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Construction of shuttle vectors expressing the *cry11Aa* gene and their mosquitocidal activity

Dae-Weon Lee, Ho-San Kim, Yeon-Ho Je, Joo-Up Kim[1], Hyo-Sok Yu and Seok-Kwon Kang*

Division of Applied Biology and Chemistry, College of Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea
[1]Department of Agronomy, College of Agriculture, Chungbuk National University, Cheongju 361-763, Korea

Abstract : For the expression of the *cry11Aa* gene highly toxic to dipteran insects, we constructed two cyanobacteria-*Escherichia coli(E. coli)* shuttle vectors, pCYASK5-1 and pCYASK5-2. These vectors were transformed into *E. coli* and selected with kanamycin. The expression of the *cry11Aa* gene in *E. coli* was characterized by SDS-polyacrylamide gel electrophoresis and Western blot analysis. Two *E. coli* transformants harboring pCYASK5-1 and pCYASK5-2 expressed the *cry11Aa* gene in size of 72 kDa and 64 kDa, respectively and showed over 89% mortality against *Culex pipiens* larvae.(Received December 8, 1997, accepted February 27, 1998)

Key words : *Bacillus thuringiensis* subsp. *morrisoni* PG-14, *cry11Aa* gene, shuttle vector, *Culex pipiens.*

Introduction

Dipteran insects including mosquitoes and black flies transmit several human and animal tropical diseases, including encephalitis, dengue fever, yellow fever and so on. The interests for *Bacillus thuringiensis* as a microbiological control agent have been intensified (Thiery *et al.*, 1991). For the control of disease vectors, *B. thuringiensis* strains toxic to dipteran insects have been used.

Among these *B. thuringiensis* strains, *B. thuringiensis* subsp. *morrisoni* PG-14 and subsp. *israelensis* produce parasporal inclusions highly toxic to dipteran insects during the sporulation (Goldberg and Marglit, 1977; Padua *et al.*, 1984).

In both subspecies, parasporal inclusions are composed of Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa and Cyt1Aa protein with molecular weight of 135, 125, 78, 72 and 28 kDa, respectively (Höfte and Whiteley, 1989; de Barjac and Southerland, 1990).

All genes encoding parasporal inclusions are located on a 72 MDa resident plasmid (Ward *et al.*, 1983; Gonzalez and Carlton, 1984). Through the analysis of parasporal inclusions, it has been reported that there are synergistic effects between the Cry -endotoxins and the Cyt1Aa protein (Tabashnik, 1992).

However, there are two defects pointed out in using dipteran-toxic parasporal inclusions. First, for the Cyt1Aa protein harboring haemolytic activity, microbial insecticides must not contain it. Second, mosquitocidal activity of parasporal inclusions is not sufficient because it sinks from the surface of water.

In this study, for the overcome of these defects, we constructed two vectors that can integrate into the genome of cyanobacteria known as mosquito larvae nutrient. We transformed them into *E. coli*, and primarily investigate the *cry11Aa* gene expression and their larvicidal activity against *Culex pipiens*.

* Corresponding author

Materials and Methods

Bacterial strains

B. thuringiensis subsp. *morrisoni* PG-14 was kindly supplied by Dr. M. Ohba (Institute of Biological Control, Faculty of Agriculture, Kyushu Univ., Japan) and grown at 28℃. *E. coli* DH5 and BL21 strain were used as a cloning and a expression host and grown at 37℃ in Luria-Bertani (LB) medium.

Construction of vectors

Plasmids pCG5 and pOV81 were generously supplied by Dr. S. S. Gill (Univ. California, USA) and Dr. Bogorad (Harvard Univ., USA), respectively.

pCG5 contains the operon structure, including *cyt1Aa* gene, *19k* gene, *cry11Aa* gene, and *20k* gene. pOV81 has genomic DNA fragment of *Synechocystis* PCC6803 (6803R and 6803L), kanamycin resistance gene, the *psbA* promoter of *Amaranthus hybridus* and multiple cloning site and can integrate into the genome of cyanobacteria by homologous recombination (Fig. 1).

To construct pCYASK5, a 6.86 kb *Sac*I-*Kpn*I fragment was excised from pCG5 and ligated into pOV81 digested by the same enzymes. For the construction of pCYASK5-1, the *cyt1Aa* gene was removed from pCYASK5 by digestion with *Sac*I and *Eco*RV and a *Sac*I-*Eco*RV fragment of pCYASK5 was treated with Klenow fragment and T4 DNA ligase. To construct pCYASK5-2, we carried out polymerase chain reaction (PCR) with the *cry11Aa*-specific primers. The pCYASK5 DNA was transferred into 0.5 ml Pre-mix (Korea Bioneer Co.) reaction tube containing PCR reaction mixture.

Amplification was accomplished by using the program set to denature at 94℃ for 2 min, anneal at 50 ℃ for 1 min 30 sec and extend at 72℃ for 2 min and the number of total PCR reaction cycle was 40. The PCR product was analyzed by agarose gel electrophoresis (Maniatis *et al.*, 1989) and treated with *Hin*dIII and *Sal*I and ligated into pOV81 digested with the same restriction enzymes. PCR primer sequences are as follows: 5' -CCCAAGCTTAGGAGGTT ATATGGAAGATAGTTCTTTAG (32mer, sense primer), 5' -ACGCGTCGACATTACAAGAGGA GCCACAATAC (32mer, anti-sense primer).

Plasmid DNA Isolation

The plasmid DNA was extracted by alkaline lysis described by Birnboim and Doly (1979). Plasmid DNAs were digested with *Kpn*I, *Sac*I, *Bgl*II, and *Spe*I, respectively and their restriction patterns were analysed by agarose gel electrophoresis.

E. coli transformation

Constructed vectors were transformed into *E. coli* BL21 by heat shock procedure (Maniatis *et al.*, 1989). And transformants were selected on the nutrient agar plate supplemented with kanamycin (10 ㎍/ml).

Sodium dodecyl sulfate-polyacrylamide gel electro-phoresis (SDS-PAGE)

SDS-PAGE was accomplished by the method of Laemmli (1970). To examine the expression of the *cry11Aa* gene in *E. coli*, all samples were grown in LB medium at 37℃ for 10 hours. 3 ml cultured cells were harvested at 7,500 rpm for 5 min and resuspended in 80 ㎕ distilled water and 20 ㎕ 5X sample buffer [60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue]. Protein samples were boiled for 5 min, transferred on ice for 2 min and loaded in SDS-PAGE gel. Protein profile was analysed by SDS-PAGE using 10% separating gel and stained with 0.1% Coomassie brilliant blue.

Antibody preparation

For the preparation of the Cry11Aa protein antibody, protein sample was prepared as described earlier and loaded in SDS-PAGE gel. The Cry11Aa protein was excised from the gel and dialyzed for 12 hours. ICR female mice were immunized with the Cry11Aa protein by injection. The first immunization of 250 ㎕ containing complete Freud' s adjuvant (Sigma Co.) was followed at 7 days interval by a series of four 500 ㎕ injection in incomplete Freud' s adjuvant (Sigma Co.).

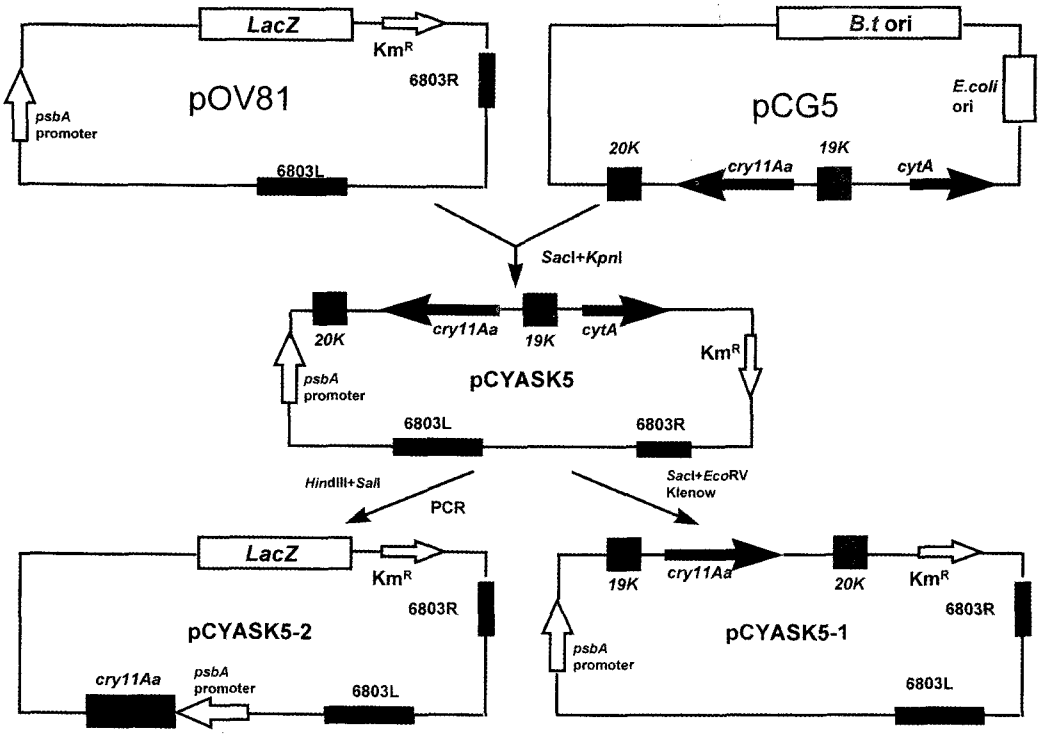

Fig. 1. Schematic diagram of integration vector construction.

Mice were bled 3 days after the last injection and sera were separated and stored at -20℃. Sera were heated at 56℃ for 30 min prior to use.

Western blot

Western blot was conducted by the method of Towin *et al.* (1979). Proteins in transformants were separated by SDS-PAGE on 10% polyacryamide gel and electro-transferred onto nitrocellulose membranes. The membrane was washed for 30 min twice in TBST buffer (10 mM Tris-HCl, 100 mM NaCl, 0.05% Tween 20) and blocked with 1% bovine serum albumin (BSA) in TBST buffer for 30 min.

The membrane was then incubated with mouse sera purified against the Cry11Aa protein for 1 hour and washed in TBST buffer. Subsequently the membrane was incubated with anti-mouse immunoglobulin G (IgG)-alkaline phosphatase (Sigma Co.) and washed with TBST buffer. Detection of the membrane was carried out with solution containing 0.01% 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.02% nitro blue tetrazolium (NBT), and 0.01 M $MgCl_2$ in 1 M Tris-HCl (pH 8.8).

Insect Bioassay

Late second instar of *Culex pipiens* were obtained from Dr. H. S. Yu (Medical Entomol. Lab., SNU) and 50 larvae per sample were used for bioassay without providing diet for 6 hours. Single larva was placed into individual wells of microtiter plate containing 150 μ l distilled water and exposed to the concentration of 5×10^7 cells/ml of *E. coli* BL21 and *E. coli* transformants. Control was in distilled water without treatment. Insecticidal activity was examined for 3 days with 3 repeats.

Results and Discussion

Construction of vectors

To construct pCYASK5, the 6.86kb *SacI-KpnI* fragment in pCG5 was ligated into pOV81 digested by the same restriction enzymes. pCYASK5-1 was generated by elimination of the *cytIAa* gene from

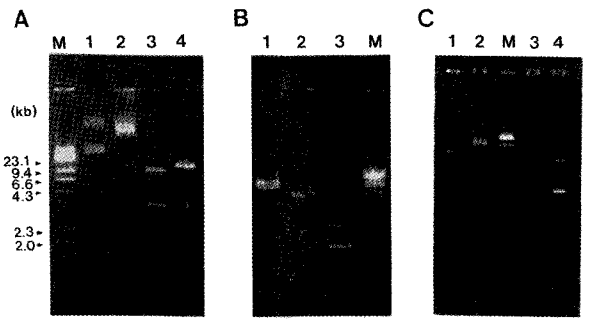

Fig. 2. Restriction endonuclease patterns of pCYASK5 (A), pCYASK5-1(B) and pCYASK5-2 (C). (A) M, -DNA digested with *Hin*dIII; Lane 1, pOV81; Lane 2, pCYASK5; Lane 3, pCYASK5 digested with *Kpn*I; Lane 4, pCYASK5 digested with *Sac*I. (B) M, -DNA digested with *Hin*dIII; Lane 1, pCYASK5-1 digested with *Bgl*II; Lane 2, pCYASK5 digested with *Kpn*I; Lane 3, pCYASK5 digested with *Kpn*I and *Spe*I. (C) M, -DNA digested with *Hin*dIII; Lane 1, pOV81 digested with *Sac*I; Lane 2, pCYASK5-2 digested with *Sac*I; Lane 3, pOV81 digested with *Pvu*II; Lane 4, pCYASK5-2 digested with *Pvu*II.

pCYASK5 because the *cytIAa* gene was reported that it does not affect other *cry* genes expression and have the haemolytic activity that is pointed out as a defect in using microbial insecticides (Delecluse *et al.*, 1991). pCYASK5-2 was constructed by ligation of the PCR-amplified *cryIIAa* gene with pOV81 (Fig. 1).

The *cryIIAa* gene expression structure in constructed vectors was different; pCYASK5 harboring the operon structure, *cytIAa* gene, *19k* gene, *cryIIAa* gene, *20k* gene, pCYASK5-1 in which the *cytIAa* gene was deleted and pCYASK5-2 in which the *cryIIAa* gene was under the control of *psbA* promoter (Fig. 1). Dervyn *et al.* (1995) reported the 373 bp *Eco*RV-*Bsp*HI fragment located in the upstream region of the *cryIIAa* gene was required for the expression of the *cryIIAa* gene, which was located in pCYASK5-1. To confirm the *cryIIAa* gene was cloned, agarose gel electrophoresis was carried out by using several restriction enzymes and all constructed vectors were found to have the predicted size, respectively (Fig. 2).

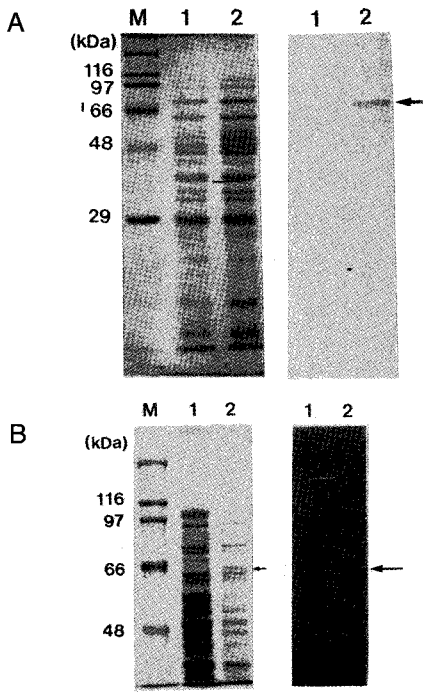

Fig 3. SDS-PAGE and Western blot analysis of *E. coli* harboring pCYASK5-1 (A) and pCYASK5-2 (B). (A) M, Protein size marker; Lane 1, *E. coli* BL21; Lane 2, *E. coli* transformed with pCYASK5-1. (B) M, Protein size marker; Lane 1, *E. coli* BL21; Lane 2, *E. coli* transformed with pCYASK5-2.

Expression of the *cryIIAa* gene

To investigate the *CryIIAa* protein expression in *E. coli* transformants, SDS-PAGE and Western blot analysis were performed. In *E. coli* transformed with pCYASK5-1, the Cry11Aa protein was not observed on SDS-PAGE gel, which was presumed that *E. coli* expressing protein was located in the place of the Cry11Aa protein in the gel. Using the Cry11Aa antibody, the Cry11Aa expression was confirmed and found 72 kDa in size. This result was consistent with the previous report (Dervyn *et al.*, 1995) that the *Eco*RV-*Bsp*HI fragment was important for the *cryIIAa* gene expression. *E. coli* containing pCYASK5-2 expressed 64 kDa in size on SDS-PAGE gel, which was somewhat smaller protein size previously reported. The small Cry11Aa protein might be a result of the

premature transcriptional termination because the PCR product using the *cry11Aa* gene-specific primer was about 2 kb (data not shown).

Transformants toxicity against *Culex pipiens* larvae

To examine the mosquitocidal effect of transformants on late second instar *Culex pipiens*, they were exposed to the concentration of 5 × 10^7 cells/ml of *E. coli* transformants. Each *E. coli* culture was harvested at the mid log phase of growth (approximately 5 × 10^7 cells/ml) and 200 μl aliquots were added to 96 well plate containing single larva. The toxicity of *E. coli* transformants was examined for 3 days (Fig. 4). It showed that larvae began to die on the first day of the exposure and *E. coli* transformed with pCYASK5-1 appeared over 90% mortality from the first day. *E. coli* harboring pCYASK5-2 were highly toxic to mosquito larvae, showing over 80% mortality on the second day.

Although the somewhat small Cry11Aa protein was expressed, its mosquitocidal activity was not affected. This result showed the premature termination of transcription was presumed to occur in the C-terminal residues that were not related to toxicity. The mortality of *E. coli* BL21 did not exceed 3%. But *E. coli* transformed with pCYASK5 was less toxic than other transformants. Though increase of the Cyt1Aa protein level in *E. coli* by 20 kDa protein was reported (Adams *et al.*, 1989; Visick and Whiteley, 1991), the increased level of the Cyt1Aa protein did not affect on the toxicity. This result was compatible with the report that the deletion of the *cyt1Aa* gene from the resident plasmid did not affect on overall toxicity (Delecluse *et al.*, 1991).

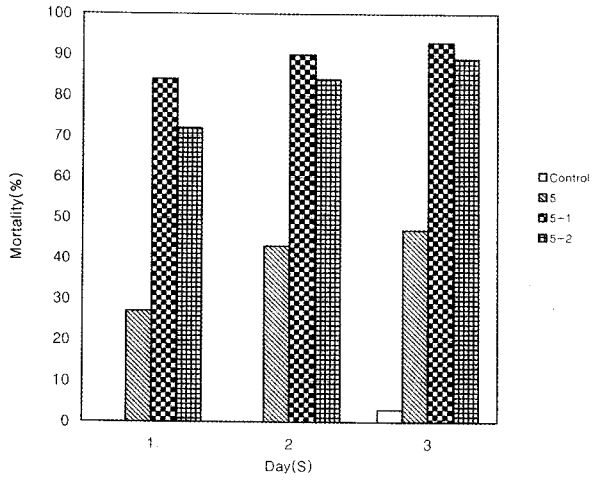

Fig. 4. Toxicity of *E. coli* transformed with pCYASK5, pCYASK5-1 and pCYASK5-2 against *Culex pipiens* larvae. The concentration of *E. coli* BL21 and transformants was 5 × 10^7 cells/ml.

These results demonstrated that *E. coli* transformed with pCYSAK5-1 and pCYASK5-2 expressed the *cry11Aa* gene effectively, showing over 85% toxicity to mosquito larvae. In addition, these transformants do not harbor the *cyt1Aa* gene, they have not the risk to occur the haemolytic activity in advanced application. Based on these results, we are now in the process of transformation of these constructs into a cyanobacterium as a nutrient used in aquatic environment and inquire the expression level of the Cry11Aa protein, its stability, its mosquitocidal effect and field evaluation.

Acknowledgements

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모기살충성 *cry11Aa* 유전자를 발현하는 벡터의 구축과 모기살충효과

이대원, 김호산, 제연호, 김주읍[1], 유효석, 강석권*(서울대학교 응용생물화학부, [1]충북대학교 농학과)

요약 : 모기에 독성을 보이는 *cry11Aa* 유전자를 발현시키기 위해 cyanobacteria와 *E. coli*에서 발현될 수 있는 두 가지 상이한 벡터 (pCYASK5-1, pCYASK5-2)를 제작하였다. 구축한 두 벡터를 *E. coli*에 형질전환하여 *cry11Aa* 유전자의 발현을 SDS-polyacrylamide gel electrophoresis (PAGE)와 Western blot analysis을 통해 조사한 결과, pCYASK5-1와 pCYASK5-2으로 형질전환된 *E. coli*는 각각 72 kDa과 64 kDa 크기의 *cry11Aa* 유전자를 발현하였다. 형질전환체의 모기살충성을 조사한 결과, pCYASK5-1과 pCYASK5-2을 가지는 형질전환체는 빨간집 모기유충*(Culex pipiens)*에 대해 각각 93%, 89%의 치사율을 보였다.

* 연락저자