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1 **Characterization of a novel insecticidal protein Cry9Cb1 from *Bacillus thuringiensis***

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ABSTRACT

In the recent decades, there have been increasing reports of insect resistance in *Bacillus thuringiensis* (Bt) crops. Alternative use of Cry toxins, with high insecticidal activity and different mechanisms of action, may be an important strategy to manage this resistance. Cry9 protein, with high toxicity to the lepidopteran pests and no cross-resistance with commercial Cry1 proteins, is a valuable relevant resource. A novel insecticidal protein MP1489, subsequently named as Cry9Cb1, with 88% amino acid sequence identity with Cry9Ca1, was identified from Bt strain SP663; it exhibited high insecticidal activity against *Plutella xylostella*, *Ostrinia furnacalis*, and *Chilo suppressalis*, and no cross-resistance with Cry1Fa in *Ostrinia furnacalis*. Its minimal active fragments against *Plutella xylostella* and *Ostrinia furnacalis* were identified to be 72^T–657^V and 68^D–655^A, respectively; food safety assessment showed no sequence homology with any known allergen, and rapid degradation and inactivation by both heat and gastrointestinal environment. Therefore, Cry9Cb1 is proposed to have brilliant prospect as an insecticidal protein in agriculture.

KEYWORDS: *Bacillus thuringiensis*, Cry9Cb1, insecticidal protein, lepidopteran pests, food safety assessment

INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive bacterium that produces insecticidal crystal proteins (ICPs) during sporulation¹. The ICPs are mostly Cry proteins, an important component of Bt biopesticides and vital tools for insect control in transgenic crops^{2, 3}. Till date, the identified Cry proteins have been classified into Cry1–Cry78 based on amino acid sequence identity (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Among these, Cry1, Cry2, and Cry9 proteins exhibit strong insecticidal activity against lepidopteran pests⁴⁻⁶. Cry1 proteins have been widely applied in transgenic cotton, corn, and soybean to control the lepidopteran pests over the last 22 years^{7, 8}. However, there has been an increasingly rapid evolution of resistance due to years of successive planting⁸, and the same toxin being expressed by Bt crops, hence creating high selection pressure on insect population. Alternatively using Cry toxins, with high insecticidal activity against major pests and involving different mechanisms of action, might help in delaying the development of insect resistance⁹. Therefore, researchers have focused on identifying Cry proteins with high toxicity and no cross-resistance with the commercial Cry proteins^{10, 11}.

In total, 37 Cry9 proteins have been classified into Cry9A–Cry9G, of which Cry9A–Cry9E are mostly characterized by high activity against lepidopteran pests. Cry9Aa has toxicity towards *Galleria mellonella*, *Thaumetopoea pityocampa*¹², and *Spodoptera exigua*¹³. Cry9Bb has insecticidal activity against *Manduca sexta*, *Velvetbean caterpillar* and *Anticarsia gemmatilis*¹⁴. Cry9Ca has a unique insecticidal spectrum that its toxicity to the members of Noctuidae family of the order Lepidoptera, such as *Agrotis segetum*¹⁵. Cry9Eb and Cry9Ee have high toxicity toward

Plutella xylostella and *Ostrinia furnacalis*⁵. Cry9 domains II and III are relatively distant from Cry1 proteins in the phylogenetic tree¹⁶, and some reports have shown Cry9A, Cry9C, and Cry9E to have no cross-resistance with the Cry1 proteins^{5, 17, 18, 19}. Taken together, Cry9 proteins with high insecticidal activity and no cross-resistance characteristics represent an important tool to manage insect resistance.

Food safety assessment of transgenic crops containing novel proteins should be addressed in two steps: (1) characterization and food safety assessment of the novel protein and (2) that of the transgenic crops²⁰. Some researchers have already worked on the food safety assessment of commercialized Cry proteins such as Cry1Ab/Ac²¹, Cry1C and Cry2A²², and their results declared all these proteins to be safe for use in food. In the current study, we focused on the first step mentioned above for food safety assessment.

We report the complete characterization of a novel insecticidal crystal protein Cry9Cb1 obtained from Bt strain SP663 by genome sequencing techniques, including protein expression, insecticidal activity, cross-resistance, minimal active fragment, and food safety assessment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The Bt strain SP663 was isolated from a soil sample collected from the Baiwang Mountain Forest Park in Beijing and preserved in the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPP-CAAS), Beijing. While *E. coli* DH5 α was used for the common transformations, *E. coli* SCS110 was used to produce the non-methylated recombinant plasmids that were subsequently used for Bt transformation. *E. coli* BL21(DE3) and acrystalliferous mutant Bt strain HD73⁻ were used as the host strains for protein expression. The vector pET-21b(+) (Novagen, Germany) was used as an expression vector in *E. coli* BL21(DE3). The vector pSTK, carrying kanamycin resistance gene and the pHT315 sequence, was used as the expression vector in HD73⁻ strain, linked with the promoter of *cry3Aa*^{23, 24}.

All *E. coli* strains were cultivated at 37 °C in Luria-Bertani (LB) medium. Bt strains were cultivated at 30 °C in either LB or 1/2 LB medium (0.5% NaCl, 0.5% tryptone, and 0.25% yeast extract). Kanamycin and ampicillin were used for bacterial strain selection at the concentration of 50 μ g/mL and 100 μ g/mL, respectively.

Q-Exactive Mass Spectrometry analysis. Bt spores and crystal mixtures dissolved in water were treated with the final concentration of 0.1 mol/L NaOH for 5 min. After that, they were analyzed by SDS-PAGE. Then bands were excised, combined and subjected to in-gel digestion with trypsin. The digested fragments were analyzed on a Q-ExactiveTM Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher), and the data retrieval using MASCOT.

Genomic DNA preparation, sequencing, and computational analysis.

Genomic DNA was extracted from Bt strain SP663 following the method described by Song *et al*²⁵. Genome sequencing was performed with PacBio RS II (Pacific Biosciences, Menlo Park, CA) sequencing platform. De-novo assembly of PacBio reads was performed by HGAP²⁶. The coding sequences (CDS) were predicted with Prodigal²⁷. A local Bt toxin database was established using the amino acid sequences in the list of delta-endotoxins on the Bt toxin nomenclature website, and used for Bt strain SP663 genome toxin gene annotation by the BLASTX program with a cutoff E-value of 10^{-5} .

Cloning MP1489 gene from Bt strain SP663.

A pair of specific primers MP1489_F and MP1489_R (Table 1) used for the amplification of MP1489 gene, was designed on the basis of SP663 genome sequence. PCR was carried out in a 50- μ L volume, containing 25 μ L PrimeSTARTM HS Premix (TaKaRa, Dalian, China), 1 μ L template DNA, 1 μ L 20 μ mol/L of each primer, and 22 μ L ultrapure water. PCR reactions were run in a PTC-100 Peltier Thermal Cycler (MJ Research) as per the following program: an initial denaturation for 5 min at 94 °C, 30 cycles each of denaturation for 1 min at 94 °C, annealing for 1 min at 54 °C, and primer extension for 3 min 30 s at 72 °C, followed by a final extension for 10 min at 72 °C. The purified PCR products were cloned into the vector pSTK between *Bam*HI and *Xho*I (TaKaRa, Dalian, China) sites and then transformed into *E. coli* DH5 α ²³. A pair of universal primers designed from pSTK vector, pSTK_F and pSTK_R (Table 1), was used for PCR to screen the positive clones, followed by restriction enzyme analysis, and finally sequencing of the positive plasmids.

Expression of the MP1489 gene in the acrySTALLiferous mutant Bt strain HD73⁻ and trypsinization. In order to demethylate plasmid DNA, the recombinant plasmid pSTK-MP1489 was transformed into *E. coli* SCS110, and subsequently extracted therefrom, to be electroporated into acrySTALLiferous mutant Bt strain HD73⁻ for protein expression. Positive clones were selected by PCR. The method for protein extraction from recombinant strains was as described by Zhou *et al*²⁸. Proteins were analyzed by SDS-PAGE and the concentrations of protein were determined by ImageJ (National Institutes of Health) using BSA as a standard. Trypsinization of the MP1489 protoxin was performed at different trypsin:protoxin ratios (1:1000, 1:500, 1:250, 1:100, 1:50, 1:25, 1:10, 1:5 (w/w)) by incubating the components at 37 °C for 2 h. After incubation, the digested proteins were analyzed by SDS-PAGE.

Toxicity bioassays. The insecticidal activity of Cry9Cb1 was evaluated against eight lepidopteran pests including *Plutella xylostella*, *Ostrinia furnacalis* (susceptible as well as resistant larvae), *Chilo suppressalis*, *Agrotis ipsilon*, *Mythimna separata*, *Helicoverpa armigera*, *Spodoptera exigua*, *Spodoptera litura*, and a coleopteran insect *Colaphellus bowringi*. Second-instar *Plutella xylostella*²⁹ larvae and *Colaphellus bowringi*³⁰ neonate larvae were examined using a leaf-dip bioassay, in which 30 insects were individually placed on each leaf cut off a 6-cm disk. Mortality was recorded after 2 days at 25 °C with a photoperiod of 16:8 h light:dark and 50% relative humidity. *Ostrinia furnacalis*, *Chilo suppressalis*, *Agrotis ipsilon*, *Mythimna separata*, *Helicoverpa armigera*, *Spodoptera exigua*, and *Spodoptera litura* were assayed by a modified method³¹ of exposing neonate larvae to the general diet of Lepidoptera (144 g/L Dry mix, 19 g/L

Agar; Bio-Serv) mixed with different concentrations of the toxin. Bioassays were performed in 24-well trays, each well containing approximately 200 mg diet. Concentrations were tested with 72 neonate larvae individually placed on each well. *Ostrinia furnacalis*, *Chilo suppressalis*, *Agrotis ipsilon*, and *Mythimna separata* were maintained at 25 °C with a photoperiod of 16:8 h light:dark and 60 to 80% relative humidity. *Helicoverpa armigera*, *Spodoptera exigua*, and *Spodoptera litura* were maintained at 25 °C with a photoperiod of 12:12 h light:dark and 50% relative humidity. Mortality was recorded after 7 days. All bioassays were repeated at least thrice. The 50% lethal concentration (LC₅₀) and 95% confidence limits were calculated using PoloPlus1.0 with Probit analysis.

Protein sequence analysis and identification of the minimal active fragment. The simulated spatial three-dimensional (3D) structure, based on MP1489 amino acid sequence, was predicted using the SWISS-MODEL workspace. The trypsin cleavage site was deduced based on the location of arginine residues. Several pairs of primers were designed to amplify the different truncated fragments of the MP1489 gene (Table 2). Meanwhile, a pair of primers to amplify the full-length MP1489 gene was also designed. The truncated fragments were subsequently cloned into the vector pET-21b(+) using the Seamless Cloning Kit (Seamless Assembly Cloning kit; Clone Smarter). A 10-μL reaction mix, containing 10 ng pET-21b(+) plasmid (linearized with *Bam*HI), 40 ng each of the PCR products, and 5 μL 2×assembly master mix, was incubated at 50 °C for 15 min. The recombinant plasmids were confirmed by PCR and sequencing. To express the full-length and truncated MP1489 proteins, the recombinant plasmids were transformed into

E. coli BL21(DE3). The method for protein extraction from recombinant strains was as described by Zhou *et al*³²; proteins were subsequently analyzed by SDS-PAGE. The insecticidal activity of full-length and truncated MP1489 proteins (50 µg/mL) was evaluated against *Plutella xylostella* and *Ostrinia furnacalis*, respectively.

Amino acid sequence homology with allergenic proteins. The MP1489 amino acid sequence homology searches were conducted in the AllergenOnline Database (<http://www.allergenonline.org/databasefasta.shtml>) and Structural Database of Allergenic Proteins (http://fermi.utmb.edu/SDAP/sdap_who.html). One search was performed to identify any known allergenic protein presenting 35% amino-acid identity with the novel protein on a window of 80 amino acids. The other search was outperformed by comparing the novel protein, subdivided into 8 amino acid blocks, with epitopes of all probable allergens.

Heat stability evaluation. The MP1489 protein was dissolved in 50 mmol/L Na₂CO₃ (pH10.0) (Sigma) buffer at a concentration of 100 µg/mL in 1.5-mL micro-centrifuge tubes. Heat stability was evaluated as described by Xu *et al*²¹. Insecticidal activity of the heat-treated proteins (20 µg/mL) was evaluated against *Plutella xylostella* and *Ostrinia furnacalis*, and mortality was calculated by one-way ANOVA with LSD test.

In-vitro digestive stability evaluation. A modified method^{21, 33} was used to evaluate the digestive stability. Briefly, 2 µL of protein (2 mg/mL), added to either 18 µL of simulated gastric fluid (SGF: 0.2 g NaCl and 0.088 g pepsin (Solarbio, Beijing, China) dissolved in 70 mL sterile water, pH adjusted to 1.2 with HCl, and sterile water added to make 100 mL) or 18 µL of simulated

167 intestinal fluid (SIF: 0.7 g KH_2PO_4 and 1 g trypsin (Solarbio, Beijing, China) dissolved in 65 mL
168 sterile water, 0.2 mol/L NaOH added to adjust to pH 7.5, and sterile water added to make 100 mL)
169 in 1.5-mL microcentrifuge tubes, were incubated at 37 °C in a water bath for 15 s, 1 min, 2 min, 5
170 min, 10 min, 30 min, and 60 min. Control sample of a 0-min digestion of the protein was also
171 prepared. SGF assay was terminated by adding 10 μL of 1.5 mol/L Na_2CO_3 , putting the sample
172 tubes on ice, and adding 5 $\times$ loading buffer. SIF assay was terminated by putting the sample tubes
173 on ice, and adding 5 $\times$ loading buffer. The resulting proteins were analyzed by SDS–PAGE.
174 Insecticidal activity of the digested protein was evaluated against those of *Plutella xylostella* and
175 *Ostrinia furnacalis* respectively, and mortality was calculated by one-way ANOVA with LSD test.

RESULTS

Initial characterization of the Bt strain SP663. Scanning electron microscopy revealed spherical crystals produced by Bt strain SP663 (Figure 1A). Total amount of sporulated protein was analyzed by SDS-PAGE (Figure 1B) and was found to contain Cry9C, Cry1I, and Cry2A, as per Q-Exactive Mass Spectrometry analysis (Table S1 of the Supporting Information).

Complete genome sequence and toxin gene annotation of Bt strain SP663. The complete genome sequence of Bt strain SP663 consists of three contigs with a mean GC content of 36.25% and a total length of 5,966,971 nucleotide base pairs. A total of 6,186 CDS, 108 tRNA, and 42 rRNAs were predicted. Toxin gene annotation of Bt strain SP663 revealed six full-length toxin genes, having high similarity with known insecticidal genes, including three *cry2A* genes, one *cryII* gene, one *cry9C* gene, and one *vip3A* gene.

Cloning and sequencing of MP1489 gene. The full-length 3,477-bp MP1489 gene encodes a polypeptide of 1,158 amino acids with a deduced molecular mass of 129.9 kDa; BLASTP search showed the highest identity of 88% with Cry9Ca1, across the NCBI protein database. The MP1489 amino acid sequence was aligned with other known Cry9 homologous proteins by Clustal W method. MP1489 was the closest to Cry9Ca1 in the phylogenetic tree (Figure S1 of the Supporting Information). This gene sequence was submitted to the NCBI GenBank (Accession No. MK005301) and named as Cry9Cb1 by the *Bacillus thuringiensis* delta-endotoxin nomenclature committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

Gene expression and trypsinization. SDS-PAGE analysis of crude spore/crystal protein of the

recombinant bacteria indicated the expressed Cry9Cb1 protein to have a molecular mass of approximately 130 kDa (Figure 2A). The Q-TOF Mass Spectrometry analysis indicated its highest score matching with that of full-length Cry9Ca1. Therefore, the *cry9Cb1* gene was successfully expressed in the acrySTALLIFEROUS mutant Bt strain HD73⁻. Scanning electron microscopy and atomic force microscopy revealed spherical crystals produced by recombinant bacteria (Figure 2B and 2C). Trypsinization of the Cry9Cb1 protoxin resulted in its digestion into two fragments, each of 70 kDa and 55 kDa (Figure 2D).

Toxicity evaluation. We chose eight agricultural lepidopteran pests and a coleopteran pest to evaluate the insecticidal activity of Cry9Cb1. Results showed high insecticidal activity of Cry9Cb1 against some lepidopteran pests including *Plutella xylostella* with LC₅₀ of 0.38 µg/mL, *Ostrinia furnacalis* with LC₅₀ of 1.28 µg/mL, and *Chilo suppressalis* with LC₅₀ of 0.50 µg/mL. In addition, Cry9Cb1 also exhibited medium activity against *Agrotis ipsilon* and *Mythimna separata* larvae with LC₅₀ of 130.22 and 244.77 µg/mL, respectively (Table 3). However, Cry9Cb1 showed no lethal effect or weight inhibition against *Helicoverpa armigera*, *Spodoptera exigua*, *Spodoptera litura*, and the coleopteran insect *Colaphellus bowringi* larvae. Furthermore, bioassay results showed Cry9Cb1 to have high toxicity, with LC₅₀ of 1.21 µg/mL against Cry1Fa 1000-fold resistant *Ostrinia furnacalis*, which is equivalent to susceptible strain (Table 3), which indicated Cry9Cb1 to have no cross-resistance with Cry1Fa in *Ostrinia furnacalis*.

Identification of the minimal active fragment of Cry9Cb1. For the future application in transgenic crops, confirming the minimal active fragment of Cry9Cb1 was considered necessary.

217 A simulated spatial 3D structure of Cry9Cb1 was generated by homology modeling, according to
218 the Cry8Ea1 3D structure (PDB code 3EB7) (Figure S2 of the Supporting Information). The
219 simulated 3D structure of Cry9Cb1 exhibited three conserved domains that might locate between
220 66^G and 658^N. In addition, the trypsin cleavage sites were deduced based on the location of arginine
221 residues, which were probably at 67^R and 661^R, when compared to Cry9Ca1¹⁵. The full-length and
222 several truncated Cry9Cb1 were expressed in *E. coli* BL21(DE3) and analyzed by SDS-PAGE
223 (Figure S3 of the Supporting Information). Insecticidal activities of the full-length and truncated
224 Cry9Cb1 against *Plutella xylostella* and *Ostrinia furnacalis* were evaluated. Information for each
225 of the truncated Cry9Cb1 was listed in Table 4. Results revealed that the minimal active fragment
226 of Cry9Cb1 against *Plutella xylostella* was located between amino acid residues 72^T and 657^V
227 while that against *Ostrinia furnacalis* lied between amino acid residues 68^D and 655^A.

228 **No sequence homology with any known allergenic proteins.** One of the key endpoints in the
229 evaluation of food safety of a novel protein is its amino acid sequence similarity with known
230 allergenic proteins. The negative search results showed Cry9Cb1 to have less than 35% identity
231 with known allergens across a length of 80 amino acids, and also possessed no 8 consecutive-
232 amino acid identity with known allergenic proteins. Therefore, theoretically, Cry9Cb1 may not be
233 a potential allergen.

234 **Heat stability.** The Cry9Cb1 protein was clearly visible at about 130 kDa at time zero on SDS-
235 PAGE. When heated in boiling-water bath for 5 min, Cry9Cb1 was partially degraded; when
236 heated for 10 min, it has completely degraded (Figure 3A), and lost its insecticidal activity against

Plutella xylostella and *Ostrinia furnacalis* (Table 5). This test suggested that Cry9Cb1 was very unstable to heat treatment.

In-vitro digestive stability. Most allergenic proteins have high stability to digestive enzymes, owing to which digestive stability can be used as an indicator of the allergenic potential of a protein³⁴. According to SDS-PAGE analysis, Cry9Cb1 protein was unstable in SGF, since the 130-kDa protein was completely digested within 15 s (Figure 3B). In SIF, it was digested into two core fragments (70-kDa and 55-kDa) and small fragments (less than 30-kDa) within 15 s; the 70-kDa fragment was further degraded to 55-kDa within another 30 min (Figure 3C). Insecticidal activity assay results showed that the Cry9Cb1 protein, digested in SGF in 15 s or in SIF in 30 min, had completely lost its insecticidal activity (Table 5). All these results indicated the instability of Cry9Cb1 in gastrointestinal fluid.

DISCUSSION

Transgenic crops is an eco-friendly alternative for insect control, but its effectiveness has been reduced by the revolution of insect resistance. It was reported that the cumulative cases of resistance to Bt crops increased from 3 in 2005 to 16 in 2016. The 16 cases represented the resistance of seven major pests in five countries to each of the nine Cry proteins produced by Bt crops: Cry1Ab, Cry1Ac, Cry1A.105, Cry1Fa, Cry2Ab, Cry3Bb, mCry3A, eCry3.1Ab, and Cry34/35Ab⁸. Therefore, insect resistance has become an urgent problem in agricultural pest control in recent years. In the present study, a novel insecticidal protein Cry9Cb1 was identified from native Bt strain SP663 which exhibited high toxicity to *Plutella xylostella*, *Ostrinia furnacalis* and *Chilo suppressalis*, especially no cross-resistance with Cry1Fa in *Ostrinia furnacalis*. This novel insecticidal protein is of great significance for the future application in transgenic crops to manage the insect resistance.

Since Cry9Cb1 shared 88% amino acid sequence identity with Cry9Ca1, we speculated Cry9Cb1 to have a similar insecticidal spectrum as Cry9Ca1. Actually, comparisons of their insecticidal spectrum indicated that both of them exhibited high activity against *Plutella xylostella*, and no activity against *Helicoverpa armigera*. As Cry9Ca1 was toxic to members of Noctuidae family, we found Cry9Cb1 also had activity against some Noctuidae pests, such as *Agrotis ipsilon* and *Mythimna separate*. However, their difference in toxicity was shown to *Spodoptera exigua* (Table S2 of the Supporting Information)¹⁵. 3D structures of Cry toxins have been found to determine the insect specificity and toxicity^{35, 36}. Since the crystal structures of Cry9 proteins have

not been determined, we analyzed the structures of Cry9Cb1 and Cry9Ca1 based on the simulated 3D structures (Figure S4-S5 of the Supporting Information)³⁷. Their highly similar structures may lead to the similar activity against some species of insects. However, we found some structural differences in the exposed loops of domain II (Figure S6 of the Supporting Information), such as Cry9Cb1-loop 2 (⁴¹⁹TALAG⁴²³) and Cry9Ca1-loop 2 (⁴¹⁹SALIG⁴²³), Cry9Cb1-loop α -8 (³¹³FNPAANVGLCRRWGNNPYNT³³²) and Cry9Ca1-loop α -8 (³¹³FNPPANVGLCRRWGTPYNT³³²). Exposed loops in domain II of Cry toxins have been reported to be responsible for receptor binding³⁵. Their structural differences in the exposed loops of domain II may result in different binding abilities to *Spodoptera exigua* midgut receptors, and further more experiments are needed to verify them.

We found that excessive trypsinization of the Cry9Cb1 protoxin could produce a 55-kDa fragment, which was the same as Cry9Ca1. Bart Lambert found the 55-kDa fragment to be non-toxic and generated by the trypsin cleavage site (Arg at position 164) in Cry9Ca1 amino acid sequence. They speculated that excessive digestion of the protoxin may affect its insecticidal activity against insects. When the trypsin cleavage site at position 164 was substituted by alanine, it could produce only one toxic fragment by trypsinization; however, it did not result in increased toxicity against *Ostrinia nubilalis*. Nevertheless, the Ala mutant of Cry9Ca1 is very valuable for the production of a stable toxic fragment by trypsinization¹⁵. Our subsequent research may focus on the trypsin cleavage-site mutant of Cry9Cb1 protoxin to evaluate the insecticidal activity against different species of insects.

Some earlier studies had suggested short Cry toxin fragments to be more stable and highly expressed in the plant³⁸. Therefore, identification of the minimal active fragments of novel Cry protoxins was considered to be particularly important prior to their application. Many studies have reported the minimal active fragment against only one or two species of insects, such as 36^L–605^L being the active fragment of Cry1Ai against *Plutella xylostella*³² and 45^L–654^P being the active fragment of Cry9Aa against *Plutella xylostella* and *Ostrinia furnacalis*³⁹. However, our result suggested Cry9Cb1 to have different minimal active fragments against different species of insects that were located between amino acid residues 72^T and 657^V against *Plutella xylostella*, while between amino acid residues 68^D and 655^A against *Ostrinia furnacalis*. We speculated that certain amino acid residues may affect the ability to bind to specific insect receptors; however, the underlying mechanism remains to be verified.

Although issues on the allergenicity of Cry9Ca in the transgenic event CBH351 for StarLink corn has faded away, the Cry9C protein can still arouse more public attention. Taylor and Hefle had earlier concluded that Cry9Ca protein in GM corn was non-allergenic, since no sequence homologous to known food or environmental allergens was available, and insufficient quantities were being used to elicit allergic sensitization⁴⁰. Some researchers worked on the food safety of Cry9Ca in animals, and their results suggested no immunotoxic activity in the CBH351-fed rats, mice, and cows^{41, 42}. There was insufficient evidence for Cry9Ca being an allergen, owing to the lack of enough experimental data, such as controlled ingestion trials in human beings. Fortunately, our result revealed that Cry9Cb1 had no sequence homology with any known allergen and was

very unstable to heat treatment and gastrointestinal fluid environment. Generally, Cry9Cb1 had a low risk of allergenic potential as per an initial food safety assessment, but further trials still need to be conducted.

This paper focused on the characterization and food safety assessment of the novel Cry9Cb1 protein; our results indicated Cry9Cb1 to have good application prospect in the management of insect resistance.

SUPPORTING INFORMATION

Supporting Information Available:

Table S1. Checked insecticidal proteins from Bt_SP663 that identified by Q-Exactive Mass Spectrometry.

Table S2. Insecticidal spectrum of Cry9Ca1 and Cry9Cb1 proteins.

Figure S1. A phylogenetic tree showing the relationships among Cry9 proteins based on their degree of amino acid identity.

Figure S2. The simulated spatial 3D structure of Cry9Cb1 protein is shown.

Figure S3. SDS-PAGE analysis of the Cry9Cb1 polypeptides expressed in *E. coli* BL21 (DE3).

Figure S4. The simulated spatial 3D structures of Cry9Ca1 and Cry9Cb1 are shown.

Figure S5. Multiple sequence alignment of Cry toxins.

Figure S6. Structural differences in the exposed loop regions of Cry9Cb1 and Cry9Ca1.

ABBREVIATIONS USED

Bt, *Bacillus thuringiensis*; ICPs, insecticidal crystal proteins; *E. coli*, *Escherichia coli*; LB, Luria-Bertani; CDS, coding sequences; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC₅₀, 50% lethal concentration; 3D, three-dimensional; Q-TOF, Quadrupole-Time of Flight; NCBI, National Center of Biotechnology Information; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SEM, scanning electron micrograph; AFM, atomic force micrograph; GMQE, global model quality estimation; MW, molecular weight.

337

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341

342 **NOTES**

343 The authors declare that there is no conflict of interest.

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Figure Captions

Figure 1. (A) Scanning electron micrograph (SEM) (Hitachi S-4800, Japan) analysis of spores and crystals and (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total sporulated protein from Bt strain SP663 are shown. M, protein marker (PageRuler Unprestained Protein Ladder, Thermo).

Figure 2. (A) SDS-PAGE analysis of the *cry9Cb1* gene expressed in the acrySTALLiferous mutant Bt strain HD73⁻. M, protein marker (PageRuler Prestained Protein Ladder, Thermo); Lane 1, HD73⁻ strain carrying the pSTK-MP1489 plasmid (target band is marked with an arrow); Lane 2, Negative control (HD73⁻ strain carrying the pSTK plasmid). (B) Scanning electron micrograph (SEM) analysis of spores and crystals from the recombinant bacterium. (C) Atomic force micrograph (AFM) (Bruker Multimode 8) analysis of Cry9Cb1 crystal protein. (D) SDS-PAGE analysis of trypsinized Cry9Cb1 protoxin. M, protein marker; Lane 1, Cry9Cb1 protoxin; Lanes 2–9: digested at different trypsin:protoxin ratios (1:1000, 1:500, 1:250, 1:100, 1:50, 1:25, 1:10, 1:5 (w/w)). The digested proteins are marked with triangles.

Figure 3. (A) SDS-PAGE analysis of heat stability. M, protein marker; Lane 1, the Cry9Cb1 protein without heat treatment; Lanes 2–8, the Cry9Cb1 protein heated in the boiling-water bath for 15 s, 1 min, 2 min, 5 min, 10 min, 30 min, and 60 min. (B) SDS-PAGE analysis of SGF-digestion. M, protein marker; Lane 1, the Cry9Cb1 protein without digestion in SGF; Lanes 2–8,

485 the Cry9Cb1 protein digested in SGF for 15 s, 1 min, 2 min, 5 min, 10 min, 30 min, and 60 min.

486 (C) SDS-PAGE analysis of SIF-digestion. M, protein marker; Lane 1, the Cry9Cb1 protein without

487 digestion in SIF; Lanes 2–8, the Cry9Cb1 protein digested in SIF for 15 s, 1 min, 2 min, 5 min, 10

488 min, 30 min, and 60 min.

489

Table 1. Primers used for gene cloning

primers	nucleotide sequences of primers (5'–3') ^a	restriction enzyme sites
MP1489_F	cgc <u>GGATCC</u> GATGAATCGAAATAATCAAAATGAATATG	<i>Bam</i> HI
MP1489_R	ccg <u>CTCGAG</u> CTACTCTTGTGTTTCAATAAACTCAATAC	<i>Xho</i> I
pSTK_F	GGGAGCTTAATTAAAGATAATATC	
pSTK_R	TGCTAGTTATTGCTCAGCGG	

^a restriction enzymes sites are underlined.

493 **Table 2. Primers used for identification of the minimal active fragment**

primers	nucleotide sequence of primers (5'–3') ^a
1F	<u>TGACTGGTGGACAGCAAATGGGTCGGATGAATCGAAATAATCAAAATGAATATG</u>
68F	<u>TGACTGGTGGACAGCAAATGGGTCGGGATGCGGTCAGACTGCACT</u>
71F	<u>TGACTGGTGGACAGCAAATGGGTCGGCAGACTGCACTTACCGTTGTT</u>
72F	<u>TGACTGGTGGACAGCAAATGGGTCGGACTGCACTTACCGTTGTTGGGA</u>
73F	<u>TGACTGGTGGACAGCAAATGGGTCGGGCACTTACCGTTGTTGGGAGAAT</u>
654R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGAATTTCAATTCGATCTATAAAATATTCAC</u>
655R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGGGCAATTTCAATTCGATCTATAAAAT</u>
656R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGAGGGGCAATTTCAATTCGATCTA</u>
657R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGTACAGGGGCAATTTCAATTCG</u>
658R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGATTACAGGGGCAATTTCAATTC</u>
660R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGCGTCGGATTACAGGGGCAAT</u>
1158R	<u>AAGCTTGTCGACGGAGCTCGAATTCGGCTCTTGTGTTTCAATAAACTCAATACTG</u>

494 ^a homologous sequences to vector pET-21b(+) are underlined.

495

496

Table 3. Toxicity of Cry9Cb1 against agricultural pests

species	LC ₅₀ (µg/mL)	95% confidence limits	slope (SE)
<i>Plutella xylostella</i>	0.38	0.32–0.46	3.64 (0.30)
susceptible <i>Ostrinia furnacalis</i>	1.28	1.01–1.61	2.10 (0.20)
Cry1Fa 1000-fold resistant <i>Ostrinia furnacalis</i>	1.21	1.05–1.40	2.53 (0.22)
<i>Chilo suppressalis</i>	0.50	0.42–0.60	2.21 (0.22)
<i>Agrotis ipsilon</i>	130.22	131.81–147.33	3.15 (0.35)
<i>Mythimna separata</i>	244.77	135.16–372.33	1.51 (0.21)
<i>Helicoverpa armigera</i>	NA ^a	-	-
<i>Spodoptera exigua</i>	NA ^a	-	-
<i>Spodoptera litura</i>	NA ^a	-	-
<i>Colaphellus bowringi</i>	NA ^a	-	-

^a NA, no activity. No lethal effect and weight inhibition were observed under 100 µg/mL protein against *Helicoverpa armigera*, *Spodoptera exigua*, *Spodoptera litura*, and *Colaphellus bowringi*.

Table 4. Characterization of Cry9Cb1 polypeptides

proteins	primers	location (amino acid)	length (amino acid)	MW (kDa)	pI	<i>Plutella xylostella</i>	<i>Ostrinia furnacalis</i>
9Cb1-a	1F/1158R	1-1158	1158	132.08	5.22	100.0% ± 0.0% ^a	100.0% ± 0.0% ^a
9Cb1-b	68F/654R	68-654	587	69.06	6.68	0.0% ± 0.0% ^b	0.0% ± 0.0% ^b
9Cb1-c	68F/655R	68-655	588	69.14	6.68	99.0% ± 1.7% ^a	98.7% ± 2.3% ^a
9Cb1-d	68F/656R	68-656	589	69.23	6.68	100.0% ± 0.0% ^a	97.3% ± 4.6% ^a
9Cb1-e	68F/657R	68-657	590	69.33	6.68	100.0% ± 0.0% ^a	98.7% ± 2.3% ^a
9Cb1-f	68F/658R	68-658	591	69.45	6.68	100.0% ± 0.0% ^a	100.0% ± 0.0% ^a
9Cb1-g	71F/660R	71-660	590	69.36	6.91	100.0% ± 0.0% ^a	1.3% ± 2.3% ^b
9Cb1-h	72F/660R	72-660	589	69.23	6.91	99.0% ± 1.7% ^a	0.0% ± 0.0% ^b
9Cb1-i	73F/660R	73-660	588	69.13	6.91	3.3% ± 3.5% ^b	0.0% ± 0.0% ^b
9Cb1-j	72F/655R	72-655	584	68.72	6.91	18.0% ± 1.7% ^c	2.7% ± 2.3% ^b
9Cb1-k	72F/656R	72-656	585	68.82	6.91	16.7% ± 5.8% ^c	0.0% ± 0.0% ^b
9Cb1-l	72F/657R	72-657	586	68.92	6.91	98.0% ± 1.7% ^a	1.3% ± 2.3% ^b

^{a, b, c} Different letters within the same column indicate significant differences (one-way ANOVA with LSD test; $p <$

0.05).

"*Plutella xylostella*" indicate corrected mortality ± standard deviation against *Plutella xylostella*; "*Ostrinia furnacalis*"

indicate corrected mortality ± standard deviation against *Ostrinia furnacalis*.

Table 5. Toxicity of heat-treated and digestive Cry9Cb1 (20 µg/mL) against *Plutella xylostella* and *Ostrinia furnacalis*

		<i>Plutella xylostella</i>		<i>Ostrinia furnacalis</i>	
treatment		Mean mortality	Corrected mortality	Mean mortality	Corrected mortality
		± Std. Deviation	± Std. Deviation	± Std. Deviation	± Std. Deviation
Cry9Cb1		100.0% ± 0.0% ^a	100.0% ± 0.0% ^a	97.8% ± 1.9% ^a	97.8% ± 1.9% ^a
Cry9Cb1	SGF:15s	2.2% ± 1.9% ^b	1.1% ± 0.0% ^b	5.6% ± 5.1% ^b	1.2% ± 3.3% ^b
	SIF:30 min	1.1% ± 1.9% ^b	1.1% ± 1.9% ^b	3.3% ± 3.3% ^b	0.0% ± 0.0% ^b
	100 °C:10 min	0.0% ± 0.0% ^b	0.0% ± 0.0% ^b	1.1% ± 1.9% ^b	1.1% ± 1.9% ^b
50 mM Na ₂ CO ₃		0.0% ± 0.0% ^b	-	0.0% ± 0.0% ^b	-
Control	SGF	1.1% ± 1.9% ^b	-	4.4% ± 1.9% ^b	-
	SIF	0.0% ± 0.0% ^b		3.3% ± 3.3% ^b	-

^{a, b} Different letters within the same column indicate significant differences (one-way ANOVA with LSD test; *p* < 0.05).

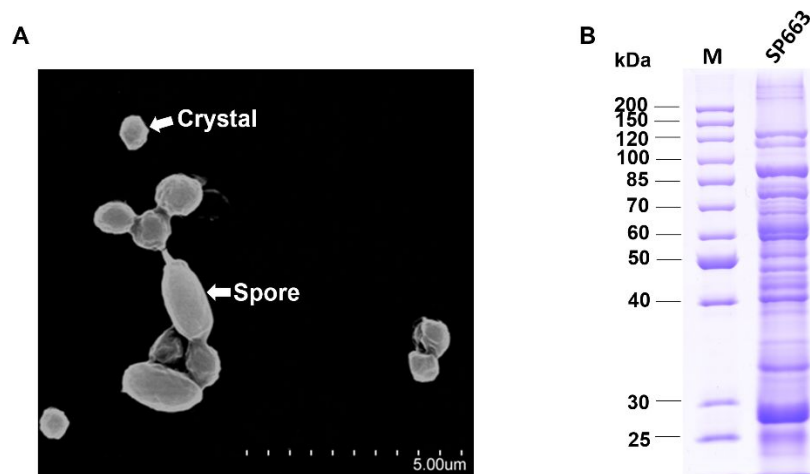
Figures**Figure 1**

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Figure 2

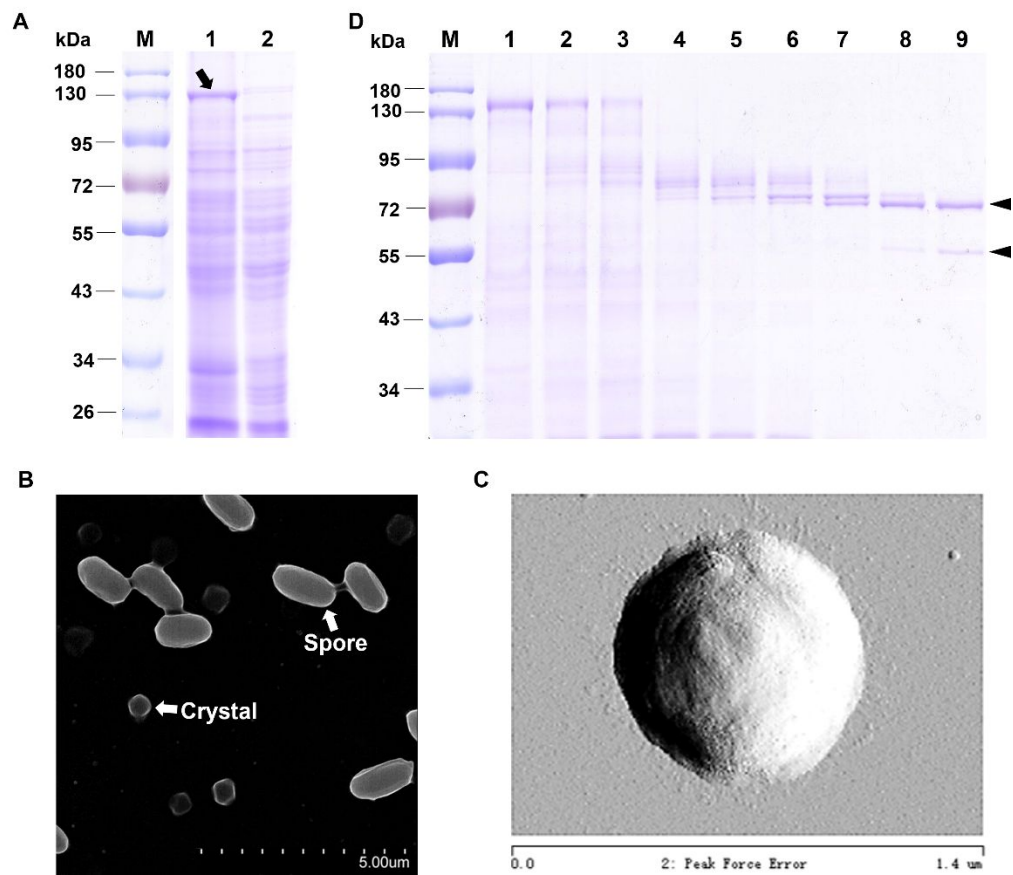


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531 1:50, 1:25, 1:10, 1:5 (w/w)). The digested proteins are marked with triangles.

532

Figure 3

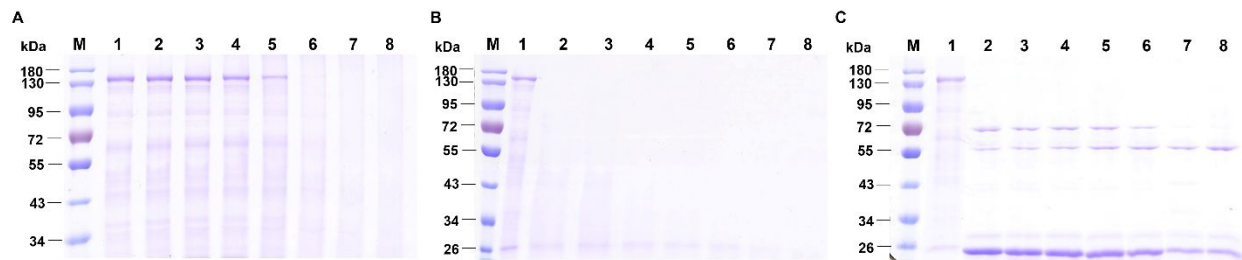
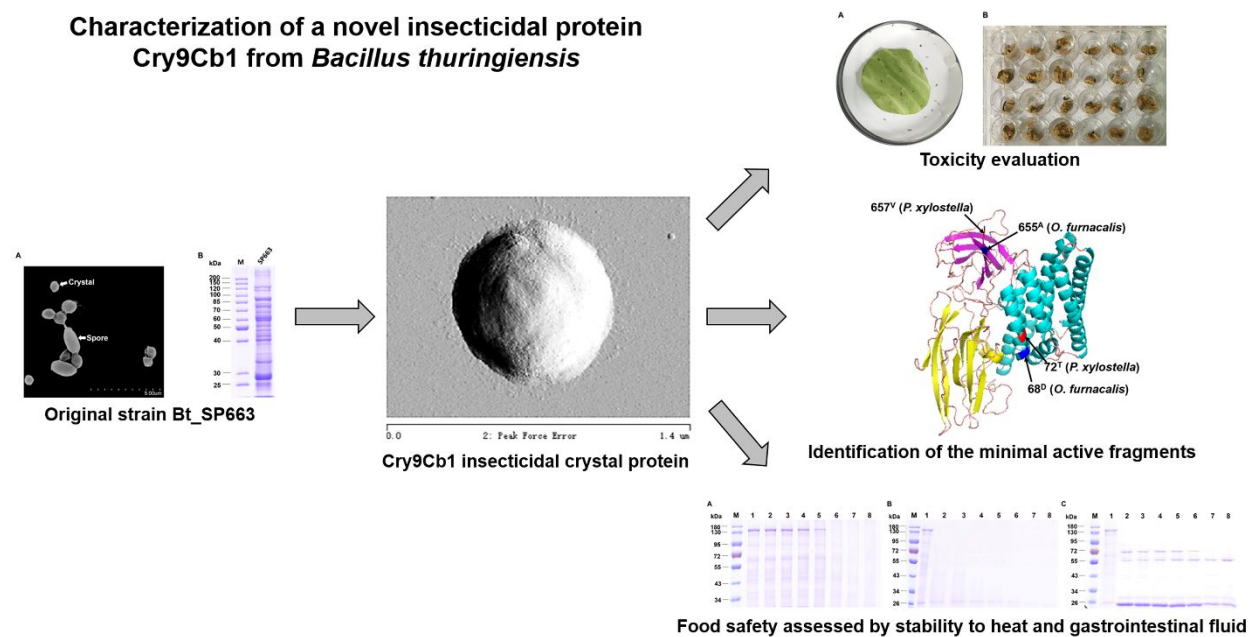
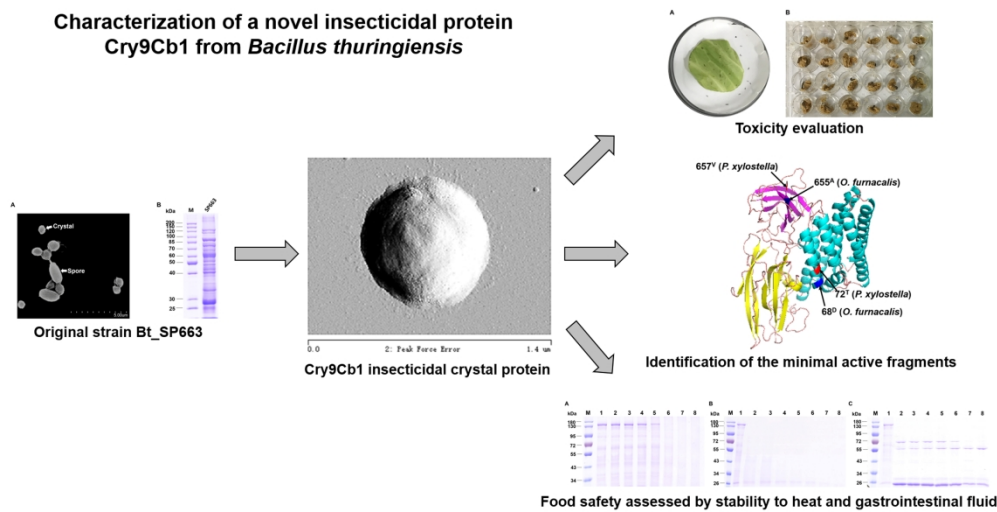


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544 TOC Graphic



545



339x175mm (300 x 300 DPI)