges. Since it exclusively infects a few species with close kinship, it is used as biologicals for controlling pests without disrupting the balance of beneficial insects and environment (Motohashi et al., 2005). Application of NPVs for controlling pests is limited owing to its short term and low efficacy in the field, compared to chemical pesticides (Yin et al., 2004, Rajendra et al., 2006). The virus efficacy was enhanced by inserting a foreign gene with a function of pest control (Luckow and Summers, 1988). Many recombined NPVs, constructed by insertion of alpha anti-insect scorpion neurotoxin gene (Chejanovsky et al., 1995), scorpion depressant toxin gene (Gershburg et al., 1998) and lepidopteran-selective neurotoxin gene (Rajendra et al., 2006) were confirmed to result in improved insect control.

Ectropis obliqua (Prout) is a major tea pest (Yin et al., 2004). A large amount of chemical pesticides were used to control this pest, resulting in serious pesticide residues in tea products. Although NPV was used to control *E. obliqua* in tea fields (Yin et al., 2004), it has not been widely acceptable because of its low virus efficacy. To enhance virus efficacy, a CHS conserved domain was cloned from *E. obliqua* and used to construct a recombined Ac*M*NPV in this study.

MATERIALS AND METHODS

Materials

The *E. obliqua* larvae were supplied by Dr Yin from Tea Research Institute of Chinese Academy of Agriculture Science (Hangzhou, China) and reared in pest rearing containers (Yaohua Glass and Plastic Instruments Factory, Haimen, China) at 27°C and 16 h light/8 h dark illumination cycle during which they were fed with fresh tea leaves (Yin et al., 2004). The TRIzol reagent, the MAX Efficiency DH10Bac^{TM} competent *E. coli,* the sf9 cells, the Bac-to-Bac Baculovirus Expression System and the vector pFastBac^{TM} I were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The PrimeScript^{TM} 1st strand cDNA Synthesis kit, the *E. coli* TG01 and the pMD18-T vector were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China). The vector pFGC5941 (stock No.CD3-447) was supplied by the Arabidopsis

Information Resource (Stanford, CA, USA). The other reagents used were supplied by the Sangon Biological Engineering Technology and Services Co, Ltd. (Shanghai, China). Primers used in the present study were listed in Table 1.

Cloning and sequencing of conserved domain

Four *E. obliqua* prepupae were ground in a mortar with liquid nitrogen. Total RNA was extracted using TRIzol reagent and first strand cDNA was synthesized using PrimeScript^{TM} 1st strand cDNA Synthesis kit as methods by Borthakur et al. (2008). A 192 bp conserved domain of *E. obliqua* CHS gene was amplified from the 1st strand cDNA by PCR using primers CS$_{00}$. The PCR product was ligated into the pMD18-T to form the pMD18-T-CHS and transformed into competent cells of *E. coli* TG01, and finally sequenced on an ABI Prism Sequencer (Applied Biosysytem, Foster, USA) using the dideoxynucleotide chain termination method (Sanger et al., 1977).

Construction of recombined bacmid with CHS dsRNAi gene

The construction of recombined bacmid with CHS dsRNAi gene was carried out as the scheme in Figure 1 according to reported methods (Detvisitsakun et al., 2007). Two amplicons with restriction sites of Asc I/Swa I or Xba I/BamH I were obtained by PCR using pMD18-T-CHS as template and primer pairs of CS$_{01}$ or CS$_{02}$ (Table1). After digesting the amplicons with restriction enzyme, the CHS conserved domain was incorporated into Asc I/Swa I and BamH I/Xba I multiple clone sites of plasmid pFGC5941 by sense and antisense insertion respectively, to construct an intermediate dsRNAi vector (pFGC5941-CHS-Intron-CHSr). The intermediate dsRNAi vector was digested by enzymes Asc I/Xba I to obtain a sequence 'CHS-Intron-CHSr' (CHS dsRNAi gene) which was used to insert into BssH II (an isocaudarner of the AscI) /Xba I multiple clone site of plasmid pFastBac^{TM} I to form transferring vector (pFastBac^{TM} I-CHS-Intron-CHSr). The pFastBac^{TM} I-CHS-Intron-CHSr was transformed into competent *E. coli* DH10Bac^{TM} which contains a bacmid DNA of Ac*M*NPV and helper plasmid encoding the transposase to form a recombined bacmid DNA with foreign CHS dsRNAi gene.

Construction and propagation of recombined Ac*M*NPV

The recombined Ac*M*NPV DNA with CHS dsRNAi gene was extracted from the above transformed *E. coli* DH10Bac^{TM} using UNIQ-10 column viral DNA extraction kit and packaged with Cellfectin® Reagent, and then transfected sf9 cells using Bac-to-Bac Baculovirus Expression System according to supplier's
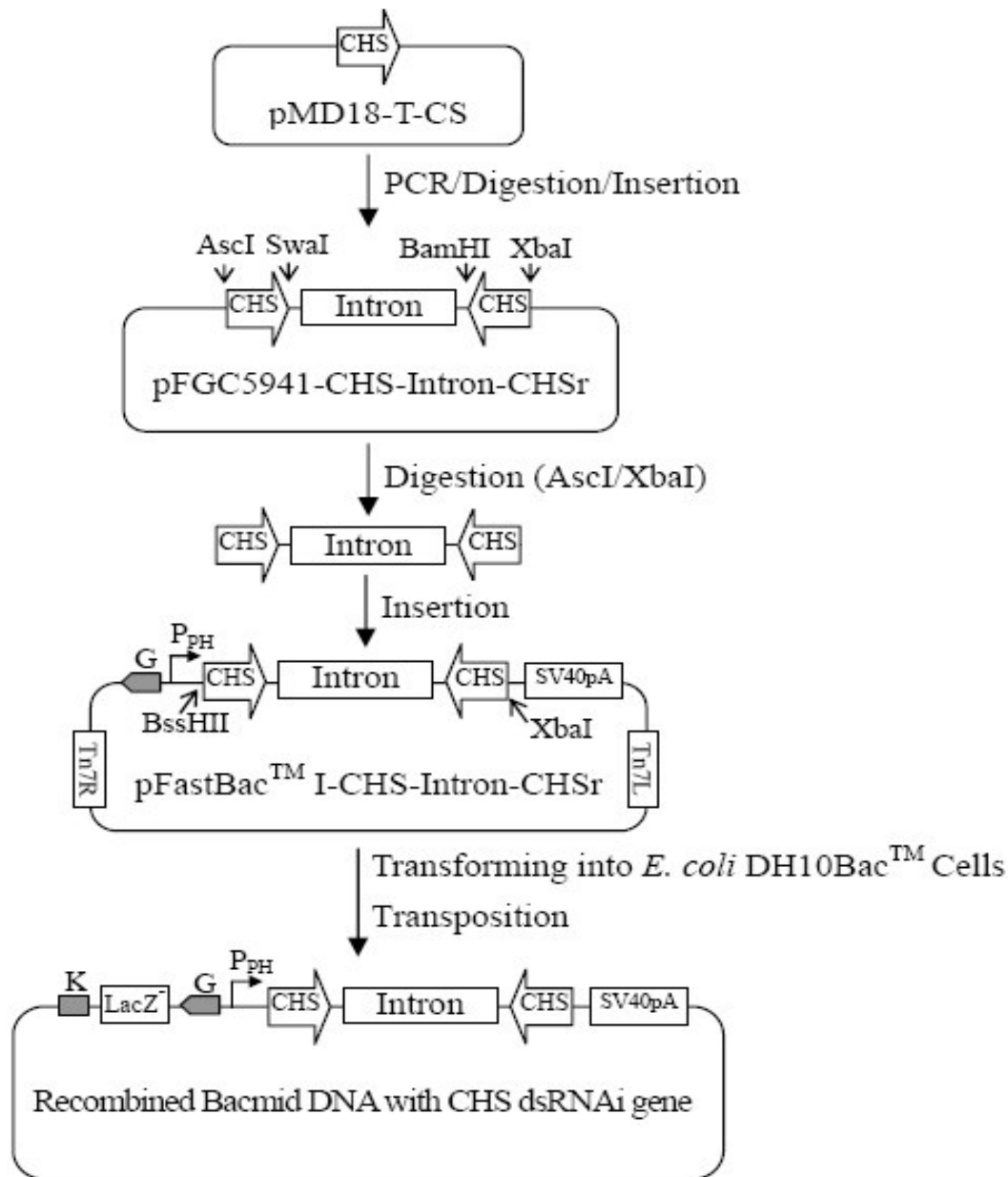
Figure 1. Scheme for constructing recombined Bacmid DNA with CHS dsRNAi gene. CHS: chitin synthase conserved domain with 192bp from *E. obliqua*. Intron: intron of the chalcone synthase A gene from petunia. P$_{PH}$: Ac*M*NPV polyhedrin promoter. G: gentamicin resistant gene. Tn7R, Tn7L: the right and left arms of Tn7 transposon. SV40pA: simian virus 40 late polyadenylation signals. K: kanamycin resistant gene. LacZ$^-$: LacZ gene interrupted by foreign gene insertion. Asc I, Swa I, BamH I, Xba I and BssH II: restriction enzyme action sites.

manual. The transfected sf9 cells were incubated in the Sf-900 II SFM medium at 28°C for 72 h, and centrifuged at 500 x g for 5 min. The supernatant was defined as P1 viral stock which was further used to infect sf9 cells to generate a high-titer P2 stock. The P2 stock was harvested after 72 h infection by centrifugation of the cultured cell and medium mixture at 500 x g for 5 min. Prior to testing the virus efficacy, viral plaque assay was performed according to the supplier's manual of the Bac-to-Bac Baculovirus Expression System.

As for control, the bacmid DNA of Ac*M*NPV was extracted from the MAX Efficiency DH10Bac™ competent *E. coli* which was not transformed by pFastBac™ I-CHS-Intron-CHSr and used to transfect the sf9 cells by the same procedure, and the virus was defined as wild type Ac*M*NPV.

Virus efficacy test of recombined Ac*M*NPV

The recombined Ac*M*NPV (2 × 10^5 pfu/ml) was injected into the hemocoel of 3rd instar *E. obliqua* larvae at a dosage 2.0 µl per
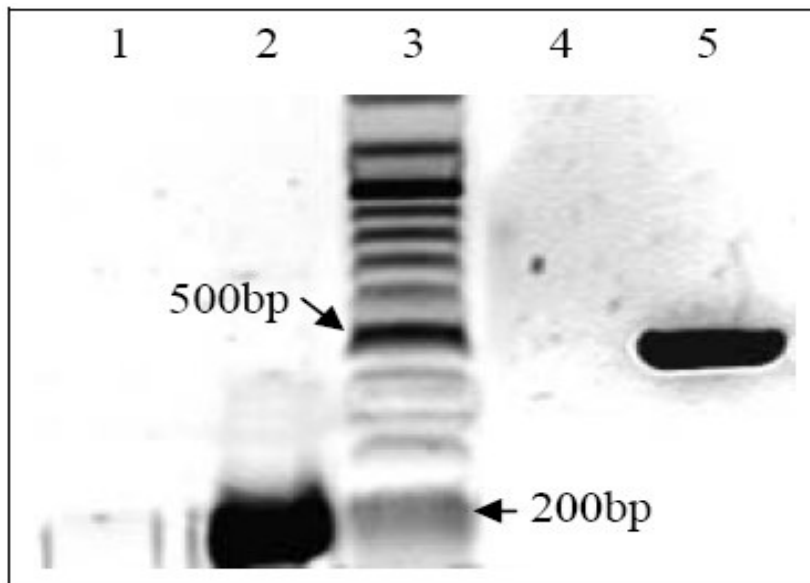
Figure 2. PCR verification of recombined bacmid DNA. Lane 1: blue clone amplified using primers CS_{00}. Lane 2: white clone amplified using primers CS_{00}. Lane 3: DNA ladder #SM0371. Lane 4: blue clone amplified using primers INT_{00}. Lane 5: white clone amplified using primers INT_{00}.

larva. The negative and positive control groups were injected with a same volume of distilled water and wild type Ac*M*NPV (2×10^5 pfu/ml), respectively. The larvae were fed with fresh tea leaf in pest-rearing container as above. There were 30 larvae in each group and the test was carried out in triplicates. After treatment, mortality and excrement of the larvae were monitored at every 24 h intervals.

RESULTS AND DISCUSSION

Recombined Ac*M*NPV

The conserved domain obtained from *E. obliqua* CHS gene was 192 bp as follows.

5'-tttgagtacgccatcggccattggctgcaaaaggcgaccgaacacatga tcggatgcgtactctgtagtcctggctgcttctcactcttcagagtaaaggcgctc atggatgataacgtcatgaagaaatacaccctcacctcacacgaggcgagg cattacgtgcgatacgaccaggggggaggatcgatgg-3'.

The domain shared 100% identity with the nucleotides 2468th to 2659th of *E. obliqua* CHS gene (GenBank accession No.EU482034). It also shared more than 80% identity with *CHS* genes in *Manduca sexta*, *Ostrinia furnacalis*, *Plutella xylostella*, *Spodoptera frugiperda*, *Spodoptera exigua* and *Tribolium castaneum*.

When pFastBac[TM] I-CHS-Intron-CHSr was transformed into competent *E. coli* DH10Bac[TM], a 192 bp amplicon and a 491 bp amplicon were amplified from the recombined bacmid DNA sample extracted from the transformed clone (in white color) by polymerase chain reaction (PCR) verification using primers CS_{00} and INT_{00}, respectively

(Figure 2). The 192 bp was the sequence by sense insertion of CHS conserved domain of *E. obliqua* and the 491 bp sequence was a part of the 'intron' which was indirectly transferred from the intermediate dsRNAi vector of pFGC5941(Figure 1). These suggested that the 'CHS-Intron-CHSr' foreign gene was transposed into the bacmid of *E. coli* DH10Bac[TM]. The recombined bacmid with CHS-Intron-CHSr gene (CHS dsRNAi gene) which could be transcribed into hairpin CHS RNA and induced the dsRNA interference was constructed as expected.

When the sf9 cells were transfected by the recombined bacmid, their growth was suppressed, compared to the control (Figure 3). Swollen cells were observed on the 3rd day incubation, and lysised cells were found on the 5th day. However, the density of control cells which were not infected by virus increased with incubation time (Figure 3). The growth of sf9 cells transfected by wild type Ac*M*NPV virus showed the similar trend as those transfected by the recombined Ac*M*NPV virus. In this case, the sf9 cells were the host of the recombined Ac*M*NPV virus. The sf9 cell lysis suggests that the recombined Ac*M*NPV virus propagated in the host sf9 cells. After one week incubation when most of the trans-fected sf9 cells were lysised, the culture solution was centrifuged and the supernatant containing the recom-bined virus was used to extract the DNA for verification test by digestion using enzymes BssH II/Xba I. It showed that the band CHS-Intron-CHSr (1.76 kb) was found in the recombined Ac*M*NPV virus sample, but not in the wild type virus sample (Figure 4). It suggested that the foreign gene of CHS-Intron-CHSr was incorporated into the Ac*M*NPV virus and amplified with the virus propagation.
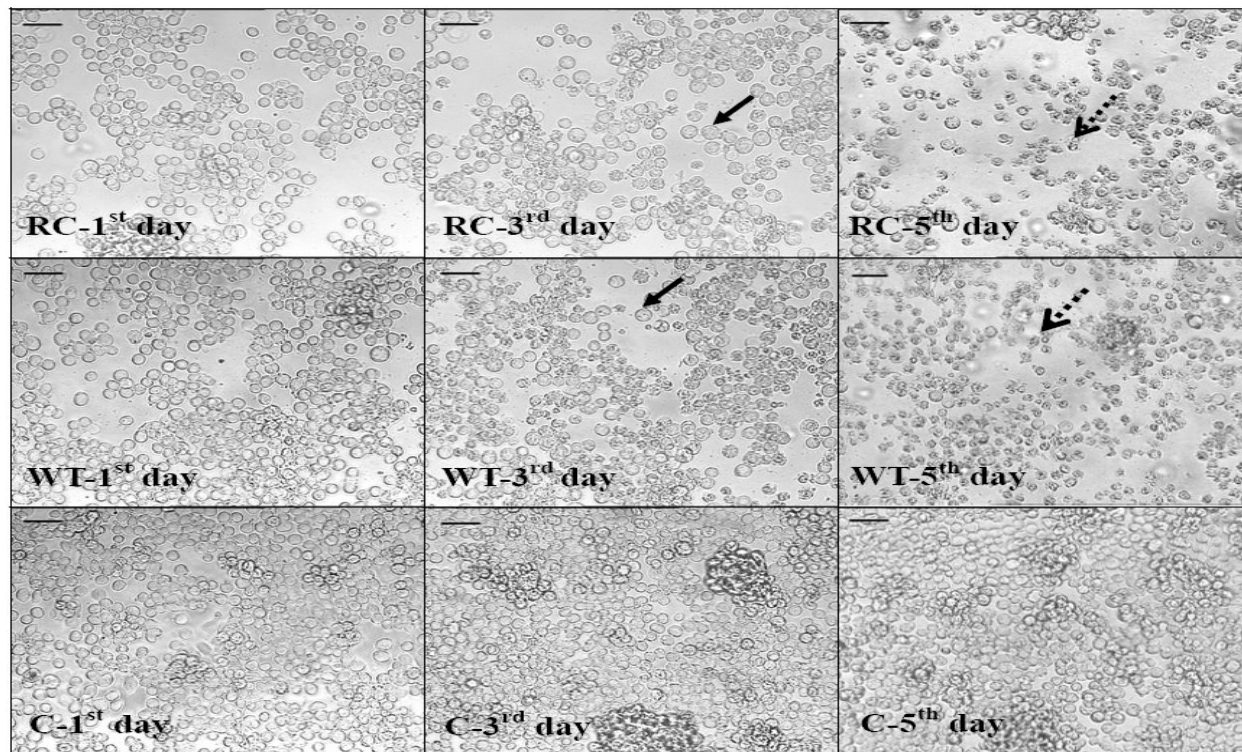
Figure 3. Propagation of recombined Ac*M*NPV virus in host cells sf9. RC: recombined Ac*M*NPV virus. WT: wild type Ac*M*NPV virus. C: control which was not transfected by virus. 1st day, 3rd day, 5th day: the day after transfection. Black bar = 50 µm. Solid arrow head: the swollen cell. Dashed arrow head: the lysised cell.
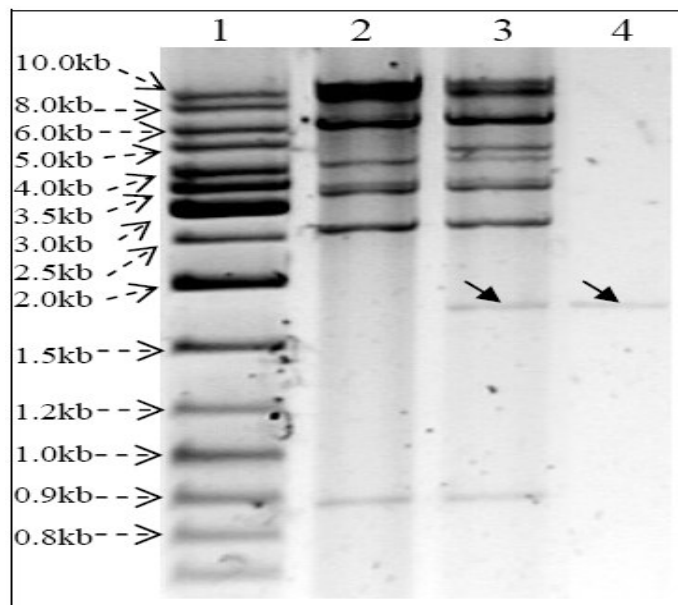

Figure 4. Verification of recombined Ac*M*NPV. Lane1: DNA ladder #SM0331. Lane2: wild type Ac*M*NPV digested by BssH II/Xba I. Lane3: recombined Ac*M*NPV digested by BssH II/Xba I. The solid arrow showed the aim band CS-Intron-CSr (1764 bp); Lane4: the purified CS-intron-CSr fragment from pFastBac I CS-intron-CSr digested by BssH II/Xba I. The solid arrow showed the aim band CS-intron-CSr (1764 bp).

Efficacy of recombined Ac*M*NPV virus

The cumulative mortality of *E. obliqua* larvae injected with the recombined Ac*M*NPV virus was 86% on the 7th day of injection, being significantly higher than those of negative control injected by distilled water and positive control injected by wild type Ac*M*NPV virus (Figure 5). The excrement of larvae treated with the recombined Ac*M*NPV virus was significantly lower than the two controls (Figure 6). The excrement was usually related to the tea leaf consumption and growth rate of the larvae (Yin et al., 2004). These suggested that the efficacy of recombined Ac*M*NPV virus was improved by the CHS dsRNAi gene insertion.

The expression of CHS genes was essential for the growth and development in insect and nematode (Zhang et al., 2005, Fanelli et al., 2005, Arakane et al., 2008). The formation of peritrophic matrix in *Aedes aegypti* was disrupted if the transcription of CHS gene was down regulated by RNAi method (Kato et al., 2006). The chitin synthesis in peritrophic matrix in *Tribolium castaneum* was strongly suppressed by injection of gene *CHS-A* dsRNAi in male or female pharate adults, resulting in less eating and lower survival (Arakane et al., 2008).NPV was used to control tea pest *E. obliqua* (Yin et al., 2004). This study showed that Ac*M*NPV had pesticidal efficacy on *E. obliqua* and the efficacy of recombined Ac*M*NPV virus
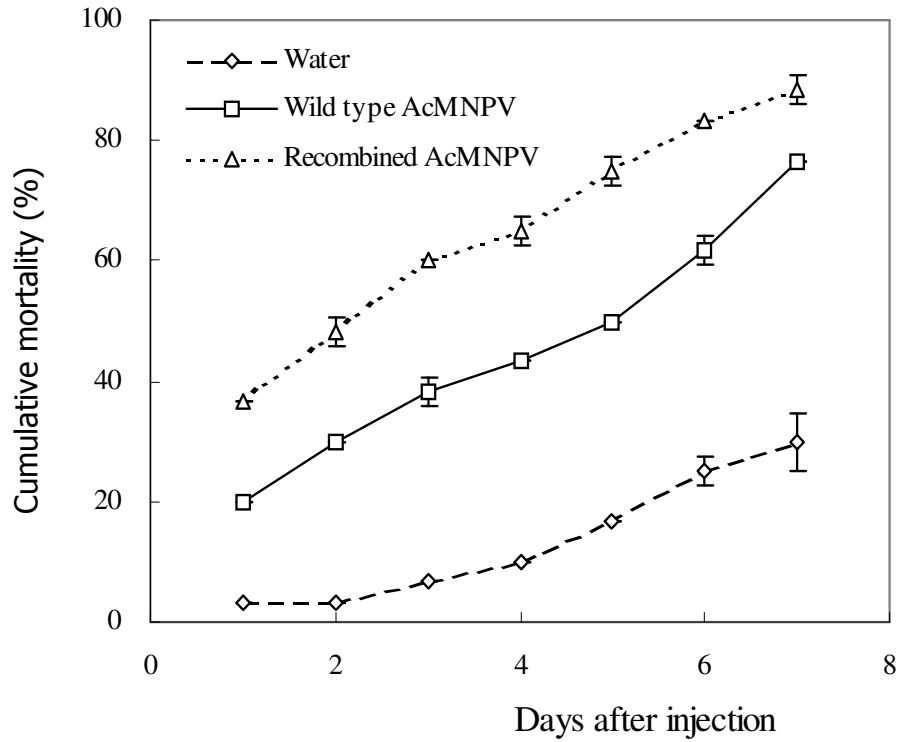
Figure 5. Cumulative mortality of *E. obliqua* after injection. There were thirty 3rd instar larvae each treatment and the test was in triplicates. Injection volume: 2 μl 2 × 10^5 pfu/ml virus solution or distilled water. The bar shows the standard deviation.
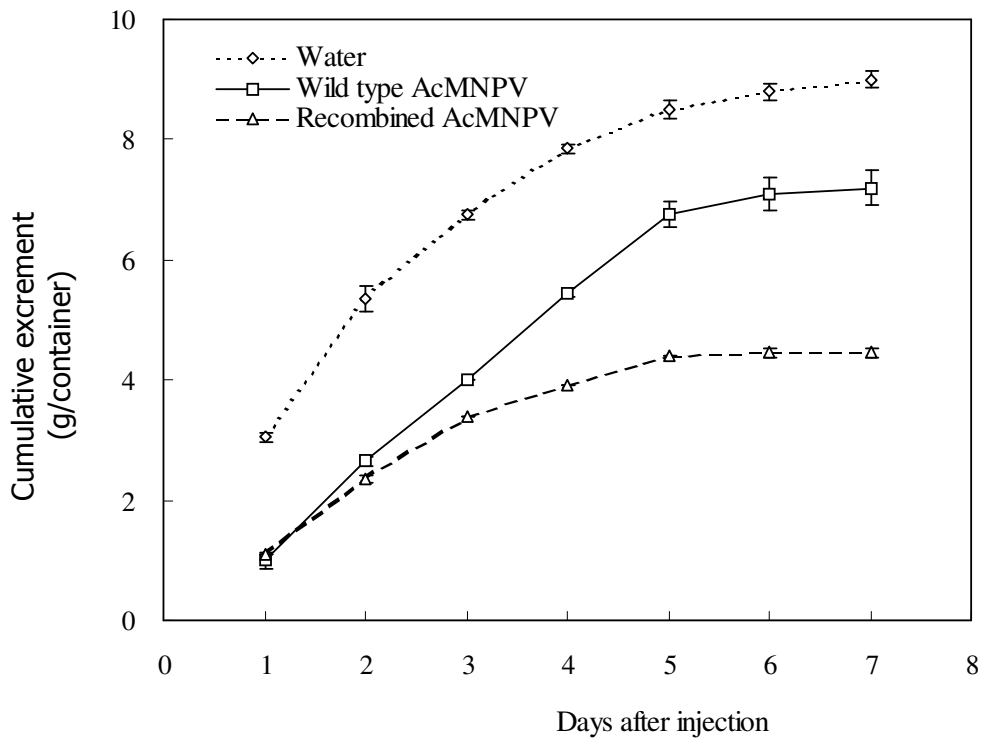

Figure 6. Cumulative excrement of *E. obliqua* after injection. There were thirty 3rd instar larvae in each treatment and the test was in triplicate. Injection volume: 2 μl 2 × 10^5 pfu/ml virus solution or distilled water. The bar shows the standard deviation.

constructed by CHS dsRNAi method was enhanced (Figures 5 and 6). This may be ascribed to the suppression of the endogenous CHS gene translation in *E. obliqua* mediated by exogenous CHS dsRNAi during infection of the recombined Ac*MN*PV. It is considered that the CHS dsRNAi will be interesting for development of alternative bio-pesticide to control the important tea pest.

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