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RESEARCH ARTICLE

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Construction of an engineering strain expressing *cry7Ab7* gene cloned from *Bacillus thuringiensis*

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Abstract The genotype of *Bacillus thuringiensis* (*Bt*) strain GW6 isolated in China was identified, and a novel *cry7Ab* gene was found based on the results of PCRs using 40 pairs of *cry* universal primers. The *cry7Ab* gene was cloned by PCR and named as *cry7Ab7* by the *Bt* Delta Endotoxin Nomenclature Committee (GenBank Accession No. FJ940776). The construction of a novel three-dimensional structure of Cry7Ab7 protein via homology modeling methods indicated that the Cry7Ab7 protein was different from other Cry7Ab proteins within the domain structures. The *cry7Ab7* gene was inserted into the *Bam*HI/*Sal*I sites of *E. coli* expression vector pET21b and the *Bt*-*E. coli* shuttle vector pSXY422b to construct the recombinant plasmids pET21b-7Ab7 and pSXY422b-7Ab7, which were then transformed into *E. coli* BL21 and *Bt* HD73cry in order to obtain the *E. coli* transformant EC7Ab7 and the engineering strain *Bt* HD7AB, respectively. The bioassay results showed that Cry7Ab7 protein inclusion bodies in EC7Ab7 and the crystal proteins in HD7AB were highly toxic to the second-instar larvae of *Henosepilachna vigintioctomaculata* with the LC_{50} values of 1.167 $\mu g \cdot \mu L^{-1}$ and 0.779 $\mu g \cdot \mu L^{-1}$, respectively. Our results clearly indicated that *cry7Ab7* gene can offer important benefits to research on *Coccinellidae* insect pest control.

Keywords *Bacillus thuringiensis*, *cry7Ab7* gene, engineering strain HD7AB, *Henosepilachna vigintioctomaculata*

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1 Introduction

The potato ladybug (*Epilachna vigintioctomaculata* Motsch. (also *Henosepilachna vigintioctomaculata* or 28-spotted potato ladybird)) belongs to the Coccinellidae family of beetles. It is a voracious and destructive insect pest of food crop plants, especially those belonging to the nightshade family (Solanaceae), which includes potatoes, tomatoes, peppers, eggplants, tobacco, and others (Chinese Academy of Medical Sciences Institute of Medicinal Plant Resources Development, 1991). In northern China and nearby regions, two or sometimes three generations of potato ladybug may hatch in a single year. Both larvae and adult beetles usually graze the leaf mesophyll tissue, leaving behind the epidermis or, in severe cases, only the veins. Often, the total tuber loss caused by potato ladybug outbreak may be 15%–20% or up to 70% in severe cases. Currently, prevention and control of this pest must rely on chemical pesticides, which results in pesticide residues in the environment and also creates difficulties in achieving long-term prevention and control effects.

Bacillus thuringiensis (*Bt*) is a very widespread, soil-dwelling, and gram-positive bacterium. Under some conditions, *Bt* can produce 27 kDa–140 kDa parasporal crystal proteins (Crickmore et al., 1998) encoded by *cry* genes or *cyt* genes (Schnepf et al., 1998) during the sporulation phase (Gill et al., 1992). *Bt* strains show specific toxicity against a variety of pests and are considered fundamentally safe (Pardo-López et al., 2009) for the environment including animals and humans. These features make them widely studied and commercially applied for the prevention and control of insect pests. However, the current application of the *Bt* insecticidal agent, toxic protein crystals, is mainly used for prevention and control of Lepidoptera pests but less for Coccinellidae pests. Therefore, it is of important practical significance to isolate and clone new *cry* genes encoding specific proteins known to be toxic to *Coccinellidae* pests. In the current study, the whole-length *cry7Ab7* gene was isolated and cloned from *Bt* strain GW6. The expression vector

containing *cry7Ab7* gene was also constructed and transformed into *E.coli* or an acrytalliferous mutant strain of *Bt* in order to construct an engineering *Bt* strain. The proteins they produced were shown to be highly toxic to the second-instar larvae of potato ladybug.

2 Materials and methods

2.1 Materials

2.1.1 Strains and plasmids

Bacillus thuringiensis GW6 and plasmid pET21b came from our laboratory. The plasmid pSXY422b (*Bt-E.coli* Shuttle vector), *Escherichia coli* SCS110, and acrystalliferous *Bt* mutant HD73cry were generously donated by the Chinese Academy of Agricultural Plant Protection.

2.1.2 Reagents

Luria-Bertani (LB) medium (Sambrook et al., 1992), DNA marker, high molecular weight standard proteins, UNIQ-10 spin column DNA gel extraction kits, IPTG, X-gal, ampicillin, and Taq Plus polymerase were from Shanghai Sangon Biologic Engineering Technology & Services Co., Ltd. Restriction enzymes and T4 DNA ligase were purchased from TaKaRa Biotechnology (Dalian, China). DNA extraction kit was purchased from TIANGEN Biotech Co., LTD (Beijing, China). Protein Quantitation kit was purchased from Bioteke Corporation (Beijing, China). Other reagents were of analytical grade.

2.1.3 Insects for bioassays

Potato ladybugs (*Henosepilachna vigintioctomaculata*) were collected from the Park of Agricultural University of Hebei, Baoding, Hebei, China.

2.2 Methods

2.2.1 DNA extraction

Bt plasmid was extracted by alkaline lysis method (Narva et al., 1991). Extraction of *E. coli* plasmids, DNA fragment recovery, and other DNA manipulations were carried out according to the methods of Sambrook et al. (1992).

2.2.2 Cloning and sequencing of *cry7* gene

Using DNA templates from *Bt* strain GW6, PCR amplifications with 40 pairs universal primers of *cry* gene (Song, 2001) were performed. The full open reading frame of the *cry7Ab* gene was amplified with a pair of PCR primers (cry7 Forward-CGGGATCCGATGAATTTAAATAATTTAGGTGGATATG; cry7 Reverse-GGGTCGACGGATTGTATGCATTTTATTTTATTTTG) and cloned using pGEM-T Easy vector following the methods of Sambrook et al. (1992).

2.2.3 Expression of *cry7Ab* gene in *E. coli* and SDS-PAGE analysis

Plasmid pGEM-7Ab7 was digested with *Bam*HI and *Sal*I. The resultant 3.4-kb fragment containing the GW6 *cry7Ab7* gene was cloned into the same sites in the pET-21b vector to generate recombinant plasmid pET21b-7Ab7. Then, pET21b-7Ab7 was transformed into *E. coli* BL21 (DE3), and the resultant transformant EC7AB7 was grown in liquid LB medium supplemented with $100\ \mu g \cdot mL^{-1}$ of ampicillin at 37°C with shaking. The cell suspension was then centrifuged to collect the cells. The bacteria were resuspended by adding $10\ mmol \cdot L^{-1}$ of Tris-HCl (pH 8.0). The cells were broken up by sonicating for 15 min, and the precipitates were collected by centrifuging and, finally, dissolved with 1/10 volume of Na_2CO_3 ($50\ mmol \cdot L^{-1}$, pH 9.5). Finally, the expressed products were separated and identified with routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1992).

2.2.4 Transformation of *B. thuringiensis* by electroporation

Plasmid pET21b-7Ab7 was digested with *Bam*HI and *Sal*I, and the resultant 3.4 kb fragment containing the *cry7Ab7* gene was cloned into the same sites in the *Bt*-*E. coli* shuttle vector pSXY422b (Song, 2001) to generate plasmid pSXY422b-7Ab7. Next, plasmid pSXY422b-7Ab7 was transformed into *E. coli* SCS110 to demethytated. Finally, pSXY422b-7Ab7 purified from *E. coli SCS110* was transformed to *Bt* acrystalliferous mutant HD73cry^{-} by electroporation (Macaluso and Mettus, 1991).

2.2.5 Morphology observation of GW6 and HD7AB strains

Bt cultures were grown on a 1/2-LB medium (tryptone 0.5%, yeast extract 0.25%, sodium chloride 0.5%, pH 7.0) at 30°C for 54 h with agitation until 70%–90% crystal off. The morphology of *Bt* strain GW6, engineering strain HD7AB, as well as their insecticidal crystals stained with saffron were observed under microscopy (Dong and Cai, 2001).

2.2.6 Growing of the three strains of *Bt*

The wild-type strain GW6, the engineering strain HD7AB, and the standard strain HD-73 were cultured in LB medium at 30°C with shaking at $220\ r \cdot min^{-1}$, and samples were collected every two hours. To examine the growth phase, the optical density at 600 nm (OD_{600}) of appropriately diluted culture was measured using a spectrophotometer.

2.2.7 Expression of *cry7Ab7* gene in HD73cry$^-$ and SDS-PAGE analyzed

The strain HD7AB was cultured in 1/2 LB medium at 30°C with agitation (Liu et al., 2009a) for 18 h, and then, the cells were collected every two hours. Cell extracts were prepared by resuspending cell pellets in 40 μL of sterilized water and 40 μL of 2 × SDS sample buffer. Samples were loaded on a denaturing gradient (10% to 5%) acrylamide gel, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1992).

2.2.8 Insect bioassays

Protein Cry7Ab7 for bioassays was expressed as inclusion bodies in *E. coli* and crystal proteins in *B. thuringiensis*. All bioassays were conducted with second-instar larvae of potato ladybug on leaf disks, as described by Song et al. (2008). Each leaf disk was immersed in a test solution for 10 s and allowed to dry at ambient temperature for 1 to 1.5 h. Control leaf disks were immersed in distilled water with Triton X-100. The leaf disks were placed in individual petri dishes (5 cm diameter) containing moistened filter paper. Ten larvae were placed in each dish, and each treatment was repeated three times. Mortality was recorded after 3 d of treatment, and LC_{50} was calculated.

3 Results and analysis

3.1 Identification and cloning of a *cry7* gene from *Bt* strain GW6

Using the genome DNA of *Bt* strain GW6 as template and only had *cry7* gene identified by PCR technique, a 1.366-kb *cry7* amplicon was cloned into pGM-T vector to construct pGM-cry7 recombinant plasmid, which was sequenced by Shanghai Sangon Biologic Engineering Technology & Services Co., Ltd. Comparing the resultant sequence of *cry7* amplicon with the sequence of other *cry* genes in GenBank database using BLAST revealed that *cry7* amplicon may be one of the *cry7Ab* genes. To isolate this gene, PCR was performed using a pair of primers designed according to the sequence of c*ry7Ab* gene. The resultant PCR products were recovered and then ligated to pGEM-T vector and transferred to *E. coli* TG1 competent cells. Positive clones were screened by alpha- complementation, and an expect 3776 base pairs DNA fragment was cloned and sequenced. The sequence results indicated that the fragment contained an open reading frame of 3414 base pairs that encoded a crystal protein of 1138 amino acids residues. The expected molecular weight was 129.64 kDa with an isoelectric point 4.97, which implied that it would be an acidic protein. The protein was named '*Bt* insecticidal crystal protein Cry7Ab7' by the Nomenclature Committee (GenBank accession number: FJ940776).

3.2 Protein sequence analysis

Cry7Ab7 protein was analyzed using the NCBI Conserved Domain Database, and the results revealed that the protein had three conserved domains. Domain I, which consists of 223 amino acid residues, starts at residue 59 and ends at residue 281 from the N-terminal. Domain II and Domain III consisting of 202 and 141 amino acid residues, respectively, are located on the region of residues 286–487 and residues 497–637 of Cry7Ab7. It is presumed that the Cry7Ab7 active domain is located on the upstream of N-terminal amino acid residues, which is No. 59–637 region. Secondary structure of Cry7Ab7 protein was analyzed based on prediction services provided by DNA-man and AnthePról software (Table 1), and its tertiary structure was constructed via homology modeling methods (Fig. 1). Compared with other Cry7 proteins, the results showed that it was nearly identical to Cry7Aa1 (M64478), Cry7Ab1 (U04367), and Cry7Ab2 (U04368), with the sequence similarity between them 94.64%, 97.10%, and 99.65%, respectively, and the number of different amino acids residues among them was 60, 32, and 4, respectively. It also shared a stunning 100% homology with Cry7Ab3 (GenBank: BI1015188) protein, suggesting that they had the closest relationship. However, the amino acids sequence similarity between Cry7Ab7 and Cry7Ba1 (ABB70817) was relatively lower, only 59.11%.

Table 1 The secondary structure of Cry7Ab7 protein

toxin	α-helix	extended strand	β-turn	random coil
Cry7Ab7	30.58%	20.21%	0	49.21%

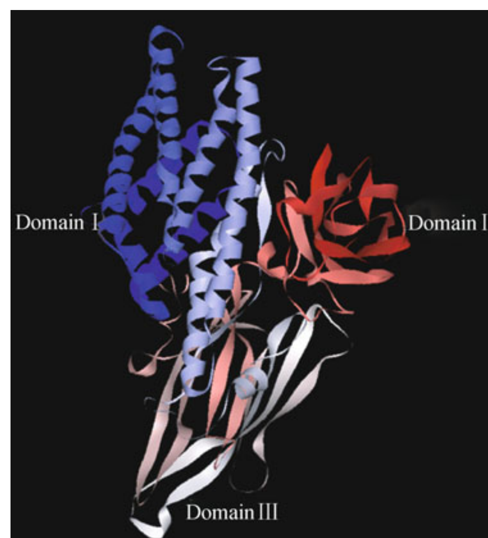

Fig. 1 The tertiary structure prediction of Cry7Ab7 protein

3.3 Expression of *cry7Ab7* gene in *E. coli*

The *cry7Ab7* gene was cloned into *E. coli* BL21 (DE3) and expressed when induced by IPTG. SDS-PAGE analysis (Fig. 2) showed that the product was in the precipitation after the ultrasonic broken cells were centrifuged. The molecular weight of expressed Cry7Ab7 was about 130 kDa, which was in agreement with the result expected. Since the expression product was not detected in the supernatant, only insoluble inclusion bodies were found.

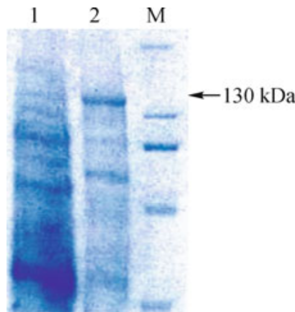

Fig. 2 Determination of the solubility of Cry7Ab7 expressed in *E. coli* EC7AB7 by SDS-PAGE analysis
Note: M represents Protein Marker (212, 116, 97, 66, 44 kDa); 1 and 2 are the soluble fraction of *E. coli* EC7AB7 and the pellets fraction of *E. coli* EC7AB7, respectively.

3.4 Inspection of the morphology of spores and crystals of Bt strain GW6 and engineering strain HD7AB

An inspection of the morphology of spores and crystals of Bt strain GW6 and engineering strain HD7AB under the light microscopy (1000×) revealed that the spores of both strains appeared as oval. Moreover, the parasporal inclusions isolated from the GW6 strain and engineering strain HD7AB each had a large number of small diamond shaped crystals, suggesting that the form of the insecticidal crystal proteins had not changed between the two strains, HD7AB and *Bt* strain GW6 (Fig. 3).

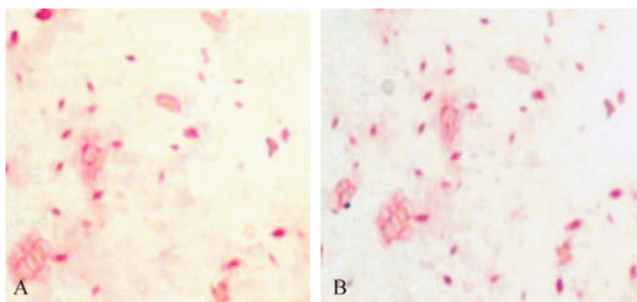

Fig. 3 The morphology of spore and insecticidal crystals of *Bt* strain GW6 and engineering strain HD7AB
Note: (A) and (B) represent GW6 B and HD7AB, respectively.

3.5 Growth characteristics of *Bt* strain GW6, engineering strain HD7AB, and standard strain HD-73

The growth curves of *Bt* strain GW6, engineering strain HD7AB and standard strain HD-73 (Fig. 4) were compared. The growth characteristics of the wild-type strain GW6 and engineering strain HD7AB showed no significant differences compared with the standard strain, HD-73. All of them could be observed in three distinct phases: lag phase (0–2 h), logarithmic phase (2–14 h), and stationary phase (after 16 h). The results showed that electroporation with pSXY422b-7Ab7 had no significant impact on growth rate of engineering strain HD7AB as compared to that of HD73.

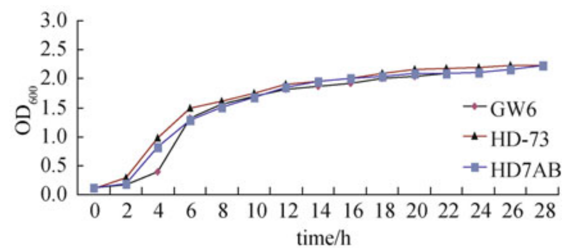

Fig. 4 The growth curve of *Bt* strain GW6, engineering strain HD7AB, and standard strain HD-73

3.6 Expression of *cry7Ab7* gene in HD73cry$^-$ and SDS-PAGE analysis

SDS-PAGE analysis results showed that the engineering strain HD7AB genetically began expression after culture for 26 h, yielding the 130 kDa protein. Expression production was gradually increasing with time going by and the target band even could be observed after 54 h in culture (Fig. 5), suggesting that *cry7Ab7* gene could be expressed in stability in engineering strain HD7AB.

3.7 Insecticidal activity

The bioassay results indicated that Cry7Ab7 protein of inclusion bodies in *E. coli* EC7AB7 and crystal protein in *B. thuringiensis* engineering strain HD7AB was highly toxic against the second-instar larvae of *H. vigintiocto-maculata* (Table 2). The mortality of *H. vigintioctoma-culata* was evaluated as LC_{50} at the concentration of 1.167 $\mu g \cdot \mu L^{-1}$ and 0.779 $\mu g \cdot \mu L^{-1}$.

Table 2 Bioassay of expression products from *cry7Ab7* gene

strains	LD-p of the regression line	LC_{50} /($\mu g \cdot \mu L^{-1}$)	95% confidence
EC7AB7	$y = 3.2119 + 1.4888x$	1.167	0.658489–2.069008
HD7AB	$y = 3.3239 + 1.8805x$	0.779	0.587222–1.032422

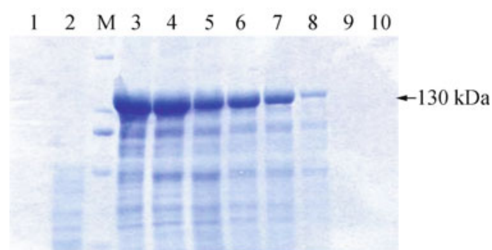

Fig. 5 Analysis of insecticidal crystal protein from HD7AB by SDS-PAGE
Note: M represents protein standard marker (212, 116, 97, 66, and 40 kDa); Lanes 1–2: HD73(cry) 22h, 54h; Lanes 3–10: HD7AB: 54h, 40h, 36h, 30h, 28h, 26h, 24h, 22h.

4 Discussion

We found that the *Bt* strain GW6, which was isolated from Baoding, China, had only the *cry7* insecticidal gene as identified by PCR technique. The *cry7* genes are not known to be effective against the members of the Coccinellidae family of insect pests. After cloning the full-length gene of *cry7Ab7* and causing its expression in *E. coli* and the engineering strain HD7AB, we conducted bioassays that showed that the Cry7Ab7 protein was toxic to the second-instar larvae of *H. vigintioctopunctata* but had no insecticidal activity against the first-instar larvae of *H. vigintioctopunctata*, *Pyrrhalta aenescens*, or *Anomala corpulenta* Motsch.

Common *Bt* Cry toxins are also toxic to potato beetle, beetle larvae, and western corn rootworm larvae. Furthermore, Cry7A toxins have a higher insecticidal activity against potato beetle (Lambert et al., 1992). However, much less is known about the effects of *Bt* toxin Cry7Ab to potato ladybug. Because the *Bt* strain GW6 only has a single virulent gene, it is of great value when used to investigate the pest resistance mechanism. The strain can also be used to screen Cry protein combinations of high virulence, and it can also be used as a naturally ideal receptor when used in constructing highly efficient engineering strains.

Cry7Ab7 toxic protein is composed of 56.67% hydrophobic amino acids, 39.45% hydrophilic amino acids, 12.74% basic amino acids, and 11.51% acidic amino acids. The proportion of leucine, serine, asparagus amide, and glutamic acid is higher than others, accounting for 8.87%, 7.46%, 7.11%, and 6.85% of the total number of amino acids, respectively, while the level of cysteine is the lowest, only 8, accounting for 0.7% of total residues. The analysis of the amino acids sequences revealed that Cry7Ab7 protein has three conserved domains. It is thought to be responsible for inserting into insect cell membrane and to be involved in pore formation, as well as to be involved in receptor binding and specificity determination (Liu et al., 2009b). Protein secondary structure analysis revealed that three conserved domains of amino acids are mainly in two forms, helix and random coil, which is a specific functional domain of toxicity (Choma et al., 1990). Tertiary structure of Cry7Ab7 was constructed via homology modeling approach, which is the basis for further research on the mechanism interaction between proteins.

Generally, other workers have found that Cry3A is expressed about 30 h after culture (Herve and Dider, 1995). In this study, *E. coli*-*Bt* promoter used for pSXY422b had a *cry3A* promoter, and the expression was around 24 h after culture, and we suggest that *cry7Ab7* gene activated the promoter in advance, which led to our results. The molecular size of Cry7Ab7 expressed in GW6 was 130 kDa, which is the same as Cry7Ab1 (Genbank: Q45708) expressed in *Bt* Dakota HD511 strain. The expression product of the *E. coli* and engineering strain was used to conduct a bioassay test based on second-larvae potato ladybug and the results showed a high insecticidal activity. However, proteins expressed in *E. coli* in fusion protein had lower insecticidal activity than *Bt* insecticidal crystal proteins, and it was mainly because proteins expressed in *E. coli* were insoluble. So, inclusion proteins cannot fold correctly, which impacts the vitality of the protein (Orsini et al., 1978). In recent years, many *cry* genes are used broadly for the construction of engineering strains and insecticidal transgenic plants, so as to increase insecticidal activity or delay insect resistance development (Huang and Lin, 2001). Furthermore, many such transgenic plants have been commercialized and have brought significant economic benefits (Zhang et al., 2002). Because *Bt* genes have profound potential effects on the construction of genetically engineering strains and transgenic plants (Tan et al., 2008), much research based on cloning and expression of *Bt* genes and construction of engineering strains has been recently published, and the topic remains a science and commercial ‘hotspot’.

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