

Cry78Ba1, One Novel Crystal Protein from *Bacillus thuringiensis* with High Insecticidal Activity against Rice Planthopper

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ABSTRACT: The rice planthopper is a very important hemipteran pest that preys on rice and substantially affects the safety of rice production. Moreover, the long-term prevention and control of these pests with chemical pesticides has led to an increase in the resistance of the rice planthopper as well as serious environmental pollution and food safety problems. *Bacillus thuringiensis* (Bt) has been used for the efficient and green control of a variety of rice pests. Therefore, based on the high-throughput screening of Bt strains that are active against the rice planthopper, we found that Bt strain B4F11 showed certain insecticidal activity against *Laodelphax striatellus* Fallén, and we have identified a novel insecticidal protein Cry78Ba1 from the Bt strain B4F11, which is expected to provide the specific and safe control of the rice planthopper. The Cry78Ba1 protein is composed of 380 amino acid residues with a molecular weight of 42.55 kDa and contains conserved Ricin_B Lectin and Toxin_10 superfamily domains. It displays high insecticidal activity against *L. striatellus* with a lethal concentration (LC_{50}) of 9.723 $\mu\text{g/mL}$. More importantly, this Toxin_10-like protein does not display sequence homology to any known allergen and can be degraded and inactivated rapidly when heated at 90 °C and in simulated gastrointestinal fluid. In summary, Cry78Ba1 has great potential for applications in the efficient and safe prevention and control of the rice planthopper.

KEYWORDS: Cry78Ba1, rice planthopper, *Bacillus thuringiensis*, insecticidal activity, food safety assessment

INTRODUCTION

The rice planthopper is one of the main pests of the main food crop in China, including the small brown planthopper (*Laodelphax striatellus* Fallén), brown planthopper (*Nilaparvata lugens* Stal), and white backed planthopper (*Sogatella furcifera* Horvath).¹ Rice planthoppers use piercing-sucking mouthparts to pierce and suck rice sap and plunder their nutrients, which substantially reduces the yield and quality of rice. In addition, the rice planthopper also spreads rice virus disease, and *L. striatellus* has even become the main hazard to Gramineae crops, such as rice, wheat, and corn. The large-scale occurrence of the rice planthopper has caused the original single virus disease to evolve into the current mixed phenomenon of pests and diseases, which also makes the control of rice planthoppers more complicated and difficult. Currently, the control of three rice planthoppers mainly relies on chemical pesticides, such as imidacloprid, buprofezin, and nitenpyram. In addition, the selection of insect-resistant rice varieties and the release of natural enemies have certain effects. Long-term use of chemical pesticides increases pest resistance and causes serious environmental pollution and food safety problems. New and efficient methods for the safe control of rice planthoppers are urgently needed.

Bacillus thuringiensis (Bt) is an entomopathogenic bacterium that produces a variety of insecticidal crystal proteins. The main active material of Bt used to kill insects is the chaperone crystal protein produced in the bud formation stage, which exerts a strong effect on pests such as Lepidoptera, Coleoptera, and Diptera but displays very low activity against spin-sucking mouth pests, such as the rice planthopper and aphid.

According to the statistical analyses presented in the literature, two types of crystal proteins present a lethal concentration of less than 10 $\mu\text{g/mL}$ in rice planthoppers: one is Cry64Ba/Cry64Ca with the MTX2 domain from the Bt1012 strain² and the other is Cry78Aa1 with Toxin_10 and Ricin_B lectin domains from the Bt C9F1 strain.³ Based on the information discussed above, the identification of Bt proteins with high insecticidal activity toward rice planthopper and no cross-resistance with known proteins has become an important aim to solve the problem of rice safety production.

Bt transgenic crops have been successfully applied in the control of agricultural pests. The newly expressed Bt protein in a genetically modified (GM) plant must undergo a comprehensive assessment using established safety assessment frameworks. In our study, we focused on the food safety assessment of the new Bt toxin Cry78Ba1. Food safety assessment includes characterization and food safety assessment of the novel protein and the transgenic crops.⁴ The food safety assessment has focused on the potential for allergenicity or toxicity of the protein using bioinformatics analysis and in vitro resistance to degradation in the presence of digestive enzymes.⁵ According to some reports, the Cry1Ab/Ac,⁶ Cry1C,⁷ and Cry2A⁸ proteins are safe for use in GM foods.

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Table 1. Primers Used for Gene Cloning

primer	sequence (5'-3') ^a
pET-gene 339-F	<u>CTGGTGGACAGCAAATGGGTGCGGTGTCAAATGAAATAATACCAAAG</u>
pET-gene 339-R	<u>GGTGCTCGAGTGCAGCGCCGCAAGT</u> GATCGAGGGATAGTCTTGTAACTG
Line-pET21b-F	CTTGCGGCGCGACTCGAGCACC
Line-pET21b-R	CCGACCCATTGCTGTCCACCAG

^aSequences that were homologous to the linearized primers of pET-21b are underlined.

Here, we performed an analysis of the homologous nucleotide sequence alignment of the *cry78Aa1* gene from 1368 Bt strains, and these strains have genome sketches. Based on the high-throughput screening of Bt strains that are active against the rice planthopper, we found that Bt strain B4F11 showed certain insecticidal activity against *L. striatellus*. Furthermore, we cloned and confirmed the novel insecticidal gene *cry78Ba1* from the genome of Bt B4F11 and completed the prokaryotic expression, insecticidal activity, and food safety assessment of this protein.

MATERIALS AND METHODS

Bioinformatics Analysis of Bt Strains that Contains the *cry78*-like Gene. The 1368 wild Bt strains was isolated from the soil sample of Baiwangshan Forest Park in Beijing and preserved at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPPCAAS), Beijing. These strains were sequenced by Dupont Pioneer, USA. The analysis of homologous nucleotide sequence of the *cry78Aa1* gene was performed using 1368 Bt strains from which genomic sketch data were obtained. The *E*-value of the BLAST alignment was set to less than 10^{-30} , and the protein sequences with a length of less than 300 amino acid residues were removed. Next, these *cry78Aa1* homologous sequences were clustered using MEGAX 10.0.2.⁹ After removing the repeated sequences, the open reading frames (ORFs) of *cry78*-like sequences were searched using Vector NTI 11.5.1 (International Business Machines Informix, USA).

Strains, Plasmid, and Growth Conditions. Bt strain B4F11 was isolated from a soil sample collected from Baiwangshan Forest Park in Beijing and preserved at IPPCAAS. The gene 339 from Bt strain B4F11 was introduced into the pET-21b (+) (Novagen) plasmid. The *Escherichia coli* DH5 α strain was used for routine transformations, while *E. coli* Rosetta (DE3) was used to express the cloned genes. All *E. coli* strains were cultured in a Luria–Bertani (LB) medium at 37 °C. Bt strains were incubated in 1/2 LB liquid medium (0.5% tryptone, 0.25% yeast extract, and 0.5% NaCl, pH 7.0) or agar plates at 30 °C. Ampicillin (Amp) and chloramphenicol (Cm) were used for bacterial selection at concentrations of 100 and 50 μ g/mL, respectively.

Extraction of Bt Crystal Proteins and Q-Exactive Mass Spectrometry Analysis. The crystal proteins of B4F11 were scanned using an optical microscopy (BX61) and an atomic force microscope (Bruker Multimode8, Germany). The SDS-PAGE analysis of crystal proteins of B4F11 was performed using the methods described by Shu et al.¹⁰ The spore–crystal mixture of B4F11 was washed successively with a 1 mol/L NaCl solution and sterile purified water. Then, the mixture was resuspended in five volumes of solubilization buffer [50 mmol/L Na₂CO₃ and 50 mmol/L EDTA; pH 10.0; add dithiothreitol (DTT; Sigma) to a final concentration of 10 mmol/L before use], and cells were sonicated in ice water at 50 W for 5 min with a 3 on and 5 s off cycle. The mixture was then shaken in an ice bath for 4 h. Next, 5 $\times$ loading buffer was added, and the samples were boiled for 10 min and centrifuged at 12,000g for 5 min. The mixture was analyzed using 10% SDS-PAGE.

The protein bands were excised, combined, and subjected to in-gel digestion with trypsin. The digested fragments were analyzed using a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo

Fisher Scientific, USA). Afterward, the data were analyzed using the MASCOT software.

Preparation of the Genomic DNA, Sequencing, and Computational Analysis. Genomic DNA from B4F11 was prepared using the procedure reported by Song et al.¹¹ Genome sequencing was performed using an Illumina HiSeq 2500 platform with a paired-end genomic library (insert size of 500 bp) containing read lengths of 125 bp. Clear reads were reassembled using SOAPdenovo.¹² All coding sequences (CDs) were predicted by GeneMark.¹³ Protein-coding sequences were annotated using the BLASTP¹⁴ program with a cutoff *E*-value of 10^{-6} by searching a local Bt insecticidal toxin database. The local database was established using the amino acid sequences of available quaternary rank Cry/Vip/Cyt toxins protein sequences listed on the website maintained by the Bt nomenclature committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).¹⁵ Conserved domains were annotated using the InterPro database.¹⁶ Homology modeling was performed to generate the three-dimensional protein structure in the SWISS-MODEL workspace.¹⁷

Cloning of the Candidate Gene from Strain B4F11. Based on the nucleotide sequence of the draft genome of strain B4F11, the primer pET-gene 339-F/R was designed to amplify candidate gene 339 (Table 1). The linearized primer targeting the pET-21b plasmid was designed based on the pET-21b sequence (Table 1), and the pET-21b plasmid was linearized by PCR (the extension time is 6 min). Sequences that were homologous to the linear primers of pET-21b were added to the 5'-termini of those primers for seamless cloning. PCR products were amplified using PrimerSTAR DNA polymerase (TaKaRa, China) in a PTC-100 Peltier thermal cycler (MJ Research, USA). PCR was performed using the following program: 30 cycles of 98 °C for 30 s, 50 °C for 15 s, and 72 °C for 1.5 min. The purified gene fragments were recombined into a linearized vector using a seamless cloning kit (Clonesmarter, USA). The recombinant products were introduced into *E. coli* DH5 α , and positive clones were screened by PCR using a pair of universal primers (T7/T7ter). Finally, the positive plasmids were sequenced with a 3730XL DNA sequencer (Applied Biosystems, USA).

Protein Expression and Purification. The correct recombinant plasmid was transferred into *E. coli* Rosetta (DE3), selected and cultured in an LB medium at 37 °C at 220 rpm until the optical density reached 0.6. Then, isopropyl β -D-thiogalactoside (IPTG; Thermo Fisher Scientific) was added at a final concentration of 0.5 mmol/L to induce protein expression. The temperature was reduced to 20 °C, and the cells were cultured for an additional 12 h at 150 rpm. Bacterial cells were collected by centrifugation at 4 °C at 7000g for 10 min. The bacterial pellet was resuspended in 20 mmol/L Tris–HCl (pH 8.0), and cells were sonicated in ice water at 65 W for 5 min with a 3 s on and 5 s off cycle. The supernatant was collected and passed through a Ni²⁺ column (GE HisTrap 5 mL) attached to an AKTA rapid protein purification system and eluted with gradient concentrations of imidazole. Buffer exchange was conducted by dialysis in 20 mmol/L Tris–HCl (pH 8.0) to remove imidazole. The purified proteins were analyzed using SDS-PAGE, and the concentrations of solubilized proteins were determined with the ImageJ software (National Institutes of Health, USA, <https://imagej.nih.gov/ij/docs/guide/index.html>) using bovine serum albumin (BSA) as a standard.

Bioassay. Three hemipteran insects, the third instar nymph of *L. striatellus*, *Bemisia tabaci* Gennadius, and *Rhopalosiphum maidis* Fitch, a lepidopteran insect, the second instar larva of *Plutella xylostella* Linnaeus, and a coleopteran insect, the newly hatched larva of

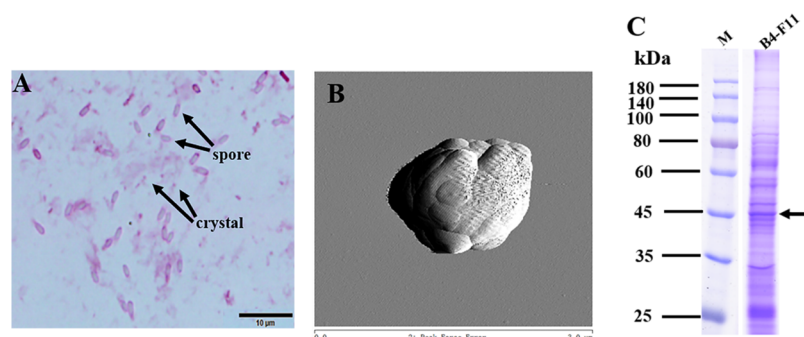


Figure 1. Images of crystals obtained using (A) optical microscopy and (B) atomic force microscopy. (C) SDS-PAGE analysis of total crystal proteins from Bt strain B4F11. M, protein marker (Blue Plus IV Protein Marker, TransGene).

Table 2. Putative Insecticidal Protein Sequences in B4F11^a

candidate toxin gene	predicted protein MW (kDa)	Bt database best match	sequence identity (%)	NCBI BLAST results (identity 100%)	expressed in B4F11
218	31.53	Vip2Ba	25.79	ADP-ribosyl transferase, partial from <i>B. thuringiensis</i> (100%)	unknown
232	25.45	Cyt2Ca	35.68	hypothetical protein from <i>B. thuringiensis</i> (100%)	+
249	81.47	Vip1Ba	24.32	hypothetical protein from <i>B. thuringiensis</i> (99.86%)	unknown
339	42.55	Cry78Aa	54.27	hypothetical protein from <i>Bacillus bingmayongensis</i> (92.37%)	+
1586	97.79	Cry22Bb	37.63	DUF5011 domain-containing protein from <i>Bacillus toyonensis</i> (100%)	–
5404	28.50	Cry46Ab	34.60	hydralysin-2 from <i>B. thuringiensis</i> (100%)	–
5493	52.63	Cry22Ba	83.98	DUF5011 domain-containing protein from <i>B. bingmayongensis</i> (90.68%)	+

^aUnknown: The predicted protein may be secreted in the fermentation broth, and thus it was not detected. +/–: The protein was/was not detectable by Q-Exactive mass spectrometry analysis in the spore–crystal mixture of strain B4F11.

Colaphellus bowringi Baly, were chosen to assess the toxicity of the candidate protein. When insecticidal activity was assayed, the 50% lethal concentration (LC₅₀) of protein in pests was analyzed by constructing dose–response curves using SPSS 22.0 (International Business Machines, USA) with a Probit analysis. Each treatment was repeated three times.

Using the method reported by Liu et al.,¹⁸ the insecticidal activity of the protein against three hemipteran pests was determined with a double parafilm membrane feeding system. Twenty-one individuals were used on one replication. Proteins were added to a liquid artificial diet at a concentration of 100 µg/mL. After 6 days of incubation at 28 °C with a light:dark photoperiod of 16:8 h and 70% relative humidity, dead insects were counted.

The bioassay methods using *P. xylostella* and *C. bowringi* were described in the studies by Tabashnik et al.¹⁹ and Yan et al.,²⁰ respectively. Thirty individuals were used on one replication. Protein concentrations of 60 and 600 µg/mL were initially used for tests with *P. xylostella* and *C. bowringi* using a leaf-dip bioassay. Mortality was recorded after an incubation of 2 days at 25 °C with a light:dark photoperiod of 16:8 h and 50% relative humidity.

Heat Stability Evaluation. The method of heat stability test refers to announcement no. 2031-17 of the Ministry of Agriculture of the People's Republic of China "Food safety detection of genetically modified organisms and derived products-Protein heat stability test".²¹ The candidate protein was dissolved in 20 mmol/L Tris–HCl (pH 8.0) (Solarbio), buffer at a concentration of 100 µg/mL in 1.5 mL of microcentrifuge tubes (100 µL per tube). They were tested for stability at a temperature of 90 °C for periods of 0.25, 0.5, 1, 2, 5, 10, 30, and 60 min. The assay was terminated by placing the protein sample tubes on ice and adding 25 µL of 5× loading buffer. Control samples of a 0 min incubation of the protein (kept at 4 °C) were also prepared. The resulting proteins were analyzed by SDS-PAGE. Insecticidal activity of the heat-treated proteins (40 µg/mL) was evaluated against *L. striatellus*, and mortality was calculated using a one-way ANOVA with the LSD test.

In vitro Evaluation of the Digestive Stability. The digestive stability evaluation method was improved from the method reported by Xu et al.^{6,22,23} First, 4 µL of protein (1 mg/mL) was added to 16 µL of simulated gastric fluid [SGF; 0.2 g of NaCl and 88 mg of pepsin (Solarbio) dissolved in 70 mL of sterile water and the pH was adjusted to 1.2 with HCl; sterile water was added to 100 mL]. Then, 2 µL of protein (1 mg/mL) was added to 18 µL of simulated intestinal fluid [SIF; 0.7 g of KH₂PO₄ and 1 g of trypsin (Solarbio) dissolved in 65 mL of sterile water, with 0.2 mol/L NaOH added to adjust the pH to 7.5 and sterile water added to 100 mL] in 1.5 mL of microcentrifuge tubes. The samples were incubated in a 37 °C water bath for 30 s or 1, 2, 5, 10, 30, or 60 min. A control sample with a 0 min digestion of the protein was also prepared. The SGF assay was terminated by placing the sample tubes on ice, adding 10 µL of 0.2 mol/L NaHCO₃ and 7.5 µL of 5× loading buffer. The SIF assay was terminated by placing the sample tubes on ice and adding 5 µL of 5× loading buffer. BSA and soybean trypsin inhibitor (STI) were used as an unstable and stable control protein, respectively. The resulting proteins were analyzed using SDS-PAGE. Insecticidal activity of the digested protein (40 µg/mL) was evaluated against *L. striatellus*, and mortality was calculated using a one-way ANOVA with the LSD test.

Allergy Safety Analysis. The homologous amino acid sequences to Cry78Ba1 were searched in the Allergen Online Database (<http://www.allergenonline.org/databasefasta.shtml>) and Structural Database of Allergenic Proteins (http://Fermi.utmb.edu/SDAP/sdap_who.html). Two methods²⁴ of sequence alignment were performed. In the first method, any known allergen proteins with 35% amino acid homology with the novel protein in a window of 80 amino acids were identified. The other method subdivides the new protein into eight amino acid blocks and then compares these blocks with the epitopes of all probable allergens.

RESULTS

Bioinformatics Analysis of Bt Strains Containing the *cry78-like* Gene. One hundred sixty-two homologous nucleotide sequences to the *cry78Aa1* gene were obtained from the Bt strain library. The results of the cluster analysis (Figure S1) showed the division of the 162 homologous nucleotide sequences to the *cry78Aa1* gene into 27 classes, and 26 new *cry78-like* genes were obtained. Seventeen candidate strains containing *cry78-like* genes were obtained. Total crystal proteins of these candidate strains were extracted (refer to the extraction method of B4F11 strain total crystal proteins) for insecticidal activity assay against *L. striatellus*, and the results showed that total crystal proteins of the candidate strain B4F11 showed certain insecticidal activity against the third instar nymph of *L. striatellus* (the data is not shown).

Initial Characterization of the B4F11 Strain. Small, approximately spherical crystals were observed using both optical microscopy (BX61) and atomic force microscopy (AFM) from the mixture of spores and crystal proteins of B4F11 (Figure 1A,B). Total crystal proteins of B4F11 were analyzed using SDS-PAGE, and one major protein with a molecular weight of approximately 45 kDa and several other minor proteins were identified (Figure 1C). Total crystal proteins of B4F11 were found to contain Cry78, Cry22, and Cyt2, as per Q-Exact mass spectrometry analysis (Table S1 and Table 2).

Draft Genome Sequence and Pesticidal Gene Annotation of B4F11. The B4F11 genome sequence has a size of 6.0 Mb and contains 94 contigs, 6,222,236 nucleotide base pairs, and a GC content of 34.74%. The predicted protein-coding sequence contained 6317 amino acids, and seven full-length sequences were successfully annotated by the Bt insecticidal protein database (Table 2). Only the protein sequence of gene 5493 was highly similar to the known Cry22Ba protein, while the other six sequences showed lower similarity to other known toxins. Among these sequences, the protein sequence encoded by gene 218 annotated by Bt insecticidal protein database shares 25.79% similarity with the Vip2Ba protein sequence that is an ADP-ribosyl transferase. The protein encoded by gene 5040 was verified by the NCBI BLASTP as a hydralysin-2 protein, which shares 51% amino acid sequence similarity with parasporin-2, which is toxic to cancer cells and is lethal to silkworms (*Bombyx mori* Linnaeus). The protein encoded by gene 339 shares 54% sequence identity with Cry78Aa1 and is the key research object in this paper.

Expression and Insecticidal Activity of the Protein Encoded by Gene 339. The full-length sequence of gene 339 was 1143 bp, and it encoded a protein of 380 amino acid residues, with a deduced molecular mass of 42.55 kDa. The putative sequence of gene 339 was successfully cloned into the pET-21b plasmid. The gene was then expressed in *E. coli* Rosetta (DE3) cells after induction by IPTG. After nickel affinity chromatography, the purified proteins were analyzed using SDS-PAGE (Figure 2). The protein encoded by gene 339 ran at a size consistent with its predicted molecular weight of 42.55 kDa.

L. striatellus, *R. maidis*, *B. tabaci*, *P. xylostella*, and *C. bowringi* were chosen to evaluate the toxicity of the gene 339 product. The results revealed the high insecticidal activity of the novel protein against *L. striatellus*, with an LC_{50} of 9.327 $\mu\text{g/mL}$ (95% CL 7.220–13.293 $\mu\text{g/mL}$) (Table 3).

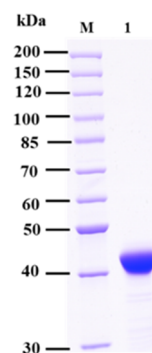


Figure 2. SDS-PAGE analysis of the purified protein encoded by gene 339. M, protein marker (PageRuler Unprestained Protein Ladder, Thermo); 1, the protein encoded by gene 339.

Table 3. Results of the Bioassay of the Protein Product of Gene 339 against Different Insects^a

insect species	LC_{50} ($\mu\text{g/mL}$)	95% confidence limits ($\mu\text{g/mL}$)	slope $\pm$ SE
<i>L. striatellus</i>	9.723	7.220–13.293	1.044 $\pm$ 0.137
<i>R. maidis</i>	N/A		
<i>B. tabaci</i>	N/A		
<i>P. xylostella</i>	N/A		
<i>C. bowringi</i>	N/A		

^aN/A, no activity. No lethal effect or weight inhibition was observed under 100 $\mu\text{g/mL}$ protein against *R. maidis*, *B. tabaci*, *P. xylostella*, and *C. bowringi*.

Molecular Characterization of Gene 339. The full-length 1143 bp gene 339 encodes a polypeptide of 380 amino acid residues with a deduced molecular mass of 42.55 kDa. NCBI BLASTP search showed the highest identity of 92% with a hypothetical protein (WP_017154552.1) from *Bacillus bingmayongensis* across the NCBI protein database and a 75% amino acid sequence similarity to a pesticidal protein sequence from Bt (WP_098568042). Based on the Align X alignment, the sequence similarity between the Cry78Ba1 and Cry78Aa1 proteins is 54%, and the corresponding nucleotide sequence similarity reaches 66%. This novel gene sequence was submitted to the NCBI GenBank (accession no. MN075151). Then, according to the systematic naming method of Bt proteins, the protein encoded by gene 339 was named Cry78Ba1 by the *B. thuringiensis* δ -endotoxin nomenclature committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

An analysis of the Cry78Ba1 sequence showed that the protein contained the core Ricin_B_lectin2 (pfam14200) and Toxin_10 (pfam05431) domains, which are located at residue positions 39–159 and 209–378, respectively (Figure 3A). The Ricin_B_lectin domain is the Lectin domain of the ricin B chain profile. The most characteristic sequence feature (though not completely conserved) of this domain is the presence of the QxW pattern.²⁵ Cry78Ba1 has three variants of QxW, namely QxF. Various insecticidal proteins have the Toxin_10 domain, such as the binary toxin BinA/BinB from *Lysinibacillus sphaericus* and the Bt toxins Cry35Ab1 and Cry78Aa1 (Figure 3A). The Toxin_10 domain in each of these four toxins is preceded by a trefoil domain.²⁶ As far as Cry78Aa1 is concerned, it also contains the QxF motif (Figure 3A). Using Cry35Ab1 (PDB 4JP0) as the template, a model of Cry78Ba1

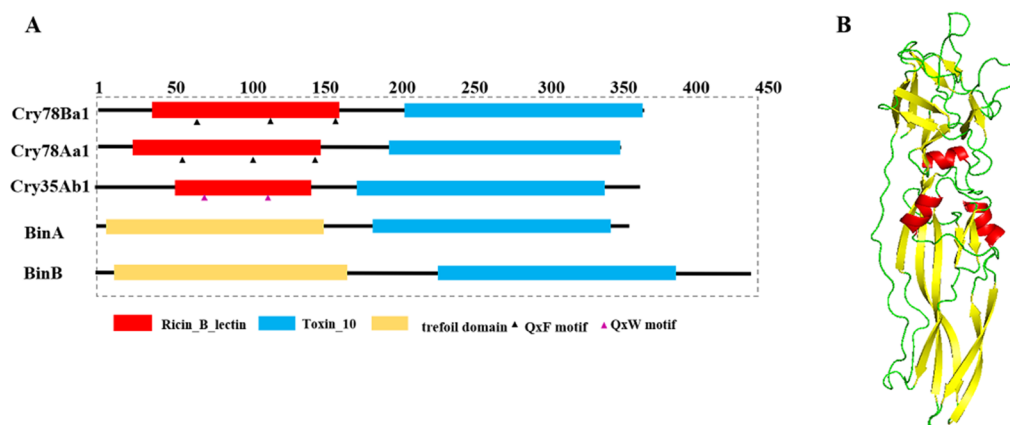


Figure 3. (A) Structure display of toxins showing a similar domain architecture as Cry78Ba1. (B) Simulated spatial structure of the Cry78Ba. Red, α -helices; yellow, β -sheets. The structure was visualized using PyMOL.

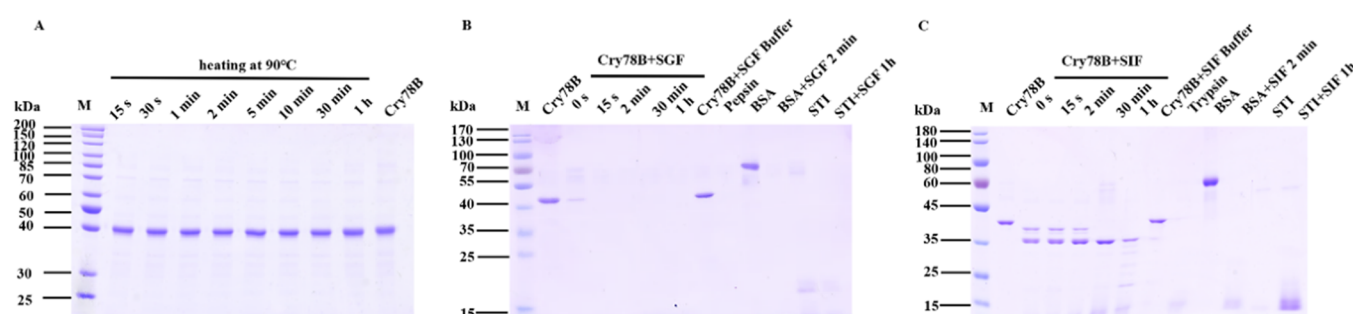


Figure 4. (A) SDS-PAGE analysis of heat stability. (B) SDS-PAGE analysis of SGF digestion. (C) SDS-PAGE analysis of SIF digestion. M, protein marker; Cry78B + SGF/SIF buffer, Cry78Ba1 protein digested in SGF/SIF buffer without pepsin/trypsin.

(GMQE = 0.53; Figure 3B) was built by the SWISS-MODEL online software.

Heat Stability of Cry78Ba1. When 100 $\mu\text{g/mL}$ Cry78Ba1 protein was heated at 90 $^{\circ}\text{C}$, no white flocculated precipitate was observed. Although the SDS-PAGE analysis showed no significant degradation bands of the Cry78Ba1 protein within 1 h of heating at 90 $^{\circ}\text{C}$ (Figure 4A), the protein completely lost its insecticidal activity against *L. striatellus* (Table 4) after heating for 10 min. Thus, Cry78Ba1 is unstable and denatured after a heat treatment.

In vitro Digestive Stability of Cry78Ba1. An in vitro simulation of digestive stability in the gastrointestinal juice is

Table 4. Toxicity of Heat-Treated and Digested Cry78Ba1 (40 $\mu\text{g/mL}$) against *L. striatellus*^a

classification	treatment	mean mortality (%)	corrected mortality (%)
Cry78Ba1	Cry78Ba1	100.0 $\pm$ 0.0a	95.2 $\pm$ 0.0a
	Cry78Ba1 + 90 $^{\circ}\text{C}$ (10 min)	7.9 $\pm$ 2.7b	3.2 $\pm$ 2.7b
	Cry78Ba1 + SGF (15 s)	7.9 $\pm$ 2.7b	1.6 $\pm$ 0.0b
	Cry78Ba1 + SIF (0 s)	98.4 $\pm$ 2.7a	90.5 $\pm$ 0.0a
	Cry78Ba1 + SIF (1 h)	96.8 $\pm$ 2.7a	88.9 $\pm$ 0.0a
	Cry78Ba1 + SIF (2 h)	7.9 $\pm$ 2.7b	0.0 $\pm$ 0.0b
control	20 mM Tris-HCl	4.8 $\pm$ 0.0b	
	SGF	6.3 $\pm$ 2.7b	
	SIF	7.9 $\pm$ 2.7b	

^aMeans $\pm$ standard deviations. Different letters within the same column indicate significant differences (one-way ANOVA with the LSD test, $p < 0.05$).

the most basic and key step in the assessment of the safety of a novel Bt toxin. According to the result of the SGF digestive test, Cry78Ba1 was completely digested within 15 s after treatment with SGF at 37 $^{\circ}\text{C}$ (Figure 4B). In the SIF digestion experiment, Cry78Ba1 and the degraded bands were not completely digested by the SIF within 1 h, but the protein was digested into two obvious fragments (40 and 35 kDa) within 2 min and further degraded to a 35 kDa fragment within 30 min (Figure 4C). Obviously, BSA was completely digested by the SGF or SIF within 2 min. The results of insecticidal activity assay showed that the Cry78Ba1 protein digested in SGF for 15 s had completely lost the insecticidal activity, but this protein still retained its original insecticidal activity after being digested for 1 h in SIF (Table 4). To sum up, if the Cry78Ba1 protein is ingested, then it will be rapidly degraded in gastric juice and has completely lost its toxicity before entering the intestine; therefore, no safety risk to humans or mammals has been identified for this protein in terms of digestion and absorption.

Allergy Safety Analysis. The homologous sequence alignment of the Cry78Ba1 protein was performed using the Allergen Online Database. Using the 80 amino acid residue sequence alignment method (Figure 5), no sequence homology with one or several sections of the 80 amino acid residue sequence of the Cry78Ba1 protein $\geq 35\%$ was observed with known allergens. At the same time, the eight consecutive amino acid residue alignment method was used to discover (Figure 5) that no eight consecutive amino acid residue sequences of the Cry78Ba1 protein perfectly matched with the sequences of known allergens. The analysis of allergic protein structures did not reveal a structure homologous to allergen

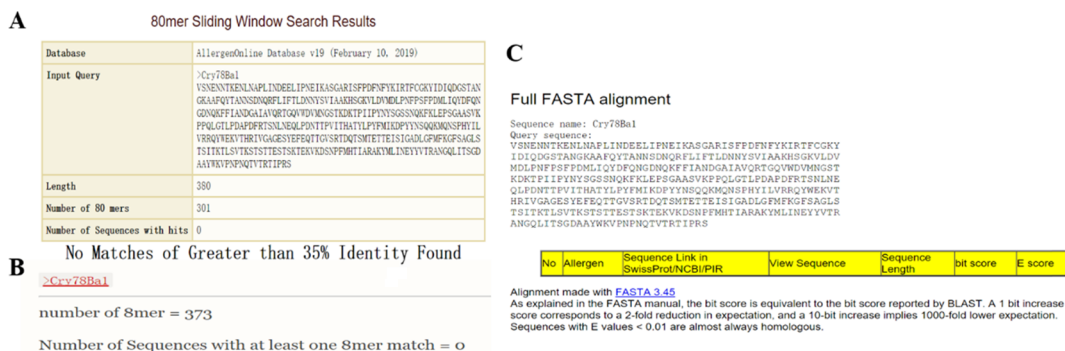


Figure 5. (A) Allergy safety analysis of the Cry78Ba1 protein using the 80 amino acid sequence alignment. (B) Allergy safety analysis of the Cry78Ba1 protein by using the eight consecutive amino acid alignment. (C) Allergic structure analysis of the Cry78Ba1 protein.

proteins (Figure 5C). A comparison of the results obtained using these three methods indicate that the Cry78Ba1 protein has low sequence homology with any known allergens and is unlikely to be a potential allergen. Therefore, theoretically, the allergenic potential of the Cry78Ba1 protein is low and it presents little allergenic risk.

■ DISCUSSION

In China, rice is the largest grain crop, and rice production is related to national food security. Therefore, the safe and efficient control of rice pests is extremely important to ensure rice yield and quality. Bt preparations and transgenic Bt rice effectively and safely control lepidopteran pests on rice, but no Bt preparations and transgenic rice have been developed to control rice planthoppers. In our study, we identified a new Bt protein with specific and effective insecticidal activity against *L. striatellus*. In a preliminary investigation, we determined the insecticidal activity of Cry78Ba1 against three other hemipteran pests, and the protein possessed insecticidal activity against *N. lugens*, *S. furcifera*, and *Nephotettix bipunctatus* Fabricius, which required further experimental determination. Due to its potential application value, we focused on the food safety assessment of the new protein. The Cry78Ba1 protein is easily inactivated after heat treatment and degraded in SGF. At the same time, Cry78Ba1 does not share homologous sequences with any known food or environmental allergens, and thus, it is not presumed to be a potential allergen. Based on these results, Cry78Ba1 has a lower potential allergic risk based on the initial food safety assessment, but its application to the transgenic plants still requires further testing.

Typical Cry proteins contain three conserved domains (known as the 3D-Cry protein) and belong to the α -pore-forming protein family. Currently, some 3D-Cry proteins have passed safety assessments and have been applied to transgenic crops. They are safe to the environment and nontoxic to humans and animals and include the proteins Cry1Ab1,^{27–29} Cry1Ac,³⁰ and Cry1C.³¹ Although Cry78Ba1 has a different structure from the 3D-Cry protein, our results show that its thermal stability, protease degradability, and allergy safety are similar to the 3D-Cry protein.^{6,32} The Cry78Ba1 protein contains the conserved Ricin_B_lectin and Toxin_10 superfamily domains, and its simulated spatial structure is very similar to the structures of Cry35Ab1³³ from Bt, BinB, and Cry49 from *L. sphaericus*;^{34,35} thus, it is a Toxin_10-like protein. By analyzing the three-dimensional structures of the Cry35Ab1³³ and BinB proteins,³⁶ the Toxin_10 domain contains five antiparallel β -sheet structures and a predicted

transmembrane domain. This spatial structure has been reported in a range of pore-forming proteins, including aerolysin, epsilon toxin, and Cry46Aa (parasporin-2).³⁷ Among these proteins, the pore-forming mechanism of the aerolysin protein has been studied in the greatest depth, and the β -sheet can be rearranged to form a barrel-like structure, which is inserted into the cell membrane to induce pore formation in the cell membrane.³⁸ Coincidentally, the C-terminal structures of ETX/MTX2 proteins, such as Cry51A³⁹ and Cry64B/C², are very similar to Toxin_10-like proteins, and these Bt insecticidal proteins display strong activity against pests with piercing-sucking mouthparts.

Although these proteins belong to the Toxin_10-like protein family, Bt Cry proteins are spatially similar to aerolysin toxins, but they are not as toxic to mammals as aerolysin. For example, Cry35Ab1 contains an aerolysin-like domain and is a member of the Toxin_10-like protein family.³³ Since 2006, millions of acres of corn expressing Cry35Ab1 have been grown annually in the United States without reported adverse effects on humans, livestock, or the environment.⁴⁰ In addition to the examples of the Toxin_10 class protein used for corn rootworm (*Diabrotica virgifera virgifera*) control in the GM maize, biopesticides containing Bt *israeliensis* Cry60Aa1 and Cry60Ba1 and β -PFPs from *L. spharicus*, Bin, MTX2, and MTX3 have been widely used to control mosquitoes in the United States and black flies and mosquitoes in Africa (including use in drinking water).⁴¹

On the other hand, many studies confirm that the Toxin_10 superfamily domain is the pore-forming domain of this class of proteins, and most of its sequence and structural diversity are located in the N-terminal domain that is likely responsible for differential receptor binding. In other words, the responsibility of sequences and domains for pore formation is generally relatively conserved in terms of the structure, while the sequences and structures of the regions responsible for receptor binding are generally diverse. In fact, nontarget insects and mammals, including humans, lack specific high-affinity Cry protein receptors on the epithelial surface of the gastrointestinal tract,^{28,42} and the receptor binding domains of the Toxin_10/aerolysin-like protein family are highly specific for different targets in prokaryotes and eukaryotes.⁴³ Additionally, many members of the toxin protein family are largely safe for many nontarget organisms, and this hypothesis should be further verified by performing an experimental safety evaluation.⁴¹

In summary, we focused on the characterization and assessment of the food safety of Cry78Ba1. The new protein

possessed highly efficient and specific insecticidal activity. Therefore, the Cry78Ba1 protein has great application prospects in the safe prevention and control of hemipteran pests.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.9b07429>.

Insecticidal proteins from Bt strain B4F11 identified by Q-Exactive mass spectrometry and the cluster analysis of 162 cry78Aa1 homologous nucleotide sequences (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

Bt, *Bacillus thuringiensis*; GM, genetically modified; *E. coli*, *Escherichia coli*; LB, Luria–Bertani; AFM, atomic force microscopy; CDSs, coding sequences; PCR, polymerase chain reaction; IPTG, isopropyl β -D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide-gel electrophoresis; BSA, bovine serum albumin; LC₅₀, 50% lethal concentration; 3D-Cry, three domain-Cry toxins; NCBI, National Center of Biotechnology Information; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; GMQE, global model quality estimation; MW, molecular weight

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Supporting Information

Cry78Ba1, One Novel Crystal Protein from *Bacillus thuringiensis* with High Insecticidal Activity against Rice Planthopper

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Table S1 Checked insecticidal proteins from Bt B4F11 that identified by Q-Exactive Mass Spectrometry

Reference*	Bt database best match	Mass	Score
gene_232-B4F11	Insecticidal crystal protein Cyt2Ca	25446	149.7
gene_339-B4F11	Insecticidal crystal protein Cry78Aa	42547	191.1
gene_5493-B4F11	Insecticidal crystal protein Cry22Ba	52622	361.8

*Based on the genome sketch data of Bt B4F11 strain, we used the B4F11 strain coding protein library as a self-built library for Q-Exactive Mass spectrometry analysis.

Figure S1

Tree scale: 100

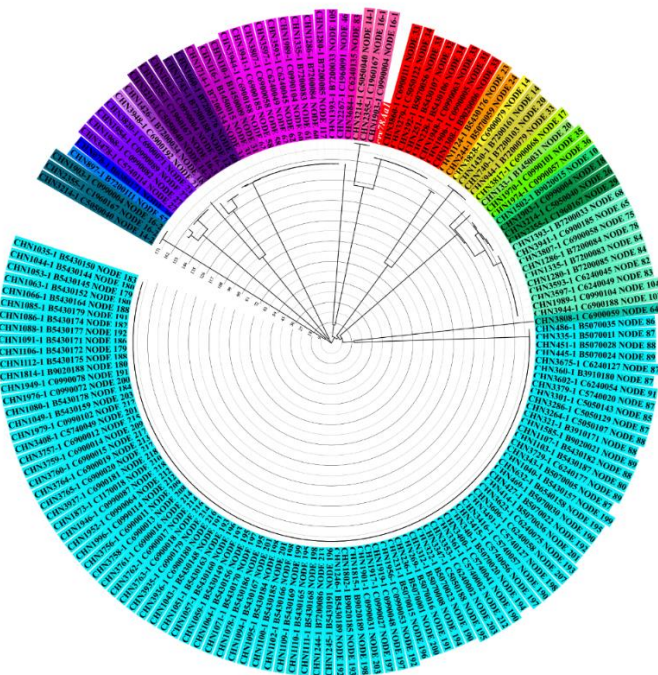


Figure S1. Results of the cluster analysis of 162 *cry78Aa1* homologous nucleotide sequences.