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Expression of a Fusion Protein with Cry1Ac Protein and a Scorpion Insect Toxin in Acrystalliferous *Bacillus thuringiensis* Strain

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Expression of a fusion protein between *B. thuringiensis* crystal protein, Cry1Ac1 and a scorpion insect toxin (AaIT, *Androctonus australis* Hector insect toxin) in acrystalliferous *B. thuringiensis* strain (Cry⁻B strain) was examined. The *cry1Ac1* gene was cloned in *B. thuringiensis-E. coli* shuttle vector, pHT3101, under the control of the native *cry1Ac1* gene promoter (pProAc) and a gene encoding AaIT was inserted in *Xho*I site in the middle of the *cry1Ac1* gene (pProAc-ScoR). *B. thuringiensis* Cry⁻B strain carrying pProAc-ScoR (ProAc-ScoR/CB) produced an inclusion body of irregular shape and the expressed fusion protein is approximately 65 kDa in size. Sporulated cells and spore-crystal mixtures of ProAc-ScoR/CB had insecticidal activity against *Plutella xylostella* larvae, showing LT_{50} of ProAc-ScoR/CB (22.59 hrs) lower than that of ProAc/CB (30.06 hrs) at 1×10^7 CFU/cm^2. These results suggest that the fusion protein including a *B. thuringiensis* crystal protein and an AaIT may be functionally expressed in *B. thuringiensis*. Moreover, we verified the additive toxicity of AaIT, which is a new feasible candidate for insect control.

Key words: *Bacillus thuringiensis*, Fusion protein, Cry1Ac, *Androctonus australis* Hector insect toxin, Cry⁻B, *Plutella xylostella*

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Introduction

Bacillus thuringiensis is a ubiquitous gram-positive, spore-forming bacterium characterized by the presence of an insecticidal parasporal inclusions (δ-endotoxins) within the cytoplasm during sporulation. Most *B. thuringiensis* strains produce one or more crystal inclusions, which are toxic to insect species among the orders Lepidoptera, Diptera and Coleoptera insects, etc (Beegle and Yamamoto, 1992). Most δ-endotoxins is encoded by *cry* genes. More than 170 *cry* genes have been cloned, sequenced and extensively studied. A general feature of the *cry* genes is their expression during sporulation period. Crystal proteins were generally accumulated in the mother cell compartment to form a crystal inclusion that can account for 20 to 30% of the dry weight of a sporulated cell. The very high level of crystal protein synthesis in *B. thuringiensis* and its coordination with the stationary phase are controlled by a variety of mechanisms occurring at the transcriptional, posttranscriptional, and posttranslational levels (Agaisse and Lereclus, 1995; Baum and Malvar, 1995). Accordingly, the *cry* promoter could be a very powerful measure for the expression of foreign genes.

The insect-specific toxin, AaIT from the venom of the scorpion *Androctonus australis* Hector consists of a single polypeptide chain of 70 amino acids cross-linked by four disulfide bridges (Zlotkin *et al.*, 1971; Darbon *et al.*, 1982). AaIT affects only insects and has been shown to have no effect on mammals even at high doses (de Dianous *et al.*, 1987; Ji *et al.*, 2002). From a strictly agrotechnical point of view the AaIT toxin by itself can not be employed as an insecticide since it is inaccessible by oral and topical applications and is weakly effective to lepidopteran larvae, the major pest of field crops (Zlotkin *et al.*, 2000). However, in plant protection, AaIT is used as a factor for the genetic engineering of insect infective bac-

uloviruses resulting in potent and selective bio-insecticides (Maeda *et al.*, 1991; Stewart *et al.*, 1991). The efficacy of the AaIT expressing, recombinant baculovirus is attributed mainly to its ability to continuously provide and translocate the gene of the expressed toxin to the insect central nervous system. Furthermore, based on the pharmacological flexibility of the voltage-gated sodium channel, AaIT has taken a role of a device for insecticide resistance management.

As an alternative approach of AaIT, it is noteworthy that the transgenic plants engineered with two genes encoding AaIT and the *B. thuringiensis* toxin respectively became insect resistant more effectively (Barton and Miller, 1993). In other words, their results suggested that AaIT could have oral toxicity to insect larvae and that the employment of neurotoxic polypeptides for insect control might be feasible. There is even the previous result that AaIT had oral toxicity to flesh flies though its oral toxicity was relatively lower than the activity by injection (Zlotkin *et al.*, 1992). Based on the previous reports, in the current work, we expressed and characterized a recombinant *B. thuringiensis* Cry toxin protein fused with AaIT for verification of the additive toxic effects due to AaIT.

Materials and Methods

Bacterial strains, culture media and genes

E. coli XL-1 blue was used as the host for transformation and amplification of the recombinant plasmids. The Cry$^-$B strain, an acrystalliferous strain *B. thuringiensis* subsp. *kurstaki,* was used as hosts for expression of recombinant plasmid between the *cry1Ac* and the scorpion insect toxin (Stahly *et al.*, 1978). *B. thuringiensis* cultures were grown at 30°C with vigorous shaking in the SPY for the plasmid preparation and the GYS for expression of the fusion protein. The spore yields were checked using a colony count and the data evaluated by standard statistical procedures.

The *cry1Ac1* gene (Adang *et al.*, 1985) was used in pProAc (Roh *et al.*, 2000). The scorpion insect neurotoxin (*Androctonus australis* Hector insect toxin; AaIT) gene was used for production of a recombinant crystal protein (Zlotkin *et al.*, 2000). The Cry$^-$B transformed with pProAc (ProAc/CB) was used as positive control.

Plasmids, oligonucleotides and PCR

An AaIT gene was amplified from the pGT-ScoR using *Xho*I-linked primers of SF (AaIT forward, 5'-AACTC-GAGATGAAAAAAAACGGCTACGC-3') and SR (AaIT reverse, 5'-GACTCGAGGTTGATGATAGTAGTGTCGC-3'). For amplification of the AaIT fragment, a PCR was performed with *Pyrobest*™ DNA polymerase according to manufacturers manual (Takara Shuzo Co., Japan). For expression of the *B. thuringiensis* fusion crystal protein, the AaIT PCR product after digestion with *Xho*I was inserted into the *Xho*I site of pProAc (Roh *et al.*, 2000) and named pProAc-ScoR. The recombinant plasmid harboring the fusion gene, pProAc-ScoR had the promoter, ribosomal binding site and the terminator of the *cry1Ac* (Fig. 1).

Other PCR amplifications were also conducted with *Taq* DNA polymerase in a PreMix®-Top (Bioneer Co., Korea) based on a 35-cycle program of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C

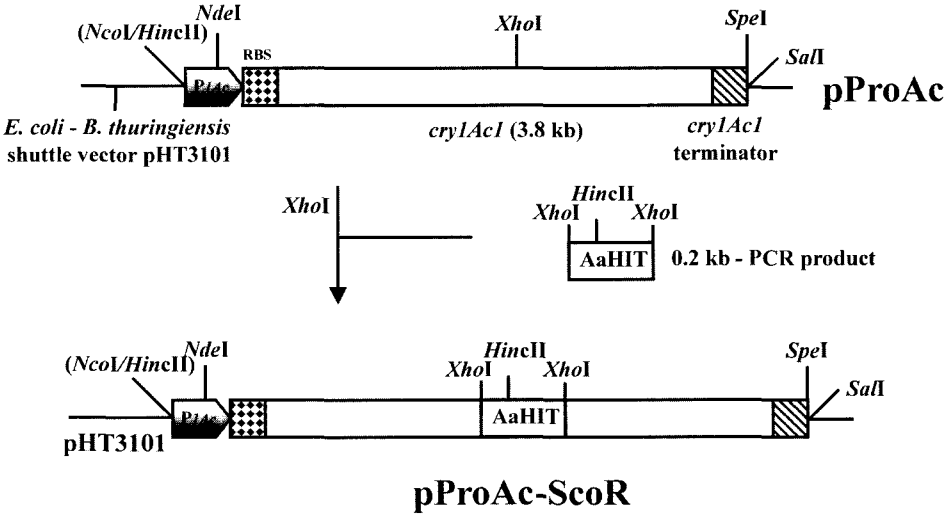

Fig. 1. Construction of a recombinant plasmid, pProAc-ScoR vector. The pProAc contains the *cry1Ac* coding region (open box), promoter (arrow box), the ribosome binding sequence (RBS, dotted box), and terminator (lined box). The PCR-amplified 0.2 kb AaIT gene represented small open box.

for 1 min. To confirm the fusion protein gene in the Cry⁻B transformant, 1Ac (*cry1Ac* specific forward, 5'-TCACTTCCCATCGACATCTACC-3') and 13' (*cry1* type-conserved region reverse, 5'-ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC-3') primers as well as the SF and SR primers were used.

Electroporation

Electroporation was performed according to the method of Lereclus *et al.* (1989), with a slight modification. *B. thuringiensis* cells were grown to an OD_{600} of 1 in 100 ml of a Brain Heart Infusion (BHI, Difco Co., USA) with shaking at 30°C. The cells were harvested and washed once in 10 ml of cold distilled water. The pellet was then resuspended in 4 ml of cold sterile polyethyleneglycol (PEG) 6000 (40%, w/v). Thereafter, cell aliquots of 0.4 ml were mixed with the plasmid DNA in 0.2 cm electroporation cuvettes (Bio-Rad Co., USA) at 4°C. The Bio-Rad Gene Pulser apparatus was set at 25 μF and 2.5 kV, and the pulse controller was set to 400 Ω. The cuvette was placed in the safety chamber and the pulse was applied once. Following electroporation, the cells were diluted in 2 ml of a pre-warmed BHI medium and incubated for 2 hrs at 30°C. After this expression period, the cells were then plated on a nutrient agar medium (Difco Co., USA) supplemented with erythromycin (25 g/ml) for growth and sporulation.

Microscopy

The development stage of *B. thuringiensis,* parasporal inclusions in sporulated cell and autolysis were monitored with a phase contrast microscope Nikon Type 104 (Nikon Co., Japan) using a × 100 oil-immersion objective. The fully-lysed *B. thuringiensis* cells were dried, coated with carbon, and stained with gold. The samples were observed by scanning electron microscope (JSM-5410LV, JEOL, USA).

SDS-PAGE

The *B. thuringiensis* Cry⁻B transformants strains were grown in GYS media, and then the spore-crystal mixtures were collected after 96 hrs after inoculation. The SDS-PAGE was performed on a 12% polyacrylamide separating gel with a 3% stacking gel.

Bioassays on *Plutella xylostella* larvae

Bioassays were performed on the third instar *P. xylostella* larvae with the sporulated cells at 36 hrs after inoculation. The treatment dosage was 1×10^7 CFU per cm^2 of Chinese cabbage leaf (2×2 cm^2). The tested larvae were reared at 25°C and 60% humidity, and the mortality was calculated by counting the dead larvae at 1×10^7 CFU/cm^2 along with time interval of 6 hrs.

Results and Discussion

The scorpion insect toxin, AaIT provides a unique tool for the study of insect neuronal excitability and chemical ecology and the design of new approaches to insect control (Zlotkin *et al.*, 2000). For agricultural approach of AaIT, the release of recombinant baculoviruses that have been genetically engineered to express the scorpion insect toxin, AaIT, has been successfully field-tested (Cory *et al.*, 1994). As an alternative strategy, in the current study, we tried to express a fusion protein with Cry1Ac and AaIT and verify additive toxic effects due to AaIT.

To construct a *B. thuringiensis* gene for the fusion protein, an AaIT gene was introduced into the *Xho*I site of the *cry1Ac* gene of pProAc (named as pProAc-ScoR). An outline describing the generation of the recombinant plasmid is shown in Fig. 1. The recombinant plasmid, pPro Ac-ScoR was electroporated into the Cry⁻B strain. The presence of a recombinant plasmid in the strain Cry⁻B after electroporation was confirmed by a PCR with 1Ac, 13, SF, and SR primers to detect the fusion of the *cry1Ac* and AaIT gene (Fig. 2). The expected PCR products were

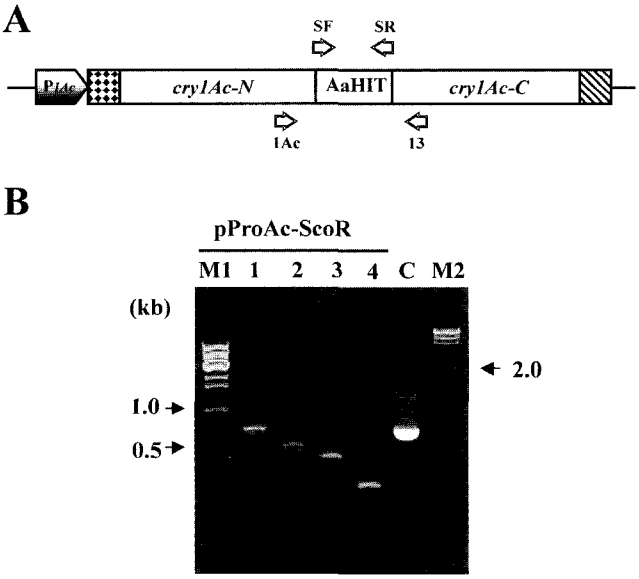

Fig. 2. Structure of a recombinant plasmid, pProAc-ScoR (A) and an electrophoresis pattern of PCR products (B) of *B. thuringiensis* Cry⁻B strain transformed with pProAc-ScoR. A: binding sites of primers shown. B: Lane 1, PCR products using 1Ac and 13 primers; lane 2, 1Ac and SR; lane 3, SF and 13; lane 4, SF and SR; lane C, PCR product from pProAc using 1Ac and 13. M1 and M2 indicate 1 kb DNA ladder and *Hin*dIII digested lambda DNA, respectively.

detected in the Cry⁻B transformants and the transformant was named ProAc-ScoR/CB.

ProAc-ScoR/CB produced irregular shaped parasporal inclusions in sporulated cells when observed by phase contrast microscopy during sporulation and by scanning electron microscopy after lysis, while ProAc/CB produced typical bipyramidal shaped inclusions (Fig. 3). The expression of the fusion protein gene in ProAc-ScoR/CB was analyzed by SDS-PAGE (Fig. 4). The protein of the ProAc-ScoR/CB exhibited three bands having a molecular mass of around 65 kDa and a main band was estimated approximately 65 kDa in size. On the other hand the ProAc/CB had 130 kDa bands as the previous report.

To compare the growth of the recombinants producing the fusion protein, each strain was cultured in the GYS medium for 24 hrs. The two transformants and Cry⁻B produced similar amounts of spores and there is no significant difference between them (Table 1). Insecticidal activities of the Cry⁻B transformants were evaluated against the third instar larvae of *P. xylostella* compared with those of the wild-type, Cry⁻B strain and ProAc/CB (Table 2). The median lethal time (LT_{50}) of ProAc-ScoR/CB (22.59 hrs) was lower than that of ProAc/CB (30.06 hrs) in the sporulated cells. However, in the view of LC_{50}, the values of two transformants were not significantly different (data not shown).

These results suggest that a fusion Cry protein may be successfully expressed in acrystalliferous Cry⁻B strain and AaIT might have the oral activity to *P. xylostella* larvae. Barton and Miller (1993) reported that transgenic plant expressing only AaIT had significant mortality relative to controls to both *Heliothis* and *Spodoptera* larvae. Fur-

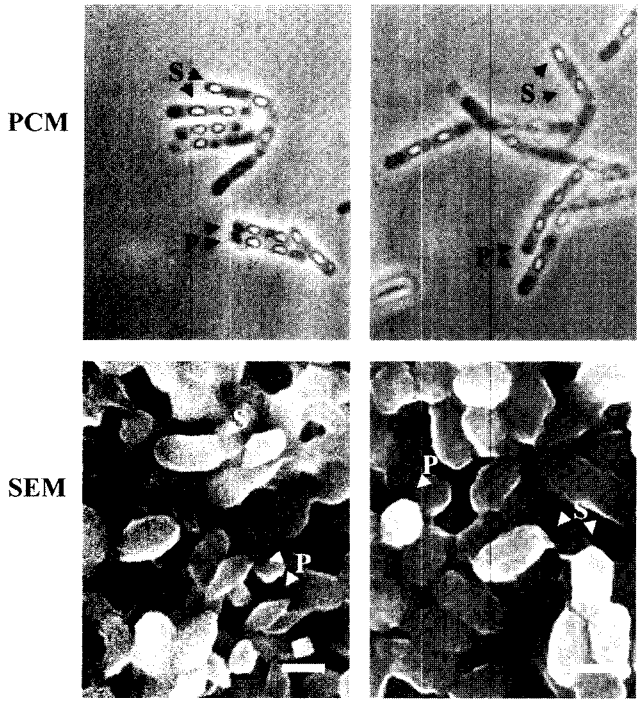

Fig. 3. Phase contrast micrographs (PCM) and scanning electron micrographs (SEM) of ProAc/CB and ProAc-ScoR/CB. S and P indicate spores and parasporal inclusions, respectively. White size bars indicate 1 μm in size.

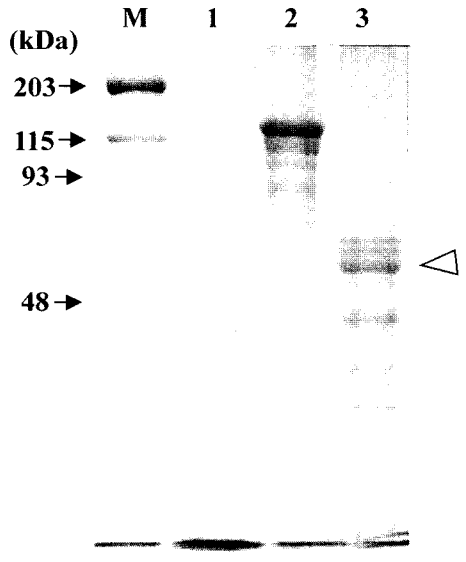

Fig. 4. SDS-PAGE of *B. thuringiensis* Cry⁻B transformants. Lane 1: Cry⁻B; 2: ProAc/CB; 3: ProAc-ScoR/CB, M: prestained molecular weight standard.

Table 1. Relative spore production of the transformed *Bacillus thuringiensis* strains in GYS

Strain	No. (of × 10^6 CFU/ml)	SD (±)
Cry⁻B	186.00a	19.16
ProAc/CB	197.33a	42.77
ProAc-ScoR/CB	131.83a	68.70

*The number with the same letter shows no significant difference by the Duncan's multiple range test ($\alpha = 0.05$)

Table 2. Insecticidal activity of the sporulated cell preparations of *B. thuringiensis* transformants against third instar larvae of *P. xylostella*

Strain	Sporulated cells	
	LT_{50}[a]	95% fiducial limits
Cry⁻B	ND[b]	
ProAc/CB	30.06	26.46-33.26
ProAc-ScoR/CB	22.59	15.46-26.89

[a]All median lethal times are presented as hours and mortalities were checked at 1×10^7 CFU/cm^2.
[b]Not determined.

thermore, the presence of both *B. thuringiensis* Cry toxin and AaIT in a plant provided at least as much toxicity to insects as either toxin individually, and a possible additive effect is indicated. Thereafter Yao *et al.* (1996) presented the transgenic tobacco expressing AaIT gene had notable insect resistant activity. Moreover, Zlotkin *et al.* (1992) reported on the oral effects of AaIT to flesh flies. According to their result, AaIT induced paralysis of flies within 1 ~ 2 hrs after oral administration, with a lethal dose (LD_{50}) of 10 μg/100 mg of body weight. In conclusion, we verified the possibility of functional fusion expression of a recombinant Cry toxin and additive toxic effects of AaIT. Finally, AaIT can be broadly used as a new approach in insect pest control. Further study is focusing on elucidation of the synergism of the two insecticidal proteins against insect larvae.

Acknowledgments

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