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Abstract

Baculoviruses have a long history of safe use as specific, environmentally friendly insecticides that provide alternatives to chemical pesticides for controlling insect pests. However, their use has been limited by several factors, particularly their slow pathogenicity. In this study, we constructed a recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) and an *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) that expressed an insect-specific cyto-insectotoxin (Cit1a) from the venom of the central Asian spider *Lachesana tarabaevi*. Cit1a is a comparatively long linear cytolytic molecule that contains a predicted α-helix structure composed of two short membrane-acting antimicrobial peptides (MAMPs) that are joined together in a "head-to-tail" shape. Cit1a fused to polyhedrin gene (*polh*) (*polh-cit1a*) was expressed in the nuclei as polyhedra in silkworm larvae, Bm5 and Sf9 cells. An early death of Bm5 and Sf9 cells by recombinant BmNPV/Polh-Cit1a and AcMNPV/Polh-Cit1a was observed compared with control viruses that lacked the toxin gene. The infected cells showed a loss of cytoplasm, membrane integrity and structural changes, suggesting that recombinant baculovirus-infected cells were killed by the necrosis caused by Cit1a. In addition, the BmNPV/Polh-Cit1a showed a significant reduction in the median lethal time (LT_{50}) against silkworm larvae compared with those of control BmNPV that lacked the *cit1a* gene.

Keywords: Cyto-insectotoxin; Recombinant baculovirus; Pathogenicity; Cytotoxicity; *Bombyx mori*

Electronic supplementary material The online version of this article (doi:) contains supplementary material, which is available to authorized users.

Introduction

Insect pests are a major cause of reduction in crop yields, and chemical insecticides are currently the dominant method for controlling pest populations (Oerke 2006). However, the emergence of insects that are resistant to chemical pesticides and their harmful effects on the environment and the potential human health risks have led to interest in alternative pest management practices. The use of baculovirus can overcome these problems inherent to chemical insecticides. Baculovirus has a long history of safe use as a specific, environmentally friendly pesticide because it has infectious particles that are preserved into the proteinaceous occlusion bodies (called polyhedra); these polyhedra favor the formulation of biological insecticides with simple application methods (Gramkow et al. 2010).

Recently, studies have been performed in the production of insecticides (Inceoglu et al. 2001) that are based on baculoviruses because of their safe use in agriculture. Baculoviruses present rod-shaped nucleocapsides within two distinguishable phenotypes, budded virus (BV) and occlusion-derived virus (ODV), in an individual cycle of infection (Smith et al. 1983). The polyhedra are discharged into the environment naturally upon insect death due to virus infection. Specific insects feed on polyhedra-contaminated foliage and are infected *per os* by OB-relief ODVs, which cause an initial infection in insect midgut cells (Slack and Arif 2007). After infection of the host midgut cells, the BV structure is formed and causes a collateral infection of all other host tissues (Wang et al. 2010). These phenomena can take several days for a week before the infected insects stop feeding. This is the principal drawback to the use of baculoviruses as insecticides in the field. Extensive crop damage can occur before the baculovirus-infected insects die.

Therefore, to apply baculoviruses in the field as insecticides, it is necessary for baculoviruses to either shorten the killing time or reduce the effective feeding time. Diverse alien genes with potential to enhance pesticidal effects have been cloned into the baculovirus

genome using genetic engineering technology (Szewczyk et al. 2006). Several insect toxin genes from bacteria, spiders, and scorpions were inserted into the baculovirus genome under a baculoviral promoter to improve pesticidal performance. The modified baculovirus killed faster than the wild type did (Chang et al. 2003; Choi et al. 2008; Gramkow et al. 2010; Stewart et al. 1991). These results indicate that insect toxins are favorable candidates to prevent extensive crop damage by reducing the time from the initial infection to the death of the baculovirus-infected insects. Recently, a recombinant baculovirus, NeuroBactrus, which can convey *Bacillus thuringiensis* crystal protein (Cry1-5) and the insect-specific neurotoxin, AaIT, from *Androctonus australis*, was constructed (Shim et al. 2013). This NeuroBactrus showed high insecticidal activity to *Plutella xylostella* larvae and prominently reduced the median lethal time against *Spodoptera exigua* larvae compared with the wild type baculovirus.

In this study, to determine the possibility of using other spider toxins to improve baculoviral insecticides, the gene of cyto-insectotoxin (Cit1a), a novel antimicrobial peptide (AMP) from the venom of the Central Asian spider (*Lachesana tarabaevi*), was inserted into the genome of *Bombyx mori* nucleopolyhedrovirus (BmNPV) and *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) as a fusion gene with a polyhedrin (Polh) gene under the polyhedrin promoter. Cit1a is a linear cationic peptide with 69 amino acid residues and represents an attractive molecule to show high antibacterial activity and anti-*Chlamydia trachomatis* activity inside infected cells (Lazarev et al. 2011; Vassilevski et al. 2008). Previously, active Cit1a was expressed as a fusion protein with EGFP in silkworm larvae and was purified (Ali et al. 2014). Though Cit1a was expressed as a fusion protein in silkworm, Cit1a was still active and showed antimicrobial activity. In this study, each recombinant baculovirus expressed Polyhedrin-Cit1a fusion protein (Polh-Cit1a) and produced polyhedra in the nuclei. The infectivity and speed of action of this virus were significantly increased

compared with the control virus that lacked the *cit1a* gene. The insecticidal activity of the recombinant baculovirus was also evaluated using silkworm larvae.

Materials and methods

Viruses, insects and insect cell lines

Both *B. mori* (Bm5) and *Spodoptera frugiperda* (Sf9) cell lines were maintained at 27°C in SF-900II medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and with an antibiotic-antimycotic mixture (Gibco, Carlsbad, CA, USA). The silkworm larvae (5^{th} instar) were purchased from Ehime Sansyu Co. (Ehime, Japan). The control BmNPV virus, BmNPV-CP$^-$-hMTP-EGFP-SH, designated as BmNPV/EGFP, harboring human microsomal transfer protein gene fused with the *egfp* gene (Kato et al. 2013), was used in the present study. Silkworm larvae were maintained in Silkmate 2S (NOSAN Co. Yokohama, Japan) at 25°C in a humidified (65%) environment chamber.

Construction of the recombinant transfer vector

The oligonucleotide sequences of the toxin gene (Cyto-insectoxins: *cit1a*, accession number FM165474) was amplified by polymerase chain reaction (PCR) using the primer set FLAG-Cit1a-F and Cit1a-xba-R (Table 1, primers 1, 2). The polyhedrin gene (*polh*) fragment was amplified using the genomic template from the AcMNPV by PCR using the primer set Polh-F and Polh-FLAG-R (Table 1, primers 3, 4). Each amplified fragment was analyzed by electrophoresis in 1% agarose gels and purified using GFX PCR and a Gel Band Purification Kit (GE Healthcare, Chicago, IL, USA) and fused to each other by PCR to obtain a Polh-Cit1a fusion gene. After 10 cycles of PCR, the two primer sets (Table 1, primers 2, 3) were added to amplify the fusion fragment (Polh-Cit1a). The fusion fragment (Polh-Cit1a) was purified using GFX PCR and a Gel Band Purification Kit (GE Healthcare, Chicago, IL, USA) and was

112 inserted into the pFastBac1 vector (Life Technologies, Carlsbad, CA, USA) by cloning, as
113 previously described (Ali et al. 2014). The heterologous insertion was confirmed by restriction
114 enzyme digestion, amplifying the target region by PCR, electrophoresis in 1% agarose gels and
115 sequencing.

116 Recombinant virus construction and toxin expression in the insect host and cell

117 The recombinant transfer vector (designed as pFastBac1polh-cit1a) harboring toxin
118 was transformed into the *E. coli* strain BmDH10Bac CP^- and DH10Bac (Life Technologies) by
119 heat shock, as previously described (Hiyoshi et al. 2007). Recombinant bacmids were selected.
120 Each bacmid was purified, and the presence of the recombinant gene was checked by PCR
121 using specific oligonucleotides (Table 1, primers 5, 6). The obtained bacmids were designated
122 as BmNPV/Polh-Cit1a and AcMNPV/Polh-Cit1a bacmids.

123 Ten micrograms of BmNPV/Polh-Cit1a was extracted by alkaline extraction with a
124 helper plasmid and mixed with 1/10 volume of 1, 2-dimyristyloxypropyl-3-dimethyl-hydroxy
125 ethyl ammonium bromide (DMRIE-C) reagent (Life Technologies) and incubated at room
126 temperature for 30–40 min. This mixture (50 µl containing 10 µg of bacmid DNA) was injected
127 into the dorsal section of the larva using a syringe with a 26-gauge beveled needle (Terumo
128 Co., Tokyo, Japan). Larval hemolymph was collected from BmNPV/Polh-Cit1a-infected
129 larvae at 96–120 h p.i. and subjected to the budded virus (BV) production analysis during
130 infection. The viruses containing hemolymph of the infected silkworm larvae were collected
131 as a virus stock and was stored at -80°C prior to use in this study. The collected fat body was
132 also stored at -80°C until use. A portion of the collected fat body was homogenized with Tris-
133 buffered saline (TBS, pH 7.4) containing 0.1% Triton X-100 (TBS-TX100), followed by
134 sonication, and the homogenate was stored at -80°C until use.

In the case of recombinant AcMNPV DNA with the Polh-Cit1a gene, DNA was packaged with Cellfectin Reagent (Life Technologies) and then transfected into Sf9 cells according to the supplier's manual. The transfected Sf9 cells were cultured in the SF-900 II SFM medium at 27°C for 72 h and were centrifuged at 1000 × g for 5 min. The supernatant was designated as P1 viral stock, which was further used to infect Sf9 cells to generate a high-titer P2 stock. The P2 stock was collected after 72 h of infection by centrifugation of the cultured cell and medium mixture at 1000 × g for 5 min and was stored at -80°C until use.

SDS-PAGE and western blot analysis

SDS-PAGE and western blot were performed to check the expression of Polh-Cit1a fusion protein from the fat body of silkworm and Sf9 cells. For the western blot, monoclonal anti-FLAG M2 conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich Japan, Tokyo, Japan) was used as an antibody to detect the Polh-Cit1a fusion protein at 1: 10,000 dilution. For secondary antibody, sheep anti-mouse IgG antibody (GE Healthcare Japan, Tokyo, Japan) was used at a 1: 10,000 dilution. The protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA).

Purification of Polh-Cit1a fusion protein from silkworm larvae

The fat body collected from 10 silkworm larvae was suspended in 25 ml of ice-cold TBS buffer (pH 7.4) and lysed by sonication 3 times for 30 s, with 1-min intervals between each. The sonication condition was 40 amplitudes with 4 output watts (Sonics and materials Inc., CT, USA). The sample was then centrifuged at 20,000 × g for 20 min, and the supernatant was filtered using a 0.45 μm filter. The collected filtrate was used for affinity purification using DDDDK tagged protein purification gel (Medical and Biological Laboratories Co., LTD, Nagoya, Japan). The DDDDK tagged protein purification gel was equilibrated with TBS buffer

prior to use. The collected supernatant was mixed with 1 ml of gel and gently stirred at 4°C for 1 h. This mixture was centrifuged at 2500 × g for 5 min, and the precipitated resin was washed with 36 ml of TBS buffer. The proteins bound to the resin were eluted with elution buffer (0.1 M glycine, pH 3.5). To check the expression of Polh-Cit1a fusion protein, 50 µg of purified fusion protein sample was digested with 1 unit of recombinant enterokinase (rEK; Novagen, Darmstadt, Germany) at room temperature for different time intervals. The product was analyzed by SDS-PAGE and silver staining according to the company's protocol (Silver Stain II Kit Wako, Wako Pure Chemical Industries, Ltd. Tokyo, Japan).

Bioassays

Twenty 5^{th}-instar silkworm larvae were injected with 50 µl of each viral stock (approximately 1×10^6 virus titer). Ten silkworm larvae were also injected with medium without virus as a negative control, and the experiment was repeated three times. The inoculated larvae were observed twice daily until death. The mortality was calculated by counting the dead larvae at 12-h intervals until all larvae were dead, and the median lethal time (LT_{50}) was determined using a Probit analysis (SPSS Base 16.0 for Windows User's Guide, SPSS Inc., Chicago, IL, USA). To study the growth inhibitory activity of Cit1a, the silkworm larvae were infected with recombinant viruses as well as control virus which has no toxin gene. The body weight of each larva were measured before infection and continued to monitor the growth upto 48 h after infection. Mock-infected silkworm larvae were also used as expected for normal growth. The growth rate (*GR*) of the recombinant baculovirus-infected insect was calculated using the following formula:

$$GR\ (\%) = \frac{(FBW - IBW)}{IBW} \times 100$$

where *IBW* and *FBW* represent the body weights of virus-infected insects at 0 and 48 h p.i., respectively. All statistical analyses were performed using the SPSS 2008 (SPSS Inc.) statistical package. Fat body was collected from the dead larvae and subjected to western blot analysis for the confirmation of the expression of its toxin.

Counting the number of polyhedra

Monolayers of Bm5 and Sf9 (5×10^6) cells were infected with recombinant baculoviruses at a multiplicity of infection (MOI) of 10. BmNPV/Polh-Cit1a-, BmNPV/EGFP-, AcMNPV-, AcMNPV/Polh-Cit1a- and mock-infected cells were collected at 72 h p.i. and washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The polyhedra formed in the cells were quantified by hemocytometry using hemocytometer (Neubaurer Improved Bright-Line, Hirschmann Laborgeräte GmbH & Co. Eberstadt, Germany), as previously described (Hong et al., 2000). Before counting polyhedra, the cells infected with virus were harvested and washed with PBS (pH 7.5). Thereafter, 1% sodium dodecyl sulfate was added to harvested cells and incubated at 37°C for 30 min. Polyhedra was released from the cells and counted by hemocytometer.

Quantification of BmNPV and AcMNPV particles

Baculoviral DNA was isolated by a High Pure Viral Nucleic Acid Kit (Roche Diagnostics K. K., Tokyo, Japan), and the virus titer was quantified by quantitative polymerase chain reaction (Q-PCR), as previously described (Kato et al. 2009; Lo and Chao 2004). Briefly, BmNPV DNA was extracted from BmNPV-infected larval hemolymph, but in case of AcMNPV DNA, the supernatant of AcMNPV-infected Sf9 cells (MOI: 10) at 72 h p.i. containing the BV was harvested, and cell debris was removed by centrifugation (1000 × *g* for 5 min). An aliquot of each supernatant was used for the DNA extraction. For the Q-PCR assays,

Brilliant II Fast SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA., USA) was used with primers 7 and 8 (Table 1). A stock of wild-type AcMNPV and BmNPV, which had been previously titered by end-point dilution, was used as a standard. The program for the DNA amplification cycle was: 95°C for 5 min for one cycle, followed by 80 cycles of amplification protocol: denaturation at 95°C for 10 s, annealing and extension at 63°C for 30 s. The PCR amplification and melting curves were analyzed using the MxPro QPCR software (Stratagene).

Microscopic analysis

For the light and fluorescence microscopy, monolayers of Bm5 cells (5×10^6) were infected at a MOI of 10. The infected cells were observed and photographed at different hours post-infection in a light microscope (LSM 700, Zeiss, Jena, Germany). Observation of the internal tissues of the recombinant baculovirus-infected larvae was performed. Four days after injection, larvae were dissected, and the silk glands were collected and examined under a light microscope (SZX16, Olympus Corporation, Tokyo, Japan). The images were analyzed using the DPC contrasted software.

Cell cytotoxicity assays

Both Bm5 and Sf9 insect cells were seeded in 6-well plates (4×10^4 cells/well) and infected at a MOI of 10 with different viruses. For the Trypan-blue cell viability assay, the medium from the virus infected cells was entirely removed; the cells were washed with PBS buffer, stained with Trypan-blue 0.4% (Invitrogen, Carlsbad, CA, USA) for 5 min, and washed with PBS (pH 7.4) three times. The number of cell deaths was determined using a hemocytometer (Neubaurer Improved Bright-Line) under a light microscope. The experiment was repeated at least three times, and Student's t-test was performed to compare two means in pairs.

Results

Construction of the recombinant virus

The *cit1a* was fused to the *polh* gene by PCR through the FLAG tag sequence and was verified by 1% agarose gel electrophoresis. The fusion gene was successfully cloned into the pFastBac1, and the generated recombinant pFastBac1Polh-Cit1a was verified by restriction enzyme digestion and by amplifying the target region using PCR, 1% agarose gel electrophoresis and sequencing. The recombinant pFastBac1Polh-Cit1a was transformed into *E. coli* BmDH10Bac CP^- and DH10Bac competent cells, and recombinant BmNPV CP^-/Polh-Cit1a and AcMNPV/Polh-Cit1a bacmids, respectively, were constructed and designated as BmNPV/Polh-Cit1a and AcMNPV/Polh-Cit1a.

Expression and purification of Polh-Cit1a in silkworm larvae and Sf9 cells

The expression of Polh-Cit1a fusion protein in both BmNPV/Polh-Cit1a bacmid injected-silkworm larvae and AcMNPV/Polh-Cit1a transfected-Sf9 cells was confirmed by western blot analysis (Fig. 1A and B). The theoretical molecular weight of the Polh-Cit1a fusion protein is approximately 40 kDa, which is consistent with the detected molecular weight of the fusion protein. No band was observed from the mock-injected silkworm (Fig. 1A, lane 2) and mock-infected Sf9 cells (Fig. 1B, lane 2). This result confirmed that the toxin was successfully expressed in the insect larvae and cultured cells. The expressed Polh-Cit1a fusion protein was purified from the fat body of the silkworm larvae using a DDDDK-tagged purification gel. A target band was detected by CBB staining (Fig. 1C) in the fraction purified from the BmNPV/Polh-Cit1a bacmid-injected larval fat body, and was also confirmed the band at 40 kDa by western blot (Fig. 1D, lane 1).

To confirm the fusion of Cit1a with Polh via the FLAG tag sequence, the purified fusion protein was treated with rEK, and the difference between the molecular weights of the rEK-treated and non-treated samples were investigated by SDS-PAGE. The FLAG tag localized between polyhedrin and Cit1a. Therefore, rEK cleaves Polh-Cit1a into polyhedrin and Cit1a. The rEK-treated product showed two bands corresponding to Polh-FLAG and Cit1a (Fig. S1). This data indicates that the active fragment of the Cit1a toxin could be released from the fusion protein by proteases, suggesting that the fusion protein will be active in the host insects.

Polyhedra formation and cell viability during recombinant baculovirus infection

In Bm5 cells and Sf9 cells, polyhedrin crystals were observed at 72 h p.i. in the nucleus of cells infected with BmNPV/Polh-Cit1a, AcMNPV, and ACMNPV/Polh-Cit1a (Fig. 2B–D), but not in the nucleus of mock-infected cells (Fig. 2A). To investigate the formation of recombinant polyhedra of the recombinant virus in insect cells via monolayer culture, Bm5 and Sf9 cells were infected with the respective virus, and polyhedra formation in the infected cells was examined at 5 days p.i. Overall, the number of polyhedra-like crystals recovered from the infected cells from the recombinant virus was similar to the wild-type AcMNPV (Fig. 2E). Morphology of cells infected with BmNPV/Polh-Cit1a at 72 h p.i. was considerably different from that of cells infected with BmNPV/EGFP and mock-infected cells. Cells infected with BmNPV/Polh-Cit1a seemed to lose its cytoplasm and outline of cells was blurred (data not shown). This result was similar to the previous paper that a theraphosid spider toxin caused early Sf21 cell death by necrosis when this peptide toxin was expressed using recombinant AcMNPV (Ardisson-Araújo et al., 2013).

In the cell viability assay using Trypan-blue, at 48 h p.i., a greater number of blue-stained cells infected with BmNPV/Polh-Cit1a was observed than that of cells infected with BmNPV/EGFP and mock-injected cells (Fig. 3A). The recombinant BmNPV expressing Polh-

Cit1a induced death in 65% (± 4.8%) of the cells at 48 h p.i., whereas for the control virus (BmNPV/EGFP), only 27.1% (± 1.5%) (P<0.01) of the cells were dead at 48 h p.i. (Fig. 3B). At 96 h p.i., almost all of the cells infected with BmNPV/Polh-Cit1a died, and approximately 40% of the cells infected with BmNPV/EGFP were alive. A similar result was found when we evaluated the AcMNPV/Polh-Cit1a infected cell line (Fig. 3C), which indicated that the expression of Polh-Cit1a promoted the cell death of Bm5 and Sf9 during baculoviral infection. The Polh-Cit1a fusion protein was detected in cells infected with BmNPV/Polh-Cit1a at different time intervals (h p.i.) by western blot (data not shown).

Bioassays

Twenty 5^{th}-instar silkworm larvae were separately inoculated with approximately 1×10^6 virus per larva of each recombinant and control virus. BmNPV/Polh-Cit1a-infected insects also exhibited strongly retarded growth, increasing their body weight by only 45% over 2 days, whereas the control insects increased their body weight by 71% and BmNPV/EGFP-infected insects increased their weight by 57% (Fig. 4). The difference in the mean larval weight values among the BmNPV/Polh-Cit1a, BmNPV/EGFP exposed and control treatments was significant ($P < 0.05$; ANOVA) at 48 h p.i. The BmNPV/Polh-Cit1a showed a LT_{50} of 76.43 h, whereas the BmNPV/EGFP had an LT_{50} of 103.43 h (Table 2). This result represents a 26% reduction of the time required to kill the virus-infected insects when using BmNPV/Polh-Cit1a compared with the control virus, BmNPV/EGFP. The constructed recombinant virus (BmNPV/Polh-Cit1a) showed improved pathogenicity against target host insects because of the expression of Polh-Cit1a fusion protein. Analysis of the recombinant protein from dead insects confirmed that the larvae died earlier because of the expression of toxin that was caused by the infection of BmNPV/Polh-Cit1a (shown in Fig. 1D, lane 3).

Discussion

The main aim of the genetic modification of wild-type baculovirus by inserting a spider venom peptide was to enhance the pathogenicity and the speed at which the target pests were killed to decrease their feeding damage by reducing the effective feeding time. Until now, several heterologous genes have been inserted into the genome of baculoviruses to improve their pathogenicity towards their hosts (Choi et al. 2008; El-Menofy et al. 2014; Gramkow et al. 2010; Osman 2012; Shim et al. 2013; Stewart et al. 1991). In this study, we fused the gene of Cit1a with polyhedrin, and represents a novel class of cytolytic molecule that shows equally potent antimicrobial and insecticidal effects. Cit1a is a membrane acting antimicrobial peptide (MAMP) with the activity prefix "M" (Vassilevski et al. 2009) and retains an α-helical motif but appear to be formed of two short MAMPs joined together in a "head-to-tail" shape (Fig. S2). This insect specific peptide toxins that lack toxicity (intracellular expression) in HEK293 cells and showed insecticidal activity against flesh fly and cockroach (Vassilevski et al. 2008). Several cytotoxic molecules have been identified from spider venoms (Vorontsova et al. 2011; Windley et al. 2012) showing capable of forming direct or indirect (by ionic channels interaction) membrane pores, and also, interfere with signal transduction and homeostasis that kill the cell (Mintz 1994; Mintz and Bean 1993; Sanguinetti et al. 1997). BmNPV/Polh-Cit1a harboring cytotoxin which causes direct or indirect membrane pores, and disrupting membrane structure causing stasis or lysis of the cell.

Polh-Cit1a was successfully expressed in the silkworm larval fat body and Sf9 cells (Figs. 1A-B) and Bm5 cells (Fig. S3). The fused Cit1a toxin could be activated in the fat body of host insects through proteolytic cleavage of the toxin by proteases. The expression of Polh-Cit1a during baculoviral infection in cultured cells and silkworm larvae enhanced the reduction of cell and larval viability. Analysis of death insect infected with BmNPV/Polh-Cit1a showed early cuticular melanization comparing to control virus infected insects (Fig. S4A). Dissection

of these larvae also showed patches of melanization on silk gland, as well as fragmentation of some of the tissues (Fig. S4C). The observed melanization of the cuticle in the recombinants BmNPV/Polh-Cit1a-infected insects may have been caused by the activation of the insect phenoloxidase enzyme, found in the form of a pro-enzyme in the hemolymph (Gramkow et al. 2010). Furthermore, Cit1a may activate the phenoloxidase cascade indirectly by damaging the basement membrane and underlying tissues. Because damaging the basement membrane activates the phenoloxidase cascade indirectly, or directly by cleaving prophenoloxidase or an upstream serine protease (Harrison and Bonning 2010). This may cause to the lower growth rate of BmNPV/Polh-Cit1a-infected larva when it was compared to control virus infected larvae (Fig. 4).

These results suggest that inserting the Polh-Cit1a gene into baculoviruses allows the baculoviruses to be used as an insecticide. These results lead to the prevention of extensive crop damage by insect pests. However, there are still several problems to solve. First, this study used injection into the hemolymph as the administration method for the recombinant baculoviruses. This method is not realistic for application in field trials. We must investigate the oral administration of recombinant baculoviruses. Polh-Cit1a considered in this study can produce polyhedra, which would be favorable for an insecticide based on baculoviruses because of its oral administration potential. Alternatively, polyhedral purified from silkworm hemolymph could be used as an insecticide peptide toxin material for field trials. Second, in this study, polyhedrin promoter was used for the expression of Polh-Cit1a in silkworm larvae and cultured cells. To kill the larvae and cultured cells faster, a constitutive promoter, such as actin A3 promoter or immediate early-2 (ie-2) promoter from *Orgyia pseudotsugata* multicapside nucleopolyhedrovirus (OpMNPV) (Douris et al., 2006), can favorably express the insectotoxin because these constitutive promoters work at all times. The expression of the toxin during baculovirus infection did not block budded virus production during the course of

infection (Table 2). This primarily occurred because the toxin versions were under the control of the polyhedrin promoter and because the budded virus was produced prior to the massive activations of the promoter (Ardisson-Araújo et al. 2013). Ba3 peptide toxin of theraphosid spider, *Brachypelma albiceps*, was expressed using AcMNPV under the control of the late and very late promoters, pSyn and pXIV, together with polyhedrin. This result suggests that Ba3 peptide has more insecticidal activity than Cit1a does. We are not sure which promoter is better for expressing Polh-Cit1a for field trials, but the method used to administer recombinant baculoviruses or polyhedral and the choice of promoters should be investigated.

In conclusion, a novel recombinant baculovirus, BmNPV/Polh-Cit1a, was constructed to develop an improved baculovirus insecticide. This recombinant BmNPV, which contains a Cit1a gene fused with a Polh gene, caused early Bm5 cell death and early 5^{th}-instar silkworm larvae death compared to the control virus, BmNPV/EGFP. Polh-Cit1a can be applied for improved recombinant baculoviruses as a bioinsecticide and can be used as an insecticidal protein.

Acknowledgements

MPA was supported by Shizuoka University Corporation Environmental Leaders Program (ELSU), Japan.

Conflict of interest The authors declare no commercial or financial conflict of interest.

References

Ali MP, Yoshimatsu K, Suzuki, T, Kato T, Park EY (2014) Expression and purification of cyto-insectotoxin (Cit1a) using silkworm larvae targeting for an antimicrobial therapeutic agent. Appl Microbiol Biotechnol 98: 6973–6982.

Ardisson-Araújo DMP, Morgado FDS, Schwartz EF, Corzo G, Ribeiro BM (2013) A new theraphosid spider toxin causes early insect cell death by necrosis when expressed in vitro during recombinant baculovirus infection. PLoS One 8: e84404.

Chang JH, Choi JY, Jin BR, Roh JY, Olszewski JA, Seo SJ, O'Reilly DR, Je YH (2003) An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin. J Invertebr Pathol 84: 30–37.

Choi JY, Wang Y, Kim YS, Kang JN, Roh JY, Woo SD, Jin BR, Je YH (2008) Insecticidal activities of recombinant *Autographa californica* nucleopolyhedrovirus containing a scorpion neurotoxin gene using promoters from *Cotesia plutellae* bracovirus. J Asia-Pac Entomol 11: 155–159.

Douris V, Swevers L, Labropoulou V, Andronopoulou E, Georgoussi Z, Iatrou K (2006) Stably transformed insect cell lines: Tools for expression of secreted and membrane-anchored proteins and high-throughput screening platforms for drug and insecticide discovery. Adv Virus Res 68: 113–156.

El-Menofy W, Osman G, Assaeedi A, Salama M (2014) A novel recombinant baculovirus overexpressing a *Bacillus thuringiensis* Cry1Ab toxin enhances insecticidal activity. Biol Proced Online 16: 7 doi:10.1186/1480-9222-16-7.

Gramkow AW, Perecmanis S, Sousa RLB, Noronha EF, Felix CR, Nagata T, Ribeiro BM (2010) Insecticidal activity of two proteases against *Spodoptera frugiperda* larvae infected with recombinant baculoviruses. Virol J 7: 143.

Harrison RL, Bonning BC (2010) Proteases as Insecticidal Agents. Toxins 2: 935-953.

Hiyoshi M, Kageshima A, Kato T, Park EY (2007) Construction of a cysteine protease deficient *Bombyx mori* multiple nucleopolyhedrovirus bacmid and its application to improve expression of a fusion protein. J Virol Methods 144: 91–97.

Hong HK, Woo SD, Choi JY, Lee HK, Kim MH, Je YH, Kang SK (2000) Characterization of four isolates of *Bombyx mori* nucleopolyhedrovirus. Arch Virol 145: 2351–2361.

Inceoglu AB, Kamita S, Hinton AC, Hung Q, Severson TF, Kang KK, Hammock BD (2001) Recombinant baculoviruses for insect control. Pest Manag Sci 57: 981–987.

Kato T, Manoha SL, Tanaka S, Park EY (2009) High-titer preparation of *Bombyx mori* nucleopolyhedrovirus (BmNPV) displaying recombinant protein in silkworm larvae by size exclusion chromatography and its characterization. BMC Biotechnol 9: 55. doi:10.1186/1472-6750-9-55

Kato T, Thompson JR, Park EY (2013) Construction of new ligation-independent clonig vectors for the expression and purification of recombinant proteins in silkworms using BmNPV bacmid system. PLoS ONE 8: e64007. doi: 10.1371/journal.pone.0064007.

Lazarev VN, Polin NF, Shkarupeta MM, Kostrjukova ES, Vassilevski AA, Kozlov SA, Grishin EV, Govorun VM (2011) Spider venom peptides for gene therapy of *Chlamydia* infection. Antimicrob Agents Chemother 55: 5367–5369.

Lo HR, Chao YC (2004) Rapid titer determination of baculovirus by Quantitative Real-Time Polymerase Chain Reaction. Biotechnol Prog 20: 354–360.

Mintz IM (1994) Block of Ca channels in rat central neurons by the spider toxin omega-Aga-IIIA. J Neurosci 14: 2844–2853.

Mintz IM, Bean BP (1993) Block of calcium channels in rat neurons by synthetic omega-Aga-IVA. Neuropharmacol 32: 1161–1169.

Oerke EC (2006) Crop losses to pests. J Agric Sci 144: 31–43.

Osman G (2012) Detection, cloning and bioinformatics analysis of vip1/vip2 genes from local *Bacillus thuringiensis*. Afr J Biotechnol 15: 11678-11685.

Sanguinetti MC, Johnson JH, Hammerland LG, Kelbaugh PR, Volkmann RA, Saccomano NA, Muelle A (1997) Heteropodatoxins: peptides isolated from spider venom that block Kv4.2 potassium channels. Mol Pharmacol 51: 491–498.

Shim HJ, Cho JY, Wang Y, Tao XY, Li, Q, Roh JY, Kim JS, Kim WJ, Woo SW, Ji, BR, Je YH (2013) NeuroBactrus, a novel, highly effective, and environmentally friendly recombinant baculovirus insecticide. Appl Environ Microbiol 79: 141–149.

Slack J, Arif BM (2007) The baculoviruses occlusion-derived virus: virion structure and function. Adv Virus Res 69: 99–165.

Smith GE, Vlak JM, Summers MD (1983) Physical analysis of Autographa californica nuclear polyhedrosis virus transcripts for polyhedrin and 10,000-molecular-weight protein. J Virol 45: 215–225.

Stewart LMD, Hirst M, Ferber ML, Weather ATM, Cayley PJ, Possee RD (1991) Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature 352: 85–88.

Szewczyk B, Hoyos-Carvajal L, Paluszek M, Skrzecz I, Lobo de Souza M (2006) Baculoviruses – re-emerging biopesticides. Biotechnol Adv 24: 143–160.

Vassilevski AA, Kozlov SA, Grishin EV (2009) Molecular diversity of spider venom. Biochemistry-Moscow- 74: 1505–1534.

Vassilevski AA, Kozlov SA, Samsonova OA, Egorova NS, Karpunin DV, Pluzhninkov KA, Feofanov AV, Grishin EV (2008) Cyto-insectotoxins, a novel class of cytolytic and insecticidal peptides from siper venom. Biochem J 411: 687–696.

Vorontsova OV, Egorova NS, Arseniev AS, Feofanov AV (2011) Haemolytic and cytotoxic action of latarcin Ltc2a. Biochimie 93: 227–241.

Wang R, Deng F, Hou D, Zhao Y, Guo L, Wang H, Hu Z (2010) Proteomics of the Autographa californica nucleopolyhedrovirus budded virions. J Virol 84: 7233–7242.

441 Windley MJ, Herzig V, Dziemborowicz SA, Hardy MC, King GF, Nicholson GM (2012)
442 Spider-venom peptides as bioinsecticides. Toxins 4: 191–227.

FIGURE LEGENDS

Fig. 1 Electrophoretic analysis of Polh-Cit1a fusion protein expressed in silkworm larvae, Sf9 cell and purification. (A) Western blot analysis of Polh-cit1a fusion protein expressed in silkworm larvae. The band at approximately 40 kDa was observed in infected larval fat body. M: molecular marker; lane 1: BmNPV/Polh-Cit1a-infected larval fat body; lane 2: mock larval fat body. (B) Western blot analysis of Polh-cit1a fusion protein expressed in Sf9 cell. Lane 1: medium; lane 2: mock cell; lane 3: AcMNPV/Polh-Cit1a transfected cell. (C) SDS-PAGE of purified Polh-Cit1a fusion protein from fat body of silkworm larvae using DDDDK-tagged purification gel. Lane 1, 2 and 3 denote elution 1, 2 and 3, respectively. (D) Western blot analysis of purified sample (lane 1), the fat body of dead BmNPV/Polh-Cit1a-infected silkworm larval (lane 3), and mock fat body (lane 2). Fat body was collected from BmNPV/Polh-Cit1a infected-dead larval samples at 96 h p.i. The protein was detected using anti-FLAG monoclonal antibody. Arrows in A–D denote Polh-cit1a fusion protein.

Fig. 2 Photographs of recombinant baculovirus-infected Bm5 and Sf9 monolayer cells by light microscopy. (A) Mock-infected cells (Sf9). (B) Cell (Bm5) was infected with the recombinant virus BmNPV/Polh-Cit1a, (C) Sf9 cell infected with AcMNPV/Polh-Cit1a expressing the toxin showing the absence of cytoplasm and polyhedra accumulated in the nucleus. (D) Sf9 cell infected with wild type AcMNPV. Photographs were taken 72 h p.i. Arrow heads indicate the polyhedra expressed in the nucleus. Bars represent 10 μm. (E) Polyhedra formation by recombinant baculoviruses in insect cell lines. The cells were infected with virus at a MOI of 10. The yield of the total released polyhedra from Sf9 infected with AcMNPV and AcMNPV/Polh-Cit1a and Bm5 infected with BmNPV/Polh-Cit1a were counted with a hemocytometer. Error bars in E indicate standard errors.

Fig. 3 Trypan-blue staining experiment of Bm5 cells infected with each recombinant BmNPV. Microscopic analysis of Bm5 cells infected by BmNPV/Polh-Cit1a (A), BmNPV/EGFP (B), and mock (C) at 48 h p.i. (D) Cell mortality of BmNPV/Polh-Cit1a (closed circles) and BmNPV/EGFP (open circles) infected Bm5 cells. Red and green arrow head show dead and live cell respectively. Cells were stained with Trypan-blue dye, and dead cells were counted by a hemocytometer. Error bars indicate the standard errors, and the asterisks centered over the error bar to indicate the relative level of the *p*-value ("**" means $p<0.01$). (E) Cell mortality of AcMNPV/Polh-Cit1a- (closed circles) and wild type AcMNPV- (open circles) infected Sf9 cells. Error bars indicate standard errors, and the asterisks centered over the error bar to indicate the relative level of the *p*-value ("*" means $p<0.05$).

Fig. 4 Effect of Cit1a on the growth of silkworm larvae during recombinant baculovirus infection. Data were analyzed using ANOVA. Error bars indicate standard errors, and the asterisks centered over the error bar indicate the relative level of the *p*-value.

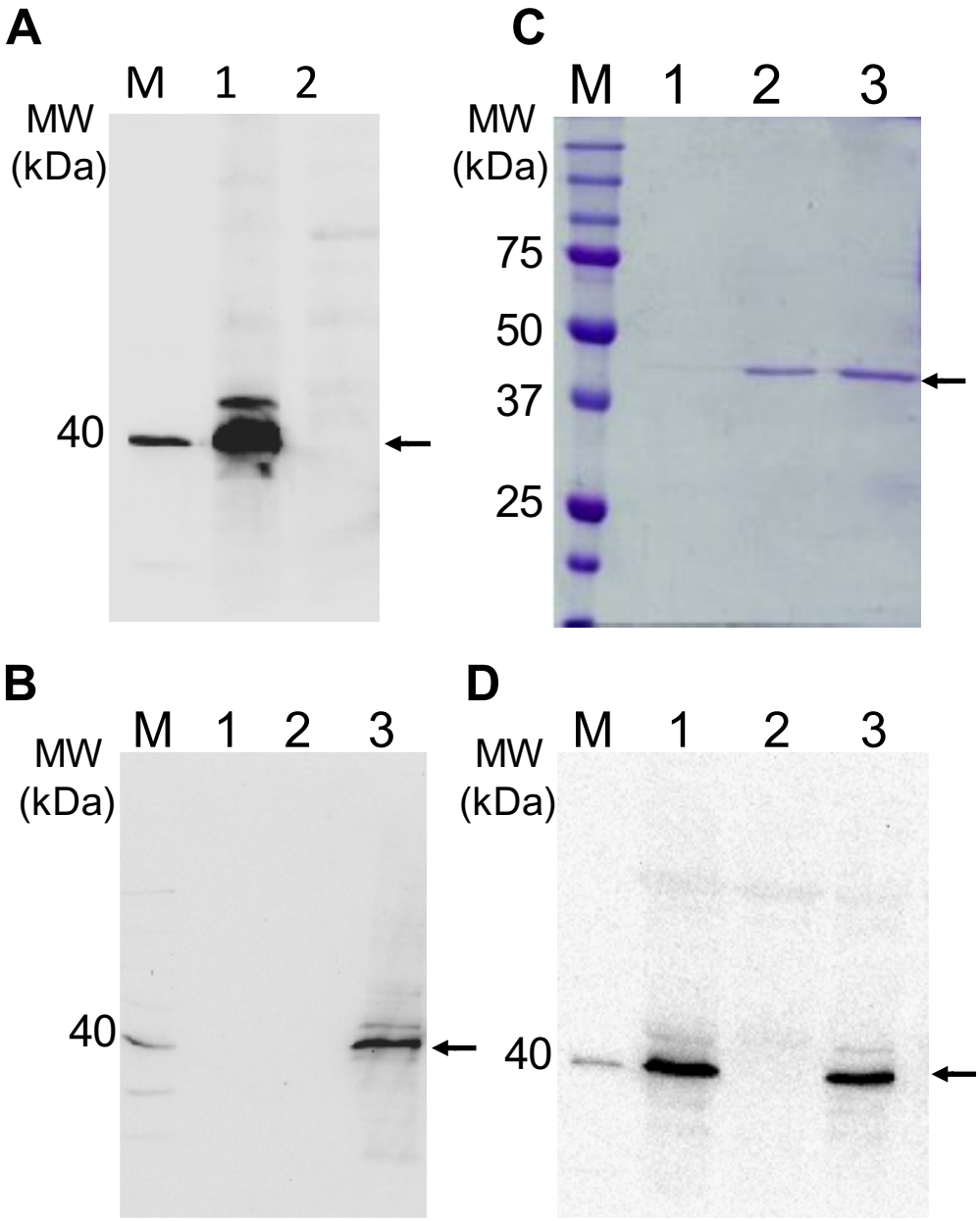
Table 1 The primers used in this study

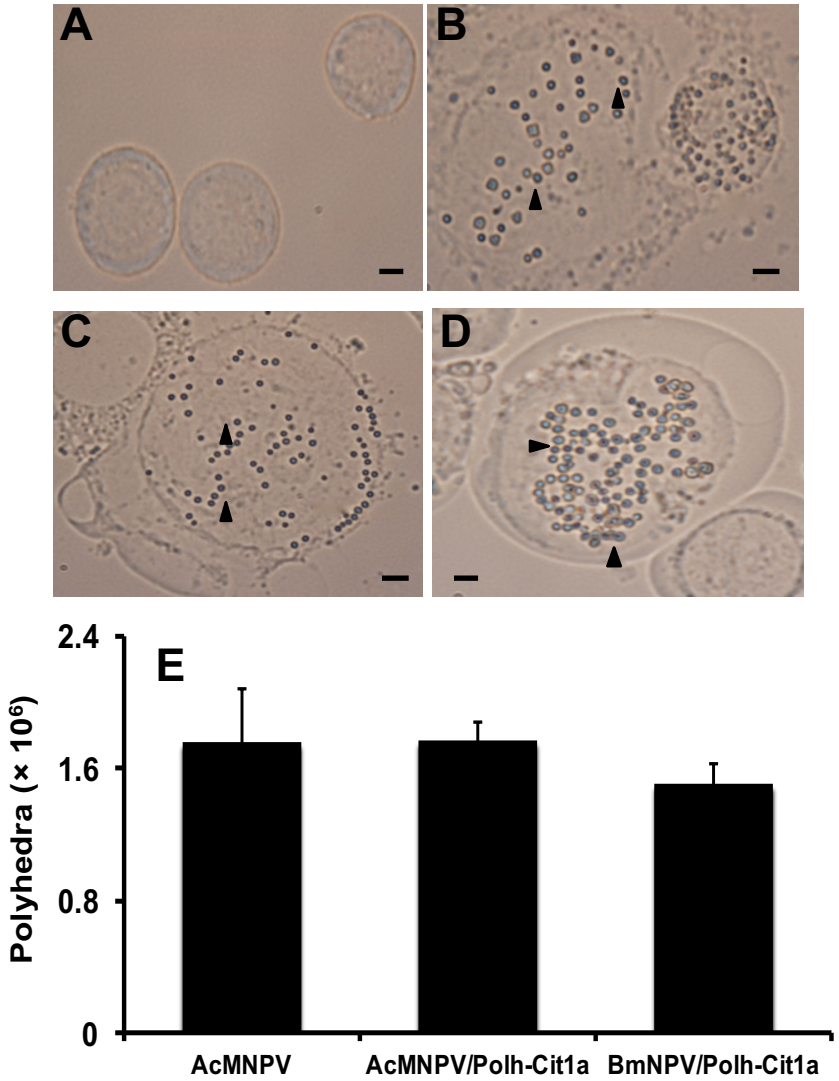
No.	Name of primer	Sequence (5' to 3')
1	FLAG-Cit1a-F	gactacaaggatgacgatgacaagggtttcttcgggaatacgtggaagaaaataaagggcaaagctgataagattatgctaaagaaagcagtaaagataatggtaaagaaagaaggaatatctaaagaagaggcg
2	Cit1a-xba-R	gctctagatcacaatttttcggacgctttttgaagagctttttttccataatacttgagtagatagagtcttatttgtttctttgacattgcatctacttttgcctgcgcctcttctttagatattcc
3	Ac-Pol-F	gcgaattcatgccggattattcataccgtc
4	Ac-Pol-FLAG-R	cttgtcatcgtcatccttgtagtcatacgccggaccagtgaacag
5	M13 Forward	cccagtcacgacgttgtaaaacg
6	M13 Reverse	agcggataacaatttcacacagg
7	Acie-1F	cccgtaacggacctcgtactt
8	Acie-1R	ttatcgagatttatttgcatacaacaag

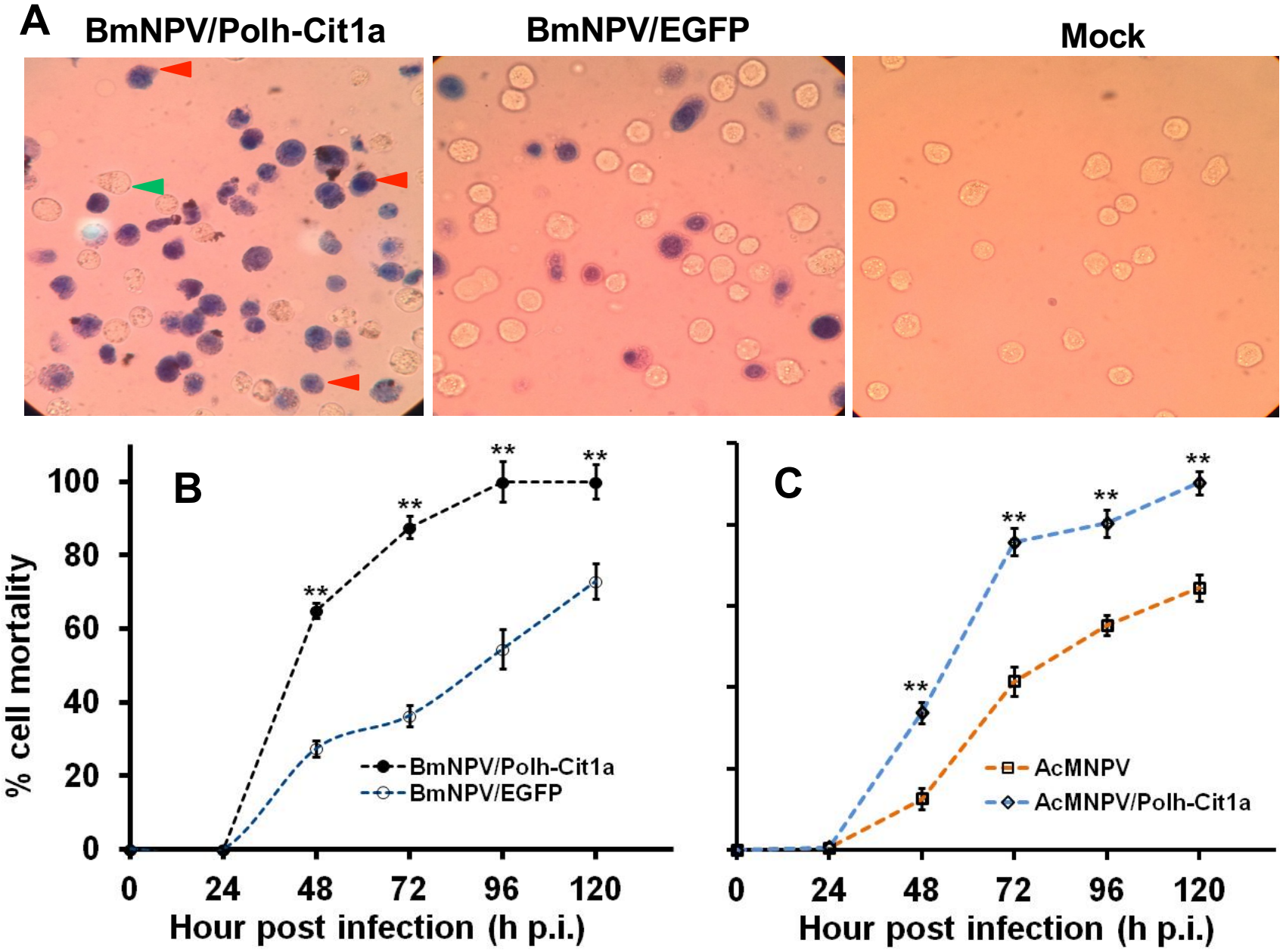
Table 2 LT_{50} values and production of budded virus for the recombinant BmNPV/Polh-Cit1a and control virus BmNPV/EGFP in 5th instar larvae of *Bombyx mori*.

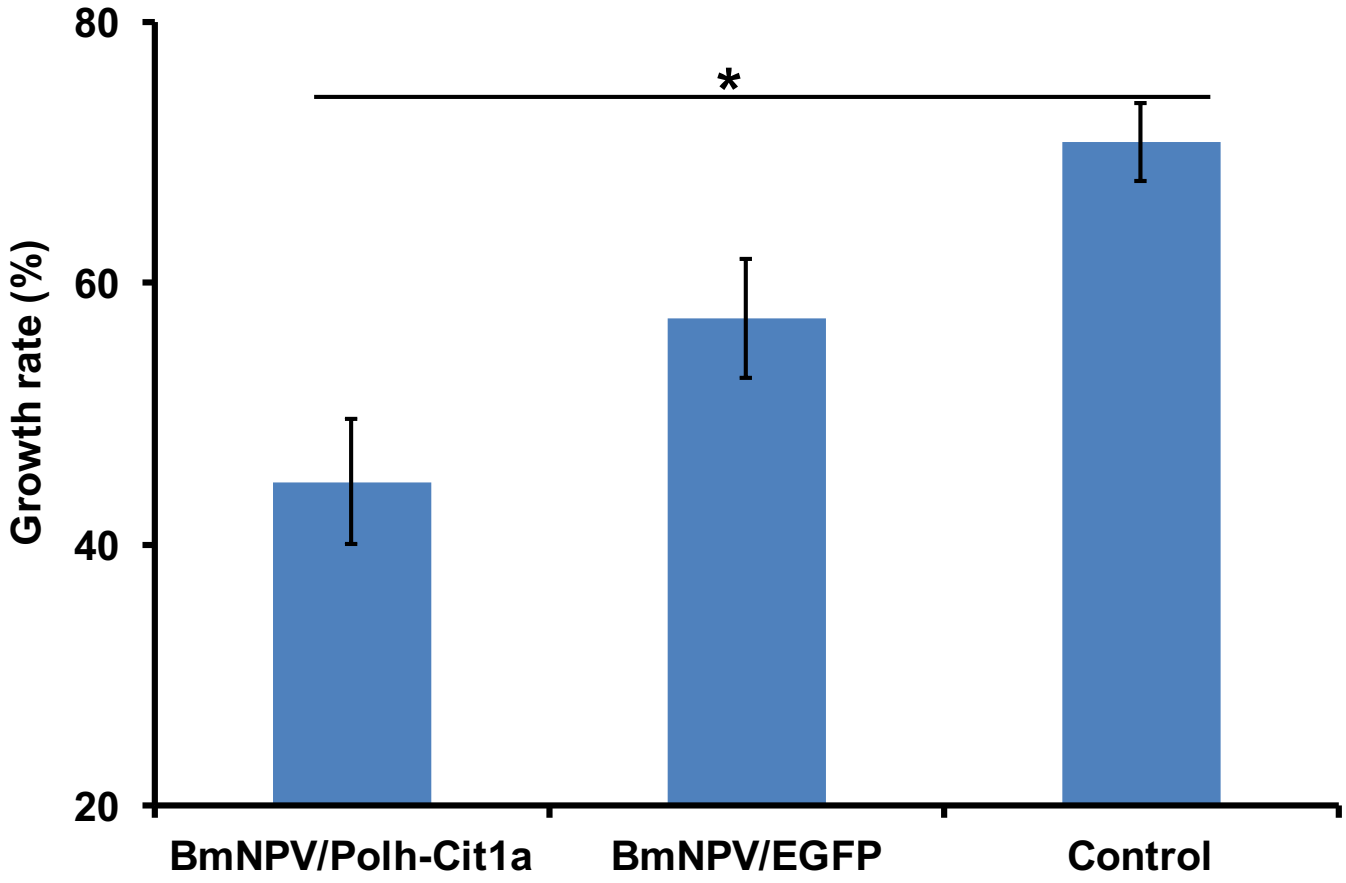
Virus	LT_{50} (h) [1,#]	Budded virus/ml hemolymph
BmNPV/Polh-Cit1a	76.63 ± 4.31**	4.72×10^{8} ± 0.74
BmNPV/EGFP	103.43 ± 4.76	4.95×10^{8} ± 0.87

[1] LT_{50} value was determined using log Probit analysis. The virus titer was calculated from the infected larval hemolymph. [#] Median lethal time (LT_{50}) values were calculated at 1×10^{5} virus titer/larva. Significant difference is indicated by ** ($p < 0.01$, *t*-test).


A
M 1 2
MW (kDa)
40
C
M 1 2 3
MW (kDa)
75
50
37
25
B
M 1 2 3
MW (kDa)
40
D
M 1 2 3
MW (kDa)
40


A
B
C
D
E
2.4
1.6
0.8
0
Polyhedra (× 10⁶)
AcMNPV
AcMNPV/Polh-Cit1a
BmNPV/Polh-Cit1a


A
BmNPV/Polh-Cit1a
BmNPV/EGFP
Mock
B
% cell mortality
100
80
60
40
20
0
0
24
48
72
96
120
Hour post infection (h p.i.)
BmNPV/Polh-Cit1a
BmNPV/EGFP
**
C
AcMNPV
AcMNPV/Polh-Cit1a
Hour post infection (h p.i.)


80
60
40
20
Growth rate (%)
*
BmNPV/Polh-Cit1a
BmNPV/EGFP
Control

Supplementary information

Improved insecticidal activity of a recombinant baculovirus expressing spider venom cyto-insectotoxin

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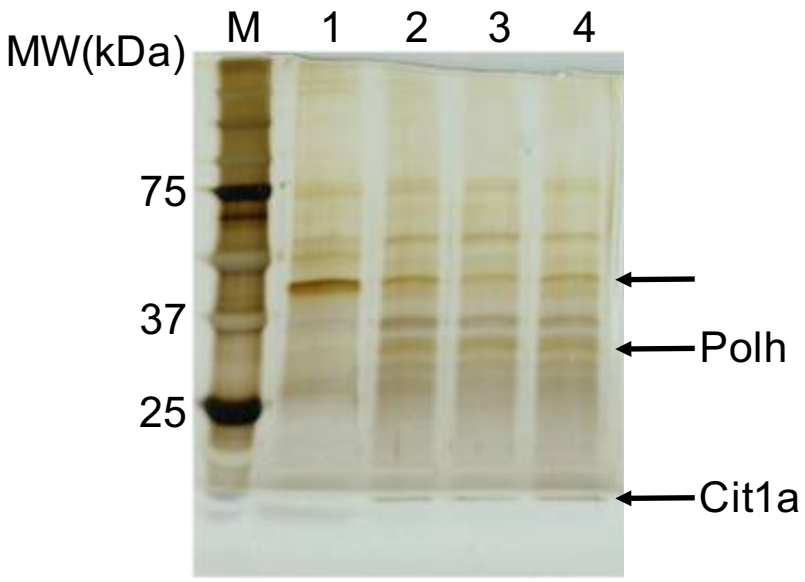

Fig. S1 SDS-PAGE of the fusion protein. Enterokinase-treated fusion protein was electrophoresed in 12% polyacrylamide gels. Polh-cit1a fusion protein was collected from silkworm fat body sample. M and lane 1 denote molecular marker and undigested sample, respectively. Lanes 2, 3 and 4 denote enterokinase-treated sample at 2, 4 and 8 h, respectively.

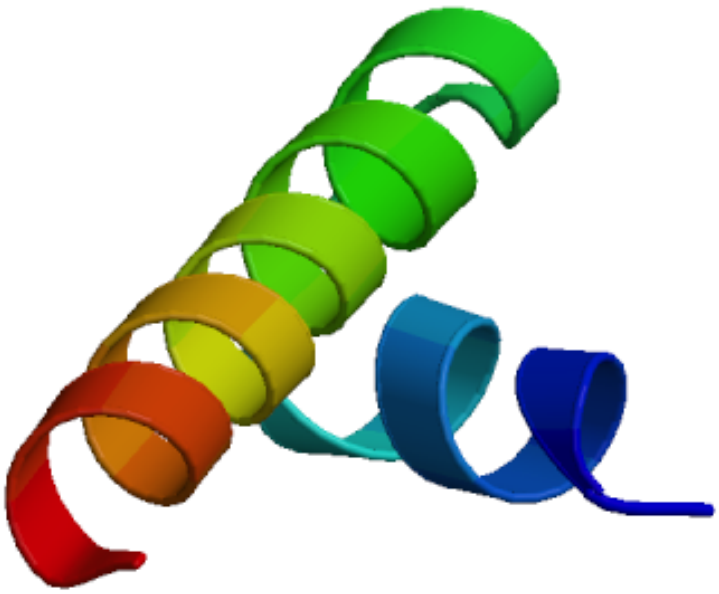

Fig. S2 The predicted structure of mature peptide (Cit1a). The Swiss-Model Server (http://swissmodel.expasy.org/) was used for protein structure prediction. The amino acid sequence was used in this model adopted from NCBI.

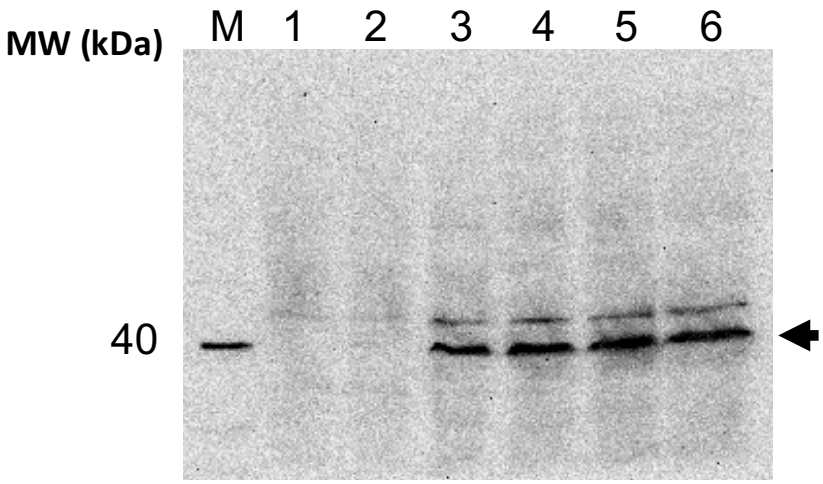

Fig. S3 Western blot analysis of Bm5 cell infected with BmNPV/Polh-Cit1a at different time interval. M: molecular marker; lane 1: mock infected cell; lanes 2–6: samples collected at 24, 48, 72, 96 and 120 h p. i.

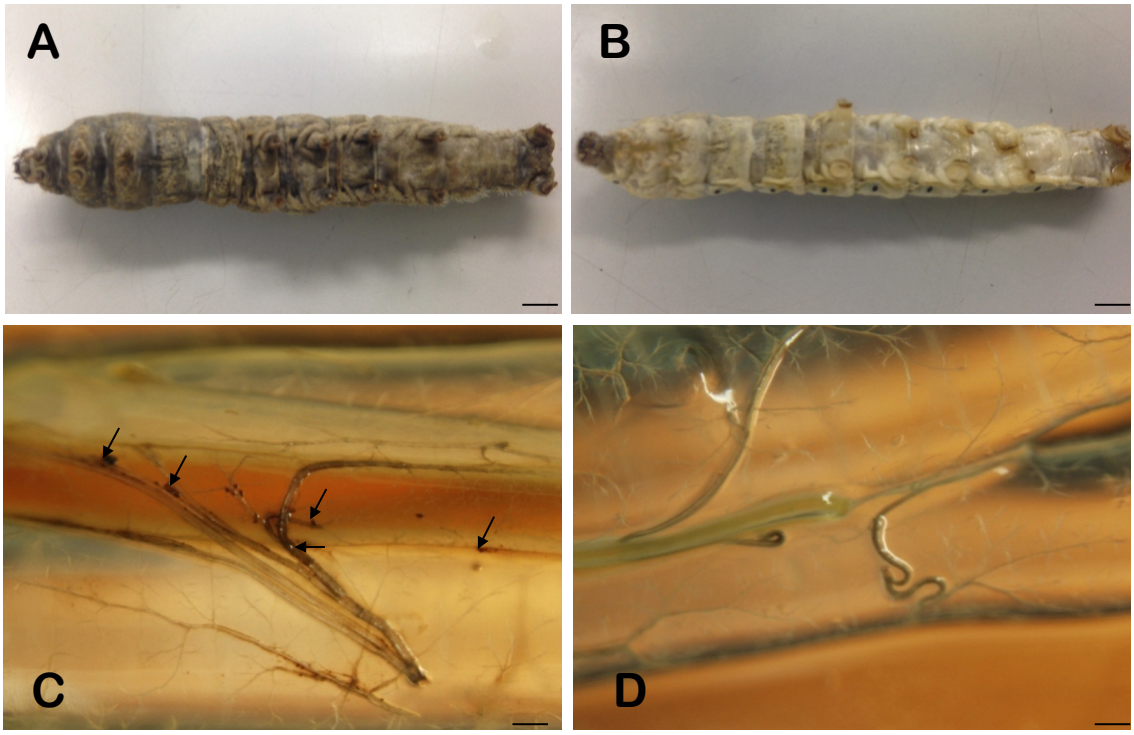

Fig. S4 Analysis of melanization of the cuticle and internal tissue of larvae of infected with recombinant viruses at 96 h p.i.. (A) Cuticular melanization of BmNPV/Polh-Cit1a-infected silkworm larvae. (B) Control virus-infected larvae. Light microscopic images of silk gland of BmNPV/Polh-Cit1a-infected silkworm larvae (C) and BmNPV-infected silkworm larvae (D). Arrows indicate deposition of melanin. Bars in (A)–(D) denote 200 μm.