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Expression of the *cry1Ac1* Gene Under the Control of the Native or the α-Amylase Promoters in an Acrystalliferous *Bacillus thuringiensis* Strain

Jong Yul Roh, In Hee Lee, Jian Hong Li[1], Ming Shun Li, Ho San Kim, Yeon Ho Je and Kyung Saeng Boo*

Graduate School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea.
[1]Department of Plant Protection, Huazhong Agricultural University, Wuhan, Hubei, 430070, P. R. China.

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Expression of the *cry1Ac1* gene of an acrystalliferous *Bacillus thuringiensis* strain under the control of the native or α-amylase gene promoter was investigated. The *cry1Ac1* gene was cloned in a *B. thuringiensis* - *E. coli* shuttle vector, pHT3101, under the control of either the native promoter (pProAc) or the α-amylase promoter from *Bacillus subtilis* (pAmyAc). These two recombinant plasmids were successfully expressed in *B. thuringiensis* subsp. *kurstaki* Cry⁻B. The first transformant (ProAc/CB), harboring pProAc, expressed an about 130 kDa protein begining 24 hr after inoculation, just as in the case of the wild type of *B. thuringiensis* subsp. *kurstaki* HD-73. The second pAmyAc-transformant (AmyAc/CB) began to express the gene just 6 hr after inoculation, but Western analysis showed that the activity of the α-amylase promoter was relatively weaker than that of the native promoter. As expected, their toxicity against *Plutella xylostella* larvae was dependent on the amount of Cry1Ac1 protein expressed.

Key words : *Bacillus thuringiensis*, Crystal protein gene, α-Amylase promoter

Introduction

The gram-positive spore-forming *Bacillus thuringiensis* is well known for its ability to produce insecticidal crystalline inclusion bodies, δ-endotoxins, during sporulation. Most *B. thuringiensis* strains produce one or several different crystal inclusions, which contain one or more insecticidal proteins that are often toxic to a variety of insect species (Kronstad and Whiteley, 1986; Visser, 1989).

The production of δ-endotoxins is developmentally regulated with being mainly dependent on sporulation except for the production of colleopteran toxic crystal proteins, Cry3 types (Hofte and Whiteley, 1989). The regulation of δ-endotoxin synthesis may share common features with the regulation of sporulation genes in *Bacillus subtilis* (Adams *et al.*, 1991). The promoter of the gene encoding δ-endotoxin is recognized by two sigma factors, σ^{35} (Brown and Whiteley, 1988) and σ^{28} (Brown and Whiteley, 1990), which share homology with two sporulation-specific sigma factors, σ^{E} and σ^{K} in *B. subtilis*.

For the production of other useful δ-endotoxins in one host strain, several researchers reported the use of sporulation-independent promoters, such as the promoter of the tetracycline resistance gene in *B. thuringiensis* or *B. megaterium* (Mettus and Macaluso, 1990) and the promoter of the α-amylase gene in *B. subtilis* or *B. stearothermophilus* (Nakamura and Imanaka, 1989). However, the expression of the resident *cry* genes was interfered or the expression level using these promoters is low.

Although the α-amylase promoter activity is relatively low, Chak *et al.* (1994) had reported the positive results showing that the expression of newly introduced *cry* gene contributed to the increase of total protein amount and this did not interfere with the production of the native Cry1Ac protein. In this work we describe the possibility of using the promoter of the α-amylase gene to express additional *cry* genes for enhancement of toxicity and/or expansion of host spectrum.

Materials and Methods

Bacterial strains and culture media

E. coli XL-1 blue was used as the host for transformation

*To whom correspondence should be addressed.
Graduate School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea. Tel: 82-31-290-2461; Fax: 82-31-296-1650; E-mail: ksboo@plaza.snu.ac.kr

of recombinant plasmids. *B. thuringiensis* subsp. *kurstaki* Cry⁻B was used as hosts for expression of the *cry1Ac1* gene. *B. thuringiensis* cultures were grown at 30°C with vigorous shaking in SPY (Kronstad *et al.*, 1983) for plasmid preparation and GYS (Nickerson *et al.*, 1974) for expression of crystal protein.

Plasmids

The α-amylase promoter of *B. subtilis* was amplified by PCR using AF (amylase promoter forward, 5'-CGCC TTTGCGGTAGTGGTGC-3') and AR (amylase promoter reverse, 5'-ACCGATGTGAAGACTGGAC3-') primers and cloned in pGEM®-T Easy (Promega Co., USA). The *cry1Ac1* full gene containing the ribosomal binding site and the terminator was cloned in pBluescript® SK(+) and this construct was designated as the pBSAc. The 3.8 kb *Bam*HI-*Nde*I fragment from pBSAc was used as the coding region for the construction of recombinant plasmids. The *E. coli* - *B. thuringiensis* shuttle vector pHT 3101 (Lereclus *et al.*, 1989) was used to clone the amylase promoter or the native promoter - *cry1Ac1* gene fusion.

Electroporation

Electroporation was performed according to Lereclus *et al.* (1989) method which was partially modified. *B. thuringiensis* cells were grown in 100 ml of the Brain Heart Infusion (BHI, Difco Co., USA) with shaking at 30°C to an OD_{600} of 1. The cells were harvested and washed once in 10 ml cold distilled water. The pellet was resuspended in 4 ml of cold sterile PEG 6000 (40%, w/v). Cell aliquots of 0.4 ml were mixed with 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, 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 pre-warmed BHI medium and incubated for 2 hrs at 30°C. After this expression period, the cells were plated on the nutrient agar (Difco Co., USA) containing erythromycin (25 μg/ml).

PCR

PCR amplification was performed with *Taq* DNA polymerase in PreMix®-Top (Bioneer Co., Korea) and with DNA Thermal Cycler (Perkin Elmer Co., USA) by a 35-cycle program, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Follwing amplification, the PCR product was analyzed by agarose gel electrophoresis. To confirm existence of the recombinant plasmid in the transformed *B. thuringiensis* strain, RBS (ribosomal binding site, 5'-CTTAATAAAAGAGATGGAGG-3'), 1Ac (*cry1Ac* specific forward, 5TCACTTCCCATCGAC ATC TACC-3') and 13' (*cry1* type-conserved region reverse, 5'-ATCACTGAGTCGCTTCGCATGTTTGAC TTTCTC-3') primers as well as AF and AR primers were used for PCR.

SDS-PAGE and immunoblot analysis

B. thuringiensis strains were grown in GYS medium and collected with to time intervals. The sporulated cell, spores and crystal proteins were harvested and washed 3 times with the washing solution (0.5 M NaCl, 2 mM EDTA). And sporulated cells were sonicated three times (22,000 cycle/sec for 1 min) and mixed with the equal volume of SDS sample buffer (4% SDS, 4% mercaptoethanol, 100 mM Tris-HCl, pH 8.0). Each sample, which was boiled for 5 min, was loaded onto SDS-10% PAGE gel. 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 and 10 kDa protein ladder (Difco Co., USA) was used as the standard.

For immunoblot analysis, SDS-PAGE was performed at 100 V for 45 min and blotted onto PVDF (polyvinylidene difluoride) membranes (Perkin-Elmer Co., USA). After blocking with 10% BSA in PBST, 1:1,000 diluted Cry1Ac polyclonal antiserum was added to the membrane. The antibody was detected with a second antibody conjugated with alkaline phosphatase and a chromogenic reaction by adding 5-bromo-4-cholro-3-indolyl phosphate disodium salt/nitroblue tetrazolium (BCIP/NBT).

Bioassay on *Plutella xylostella* larvae

Bioassays were performed with the third instar diamondback moth, *P. xylostella*, larvae and samples collected at 12 hrs and 48 hrs after inoculation. Treatment dosages (1×10^7 CFU/cm^2) were applied uniformly to the surface of a 2×2 cm^2 section of Chinese cabbage leaf. Mortality was calculated by counting the dead larvae after 24 hrs.

Results

Cloning of the *cry1Ac1* gene under the α-amylase or the native promoter into pHT3101

An outline describing the preparation of the two recombinant plasmids is shown in Fig. 1. The *Bam*HI-*Nde*I (3.8 kb) fragment containing the *cry1Ac1* coding, ribosomal binding and terminator sequences was digested from pBSAc and ligated under the α-amylase promoter (named

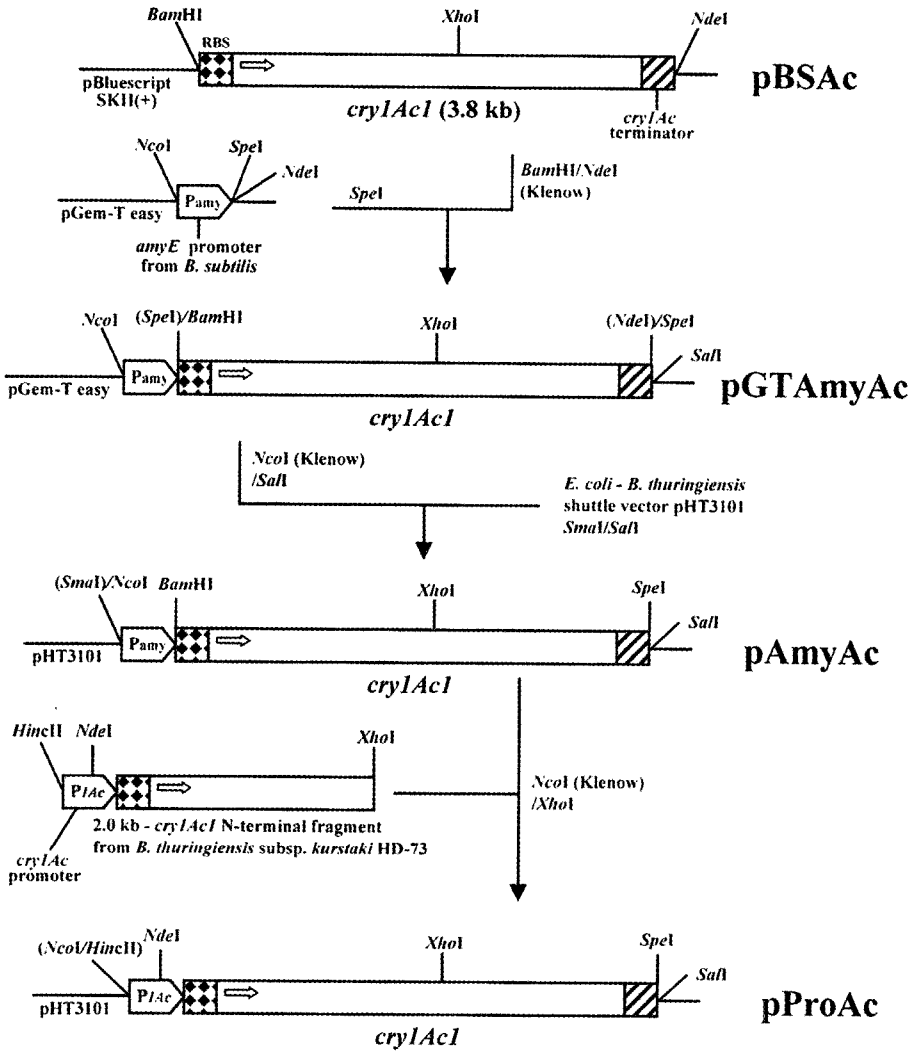

Fig. 1. Construction of the pAmyAc and pProAc vectors. A 3.8 kb *cry1Ac1* fragment contained the *cry1Ac1* coding region (open box), ribosomal binding sequence (RBS, dotted box), and the terminator (lined box). The open arrowhead in each plasmid drawing indicates the reading orientation of the *cry1Ac1*.

as pGTAmyAc). The *Nco*I (fill in with Klenow) - *Sal*I 4.1 kb of recombinant plasmid pGTAmyAc, containing the α-amylase promoter - *cry1Ac1* gene fusion, was cloned into the *Sma*I and *Sal*I sites of pHT3101 and this construct was designated as the pAmyAc.

For the replacement of the α-amylase promoter with the native *cry1Ac1* promoter, the N-terminal region (*Hinc*II-*Xho*I) of the *cry1Ac1* gene containing the two promoters (BtI and BtII) was electroeluted from total plasmid DNA of *B. thuringiensis* subsp. *kurstaki* HD-73. This N-terminal region was replaced by *Nco*I (fill in with Klenow) - *Xho*I 2.0 kb of pAmyAc to yield pProAc.

The sequences of the promoter regions in the two recombinant constructs are shown in Fig. 2. Two promoter regions had the same sequence from the ribosomal binding site to the terminator region (154 bp downstream of stop codon) of the *cry1Ac1* gene.

Electroporation of pAmyAc and pProAc into *B. thuringiensis* Cry⁻B

Recombinant plasmids, pAmyAc and pProAc, were extracted from *E. coli* XL1-Blue and electroporated into the plasmid-negative strain, *B. thuringiensis* subsp. *kurstaki* Cry⁻B. The presence of a recombinant plasmid in the strain Cry⁻B after electroporation was confirmed by PCR with a set of oligonucleotide primers to detect the presence of the promoter and the *cry1Ac1* gene (Fig. 3). The expected PCR products for the promoter and the *cry1Ac1* gene were detected in each transformant. Two *B. thur-*

A

Amy-For

GCCTTTGCGG TAGTGGTGCT TACGATGTAC GACAGGGGGA TTCCCCATAC
ATTCTTCGCT TGGCTGAAAA TGATTCTTCT TTTTATCGTC TGCGGCGGCG
TTCTGTTTCT GCTTCGGTAT GTGATTGTGA AGCTGGCTTA CAGAAGAGCG
GTAAAAGAAG AAATAAAAAA GAAATCATCT TTTCTGTTTG GAAAGCGAGG
GAAGCGTTCA CAGTTTCGGG CAGCTTTTTT TATAGGAACA TTGATTTGTA

-35 -10

TTCACTCTGC CAAGTTGTTT TGATAGAGTG ATTGTGATAA TTTTAAATGT

amyO Amy-Rev (*Spe*I)/*Bam*HI

AAGCGTTAAC AAAATTCTCC AGTCTTCACA TCGGTAATCA CTAGGATCCC

RBS Met

CCGGAGGTAA CTTATG. . . [*cry1Ac1* coding region]

B

(*Nco*I/*Hinc*II)

CCATGAACAC CCTGGGTCAA AAATTGATAT TTAGTAAAAT TAGTTGCACT

BtII Ec BtI

TTGTGCATTT TTTCATAAGA TGAGTCATAT GTTTTAAATT GTAGTAATGA

*Nde*I RBS

AAAACAGTAT TATATCATAA TGAATTGGTA TCTTAATAAA AGAGATGGAG

Met

GTAACTTATG. . . [*cry1Ac1* coding region]

Fig. 2. Nucleotide sequences of the promoter regions of the pAmyAc (A) and the pProAc (B) vectors. Amy-For and Amy-Rev indicate the position of forward and reverse PCR primers. The -10 and -35 regions and the operator (*amyO*) region of the α-amylase gene promoter are shown. BtI, BtII and Ec indicate two promoters (-10 regions) of the *cry1Ac1* and the promoter of *E. coli*, respectively. The initiation codon of the cry1Ac1 gene is also shown. The thick lines are restriction enzyme sites.

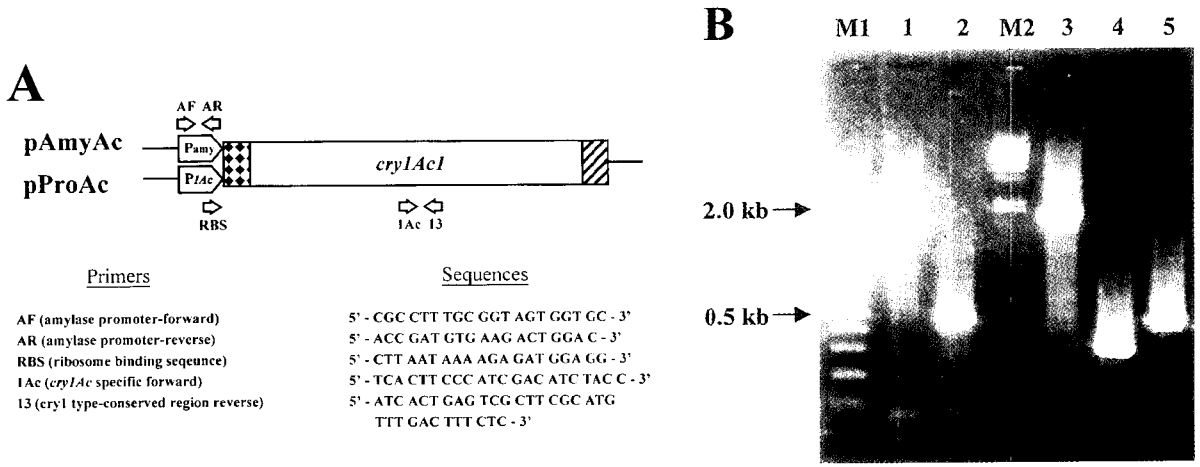

Fig. 3. Oligonucleotide sequences of primers (A) and PCR analysis of plasmid DNAs of *B. thuringiensis* Cry⁻B strains transformed with the pAmyAc and pProAc (B). Lanes 1 and 3, PCR product using primers RBS and 13; Lanes 2 and 5, primers 1Ac and 13; Lane 4, primers AF and AR. M1 and M2 indicate 100 bp DNA ladder and lambda DNA digested with *Hind*III, respectively.

ingiensis Cry⁻B transformants were named as AmyAc/CB and ProAc/CB, respectively.

Expressions in *B. thuringiensis* of the *cry1Ac1* gene

Expression of the *cry1Ac1* gene in *B. thuringiensis* Cry⁻B recombinant clones was analysed by SDS-PAGE and immunoblot analysis (Fig. 4). Antibody raised against the Cry1Ac1 crystals from *B. thuringiensis* subsp. *kurstaki* HD-73 strain was used.

Expression of the *cry1Ac1* gene under the control of the native promoter (ProAc/CB) began from 24 hrs after inoculation, just as in the case of the wild type of *B. thuringiensis* subsp. *kurstaki* HD-73. During sporulation period, the native *cry1Ac1* promoter was operated and the expressed proteins were accumulated in sporulated cells. On the other hand, the recombinant, AmyAc/CB produced a 135-kDa protein just 6 hrs after inoculation and its expression level increased until 36 hrs. However, after 36 hrs, expressed proteins were gradually degraded. The ProAc/CB produced Cry1Ac1 over the 10-fold more than by the AmyAc/CB.

Insecticidal activity of recombinant *B. thuringiensis* strains

Insecticidal activities of the two recombinants were evaluated against second or third instar larvae of *P. xylostella* and compared with these of *B. thuringiensis* subsp. *kurstaki* HD-73 and Cry⁻B strains (Fig. 5). Spore and crystal protien mixtures were collected at 12 hrs (exponential phase) and 48 hrs (mid-sporulation phase) and bioassay was performed at 1×10^7 CFU/cm^2 concentration. With samples collected at 12 hrs, AmyAc/CB gave about 57% mortality while ProAc/CB and HD-73 had lower than that of AmyAc-CB. However, the toxicity of ProAc/CB and HD-73 was remarkably enhanced with samples collected at 48 h but that of AmyAc/CB was slightly decreased.

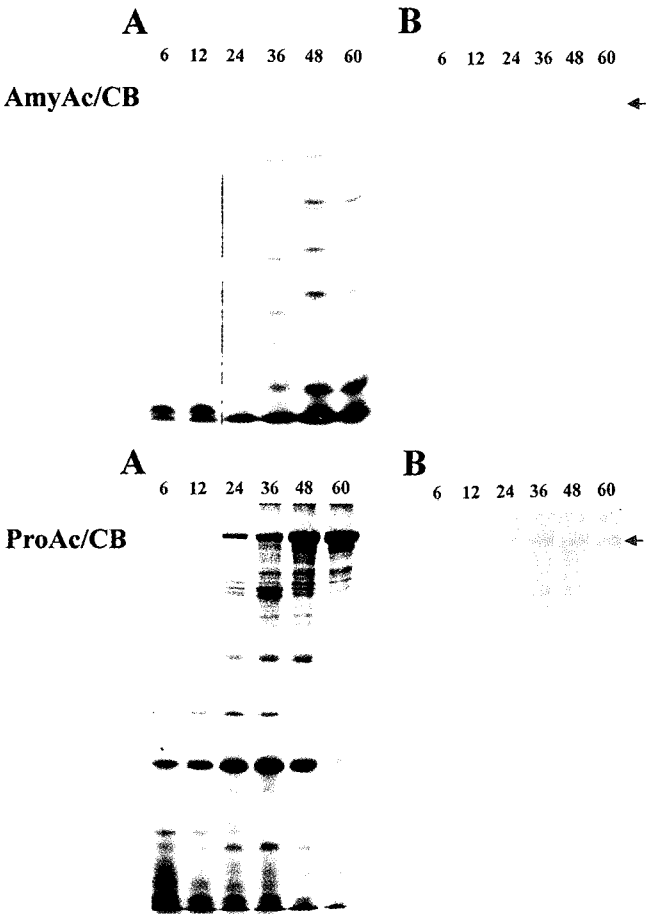

Fig. 4. SDS-PAGE (A) and immunoblot (B) analysis of two *B. thuringiensis* Cry⁻B transformants (AmyAc/CB and ProAc/CB) at times sampled. Arrow indicates the Cry1Ac1 protein. Antiserum was also raised against Cry1Ac protein from *B. thuringiensis* subsp. *kurstaki* HD-73.

Discussion

Alternative host organisms for expression of *B. thuringiensis* δ-endotoxins, to study the organization and expression regulation of individual *cry* gene and to identify gene products, were searched. Although related microorganisms, such as *B. subtilis*, *B. cereus*, and *B. megaterim* have been explored, the *cry* genes were poorly expressed (Calogero *et al.*, 1989; Gonzalez *et al.*, 1982; Sakanyan *et*

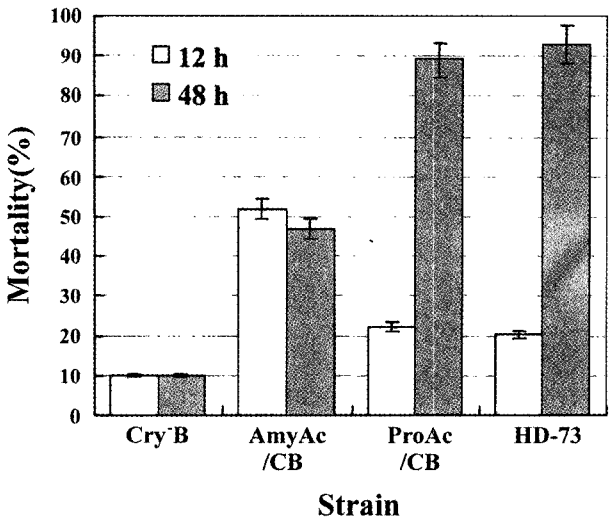

Fig. 5. Mortality of *Plutella xylostella* due to two *B. thuringiensis* Cry⁻B transformants (AmyAc/CB and ProAc/CB) at two harvest times. Collection times are 12 hrs (before sporulation) and 48 hrs (after sporulation). *B. thuringiensis* Cry⁻B and HD-73 strains were used as the controls.

al., 1982). In recent years, acrystalliferous mutant *B. thuringiensis* strains and the natural strains containing relatively simple *cry* gene composition were used as a recipient (Bone and Ellar, 1989; Lereclus *et al.*, 1989; Mahilon *et al.*, 1989; Chak *et al.*, 1994). In this study, we tried to express *cry* gene in acrystalliferous *B. thuringiensis* strain and compared their expression under the sporulation-independent and -dependent promoters. Re- cently, sporulation-independent promoters, such as the promoter of the tetracycline resistance gene (Macaluso and Mettus, 1990) or the α-amylase promoter (Chak *et al.*, 1994), were used for vegetative expression. The *tet* promoter system for expression of *cry1Ac* and *cry2A* in *B. thuringiensis* HD73-26 strain which is a crystal-negative derivative, had a low expression level, because the early expression disturbs vegetative growth, as well as the developmental processes. However, the α-amylase promoter for expression of *cry1C* in *B. thuringiensis* HD-73 had relatively high (about 40% of that for the native resident crystal protein, Cry1Ac). Also, our results showed that the α-amylase promoter system and the native promoter system were successfully expressed. However, the expression level of the former was significantly higher than that of the latter (about more than 10-fold). Nevertheless, the α-amylase promoter system had several advantages to express the sporulation-dependent *cry* gene in *B. thuringiensis* strains. The α-amylase promoter required a different sigma factor (σ^{43}) with the native promoter (σ^{28} and σ^{35}) and because the activity of this promoter was very weak, cell growth was not affected by high-level expression of the newly introduced *cry* gene. Furthermore, the difference in timing for expression of the *cry* genes minimized the interference in gene expression between the resident and introduced *cry* genes. In conclusion, the two recombinant plasmids constructed in this study can be successfully introduced into *B. thuringiensis* strains lacking the *cry1Ac* gene, resulting in enhancement of their toxicity and/or in expansion of their host spectrum.

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