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〈論 文〉

Expression and Morphology of Crystal Proteins in *Bacillus thuringiensis* subsp. *kurstaki* Cry⁻*B*

Hyun Woo Park, Ho San Kim, Young Hoon Kim, Byung Rae Jin and Seok Kwon Kang

College of Agriculture and Life Sciences
Seoul National University, Suwon, Korea

Abstract

To investigate the morphology of *Bacillus thuringiensis* crystal proteins, two type crystal protein genes, *cryIA(c)* gene under the control of *cryIA(b)* gene promoter and *cryIIA* gene under the control of its own promoter, were transformed in *B. thuringiensis* acrystalliferous mutant strain and the transformants were characterized by SDS-PAGE and scanning electron microscopy. The expression and formation of crystal proteins in *B. thuringiensis* subsp. *kurstaki* Cry⁻*B* revealed that crystal proteins appear to have same molecular weight and morphology to those of wild type strain's, suggesting that the expression and formation of crystal proteins affected not by host cell or recombination of *cryIA(c)* gene under the control of *cryIA(b)* gene promoter but by only structural fragment of protoxin.

Key words : *Bacillus thuringiensis*, crystal protein gene, *cryIA(b)*, *cryIA(c)*, *cryIIA*, expression, morphology

INTRODUCTION

B. thuringiensis is a gram positive soil bacterium characterized by its ability to produce crystalline inclusion bodies during sporulation. These inclusions contain one or more proteins that have a highly specific insecticidal activity (Whiteley and Schnepf, 1986). Because of their insecticidal activity, many *B. thuringiensis* strains have been isolated and characterized. These isolates are classified into 34 subspecies differentiated by the antigenic properties of their flagella as well as variety of biochemical tests (de Barjac and Frachon, 1990). Most strains are active against larvae of Lepidopteran, but some show toxicity against Diptera (Federici *et al.*, 1990) or Coleoptera (Krieg *et al.*, 1983). For some crystal producing strains, no toxic activity has also been demonstrated.

To date, nucleotide sequences have been reported for 42 *B. thuringiensis* crystal protein genes. These genes have been divided into four major classes and several subclasses characterized by both the structural similarities and the insecticidal spectra of the encoded proteins (Höfte and Whiteley, 1989). Type I genes encode 130 kDa proteins that are normally active only against Lepidoptera. Type II genes encode 70 kDa proteins that also maintain Lepidoptera activity. At least one type II gene product, CryIIA, is active against both Lepidoptera and Diptera. Type III gene products are active on Coleoptera, and their proteins are also about 70 kDa. Type IV gene products, including both 70 kDa and 130 kDa crystal proteins, were originally isolated from *B. thuringiensis* subsp. *israelensis* that is highly active against mosquito and blackfly larvae. Also found in *B. thuringiensis* subsp. *israelensis* is a cytolytic crystal protein, CytA, that has a molecular weight of approximately 27 kDa.

Most *B. thuringiensis* isolates produce crystal proteins in the shape of bipyramid, a configuration composed of two pyramids bound together at the base. The crystal proteins that form bipyramidal shapes are typically 130 kDa in size and several closely related proteins may be present in a single crystal. Many *B. thuringiensis* isolates, however, produce proteins that crystallize in different shapes, such as cuboidal, rhomboidal and ovoidal crystals. However, when each of the crystal protein genes was expressed in *B. thuringiensis* strains, some of these show unclear morphology (Yamamoto and Powell, 1993).

In this study, to investigate the shape formation of *B. thuringiensis* crystal proteins, two crystal protein genes, *cryIA(c)* gene under the control of *cryIA(b)* gene promoter and cryIIA gene under the control of its own promoter, were expressed in *B. thuringiensis* acrystalliferous mutant strain, and the shape formation of crystal proteins were characterized.

MATERIALS AND METHODS

1. Bacterial strains, medium and plasmids

B. thuringiensis subsp. *kurstaki* Cry *B* and *E. coli* DH5α [*supE*44 *lacU*169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *gyrA*96 *thi*-1 *relA*1] were used for expression and cloning of crystal protein genes. For obtaining crystal proteins of *B. thuringiensis*, G.Y.S. (Glucose Yeast extract Salt) medium was used. Commercially available plasmids pUC19 and pGEM-7Zf were used for cloning. The shuttle vector pHT3101 was kindly provided by Dr. S. S. Gill (Dept. of Entomology, University of California, Riverside, U.S.A.).

2. Construction of the expression vector pKC1 A and pKC2A

For the construction of pKC1A, the *cryIA(b)* promoter contained on a 370 bp *Nde*I fragment of the plasmid pSK3 (Je *et al.*, 1993) was eluted from the agarose gel and cloned into pUC19 *Nde*I site, which was then partially digested with *Nde*I. The resulting fragment was ligated with 3.7 kb *Nde*I fragment of *cryIA(c)* gene obtained from pN6.6 (Adang *et al.*, 1985) to yield pBP3. The *cryIA(c)* gene and *cryIA(b)* promoter contained on a 4.0 kb *Sma*I fragment were inserted into pHT3101 *Sma*I site to yield expression vector pKC1A.

For the construction of pKC2A, the plasmid pSKIIA (Kim *et al.*, 1993), which contains *cryIIA* gene of *B. thuringiensis* subsp. *kurstaki* HD-1, was digested with *Bam*HI and *Hin*dIII. The *cryIIA* contained on a 4.0 kb *Bam*HI and *Hin*dIII fragment was ligated into pGEM-7Zf, which was then digested with *Sac*I and *Eco*RI. The resulting fragment was inserted into *Sac*I and *Eco*RI site of pHT3101 to yield expression vector pKC2A.

3. Electroporation

To introduce expression vectors, pKC1A and pKC2A, into *B. thuringiensis* subsp. *kurstaki* Cry *B*, electroporation was performed by partially modified Bone and Ellar's method (1989). *B. thuringiensis* cells were grown with shaking in 200 ml of Brain Heart Infusion (BHI, Biolife) at 37℃ overnight. The cells were harvested and resuspended in 10 ml of cold sterile HG buffer (1 mM HEPES, pH 7.0; 10% glycerol). The resuspened cells were mixed with 1 μg of plasmid DNA in a precooled 0.2 cm electroporation cuvettes (Bio-Rad). Electroporation was carried out using a Bio-Rad Gene Pulser™ coupled to a parallel resistance selector (Bio-Rad Pulse Controller). The DNA/cells were incubated on ice for 10 min and then electroporated with a single pulse (capacitance 25 μF, set voltage 2.5 kV, resistance 200 Ω). After the pulse, the electroporated cells were incubated on ice for 10 min. One milliliter of L.B. was added to the electroporated cells and the cells were transferred to a sterile tube for incubation at 37℃ for 1 hr prior to plating out on nutrient agar containing 25 μg/ml erythromycin.

4. SDS-Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed on a 10% polyacrylamide separating gel with a 3% stacking gel as described by Laemmli (1970). The gel was stained with Coomassie brilliant blue. Molecular weight markers (Sigma) were used as standards. For the protein analysis of transformants, cells were cul-

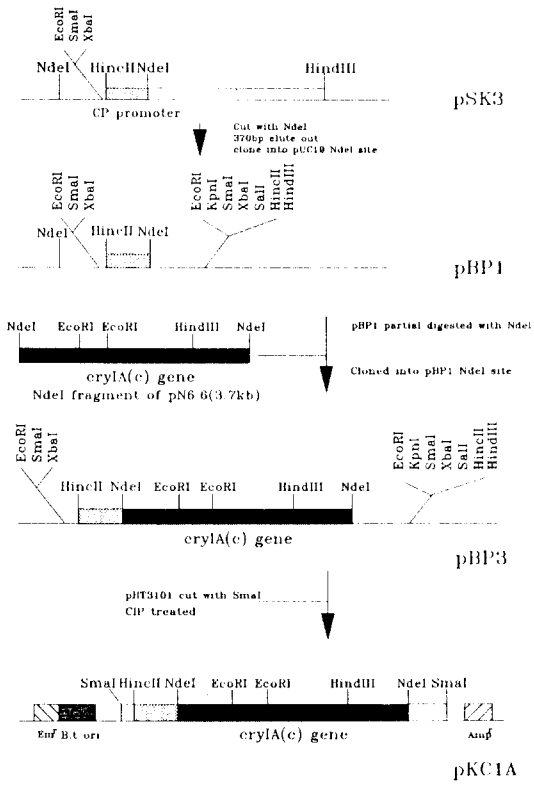

Fig. 1. *Schematic diagram of construction of expression vector pKC1A. Expression vector pKC1A was constructed by ligation of cryIA(c) gene under the control of cryIA(b) promoter.*

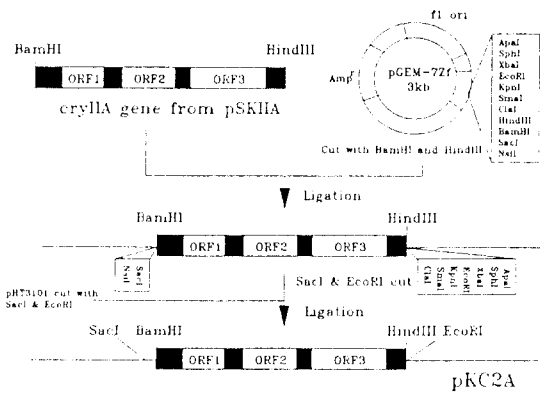

Fig. 2. *Schematic diagram of construction of expression vector pKC2A. Expression vector pKC2A was constructed with cryIIA gene and its own promoter.*

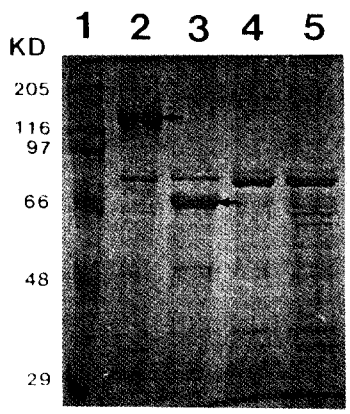

Fig. 3. *SDS-PAGE analysis of crystal proteins of transformed B. thuringiensis subsp. kurstaki Cry B strains. Lane 1, protein size marker ; Lane 2, Cry B strain transformed with pKC1A ; Lane 3, Cry B strain transformed with pKC2A; Lane 4, Acrystalliferous mutant strain, Cry B; Lane 5, Cry B transformed with pHT3101.*

tured in G.Y.S. medium containing 25 μg/ml erythromycin and harvested by centrifugation at time points up to 5 days. The samples were prepared by boiling for 5 min in gel sample buffer.

5. Electron microscopy

B. thuringiensis strains were cultured in G.Y.S. medium for 5 days at 30℃ to ensure sporulation and complete autolysis. All samples were air dried, coated with carbon, and stained with gold. The samples were observed by scanning electron microscope (Cambridge Stereo Scan 250 Mk2).

RESULTS AND DISCUSSION

To investigate the expression and morphology of crystal proteins in *B. thuringiensis* subsp. *kurstaki* Cry B, expression vectors, pKC1A and pKC2A, were constructed, respectively. The expression vector pKC1A was constructed by ligation of *cryIA(c)* gene under the control of *cryIA(b)* promoter. The *cryIA(b)* promoter contained on a 370 bp *Nde*I fragment of the plasmid pSK3 (Je *et al.*, 1993) was ligated with 3.7 kb *Nde*I fragment of *cryIA(c)* gene obtained from pN6.6 (Adang *et al.*, 1985) to yield pBP3. For the expression in *B. thuringiensis*, *cryIA(c)* gene and *cryIA(b)* promoter contained on a 4.0 kb *Sma*I fragment were inserted into pHT3101 *Sma*I site. The expression vector

was named pKC1A (Fig. 1).

The expression vector pKC2A was constructed with *cryIIA* gene under the control of its own promoter. The *cryIIA* gene and its promoter obtained from pSKIIA (Kim *et al.*, 1993) was ligated into pHT3101 to yield expression vector pKC2A (Fig. 2).

For the expression in *B. thuringiensis* subsp. *kurstaki* Cry$^-$*B*, the expression vector pKC1A and pKC2A were introduced into *B. thuringiensis* subsp. *kurstaki* Cry B by electroporation, respectively, and transformed cells were selected by erythromycin containing nutrient agar plate. The transformants were incubated at 30℃ for 5 days, and the transformed cell lysates were analysed by SDS-PAGE (Fig. 3). Lane 2 shows the crystal protein produced in *B. thuringiensis* subsp. *kurstaki* Cry$^-$*B* strain transformed with pKC1A, while lane 3 shows the crystal protein produced in *B. thuringiensis* subsp. *kurstaki* Cry *B* strain transformed with pKC2A. The *B. thuringiensis* subsp. *kurstaki* Cry *B* and *B. thuringiensis* subsp. *kurstaki* Cry$^-$*B* transformed with pHT3101 were used as a negative controls (Lane 4 and 5). The result indicated that crystal proteins expressed in *B. thuringiensis* subsp. *kurstaki* Cry *B* had their own molecular weight, 133 and 71 kDa, respectively. Actually, Lereclus *et al.* (1989) reported that the *cryIA(a)* gene cloned in pHT3101 was expressed in *B. thuringiensis* subsp. *kurstaki* Cry$^-$*B*. Therefore, the aforementioned results demonstrated that the *cryIA(c)* or *cryIIA* genes in *B. thuringiensis* subsp. *kurstaki* Cry$^-$*B* are also expressed and the expression of crystal protein was not affected to recombination of *cryIA(c)* gene under the control of *cryIA(b)* promoter.

Furthermore, morphology of crystal proteins produced by transformants was observed by scanning electron microscopy (Fig. 4). The results revealed that the pKC1A is produced bipyramidal crystals and pKC2A is produced cuboidal crystals, demonstrating that the transformants have their own crystal shapes.

These observations suggest that formation of crystal proteins affected not by host cells or other plasmids co-existed but by only C-terminal structural fragment of protoxin. Also, the expression and formation of crystal protein were not affected to recombination of *cryIA(c)* gene under the control

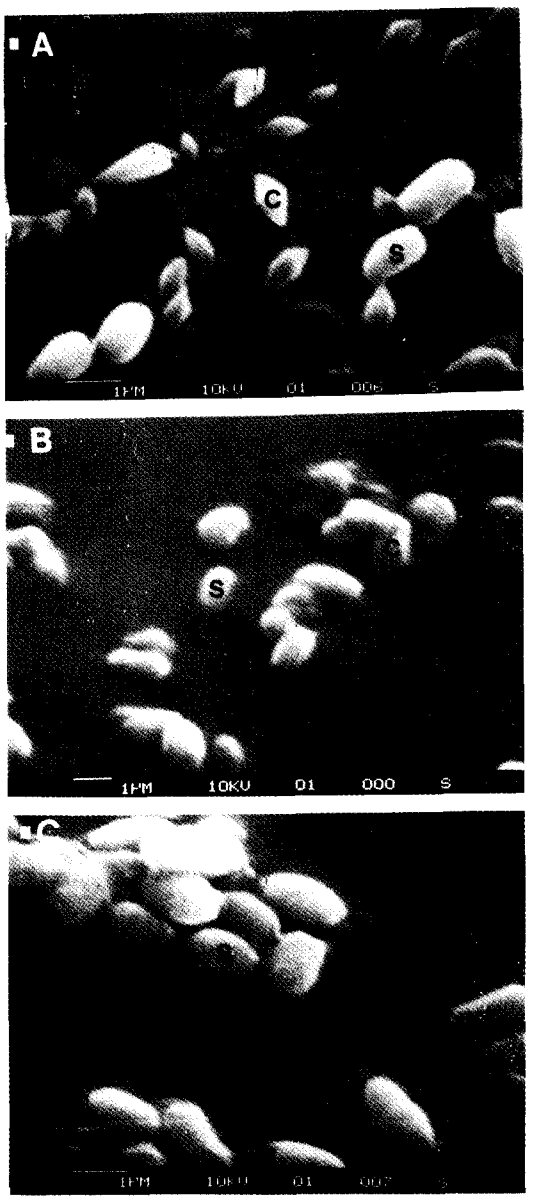

Fig. 4. *Scanning electron microscopy of crystal proteins expressed in B. thuringiensis subsp. kurstaki Cry$^-$B strain transformed with pKC1A and pKC2A. S and C indicate spore and crystal protein, respectively. Panel A, Cry B strain transformed with pKC1A ; Panel B, Cry$^-$B strain transformed with pKC2A ; Panel C, Acrystalliferous mutant strain, Cry B.*

of *cryIA(b)* promoter. It may be suggested that C-terminal fragment play a role in the formation of parasporal inclusions (Aronson *et al.*, 1986; Höfte and Whiteley, 1989). In conclusion, the present study demonstrated that expression vectors, pKC 1A and pKC2A, in *B. thuringiensis* subsp. *kurstaki* Cry *B* are also expressed with their own morphology and molecular weight, and the recombination of *cryIA(c)* gene under the control of *cryIA(b)* promoter does not affected to the expression and formation of crystal protein. To develop the efficient *B. thuringiensis* pesticide, therefore, genetic engineering of *B. thuringiensis* and crystal protein genes will be expected as an interesting and promising source of insecticide.

摘 要

B. thuringiensis subsp. *kurstaki* Cry *B*에서 *cryIA (b)* 유전자 promoter 조절을 받는 *cryIA(c)* 유전자와 그 자신의 promoter 조절을 받는 *cryIIA* 유전자의 발현 여부와 내독소 단백질의 형태를 관찰하기 위하여, 이들 두 내독소 단백질 유전자를 *B. thuringiensis* - *E. coli* shuttle vector를 이용하여 발현벡터 pKC1A와 pKC2A를 각각 제작하였다. 발현벡터 pKC1A와 pKC2A를 *B. thuringiensis* subsp. *kurstaki* Cry *B* 균주에 형질전환시키고, 이들 형질전환체로부터 각각 bipyramid형과 cuboid형의 정상적인 내독소 단백질이 발현되었음을 확인하였다.

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