韓應昆誌 35(1) : 85~90 (1996)
Korean J. Appl. Entomol.

Synergistic Effect in Mosquitocidal Activity of Crystal Proteins from *Bacillus thuringiensis* NT0423 Transformed with *cryIVD* Gene

cryIVD 유전자로 형질전환된 *Bacillus thuringiensis* NT0423 균주 내독소 단백질의 모기 유충에 대한 독성의 상승효과

Hyun Woo Park[1], Yong Man Yu[1], Hyun Woo Oh[2], Byung Rae Jin[1], In Ki Kim[3] and Seok Kwon Kang[1,*]

박현우[1] · 유용만[1] · 오현우[2] · 진병래[1] · 김인기[3] · 강석권[1,*]

ABSTRACT *Bacillus thuringiensis* NT0423 produces quite a typical bipyramidal crystals of a common major band of ca. 130 kDa, and has dual specificity against Lepidoptera and Diptera. To enforce the Diptera-toxicity of *B. thuringiensis* NT0423, *cryIVD* gene was transformed into *B. thuringiensis* NT0423. The transformant *B. thuringiensis* PT1227 was obtained from introduction of pCG10 into *B. thuringiensis* NT0423 by electroporation. The result showed that *cryIVD* and resident crystal protein genes in transformant were stably expressed with its own shape. Furthermore, the toxicity of *B. thuringiensis* PT1227 against Diptera was highly enforced, suggesting that the enforced toxicity of *B. thuringiensis* PT1227 was due to synergistic effect of both introduced and resident crystal proteins in transformant.

KEY WORDS *Bacillus thuringiensis, cryIVD*, synergistic effect

초 록 국내에서 분리된 *Bacillus thuringiensis* NT0423은 나비목과 파리목 곤충에 독성을 보이는 130 kDa의 전형적인 다이아몬드형 내독소 단백질을 생성한다. 이 균주의 파리목에 대한 독성을 강화하기 위하여, *B. thuringiensis* NT0423에 모기 유충에 강한 독성을 보이는 *B. thuringiensis* subsp. *morrisoni* PG-14의 *cryIVD* 유전자를 가지고 있는 pCG10 플라스미드를 electroporation 방법을 이용하여 형질전환하였다. 형질전환체인 *B. thuringiensis* PT1227내에서 *cryIVD*와 숙주가 생성하는 원래의 다이아몬드형 130 kDa 내독소 단백질 유전자는 그 자신의 형태로 잘 발현되었다. 형질전환체의 모기 유충에 대한 독성은 원래 숙주의 내 독소 단백질과 도입된 CryIVD의 상승효과에 의해 현저히 증가하였다.

검색어 *Bacillus thuringiensis, cryIVD*, 상승효과

Bacillus thuringiensis is a gram positive bacterium characterized by its ability to produce crystalline inclusions during sporulation. These inclusions consist of proteins exhibiting a highly specific insecticidal activity (Whiteley & Schnepf 1986). Although most subspecies of *B. thuringiensis* are active against Lepidoptera, several *B. thuringiensis* subspecies including *israelensis, darmstadiensis, kyushuensis* and *morrisoni* PG-14 are toxic to Diptera such as mosquitoes (Gill *et al.* 1987).

In recent years, our increased understanding of the molecular genetic basis of *B. thuringiensis* insecticidal activity has enabled the use of novel *B. thuringiensis* strains improvement approaches that have considerably greater potential than the more conventional strain isolation and bioassay screening program. These strain improvement approaches have involved the genetic manipulation of a *B. thuringiensis* strain's plasmid coding

[1] The Research Center for New Bio-Materials in Agriculture and Department of Agricultural Biology, Seoul National University, Suwon 441-744, Korea (서울대학교 농생물학과 및 농업생물신소재연구센터)
[2] Korea Research Institute of Bioscience & Biotechnology, KIST, Taejon 305-606, Korea (KIST 생명공학연구소)
[3] Sun Yang Co., Ltd., Yongin-Gun, Kyunggi-do 449-800, Korea ((주)선양)
* Corresponding author

for crystal protein, and have utilized both non-recombinant and recombinant methodologies. Such methodologies can alter a *B. thuringiensis* strain's genetic information with regard to insecticidal crystal protein production and can result in distinct improvement in a strain's insecticidal activity potency and spectrum (Yamamoto & Powell 1993).

Numerous alternative host organisms such as *B. megaterium, B. cereus* and *B. subtilis* have been explored for expression of *B. thuringiensis* crystal protein genes, but *B. thuringiensis* crystal protein genes in these organisms were poorly expressed (Calogero *et al.* 1989, Gonzalez *et al.* 1982, Sakanyan *et al.* 1982). Recent studies of gene transfer to *B. thuringiensis* strains have been mainly performed with acrystalliferous mutant strains. Recently, three crystal protein genes of *B. thuringiensis*-one *cryIIIA* and two *cryIA(c)*-were respectively introduced in an acrystalliferous mutant strains by conjugational transfer (Carlton & Gawron-Burke 1993). Futhermore, transformation of *B. thuringiensis* crystal protein genes into the strains that produce their own crystal proteins has also been reported, it referred only transformation efficiency (Bone & Ellar 1989; Mahilon *et al.* 1989). In the previous study, we expressed three types of crystal protein genes in *B. thuringiensis* (Park *et al.* 1995). In this study, to enforce the Diptera-toxicity of *B. thuringiensis* NT0423 which has dual specificity against lepidopteran and dipteran insects, *cryIVD* gene of *B. thuringiensis* subsp. *morrisoni* PG-14 was transformed and characterized the expression of crystal proteins in *B. thuringiensis* NT0423.

MATERIALS AND METHODS

Bacterial strains and culture media

B. thuringiensis NT0423 (Kim *et al.* 1993), *B. thuringiensis* subsp. *morrisoni* PG-14, and *B. thuringiensis* subsp. *kurstaki* HD-1 were used in this study. For obtaining crystal proteins and for extraction of plasmid DNA of *B. thuringiensis*, G.Y.S. and S.P.Y. media were used.

Plasmid

The pCG10 was kindly provided by Dr. S. S. Gill (Dept. of Entomology, University of California, Riverside, U.S.A.). Plasmid, pCG10 was contained *cryIVD* gene of *B. thuringiensis* subsp. *morrisoni* PG-14, which was also contained origins of replication in both *B. thuringiensis* and *E. coli*, Em^r and Amp^r as selectable marker and a multiple cloning site in the *lacZ* gene (Chang *et al.* 1993). Plasmid DNAs of *B. thuringiensis* strains were isolated by partially modified alkaline lysis method (Birnboim & Doly 1979).

Southern blot

Southern hybridization (Southern 1975) of total plasmid DNA of all *B. thuringiensis* strains were carried out according to the method recommened by supplier using the nonradioactive DNA labelling and detection kit (Boehringer Mannheim Co.). Plasmid profiles of all *B. thuringiensis* strains were performed by running undigested total plasmid DNA on a 0.7% agarose gel. The 2.85 kb *Eco*RI fragment of pCG10 containing *cryIVD* gene was used as a probe.

Electroporation

The introduction of *B. thuringiensis* crystal protein gene into *B. thuringiensis* host was performed by partially modified Bone and Ellar's electroporation method (1989). 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). After the pulse, the electroporated cells were incubated on ice for 10 min. One milliliter of medium was added to the electroporated cells and the cells were transferred to a sterile tube for incubation at 37°C for 1 hr prior to plating out on nutrient agar containing 25 μg/ml erythromycin (Sigma).

Purification of parasporal inclusions

Purification of parasporal inclusions was performed by partially modified method of Thomas and Ellar (1983). *B. thuringiensis* strains were cultured in G.Y.S.

medium for 5 days at 30℃ to ensure sporulation and complete autolysis. The spore-parasporal inclusion mixtures were thoroughly washed with 1 M NaCl - 0.01% Triton X-100 and sedimented by centrifugation at 15, 000 g for 10 min. The pellets were resuspended in distilled water, sonicated three times (22,000 cycle/sec for 30 sec), loaded onto a discontinuous 45 to 87 % sucrose step gradient, and centrifuged at 80,000 g for 2 hr.

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

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

Electron microscopy

The transformant was grown on nutrient agar plate containing 25 μg/ml erythromycin. For the scanning electron microscopy of *B. thuringiensis* parasporal inclusions, the purified parasporal inclusions were air dried, coated with carbon, and stained with gold. The samples were observed by scanning electron microscope (Phillips SEM 515).

Insect bioassay

The toxicity of the transformant was determined against dipteran and lepidopteran larvae. To determine the mosquitocidal activity of purified inclusions from the *B. thuringiensis* strains, bioassay against second-instar *Culex pipiens* larvae was applied to 5 different inclusion concentrations (1 to 2,000 ng/ml) with three replicates per concentrations. The mortality was determined at 24 hr pi, and LC_{50} values were calculated by Bradford method (Bradford 1976). The larvicidal activity against *Spodoptera exigua* was also measured by overlay technique. The surface of an artificial diet was covered with a five diluents (1 to 1,000 ng/ml) of *B. thuringiensis* parasporal inclusions, inoculated to third-instar *S. exigua* larvae and the LC_{50} values were calculated by same method.

RESULTS AND DISCUSSION

To enforce the Diptera-toxicity, pCG10 containing *cryIVD* gene was introduced into *B. thuringiensis* NT 0423 by electroporation and colonies grown for 5 days onto the nutrient agar plates were observed by phase contrast microscope to confirm the two type crystal formation (data not shown). Futhermore, transformation of pCG10 into *B. thuringiensis* NT0423 was confirmed by Southern blot analysis (Fig. 1). The transformant was named PT1227.

To determine the expression of *cryIVD* gene in *B. thuringiensis* NT0423, transformed cell lysate was collected at 48 hr and then analysed by SDS-PAGE (Fig. 2). *B. thuringiensis* NT0423 showed unique 130 kDa

Fig. 1. Southern hybridization analysis of *B. thuringiensis* PT1227. The undigested total plasmid DNA of *B. thuringiensis* strains were electrophoresed on a 0.7% agarose gel (Panel A). The DNAs were hybridized with labelled 2.85 kb *Eco*RI fragment containing *cryIVD* gene (Panel B). Lane 1, *B. thuringiensis* PT1227 (*B. thuringiensi* NT0423 transformed with pCG10); Lane 2, *B. thuringiensis* NT0423

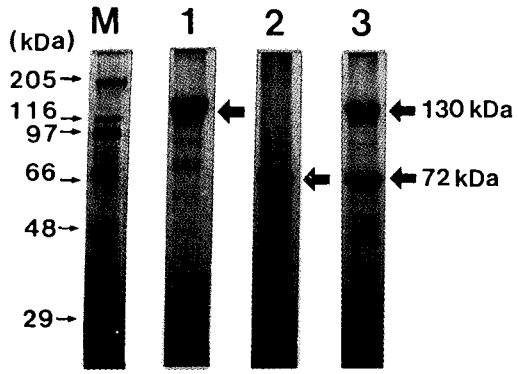

Fig. 2. SDS-polyacrylamide gel elecrophoresis analysis of *B. thuringiensis* crystal proteins. Lane 1, *B. thuringiensis* NT0423; Lane 2, *B. thuringiensis* subsp. *kurstaki* Cry*B* transformed with pCG10; Lane 3, *B. thuringiensis* PT1227. M indicates protein size marker (Sigma).

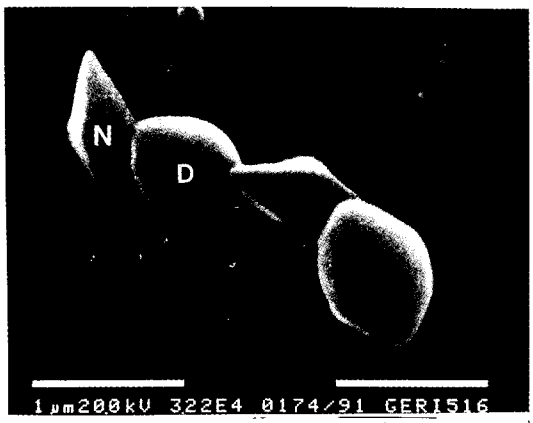

Fig. 3. Scanning electron microscopy of crystals of *B. thuringiensis* PT1227. The production of CryIVD (D) and NT0423 crystal protein (N) in transformant, *B. thuringiensis* PT1227 were observed by SEM.

band as expected in Lane 1. Production of *CryIVD* in *B. thuringiensis* PT1227 by pCG10 was as the 72 kDa band in Lane 3. Also, the *cryIVD* gene in *B. thuringiensis* PT1227 was well expressed as produced in *B. thuringiensis* subsp. *kurstaki* Cry*B*.

In addition, the morphology of parasporal inclusions in *B. thuringiensis* PT1227 was observed by scanning electron microscope (Fig. 3). The result showed that CryIVD in *B. thuringiensis* PT1227 has its own ovoidal shapes while crystal protein of *B. thuringiensis* NT0423 is typical bipyramidal crystals.

The *cryIVD* gene encodes a 72 kDa protein which is

Table 1. Insectcidal activities of *B. thuringiensis* crystal proteins

strains	LC_{50} in ng/ml after 24 hr	
	Culex pipiens	*Spodoptera exigua*
NT0423	1040	286
subsp. *kurstaki* HD-1	—[a]	11.8
subsp. *morrisoni* PG-14	1.5	—
pCG10[b]	38.7	ND[c]
PT1227	10.5	375

a: not tested.

b: *B. thuringiensis* subsp. *kurstaki* Cry*B* transformed with pCG10.

c: LC_{50} was not determined until the protein concentration was reached to 1,000 ng/ml.

a major component of the *B. thuringiensis* subsp. *israelensis* and subsp. *morrisoni* PG-14 (Federici *et al.* 1990). This crystal protein, unlike all other known *cry*-encoded proteins, is proteolytically converted into an active fragment of ca. 30 kDa (Chilcott & Ellar 1988). The exact localization of this fragment in the intact protein is not known and shape of this crystal protein has been unclear. The toxicity of each of CryIV-type proteins is from 10- to 100-fold less than that of the parasporal body, and to explain this it has been proposed that the proteins potentiate each other (Wu & Chang 1985).

Insecticidal activity of *B. thuringiensis* PT1227 was assayed against Diptera and Lepidoptera (Table 1). In case of mosquitocidal activity, *B. thuringiensis* subsp. *morrisoni* PG-14 was used as a control. *B. thuringiensis* PT1227 had much higher toxicity against dipteran larvae, *C. pipiens* than that of the *B. thuringiensis* NT0423 native strain. The LC_{50} value of transformant was enhanced approximately 100- and 3.7-fold than that of wild type and transformed *B. thuringiensis* subsp. *kurstaki* Cry*B*, respectively. This was due to synergistic effect of both introduced and resident crystal proteins in transformant. However, larvicidal toxicity of *B. thuringiensis* PT1227 against *S. exigua* was slightly lower than that of wild type. The result may implicate that resident crystal protein production was interfered with introduced crystal protein gene (Park *et al.* 1995). In order to develope *B. thuringiensis* strain that produce δ-endotoxin extremely toxic

to both lepidopteran and dipteran larvae, therefore, it is necessary to introduce both Lepidoptera- and Diptera-toxic genes.

In conclusion, 2 types of crystal protein, host and introduced crystal protein genes, are stably expressed in *B. thuringiensis* PT1227, and Diptera-toxicity is enforced by synergistic effect. Above-mentioned results suggesting that *B. thuringiensis* strain by genetic engineering will be expected as an interesting and promising source of insecticides.

ACKNOWLEDGEMENTS

This work was supported by grants from the Korea Ministry of Science and Technology (U.R. project) and from the Research Center for New Bio-Marerials in Agriculture, Seoul National University.

REFERENCES

Birmboim, H. C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**: 1513-1523.

Bone, E. J. and D. J. Ellar. 1989. Transformation of *Bacillus thuringiensis* by electroporation. *FEMS Microbiol. Lett.* **58**: 171-178.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Calogero, S., A. M. Albertini, C. Fogher, R. Marzari and A. Galizzi. 1989. Expression of a cloned *Bacillus thuringiensis* delta-endotoxin gene in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **55**: 446-453.

Carlton, B. C. and C. Gawron-Burke. 1993. Genetic improvement of *Bacillus thuringiensis* for bioinsecticide development, pp. 43-61. *In* L. Kim (ed.), *Advanced Engineered Pesticides*, Marcel Dekker, Inc., New York.

Chang, C., Y. M. Yu, S. M. Dai, S. K. Law and S. S. Gill. 1993. High level *cryIVD* and *cytA* gene expression in *Bacillus thuringiensis* does not require the 20-kilodalton protein, and the coexpressed gene products are synergistic in their toxicity to mosquitoes. *Appl. Environ. Microbiol.* **59**: 815-821.

Chilcott, C. N. and D. J. Ellar. 1988. Comparative study of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. *J. Gen. Microbiol.* **134**: 2551-2558.

Federici, B. A., P. Luthy and J. E. Ibarra. 1990. Parasporal body of *Bacillus thuringiensis israelensis*, pp. 16-44. *In* de Barjac H. and D. J. Surtherland (ed.), *Bacterial Control of Mosquitoes & Black Flies*. Rutgers University Press, New Brunswick.

Gill, S. S., J. M. Hornung, J. E. Ibarra, G. J. P. Singh and B. A. Federici. 1987. Cytolytic activity and immunological similarity of the *Bacillus thuringiensis subsp. israelensis Bacillus thuringiensis* subsp. *morissoni* isolate PG-14 toxins. *Appl. Environ. Microbiol.* **53**: 1251-1256.

González, J. M. Jr., B. J. Brown and B. C. Carlton. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci. U.S.A.* **79**: 6951-6955.

Kim, H. S., H. W. Park, S. H. Kim, Y. M. Yu, S. J. Seo and S. K. Kang. 1993. Dual specificity of δ-endotoxins produced by newly isolated *Bacillus thuringiensis* NT0423. *Korean J. Appl. Entomol.* **32**: 426-432.

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

Mahilon, J., W. Chungjatupornchai, J. Decock, S. Dierickx, F. Michiels, M. Peferoen and H. Joos. 1989. Transformation of *Bacillus thuringiensis* by electroporation. *FEMS Microbiol. Lett.* **60**: 205-210.

Park, H. W., H. S. Kim, D. W. Lee, Y. M. Yu, B. R. Jin and S. K. Kang. 1995. Expression and synergistic effect of three types of crystal protein genes in *Bacillus thuirngiensis*. *Biochem. Biophys. Res. Comm.* **214**: 602-607.

Sakanyan, V. A., T. V. Tsoi, G. V. Sezonov and S. I. Alikhanian. 1982. Expression of enterobacterial gene for antibiotic resistance under control of regulatory signals of *Bacillus thuringiensis* in gram-negative and gram-positive bacteria. *Genetika.* **18**: 1825-1834.

Southern, E. M. 1975. Detection of specific sequence among DNA fragment separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517.

Thomas, W. E. and D. J. Ellar. 1983. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin : Effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.* **60**: 181-197.

Whiteley, H. R. and H. E. Schnepf. 1986. The molecular

biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Annu. Rev. Microbiol.* **40**: 549-576.

Wu, D. and F. N. Chang. 1985. Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. *FEBS Lett.* **190**: 232-236.

Yamamoto, T. and G. K. Powell. 1993. *Bacillus thuringiensis* crystal proteins : Recent advances in understanding its insecticidal activity, pp. 3-42. In L. Kim (ed.), *Advanced Engineered Pesticides*, Marcel Dekker, Inc., New York.

(Received December 6, 1995)