Int. J. Indust. Entomol. Vol. 14, No. 1, 2007, pp. 33~36

International Journal of *Industrial Entomology*

Expression and Synergistic Effect of *Bacillus thuringiensis* Cry1Ac in Lepidopteran Toxic Strain to *Plutella xylostella*

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(Received 30 December 2006; Accepted 5 February 2007)

To improve insecticidal activity of *B. thuringiensis* 2385-1 (Bt 2385-1), a recombinant plasmid, pHT1K-1Ac, was introduced into lepidopteran toxic Bt 2385-1 by electroporation. The presence of the recombinant plasmid in Bt 2385-1 after electroporation was confirmed by PCR. Bt 2385-1 transformant was named as Bt pHT1K-1Ac/2385-1 (1K-1Ac/2385-1). The 1K-1Ac/2385-1 transformant produced bipyramidal-shaped parasporal inclusion as like the wild-type strain, Bt 2385-1, and showed an 130 kDa band of Cry1Ac protein. The insecticidal activity of 1K-1Ac/2385-1 against *S. exigua* was similar to that of Bt 2385-1 but the LC_{50} value of transformant against *P. xylostella* was 1.8 times lower. Through these bioassay results, it was confirmed that toxicity of Bt 2385-1 transformant showed synergistic effect by introducing Cry1Ac. These results suggested that the multiple expressions of Cry proteins in a promising Bt strain may interact synergistically in insect midgut, resulting in increase of toxicity and expansion of host spectrum.

Key words: *Bacillus thuringiensis*, Bt 2385-1, *cry1Ac* gene, synergistic effect

Introduction

B. thuringiensis is a Gram-positive and spore-forming soil bacterium that produces more than one type of insecticidal crystal proteins (Cry proteins) during sporulation stage. The Cry proteins of 130 to 140 kDa are assembled as bipyramidal crystals and in lepidopteran larval midguts, and these protoxin molecules undergo specific proteolytic cleavage, yielding the toxin of 60 to 65 kDa (Aronson, 1993). It has been suggested that a mixture of different toxins could be more effective than a single toxin and might delay development of resistance (Gill *et al.*, 1992; Van Rie *et al.*, 1990). *B. thuringiensis* subsp. *kurstaki* HD-1 strain contains Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2B, showing different insecticidal specificities to several lepidopteran insects (Hofte and Whiteley, 1989) and the synergistic interaction of the HD-1 toxins were reported (van Frankenhuyzen *et al.*, 1991). *B. thuringiensis* subsp. *israelensis*, which is active against dipteran larvae, contains also a functionally diverse group of insecticidal proteins, including Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa and Cyt1Aa (Hofte and Whiteley, 1989). Wu and Chang reported that mixtures of the 27 and 65 kDa proteins were more toxic than expected on the basis of their individual toxicities (Wu and Chang, 1985).

The 2385-1 strain of *Bacillus thuringiensis* (Bt 2385-1) isolated from Korean soil sample and was identical to the H-serotype of subsp. *kenyae* (H4a4c), and its crystal was bipyramidal-shaped. However, Bt 2385-1 showed much higher toxicity against *Plutella xylostella* and *Spodoptera exigua* larvae than subsp. *kenyae* since it has more toxin genes than subsp. *kenyae*. In this study, we used pHT1K vector (Kang *et al.*, 2005) for introducing *cry1Ac* gene to confirm synergistic effect of Cry1Ac in lepidopteran *B. thuringiensis* strain.

Materials and Methods

Bacterial strains and culture

B. thuringiensis strains used in this study were HD-73, a

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wild-type isolate of *B. thuringiensis* subsp. *kurstaki* containing only the *cry1Ac* gene (Adang *et al.*, 1985); 2385-1, a wild-type isolate of *B. thuringiensis* subsp. *kenyae* showing high toxicity against *P. xylostella* and *S. exigua*. The constructed plasmids, pHT1K-1Ac (Kang *et al.*, 2005) were amplified in *E. coli* strain TOP10 (Invitrogen, USA). *B. thuringiensis* was grown at 28°C with vigorous shaking in SPY medium for plasmid preparation and GYS medium for expression of crystal proteins.

PCR

Plasmid DNA was extracted from *B. thuringiensis* strains using Qiagen® Plasmid Midi kit (Qiagen, Germany) adding lysozyme treatment step according to the instruction of manufacturer. *Taq* DNA polymerase in PreMix-Top (Bioneer, Korea) was used to confirm *cry* gene of the transformed *B. thuringiensis* strains, and PCR amplification was employed for 33 cycles as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. All PCR amplifications were performed using Mastercycler gradient (Eppendorf, Germany). The primers used to detect *cry* genes were cry1Ac, 5'-TCACTTCCCATCGACATCTACC-3'; cryl-3 (universal 3' primer of *cryl*-type gene), 5'-ATCACTGAGTCGCTTCGCATGTTTGACTTTCTC-3'.

Electroporation

Electroporation was performed according to the method of Lereclus *et al.* (Lereclus *et al.*, 1989). The Bio-Rad Gene Pulser apparatus (Bio-Rad, USA) was set at 25 μF and 2.5 kV, and the pulse controller was set to 400 Ω. The electroporated cells were plated on a nutrient agar medium (Difco, USA) supplemented with erythromycin (25 μg/ml) for growth and sporulation.

Microscopy and SDS-PAGE

The development stages of *B. thuringiensis* and parasporal inclusions during sporulation and autolysis were monitored with a phase contrast microscope, Nikon OPTIPHOT-2 (Nikon, Japan) using a×100 oil-immersion objective.

B. thuringiensis strains were grown in GYS medium containing 25 μg/ml erythromycin and harvested by centrifugation at the point up to 5 days. The samples were washed 3 times with a washing solution (0.5 M NaCl, 2 mM EDTA) and then applied to a 10% polyacrylamide separating gel with a 3% stacking gel, as described by Laemmli (Laemmli, 1970).

Bioassay

Bioassays were performed on the third instar *P. xylostella* larvae and second instar *S. exigua* larvae at 25°C in 60 to 70% humidity with a 16 h : 8 h L/D cycle. To determine the median lethal concentrations (LC_{50}) against the 50 of *S. exigua* and 50 of *P. xylostella*, samples were treated uniformly on a disc of Chinese cabbage leaf (2×2 cm^2) and air-dry. The larvae were exposed by feeding on each treated cabbage leave plugs, respectively. All tests were performed 3 times with spore-parasporal inclusion suspensions. The mortality for *P. xylostella* was checked every 12 h for 2 days, and for *S. exigua* every 24 h for 3 days. LC_{50} was calculated using a Probit analysis (Finney, 1971).

Results and Discussion

Expression of Cry1Ac in *B. thuringiensis* 2385-1

To improve insecticidal activity of *B. thuringiensis* 2385-1 (Bt 2385-1) by synergistic effect, a recombinant plasmid, pHT1K-1Ac, was introduced into lepidopteran toxic Bt 2385-1 by electroporation. The presence of the recombinant plasmid after electroporation was confirmed by PCR with a set of oligonucleotide primers specific to *cry1Ac* gene (Fig. 1). The expected PCR product for the *cry1Ac* gene was detected in a transformant. Bt 2385-1 transformant was named as Bt pHT1K-1Ac/2385-1 (1K-1Ac/2385-1). The expressed crystal proteins during sporulation period and lysed cells were observed by phase contrast microscopy (Fig. 2). The 1K-1Ac/2385-1 transformant produced bipyramidal-shaped parasporal inclusion as like the wild-type strain, Bt 2385-1. In 1K-1Ac/2385-1 cells, a distinct Cry1-type crystal was easily

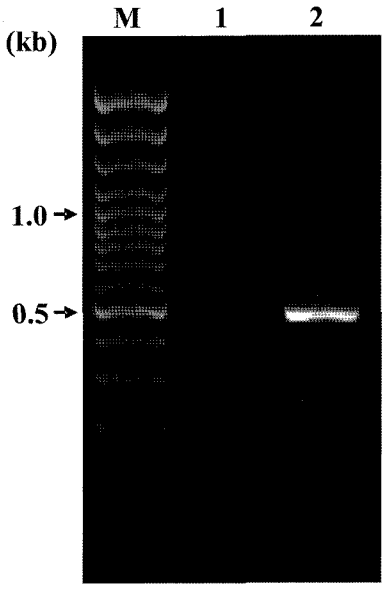

Fig. 1. PCR amplification of *cry1Ac* gene fragment from transformed *B. thuringiensis* 2385-1. The fragment was amplified by oligonucleotide primer set, cry1Ac/cryI3 and the predicted size of fragments is 550 bp. Lane M, 100 bp DNA Ladder; 1, wild-type Bt 2385-1 isolate; 2, 1K-1Ac/2385-1.

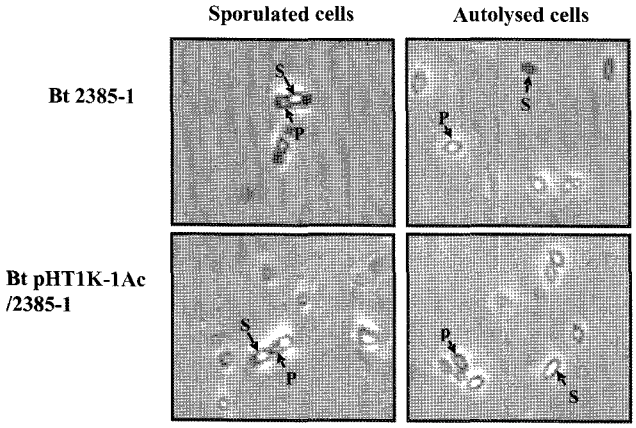

Fig. 2. Phase-contrast micrographs of wild-type and transformed *B. thuringiensis* strains during two different stages, sporulation (A) and autolysis (B). S and P indicate spore and parasporal inclusion, respectively.

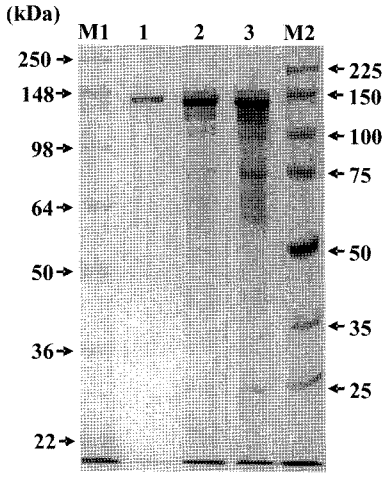

Fig. 3. SDS-PAGE of crystal proteins produced by *B. thuringiensis* 2385-1 transformed with the pHT1K-1Ac. Lane M1, protein molecular weight marker (Novagen); 1, Bt subsp. *kurstaki* HD-73; 2, wild-type 2385-1; 3, 1K-1Ac/2385-1; M2, Protein molecular weight marker (Invitrogen).

Table 1. Insecticidal activity of spore-crystal mixture of transformed *B. thuringiensis* 2385-1 strain against *P. xylostella* and *S. exigua*

Strain	*P. xylostella*[a]		*S. exigua*[b]	
	LC_{50}[c]	FL_{95}[d]	LC_{50}	FL_{95}
Bt 2385-1	5.53	(4.43-6.66)	4.40	(3.65-5.81)
1K-1Ac/2385-1	3.05	(2.21-4.00)	3.82	(3.14-4.49)

[a]The 3rd instar larvae were used.
[b]The 2nd instar larvae were used.
[c]All median lethal concentrations are presented as $\times 10^5$ CFU/cm^2.
[d]FL means 95% fiducial limits.

observed. Expression of the *cry1Ac* gene in Bt 2385-1 transformant was analyzed by SDS-PAGE (Fig. 3). The 1K-1Ac/2385-1 had an 130 kDa band of Cry1Ac protein.

Improvement of insecticidal activity of transformed *B. thuringiensis* 2385-1 by synergistic effect

To evaluate the insecticidal activity of the transformant, bioassay was performed against 3rd instar larvae of *P. xylostella* and 2nd instar larvae of *S. exigua* (Table 1). The insecticidal activity of 1K-1Ac/2385-1 against *S. exigua* was similar to that of Bt 2385-1 but the LC_{50} value of transformant against *P. xylostella* was 1.8 times lower than that of Bt 2385-1. Through these bioassay results, it was confirmed that toxicity of Bt 2385-1 transformant showed synergistic effect by introducing Cry1Ac.

Several workers have reported the possibility of synergism between individual toxins within a crystal inclusion (Chilcott and Ellar, 1988; Wu and Chang, 1985). Furthermore, synergism has been established between different toxin combinations against both *Culex pipiens* and *Anopheles stephensi* (Delecluse *et al.*, 1993; Poncet *et al.*, 1995). However, little is known about the mechanism of this synergistic interaction. The expression of novel crystal protein combinations within a strain might be an effective strategy for investigation of possible synergisms.

In this study, to investigate synergism, pHT1K-1Ac was introduced into Bt subsp. *kenyae* 2385-1. In newly constructed transformant, 1K-1Ac/2385-1, the host spectrum of the parent strain was maintained and furthermore, insecticidal activity against *P. xylostella* was improved. These results suggested that the multiple expressions of Cry proteins in a promising Bt strain may interact synergistically in insect midgut, resulting in increase of toxicity and expansion of host spectrum.

Acknowledgement

This work was supported by grants from Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea, and BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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