大韓바이러스學會誌 : 第 29 卷 第 3 號 1999
J. Korean. Soc. Virology, Vol. 29, No. 3, 1999

Construction of a Novel Recombinant Baculovirus Producing Polyhedra with a *Bacillus thuringiensis* Cry1Ac Crystal Protein

Yeon Ho Je[1]*, Byung Rae Jin[2], Jong Yul Roh[1], Jin Hee Chang[1] and Seok Kwon Kang[1]

The Research Center for New Bio-Materials in Agriculture, and Division of Applied Biology and Chemistry, College of Agriculture & Life Sciences, Seoul National University[1], Suwon 441-100, Korea College of Natural Resources and Life Science, Dong-A University[2], Pusan 604-714, Korea

=Abstract=

We have now constructed a novel recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) producing polyhedra with *Bacillus thuringiensis* (Bt) CryIAc crystal protein. The recombinant polyhedra produced by the recombinant baculovirus, Btrus, in insect cells was characterized. The recombinant baculovirus has two independent transcription units in opposite orientations with two promoters, p10 or polyhedrin gene promoter each initiating transcription of either native polyhedrin or fusion protein with polyhedrin and Bt Cry1Ac crystal protein. Surprisingly, this recombinant baculovirus stably produced recombinant polyhedra which were nearly similar to those of wild-type AcNPV. The immunogold staining experiment showed that the recombinant polyhedra were assembled with polyhedrin and Bt Cry1Ac crystal protein, and contained virus particles. Insecticidal toxicity of recombinant polyhedra of Btrus to the fall webworm, *Hyphantria cunea*, was strikingly improved in comparison with the wild-type AcNPV.

Key Words: Baculovirus, Polyhedrin, *Bacillus thuringiensis*, CryIAc crystal protein, Insect cells, Recombinant polyhedra

INTRODUCTION

Baculoviruses are pathogenic to insects, so that they are proliferated to kill the hosts. In addition, baculoviruses are nonpathogenic to the vertebrate and maintain their activity for a long time in fields. Owing to these advantage, as many as 20 viruses are developed as commercially available insecticides [3,21]. However, baculoviruses have a limited host range and the insecticidal effects are relatively slow. These disadvantages have limited their use as control agents of insect pests [4,10].

Recently, active research has been directed to the improvement of baculoviruses in usability as a specific pest control agent. In this regard, genetic engineering technology provides a useful means. A number of attempts have been made to produce the baculoviruses which are

접수 : 1999년 7월 19일

*; Corresponding author: Yeon-Ho Je, The Research Center for New Bio-Materials in Agriculture, and Division of Applied Biology and Chemistry, College of Agriculture & Life Sciences, Seoul National University, Suwon 441-100, Korea; Tel: 0331-290-2492; e-mail: kskipl@plaza.snu.ac.kr

improved in killing speed, by introducing various foreign genes into the baculoviruses. Such foreign genes include those encoding for the insect-specific toxins, hormones or other gene products [5,7,17,19].

There were noticeable studies on the improvement of viral insecticidal activity by expressing the *Bacillus thuringiensis* (Bt) δ-endotoxin protein in baculoviruses [11-13,15]. However, there was observed no enhancement in pathogenicity of the recombinant baculoviruses although they produce a large amount of the Bt δ-endotoxin proteins in the hemolymph and fat body of the insects. This results from no consideration for the insecticidal mechanism of Bt δ-endotoxin which is fulfilled in the presence of the proteases and the epithelial cells in the midgut. That is, the single Bt δ-endotoxin protein which is expressed under the control of the p10 or polyhedrin promoter in the hemolymph and fat body, is not activated as in the midgut.

In this study, therefore, we have now constructed a novel recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) producing polyhedra with Bt CryIAc crystal protein. The recombinant polyhedra produced by the recombinant baculovirus in insect cells was characterized.

MATERIALS AND METHODS

Cell lines and viruses

Spodoptera frugiperda (Sf9) cells used in this study were maintained at 27℃ in TC-100 medium (Gibco, USA) supplemented with 10% heat-inactivated (56℃, 30 min) fetal bovine serum (FBS) (Gibco, USA). Wild-type AcNPV and recombinant virus were propagated in Sf9 cells. The titer of viruses was determined by plaque assay in Sf9 cells as described previously [9,14,18].

Construction of baculovirus transfer vector

The synthetic primers were prepared to 5'-ATGCCGGATTATTCATACCGTCCC-3' for the translational start sequence region of AcNPV polyhedrin gene and 5'-CTCGAGATACGCCGGACCAGTGAAC-3' for the 3' coding region of AcNPV polyhedrin gene which added *Xho* I site for the cloning, and removed termination codon TAA. Two primers specific for regions within the polyhedrin gene were used to amplify a 741 bp polyhedrin gene region from AcNPV (All PCR reactions comprised 35 cycles of 94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min.). The amplified polyhedrin gene product was treated with Klenow fragment, and inserted into pAcUW31 (Clontech, USA) digested with *Bam*H I and blunt ended with Klenow fragment to yield pAcUW-P. In the second step, AcNPV polyhedrin gene was amplified from AcNPV by two synthetic primers, 5'-AAAGATCTAATATGCCGGATTATTCATACC-3' for the translational start sequence region of polyhedrin gene containing the *Bgl* II site and 5'-ATGAATTCTTAATACGCCGGACCAGTG-3' for the 3' coding region of polyhedrin gene which added *Eco*R I site for the cloning. The amplified AcNPV polyhedrin gene was digested with *Bgl* II and *Eco*R I, and introduced into the *Bgl* II and *Eco*R I sites of pAcUW-P to yield pAcUW-PP. Finally, the N-terminal fragment of *cryIAc* crystal protein gene was amplified from Bt subsp. *kurstaki* HD-73 *cryIAc* crystal protein gene of pN 6.6 [1] by two synthetic primers, 5'-AACTCGAGATGGATAACAATCCGAAC-3' for the translational start sequence region of *cryIAc* crystal protein gene containing the *Xho* I site and 5'-TCAGCCTCGAGTGTTGCAGTAAC-3' for the N-terminal specific region of *cryIAc* crystal protein gene. The amplified *cryIAc* N-terminal fragment was digested with *Xho* I, and inserted into *Xho* I sites of pAcUW-PP to yield pBtrus.

Polymersae chain reaction (PCR)

The introduction of fusion gene under the control of polyhedrin gene promoter and polyhedrin gene under the control of p10 gene promoter was analyzed by PCR from pBtrus

plasmid DNA using primers (PF: 5'-ATGCCG-GATTATTCAT-3'; PR: 5'-ATACGCCGGACC-AGTG-3'; CF: 5'-ATGGATAACAATCCGAAC-3'; CR: 5'-TGTTGCAGTAACTGGAAT-3'; BF: 5'-ACGGGCAGACATGGCCTG-3'; BR: 5'-AA-TACGTACAACAATTG-3'). After a 35-cycle amplification (94℃ for 1 min; 55℃ for 1 min; 72℃ for 1 min), PCR products were ethanol precipitated, centrifuged at 10,000 ×g for 30 min, and rinsed with 70% ethanol. These DNAs were analyzed by agarose gel electrophoresis.

Construction of recombinant AcNPV

The cell culture dish (35-mm diameter) seeded with 1.0×10^6 Sf9 cells was incubated at 27℃ for 1 hr to allow the cells to attach. One microgram of BacPAK6 viral DNA (Clontech, USA), 5 μg of pBtrus plasmid DNA in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystyrene tube. Fifty μl of 100 μg/ml Lipofectin™ (Gibco, USA) were gently mixed the DNA solution, and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC-100 medium (Gibco, USA). Serum-free TC-100 (1.5 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 27℃ for 5 hr, 1.5 ml TC-100 containing antibiotics and 10% FBS was added to each dish and the incubation at 27℃ continued. At 5 days postinfection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min, and stored at 4℃ before plaquing on Sf9 cells. To plaque purify recombinant AcNPV, 1.5×10^6 Sf9 cells were seeded per well on a 6-well plate and the supernatant was added to each well. Recombinant AcNPV was plaque purified in Sf9 cells.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot

Sf9 cells were mock infected or infected with wild-type AcNPV and recombinant AcNPV in a 35-mm diameter dish (1.0×10^6 cells) at a MOI of 5 plaque forming unit (PFU) per cell. After incubation at 27℃, cells were harvested at 1, 2, and 3 days p.i. For SDS-PAGE of cell lysates, uninfected Sf9 cells and cells infected with virus were washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and mixed with an equal amount of 2 × sample buffer (5% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 20% glycerol). Samples were boiled for 5 min and clarified by centrifugation (10,000 ×g for 1 min). The total cellular lysates were subjected to 10% SDS-PAGE [8], electroblotted and incubated with AcNPV polyhedrin or crystal protein antibody [20]. SDS-PAGE molecular weight standards were used as size marker (Sigma, St. louis, USA).

Microscopy

Microscopy of Sf9 cells infected with recombinant virus were performed using inverted phase contrast microscope (Hund, Wilovert S, Germany).

Immunogold staining

Samples were fixed for 3 hr in a mixture of 4% formaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 mM $CaCl_2$ and 0.45 M sucrose (FM). Fixation was completed by incubating overnight in FM (pH 10.4) without glutaraldehyde. The samples were rinsed in 0.1 M sodium phosphate buffer (pH 7.5) and then dehydrated in a graded ethanol series (up to 95%) and embedded in Lowicryl K4M (Polysciences, Warrington, PA). Ultrathin sections mounted on formvar-coated nickel grids were treated for 10 min with Tris-buffered saline (TBS; 0.02 M Tris-HCl, pH 7.5 containing 0.5 M NaCl). The sections were etched with 3% H_2O_2 in double distilled H_2O for 5 min and then blocked with 3% BSA in TBS for 30 min. Double immunogold staining was performed as described [2].

The sections were incubated with primary antiserum against Bt toxin at a dilution of 1:200, in TBS/Tween 3 times for 15 min with gentle agitation, and the sections were exposed to gold-goat anti-mouse IgG (30 nm: BioCell, cardiff, UK) diluted 1:10 in TBS/Tween for 60 min. After washing with 0.3% BSA in TBS, the grids were dried and the reverse of the grid incubated with antiserum against polyhedrin (1:300) and gold-goat anti-mouse IgG (10 nm: Zymed, CA, USA) diluted 1:30 in TBS/Tween. After the double immunostaining, the sections were post stained with 2% uranyl acetate followed by 0.2% lead citrate. The sections were observed with a Hitachi M-600 transmission electron microscope operating at 75 KV.

Bioassay against *Hyphantria cunea* larvae

Refusing diet assay [13] of the recombinant baculovirus Btrus was performed with *H. cunea.* Fifty second-instar *H. cunea* larvae were fed on dilutions (6000, 1500 and 375 polyhedra per larva) of each virus stock on a small plug of artificial diet for 24 hr. The insects that consumed the plugs were then given fresh diet and monitored until death or pupation.

RESULTS

Construction of recombinant AcNPV

To generate the recombinant baculovirus producing recombinant polyhedra with AcNPV

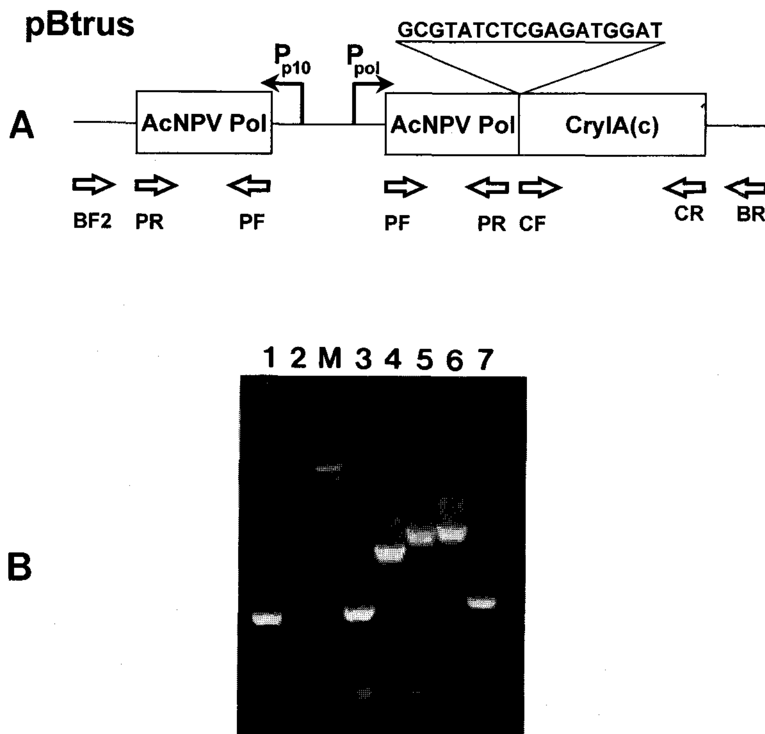

Figure 1. The baculovirus transfer vector map used to generate recombinant virus (A) and PCR analysis of transfer vector pBtrus plasmid DNA (B). The pBtrus has two independent transcription units in opposite orientations. The introduction of polyhedrin or fusion gene under the control of p10 or polyhedrin gene promoter was analyzed by PCR using wild-type AcNPV (lanes 1 and 2) and transfer vector pBtrus (lanes 3, 4, 5, 6 and 7). Lanes 1 and 3, primers PF and PR; Lanes 2 and 4, primers CF and CR; Lane 5, primers PF and CR; Lane 6, primers PF and BR; Lane 7, primers PF and BF. Solid arrow indicates baculovirus p10 or polyhedrin gene promoter and orientation. Open arrows indicate primer positions of PCR.

polyhedrin and Bt Cry1Ac crystal protein, double expression vector was constructed as shown in Figure 1A. The N-terminal domain of *cry1Ac* gene of Bt subsp. *kurstaki* HD-73 was fused at the back of polyhedrin gene. The transfer vector pBtrus was constructed with two promoters, p10 or polyhedrin gene promoter, each initiating transcription of either native polyhedrin or fusion protein with polyhedrin and Bt Cry1Ac crystal protein. The transfer vector pBtrus has two independent transcription units in opposite orientations. The recombinant virus by pBtrus was named as Btrus.

To confirm the introduction of polyhedrin or fusion gene under the control of p10 or polyhedrin gene promoter, respectively, the transfer vector pBtrus was analyzed by PCR using specific primers (Figure 1B). Bands expected for the polyhedrin or fusion genes were detected in the transfer vector pBtrus.

Expression of the fusion gene in insect cells

In order to examine the expression of polyhedrin and fusion protein by recombinant virus in Sf9 cells, the protein synthesis in Sf9 cells infected with Btrus was initially analyzed by SDS-PAGE and Western blot (Figure 2). Wild-type AcNPV-infected cells showed a pattern of protein synthesis typical of AcNPV-infected cells. Fusion protein band was not detected in cells infected with wild-type AcNPV or from mock-infected cells. The fusion protein expressed by the fusion gene was present as a band of about 98 kDa in cells infected with recombinant virus, Btrus, which was absent in cells infected with wild-type AcNPV. The cells infected with Btrus also produced 33 kDa polyhedrin as expected. Immunoblotting with polyhedrin or Cry1Ac crystal protein antibody was used to confirm that the 98 kDa band was fusion protein with a polyhedrin and Bt Cry1Ac crystal protein.

In the light microscopy of Sf9 cells infected with Btrus at 4 days p.i., Sf9 cells produced re-

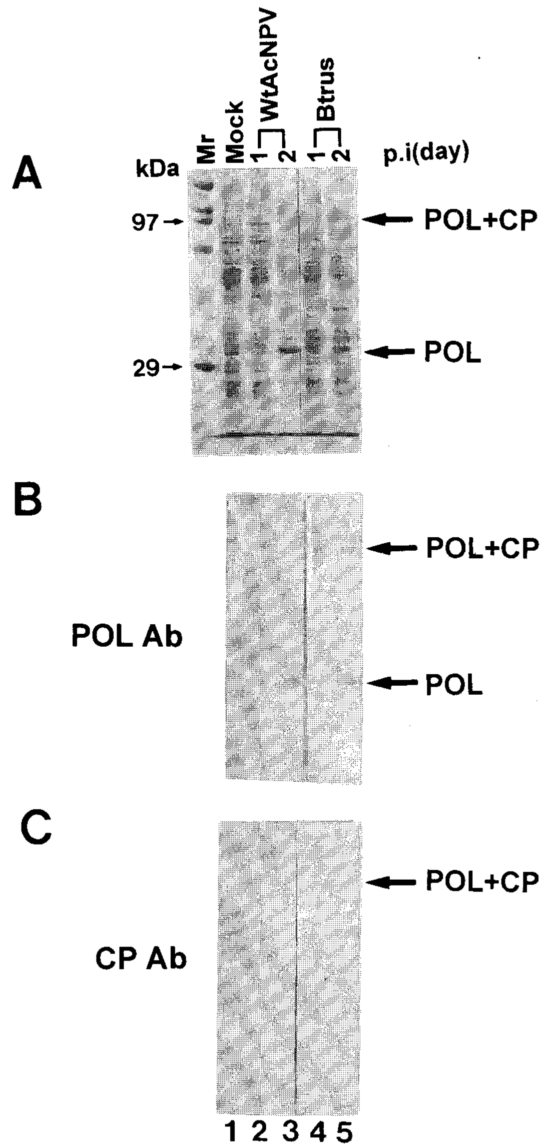

Figure 2. SDS-PAGE and Western blot analysis of the fusion protein expression of recombinant virus Btrus in Sf9 cells. Sf9 cells were mock-infected (lane 1) or infected with wild-type AcNPV (lanes 2 and 3) and Btrus (lanes 4 and 5) at MOI of 5 PFU per cell. Cells were collected at 1 (lanes 2 and 4) and 2 (lanes 3 and 5) days p.i. Total cellular lysates were subjected to 10% SDS-PAGE (**panel A**), electroblotted and incubated with polyhedrin (**panel B**) or crystal protein (**panel C**) antibody. Fusion protein and polyhedrin bands are indicated on the right of each panel. Molecular weight standards were used as size marker (lane M).

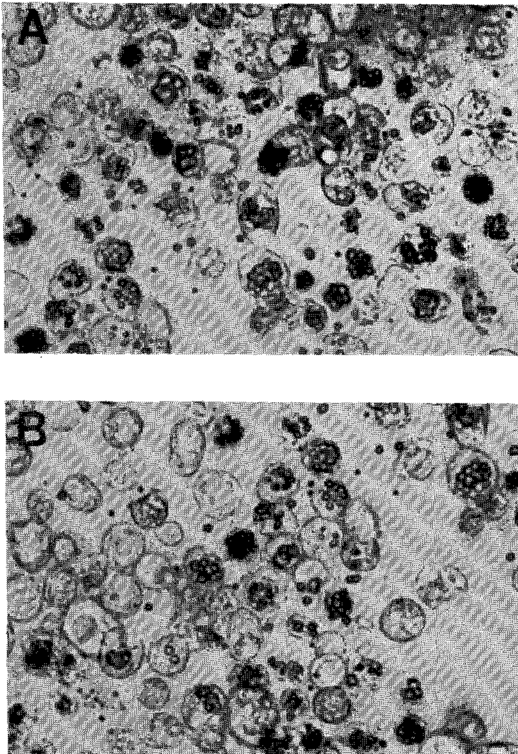

Figure 3. Microscopy of polyhedra produced in Sf9 cells infected with recombinant virus Btrus. Cells infected with wild-type AcNPV (**A**) or recombinant virus, Btrus (**B**) were observed by light microscope (× 1,000).

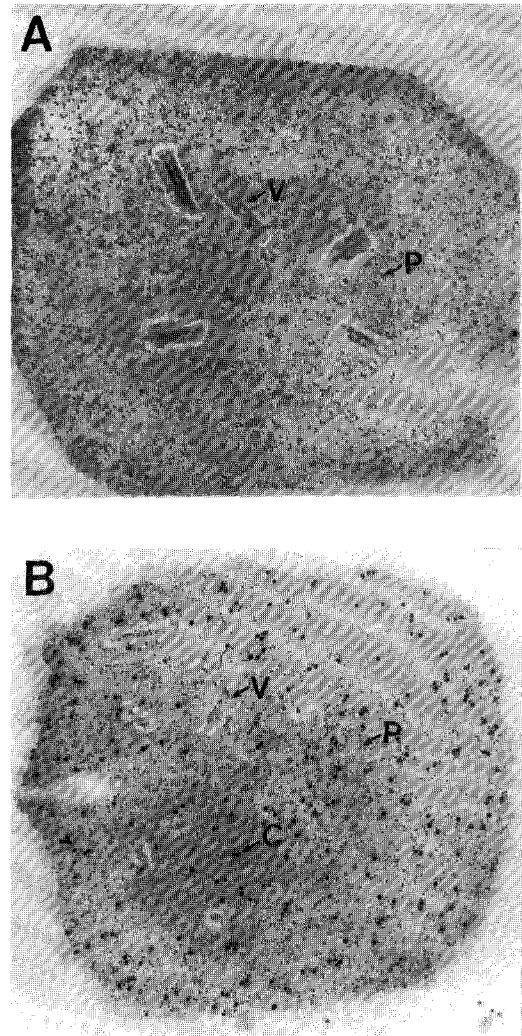

Figure 4. Immunogold staining of recombinant polyhedra produced by recombinant virus Btrus. Polyhedra sections of wild-type AcNPV (**A**) and Btrus (**B**) were incubated with antiserum to the polyhedrin or Bt Cry1Ac crystal protein, and then incubated with goat anti-rabbit colloidal gold conjugates. Gold particles with polyhedrin (P) or Bt Cry1Ac crystal protein (C) were observed by transmission electron microscope. Virions (V) are indicated. Size bars represent 1 μm.

combinant polyhedra, which were nearly similar to those of wild-type AcNPV (Figure 3). To determine the fusion with polyhedrin and foreign gene products, therefore, recombinant polyhedra of Btrus were examined by immunogold staining (Figure 4). Gold particles with polyhedrin and Bt Cry1Ac crystal protein antibodies were concurrently observed in the recombinant polyhedra, whereas gold particles with Bt Cry1Ac crystal protein antibody were not detected in wild-type polyhedra. This result revealed that recombinant polyhedra by Btrus were assembled with polyhedrin and Bt Cry 1Ac crystal protein, and virus particles were contained in the recombinant polyhedra.

Bioassay against *H. cunea* larvae

Insecticidal toxicity of the recombinant polyhedra of Btrus was evaluated against second-instar larvae of *H. cunea* and compared with wild-type AcNPV (Table 1). *H. cunea* larvae fed the equivalent of 6,000 recombinant polyhedra of Btrus refused to consume the contaminated diets. When this dose was reduced

Table 1. Biological activity of Btrus and AcNPV against *H. cunea larvae*

Virus	Larvae* refusing diet (%)		
	6000†	1500	375
Mock	0	0	0
wt AcNPV	11.9	10.4	5.1
Btrus	100	97.6	48.2

*Second instar *H. cunea* larvae; approximately 50 insects per dose

†Number of polyhedra equivalents per larva (50 μl per diet plug)

to 1,500 recombinant polyhedra, 97.6% of the *H. cunea* larvae refused to feed, and when 375 recombinant polyhedra were used, 48.3% declined the diets. The result showed that such feeding inhibition in the very low doses was due to the crystal protein of recombinant polyhedra.

DISCUSSION

We have tried to construct a novel AcNPV recombinant that produces polyhedra which display foreign gene product. The strategy was to create the recombinant virus Btrus described in Figure 1A. The Btrus was constructed with two promoters, p10 or polyhedrin gene promoter, each initiating transcription of either native polyhedrin or fusion protein with polyhedrin and Bt Cry1Ac crystal protein. Thus, all of the cells infected with Btrus produced stably recombinant polyhedra which were similar in morphology and size to wild-type AcNPV polyhedra. Consistent with the immunogold labeling of purified Btrus polyhedra, the recombinant polyhedra consisted of the 98 kDa polyhedrin-Bt Cry1Ac fusion protein, the 33 kDa native polyhedrin, and virions. On the basis of our current knowledge this Btrus system, the most likely mechanism for the assembly of recombinant polyhedra is that the polyhedra are formed by coassembly of both the fusion protein and the native polyhedrin expressed in every infected cell.

The most striking result in this study is that crystal protein within the recombinant polyhedra of Btrus is functional in bioassay. We found that the insects fed on the recombinant polyhedra of Btrus refused to consume the diet, indicating that such feeding inhibition was due to the crystal protein of the recombinant polyhedra [6,16]. The result suggests that enhanced speed to kill might alleviate the speed problem in the baculovirus insecticides, and thus the recombinant polyhedra would be more effective in reducing crop damage. Several previous reports have described the generation of recombinant baculoviruses expressing Bt toxin, but the insecticidal toxicities of these recombinant baculoviruses were not significantly improved [11-13,15]. It seems likely that our approach has been successful because the Bt toxin is delivered to the gut of the insect, its normal action site. With previous Bt-toxin expressing recombinant baculoviruses, the toxin would have been produced in infected cells within the insect.

The use of the recombinant polyhedra of Btrus for baculovirus insecticide offers many advantages over the wild-type AcNPV. A baculovirus insecticide of the recombinant polyhedra of Btrus employed both the mode of action of Bt crystal protein and viral replication of AcNPV in insects, improving its pathogenicity and host range. Thus, it should provide a powerful means for the pest management.

SUMMARY

A novel recombinant baculovirus producing polyhedra with Bt CryIAc crystal protein was constructed. The recombinant baculovirus, Btrus, has two independent transcription units in opposite orientations with two promoters, p10 or polyhedrin gene promoter each initiating transcription of either native polyhedrin or fusion protein with polyhedrin and Bt Cry1Ac crystal protein. The recombinant polyhedra of Btrus were assembled with polyhedrin and Bt Cry1Ac

crystal protein, and contained virus particles. Insecticidal toxicity of recombinant polyhedra of Btrus in fall webworm, *H. cunea*, was strikingly improved in comparison with the wild-type AcNPV. In conclusion, our results suggest that the novel baculovirus producing the recombinant polyhedra may be useful as a powerful baculovirus insecticide in the control of insect pests.

Acknoweedgments

This work was supported by Korea Research Foundation grant (KRF 98-019-G00001).

REFERENCES

1) **Adang MJ, Staver MJ, Rocheleau TA, Leighton J, Barker RF, Thomson DV:** Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. *Gene* **36:** 289-300, 1985.

2) **Bendayan M:** Double immunocytochemical labelling applying the protein A-gold technique. *J Histochem Cytochem* **30:** 81-86, 1982.

3) **Bonning BC, Hammock BD:** Development of recombinant baculoviruses for insect control. *Annu Rev Entomol* **41:** 191-210, 1996.

4) **Granados RR, Federici BA:** The biology of baculoviruses, vols 1 and 2. Boca Raton. CRC Press, 1986.

5) **Hammock BD, Bonning BC, Possee RD, Hanzlik TN, Maeda S:** Expression and effects of the juvenile hormone esterase in a baculovirus vector. *Nature* **344:** 458-461, 1990.

6) **Hofte H, Whiteley HR:** Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev* **53:** 242-255, 1989.

7) **Hughes PR, Wood HA, Breen JP, Simpson SF, Duggan AJ, Dybas JA:** Enhanced bioactivity of recombinant baculoviruses expressing insect-specific spider toxins in lepidopteran crop pests. *J Invertebr Pathol* **69:** 112-118, 1997.

8) **Laemmli UK:** Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227:** 680-685, 1970.

9) **Lee HH, Miller LK:** Isolation and initial characterization of temerature-sensitive mutants of *Autographa californica* nuclear polyhedrosis virus. *J Virol* **31:** 240-252, 1979.

10) **Maeda S:** Further development of recombinant baculovirus insecticides. *Cur Opi Biotech* **6:** 313-319, 1995.

11) **Martens JWM, Honee G, Zuidema D, Van lent JWM, Visser B, Vlak JM:** Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. *Appl Environ Microbiol* **56:** 2764-2770, 1990.

12) **Martens JWM, Knoester M, Weljts F, Groffen SJA, Hu Z, Bosch D, Vlak JM:** Characterization of baculovirus insecticides expressing tailored *Bacillus thuringiensis* CryIA (b) crystal proteins. *J Invertebr Pathol* **66:** 249-257, 1995.

13) **Merryweather AT, Weyer U, Harris MPG, Hirst M, Booth T, Possee RD:** Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. *J Gen Virol* **71:** 1535-1544, 1990.

14) **O'Reilly DR, Miller LK, Luckow VA:** Baculovirus expression vectors: a laboratory manual. W. H. Freeman & Co., New York. 1992.

15) **Ribeiro BM, Crook NE:** Expression of full-length and truncated forms of crystal protein genes from *Bacillus thuringiensis* subsp. *kurstaki* in a baculovirus and pathogenicity of the recombinant viruses. *J Invertebr Pathol* **62:** 121-130, 1993.

16) **Schnepf E, Crickmore N, Van rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH:** Bacillus thuringiensis and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62:** 775-806, 1998.

17) **Stewart LMD, Hirst M, Ferber ML, Merryweather AT, Cayley PJ, Possee RD:** Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature* **352:** 85-88, 1991.

18) **Summer MD, Smith G:** A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agric Exp Station Bull* **1555:**

1-56, 1987.

19) **Tomalski MD, Miller LK:** Insect paralysis by baculovirus-mediated expression of a mite neurotoxin gene. *Nature* **352:** 82-85, 1991.

20) **Towbin H, Staehelin T, Gordon J:** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350-4354, 1979.

21) **Wood HA, Granados RR:** Genetically engineered baculoviruses as agents for pest control. *Annu Rev Microbiol* **45:** 69-87, 1991.