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Cry64Ba and Cry64Ca, Two ETX/MTX2-Type *Bacillus thuringiensis* Insecticidal Proteins Active against Hemipteran Pests

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ABSTRACT Genetically modified crops that express insecticidal *Bacillus thuringiensis* (Bt) proteins have become a primary approach for control of lepidopteran (moth) and coleopteran (beetle) pests that feed by chewing the plants. However, the sap-sucking insects (Hemiptera) are not particularly susceptible to Bt toxins. In this study, we describe two Cry toxins (Cry64Ba and Cry64Ca) from Bt strain 1012 that showed toxicity against two important hemipteran rice pests, *Laodelphax striatellus* and *Sogatella furcifera*. Both of these proteins contain an ETX/MTX2 domain and share common sequence features with the β-pore-forming toxins. Coexpression of *cry64Ba* and *cry64Ca* genes in the acrystalliferous Bt strain HD73$^-$ resulted in high insecticidal activity against both hemipteran pests. No toxicity was observed on other pests such as *Ostrinia furnacalis*, *Plutella xylostella*, or *Colaphellus bowringi*. Also, no hemolytic activity or toxicity against cancer cells was detected. Binding assays showed specific binding of the Cry64Ba/Cry64Ca toxin complex to brush border membrane vesicles isolated from *L. striatellus*. Cry64Ba and Cry64Ca are Bt Cry toxins highly effective against hemipteran pests and could provide a novel strategy for the environmentally friendly biological control of rice planthoppers in transgenic plants.

IMPORTANCE In Asia, rice is an important staple food, whose production is threatened by rice planthoppers. To date, no effective *Bacillus thuringiensis* (Bt) protein has been shown to have activity against rice planthoppers. We cloned two Bt toxin genes from Bt strain 1012 that showed toxicity against small brown planthoppers (*Laodelphax striatellus*) and white-backed planthoppers (*Sogatella furcifera*). To our knowledge, the proteins encoded by the *cry64Ba* and *cry64Ca* genes are the most efficient insecticidal Bt Cry proteins with activity against hemipteran insects reported so far. Cry64Ba and Cry64Ca showed no toxicity against some lepidopteran or coleopteran pests. These two proteins should be able to be used for integrated hemipteran pest management.

KEYWORDS *Bacillus thuringiensis*, planthopper, hemipteran pests, insecticidal toxins

Bacillus thuringiensis (Bt) is a common insect pathogen that is widely distributed in various ecological niches, including, but not limited to, water, soil, insects, and plant surfaces (1). The main virulence factors of Bt are insecticidal crystal proteins (ICPs) including Cry and Cyt toxins that are produced during the sporulation stage (2). When insects ingest the ICPs, the crystals are solubilized in the alkaline midgut lumen and are activated by gut proteases to yield mature toxins. The activated toxins bind with receptors located on the plasma membrane of the midgut epithelium of the target insects, resulting in pore formation, disturbance of the osmotic balance, and cell lysis,

Received 10 September 2017 **Accepted** 6 November 2017

Accepted manuscript posted online 17 November 2017

Citation Liu Y, Wang Y, Shu C, Lin K, Song F, Bravo A, Soberón M, Zhang J. 2018. Cry64Ba and Cry64Ca, two ETX/MTX2-type *Bacillus thuringiensis* insecticidal proteins active against hemipteran pests. Appl Environ Microbiol 84:e01996-17. https://doi.org/10.1128/AEM.01996-17.

Editor Janet L. Schottel, University of Minnesota

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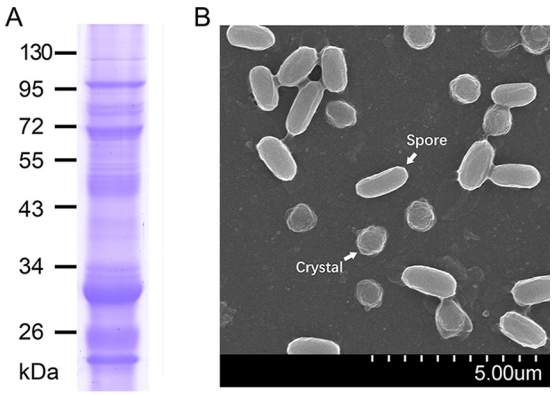

FIG 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (A) and scanning electron micrograph (SEM) (B) analyses of spores and crystal mixtures from Bt strain 1012 are shown.

finally causing insect death (3). Due to the high specificity of Cry toxins, these proteins have been shown to be safe for humans or other vertebrates. Thus, some Bt strains have been used as biological pesticides and some *cry* genes have been employed for the construction of transgenic crops that resist insect attack (4).

Cry toxins with considerable insecticidal activity against lepidopteran, dipteran, and coleopteran pests have been described. However, hemipteran insects are not particularly susceptible to Bt toxins (5). To date, only a few examples of poorly active Bt strains or Bt toxins have been reported (6–8). Among these, we can mention the Cry51Aa2 toxin (Cry-ETX/MTX type) that showed a median lethal concentration (LC_{50}) of 72.9 μg/ml against *Lygus hesperus* (6) and the Cry41Aa-related protein with an LC_{50} of 32.7 μg/ml against *Myzus persicae* (7). On the other hand, engineered Bt toxins with improved toxicity to sap-sucking pests have been described. The addition or replacement of a pea aphid gut-binding peptide in specific loop regions of Cyt2Aa effectively promoted the toxicity of this hybrid toxin against *Acyrthosiphon pisum* with an LC_{50} of 9.55 to 28.74 μg/ml, which was significantly lower than the LC_{50} of the wild type Cyt2Aa ($>$150 μg/ml) (5). A similar strategy was applied for the molecular modification of Cry1Ab, retargeting its toxicity against *Nilaparvata lugens* (brown planthopper) (9). Another example is the Cry51Aa2 variant obtained by combinatorial and saturation mutagenesis that resulted in high toxicity against *Lygus* spp. (LC_{50} of 0.3 to 0.85 μg/ml). This toxin had been expressed in cotton plants, causing an effective decrease of the *Lygus* spp. populations in field trials (10). All of these cases fully demonstrated the potential of Bt proteins for the control of hemipteran pests.

Here, we report novel features of two Bt Cry toxins, Cry64Ba and Cry64Ca, which were previously identified from Bt isolates showing toxicity against HepG2 cancer cells (11). We cloned both genes and confirmed that Cry64Ba and Cry64Ca proteins expressed together in an acrystalliferous Bt strain showed high insecticidal activity against rice planthoppers but no cytotoxicity against HepG2 cells. To our knowledge, these toxins are the most toxic Bt Cry proteins against hemipteran pests described so far.

RESULTS

Isolation of Bt strain 1012 and draft genome sequence. Screening of Bt strains for toxicity against hemipteran insects identified Bt strain 1012 (given the internal lab code IPPBIOTSUC1012), whose crude spore/crystal protein extract (Fig. 1A) had high insecticidal activity against *Laodelphax striatellus* (small brown planthopper). Scanning electron microscope observation of crystals produced by Bt 1012 revealed amorphous crystals (Fig. 1B). In order to identify the virulence factors produced by Bt 1012, genomic DNA was isolated and sequenced by a high-throughput sequencing technology. After the processing of raw sequence data, a total of 5,688,491 nucleotide base pairs (G+C content, 34.95%) constituted 39 scaffolds which were sized 5.51 MB, and 6,063 protein-coding sequences were predicted. Draft genome annotation using a local

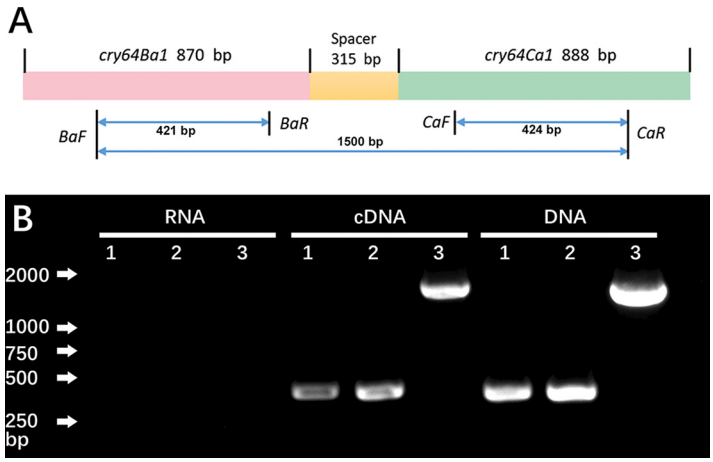

FIG 2 Transcriptional analysis of *cry64Ba* and *cry64Ca*. (A) Shown here is a schematic diagram of distributions of *cry64* genes and primers used for transcription analysis. (B) The electrophoresis pattern of the RT-PCR analysis for the *cry64* operon is shown. Lane 1, fragments amplified by BaF/BaR; lane 2, fragments amplified by CaF/CaR; lane 3, fragments amplified by BaF/CaR, a PCR product that covered the gap between *cry64Ba* and *cry64Ca*. DNase-digested total RNA was used as the negative control while genomic DNA was used as the positive control. The cDNA was synthesized from total RNA and used for the transcriptional analysis.

Bt toxin database revealed two putative *cry* toxin genes. The sequences of the two *cry* genes were identical with the previously reported *cry64Ba* and *cry64Ca* genes, respectively (GenBank accession no. KC960014) (11). In addition, we conclude that the *cry64Ba* and *cry64Ca* genes are plasmid encoded since these genes were located at scaff_269 in the genome draft sequence and a BLASTN search revealed that scaff_269 shows 99% identity with plasmid pYGD98 from the *Bacillus thuringiensis* strain YGd22-03.

Molecular characterization of Cry64Ba and Cry64Ca. The lengths of the *cry64Ba* and *cry64Ca* genes were 870 bp and 888 bp, respectively; *cry64Ba* and *cry64Ca* encoded proteins of 289 and 295 amino acids, respectively, with deduced molecular masses of 31.5 and 32.7 kDa, respectively, which agree with the protein bands observed in the spore/crystal suspension (Fig. 1A). It is worth mentioning that the two genes were adjacent to each other, with a 315-bp sequence gap between them (Fig. 2A). There is a stop codon after the *cry64Ba* gene and an initiation codon before the *cry64Ca* gene. The upstream sequences of *cry64Ba* and *cry64Ca* had predicted −10 and −35 promoter regions, respectively. A sequence (TTA CAT GTT TGT AAA ATA TGT GA) with rho-independent terminator features was found downstream of the stop codon of *cry64Ca* with a predicted stem-loop structure (ΔG° of −25.5 kJ/mol) followed by 10 A-T base pairs. Nevertheless, no apparent terminator sequences were found at the spacer sequence between the *cry64Ba* and *cry64Ca* genes, suggesting that these two genes are cotranscribed as a single operon. RT-PCR experiments were performed to determine whether the two genes were transcribed as a single transcript. The results showed that both genes were detected as a single PCR product from a cDNA sample synthesized from total RNA of Bt 1012 when the upstream primer of *cry64Ba* (primer BaF) and the downstream primer of *cry64Ca* (primer CaR) were used (Fig. 2B, lane 3). These data indicate that the two genes are located in the same transcription unit.

The Cry64Ba and Cry64Ca proteins shared 51% amino acid sequence identity with each other. Cry64Ca shared 45%, 36%, and 31% identity with Cry64Aa1, Cry33Aa1, and Cry15Aa1, respectively. A conserved ETX/MTX2 domain (PF03318) was found located at positions 57 to 270 or 33 to 266 in the Cry64Ba and Cry64Ca proteins, respectively. The ETX/MTX2 domain is a member of the Aerolysin_ETX clan (CL0345 [http://pfam.xfam.org/clan/Aerolisin_ETX]). Multiple sequence alignments with different Cry-ETX/MTX-type Bt toxins revealed an amphipathic transmembrane β-hairpin domain flanked by two serine-rich and threonine-rich hydrophilic regions in both Cry64Ba and Cry64Ca (Fig. 3A). Phylogenetic analysis showed limited homology with the related Cry se-

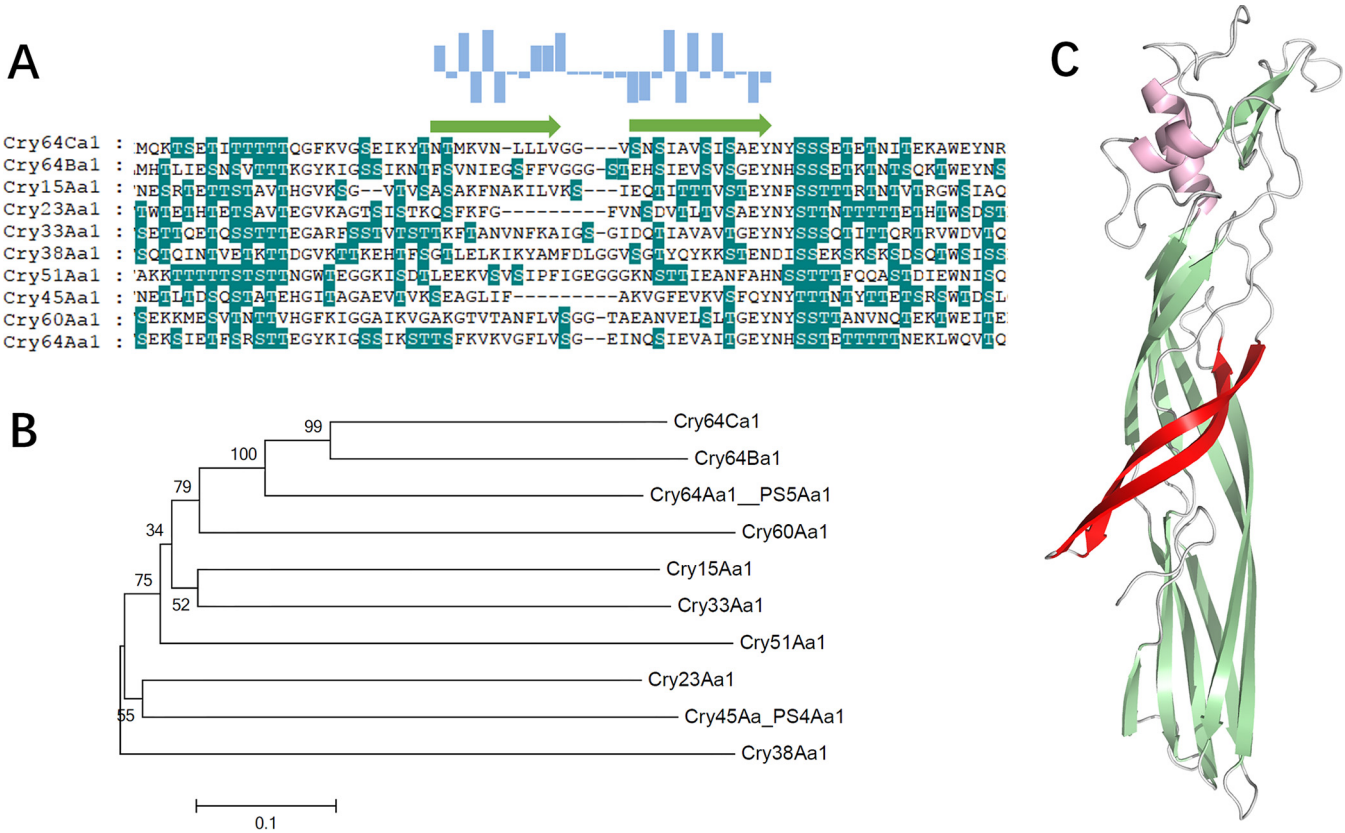

FIG 3 Sequence analysis of Cry64Ba and Cry64Ca proteins. (A) Multiple sequence alignments of the putative transmembrane domains (TMDs) of the Cry-ETX/MTX-like Bt toxins are shown. The putative transmembrane domain was predicted by structure simulation and is indicated by a green arrow. The hydropathy profile plot of the Cry64Ba TMD is exhibited above the amino acid sequences. The hydrophilic/hydrophobic amino acids were referred to Kyte-Doolittle methodology. Serine and threonine residues in these regions are shown in blue. (B) A phylogenetic tree of Cry proteins containing the ETX/MTX2 domain is shown. The phylogenetic tree was constructed by MEGA 7 using a neighbor-joining method with a bootstrap of 1,000 replications. (C) The simulated spatial 3D structure of Cry64Ba is shown. Cry51Aa2 (PDB code 5HD2) was selected as a model for homology modeling in SWISS-MODEL (global model quality estimation [GMQE] 0.63). Pink, α-helices; green, β-sheets; red, putative transmembrane segments. The structure was obtained by using PyMOL.

quences analyzed (Fig. 3B). A three-dimensional (3D) structure model of Cry64Ba was generated by homology modeling according to the Cry51Aa2 3D structure (PDB code 5HD2) (Fig. 3C). Although only 24% amino acid identity between Cry51Aa2 and Cry64Ba is shared, highly similar structures were revealed with a TM score of 0.93 calculated by the TM-align server (https://zhanglab.ccmb.med.umich.edu/TM-align/).

Cry64Ba and Cry64Ca insecticidal activity. To explore if Cry64Ba and Cry64Ca are the virulence factors of Bt strain 1012 against rice planthoppers, expression of the two genes was achieved in Bt strain HD73$^-$ (Fig. 4A). The complete putative operon of Cry64Ba/Cry64Ca as well as the individual genes for each toxin were amplified from the genomic DNA of Bt 1012, cloned into the expression vector, and introduced into strain HD73$^-$. SDS-PAGE analysis of total proteins extracted from each one of the recombinant strains indicated that these genes could produce proteins of the expected size only when they were cloned together in the same plasmid as one operon. We were incapable of detecting protein expression when these two genes were cloned separately (Fig. 4B). The Cry64Ba and Cry64Ca proteins expressed in HD73$^-$ were extracted and purified by ion exchange followed by gel filtration chromatography, to evaluate their molecular masses (Fig. 4C). A single elution absorbance peak of the proteins appeared earlier than the peak of the bovine serum albumin (BSA) control, indicating that the Cry64Ba and Cry64Ca proteins form a multimeric structure with a molecular mass higher than 66 kDa (Fig. 4C). The bioassays were performed with this sample containing the Cry64Ba/Cry64Ca complex since the two individual proteins could not be separated.

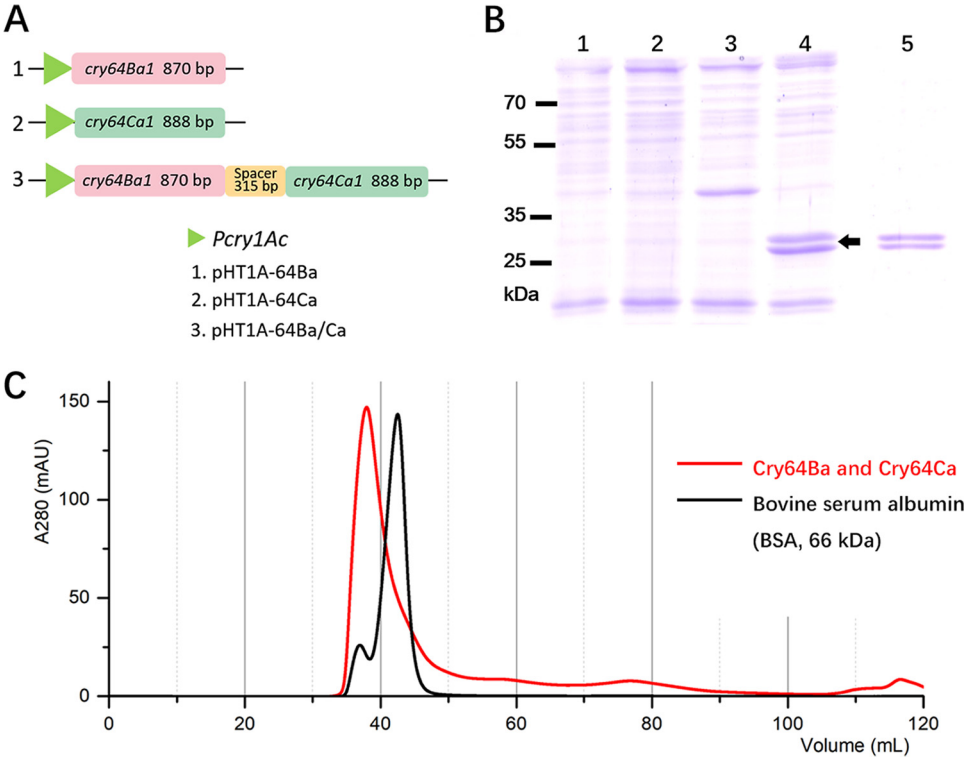

FIG 4 Expression and purification of Cry64Ba and Cry64Ca in HD73$^-$. (A) Physical maps of recombinant plasmids containing *cry64Ba* and *cry64Ca* clones. (B) SDS-PAGE analysis of Cry64Ba and Cry64Ca proteins expressed in HD73$^-$ is shown. Lane 1, HD73$^-$; lane 2, HD73$^-$ harboring pHT1A-64Ba; lane 3, HD73$^-$ harboring pHT1A-64Ca; lane 4, HD73$^-$ harboring pHT1A-64Ba/Ca (target bands are marked with an arrow); lane 5, purified Cry64Ba and Cry64Ca proteins. (C) Gel filtration chromatography of purified Cry64Ba and Cry64Ca proteins is shown. BSA (66 kDa) was used as the molecular mass standard.

The toxicity of the Cry64Ba/Cry64Ca complex was evaluated against two hemipteran pests (*L. striatellus* and *Sogatella furcifera*), two lepidopteran pests (*Ostrinia furnacalis* and *Plutella xylostella*), and a coleopteran insect (*Colaphellus bowringi*). No lethal effect or any negative effect on growth or development was induced by Cry64Ba/Cry64Ca against the two lepidopterans or the coleopteran that was tested (Table 1 and data not shown). In contrast, toxicity against the two hemipteran pests was observed with LC_{50} values of 3.15 and 2.14 μg/ml against *L. striatellus* and *S. furcifera*, respectively (Table 1). The ultrastructure of *L. striatellus* midgut tissue was examined after feeding the insects with a dose of 5.0 μg of Cry64Ba/Cry64Ca per ml of diet. Serious damage of midgut epithelial cell microvilli was observed after the treatment with these toxins (Fig. 5). In the process of Cry64Ba/Cry64Ca protein exposure from 48 to 96 h, the damage gradually became more serious. The vacuolization of the cytoplasm was obvious at 72 h while the microvillus matrix was completely destroyed at 96 h, in contrast with the control insects that were fed with artificial diet (Fig. 5).

TABLE 1 Toxicity of purified Cry64Ba-Cry64Ca against five agricultural pests

Species	LC_{50}	95% confidence interval[a]	Slope (SE)
Laodelphax striatellus	3.15 μg/ml	2.12–3.80	3.02 (0.26)
Sogatella furcifera	2.14 μg/ml	1.18–3.15	2.89 (0.32)
Ostrinia furnacalis	>300 μg/g	ND	ND
Plutella xylostella	>300 μg/g	ND	ND
Colaphellus bowringi	>300 μg/g	ND	ND

[a]ND, not detected. No lethal effect and weight inhibition was observed under 300 μg/g Cry64Ba-Cry64Ca protein against *O. furnacalis*, *P. xylostella*, and *C. bowringi*.

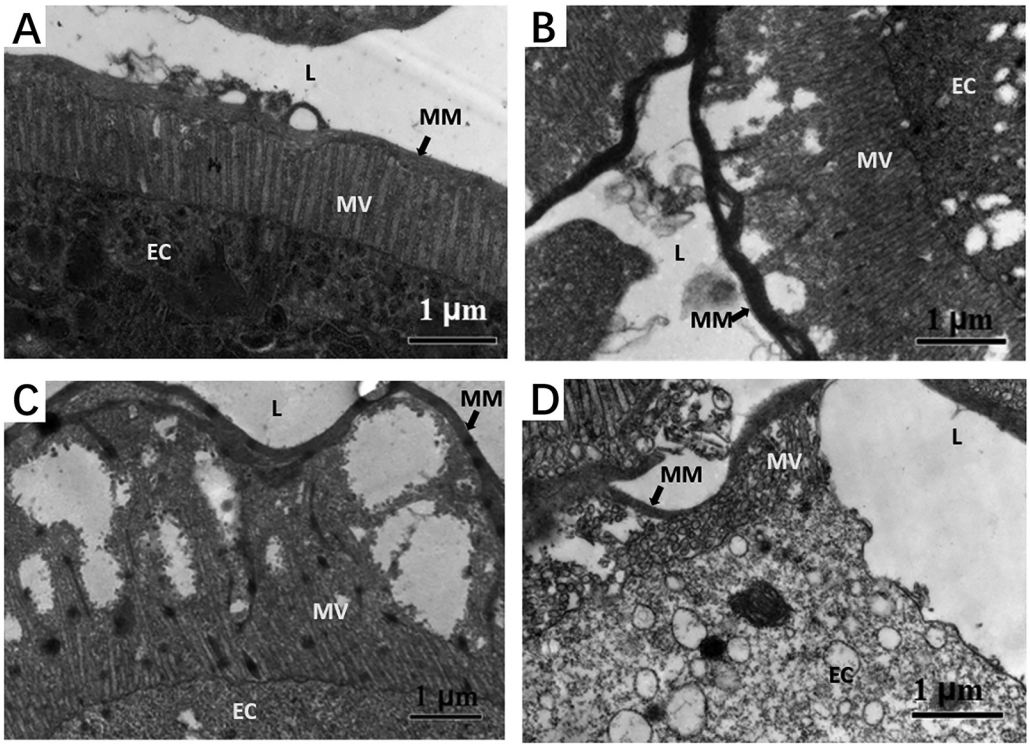

FIG 5 Histopathological effect of Cry64Ba/Cry64Ca in the midgut of *L. striatellus*. (A) Midgut tissue section of control group *L. striatellus* fed on pure artificial diet (96 h) is shown. Midguts from *L. striatellus* fed on 5.0 μg/ml Cry64Ba/Cry64Ca for 48 h (B), 72 h (C), and 96 h (D) are shown. L, lumen; MV, microvillus; EC, gut epithelial cell; MM, microvillus matrix.

Cytotoxicity of Cry64Ba/Cry64Ca complex against HepG2 and red blood cells. Previously, it was reported that solubilized crystal proteins produced by five Bt strains that were isolated from samples directly collected from the rectums of cattle showed toxicity against HepG2 cells (11). Genomic analysis of these strain allowed the identification of the *cry64Ba* and *cry64Ca* genes, and the authors concluded that HepG2 toxic component in these strains corresponds to the Cry64 proteins (11). We analyzed the cytotoxicity of Cry64Ba/Cry64Ca purified proteins from the transformed HD73$^-$ strain against HepG2 cells. Our data showed less than 10% cell mortality when HepG2 cells were exposed to 10 μg of Cry64Ba/Cry64Ca per ml while 87% cell viability was inhibited by 10 μmol/liter doxorubicin. In addition, hemolysis was also evaluated and showed no effect on red blood cells at the highest tested concentration of Cry64Ba/Cry64Ca (100 μg/ml).

Binding of Cry64Ba/Cry64Ca proteins to brush border membranes from *L. striatellus*. Saturation and competition binding assays were performed to estimate if these two proteins have specific binding sites on brush border membranes (BBMV) from *L. striatellus* nymphs. Cry64Ba/Cry64Ca showed dose-dependent binding with the BBMV. Binding saturation occurred at a concentration of around 300 nmol/liter Cry64Ba/Cry64Ca, indicating a limited number of binding sites on the BBMV (Fig. 6A). Cry64Ba/Cry64Ca competed in binding of the fluorescein DyLight 488-labeled Cry64Ba/Cry64Ca protein in homologous competition assays, suggesting specific binding of Cry64 proteins to BBMV from *L. striatellus*. In contrast, no competition was observed when Cry1Ac was used as a competitor (Fig. 6B).

DISCUSSION

Cry proteins are crucial virulence factors of Bt strains. They have been subdivided into several groups, such as three-domain, ETX/MTX-like, and Bin-like toxins (12). Here, we report two ETX/MTX type Cry proteins from Bt strain 1012. The Cry64Ba and Cry64Ca proteins presented here were successfully expressed in Bt HD73$^-$ only when they were

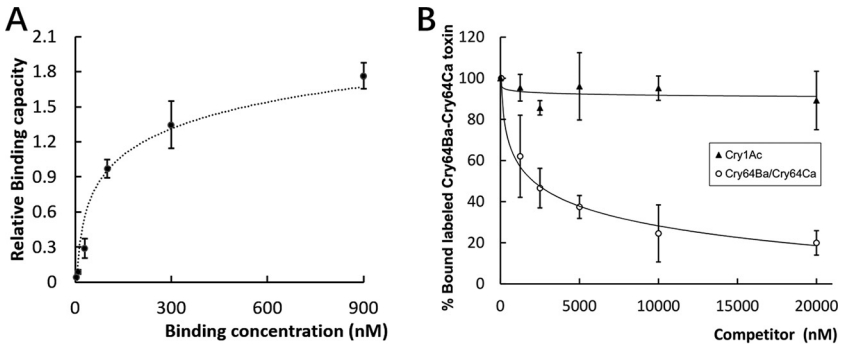

FIG 6 Binding assay of fluorescein-labeled Cry64Ba-Cry64Ca to BBMV of *L. striatellus*. (A) Saturation binding assay of Cry64Ba/Cry64Ca to *L. striatellus* BBMV is shown. A fixed amount of BBMV proteins (10 μg) was used to bind Cry64Ba/Cry64Ca (0 to 900 nmol/liter). (B) Competition binding assay of Cry64Ba/Cry64Ca to *L. striatellus* BBMV is shown. The binding of 50 nmol/liter labeled Cry64Ba/Cry64Ca to *L. striatellus* BBMV was tested with increased concentrations of unlabeled Cry64Ba/Cry64Ca or Cry1Ac as a competitor.

cloned simultaneously as one operon. The coexpression protein product (Cry64Ba/Cry64Ca) showed efficient and high insecticidal activity against important sap-sucking pests (Table 1). It is unclear why the two components are essential for their high expression. One possibility is that the cotranscription of these two genes may promote the stability of the mRNA. Another possibility is that the interaction of the two proteins may stabilize their physical and chemical properties or confer resistance to endogenous protease digestion. Gel filtration chromatography showed that both Cry64Ba and Cry64Ca proteins form a complex with a molecular size higher than 66 kDa (Fig. 4). Further investigations are needed to reveal the details of the regulation and expression of the Cry64Ba and Cry64Ca proteins.

Since it was not possible to separate these proteins, it still remains to be determined if the two proteins have toxicity separately or if they act as a binary toxin in which both components are necessary for toxicity. A bioassay performed against different insect orders indicated that Cry64Ba/Cry64Ca proteins showed no toxicity against the lepidopterans *Ostrinia furnacalis* and *Plutella xylostella* or the coleopteran *Colaphellus bowringi* (Table 1). However, Cry64Ba/Cry64Ca showed significant toxicity against the rice planthoppers *Laodelphax striatellus* and *Sogatella furcifera* (Table 1). The data indicate that the Cry64Ba/Cry64Ca proteins have the highest toxicity against hemipteran pests reported so far compared to other Bt Cry proteins.

Members of the family of Cry-ETX/MTX proteins show variable toxicity against different insect species. The Cry15, Cry23, Cry33, and Cry38 toxins are toxic to coleopteran larvae, while other Cry-ETX/MTX proteins such as the Cry60 toxin are active against dipteran larvae (13, 14). The Cry51 toxin is toxic against both coleopteran and hemipteran larvae (15). Cry45Aa and Cry64Aa belong to this family, but they are also named Parasporin4A1 and Parasporin5A1 due to their toxicity against human cancer cells (15–17). Interestingly, some Cry-ETX/MTX toxins are accompanied by additional proteins. For example, the Cry15Aa toxin is associated with a 40-kDa protein, but Cry15Aa alone showed hemolytic and insecticidal activities against *Manduca sexta*, indicating that the 40-kDa protein is not required for toxicity (18, 19). Cry33 is produced in the same operon with a noninsecticidal 32-kDa protein named NT32KD (20). Cry23 associates with Cry37, forming a binary toxin (PDB code 4RHZ) that shows pore formation activity (21). Other examples of Cry proteins that are expressed in the same operon with additional proteins are the Cry16 and Cry17 proteins, which are expressed with two additional proteins named Cbm17.1 and Cbm17.2 in *Clostridium bifermentans* (22). It was shown that individual Cry16 and Cry17 proteins are not active against mosquitoes, while mosquitocidal activity was observed when the four proteins were expressed together, indicating that the four proteins are required for toxicity against *Aedes aegypti* (22).

At the primary sequence level, the Cry-ETX/MTX toxins share limited identity, but at the structural level, all of these proteins showed significant similarities to proteins from the aerolysin-like β-PFT group. Cry-ETX/MTX showed high similarities to the tail region of other aerolysin-like β-PFT proteins, and it has been proposed that the tail region of aerolysin-like β-PFT proteins is responsible for oligomerization and pore formation (23). In contrast, Cry-ETX/MTX showed lower structural similarities to the head region of aerolysin-like β-PFT proteins. The head region of the aerolysin-like β-PFT proteins is responsible for binding with receptors and confers toxin specificity (24). Sequence analysis and the model of the three-dimensional structure of Cry64Ba indicated that Cry64 proteins also belong to the aerolysin-type β-PFT family.

In a previous study, the *cry64Ba* and *cry64Ca* genes were found in five Bt strains that showed toxicity against HepG2 cells (11). Our data suggest that the cytotoxicity of the five Bt strains against HepG2 cells was not caused by the Cry64Ba and Cry64Ca toxins, since we found that the purified cloned proteins were not toxic to HepG2. Interestingly, Cry64Ba and Cry64Ca were not hemolytic, in contrast to most proteins of the aerolysin family (25).

In summary, we isolated Bt strain 1012 with high toxicity against rice planthoppers and identified the virulence factors as the Cry64Ba/Cry64Ca proteins. Pure Cry64Ba/Cry64Ca proteins showed efficient and highly specific insecticidal activity against *L. striatellus* and *S. furcifera*. These Cry proteins could provide an alternative approach for integrated pest management of rice planthoppers.

MATERIALS AND METHODS

Strains and plasmids. Bt strain 1012 (IPPBIOTSUC1012, China General Microbiological Culture Collection Center [CGMCC] strain 10783) was isolated from a soil sample from Baiwangshan Forest Park in Beijing and preserved at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPPCAAS). Scanning electron microscopy of the spore-crystal mixture of Bt strain 1012 was performed following the process described by Shu et al. (26). The spore-crystal mixtures were washed in sterile distilled water and then resuspended in 5 volumes of solubilization buffer (50 mmol/liter Na_2CO_3-$NaHCO_3$ and 50 mmol/liter EDTA; pH 10.0) for 1 h at 37°C. Then 2× Laemmli sample buffer was added, and the samples were boiled for 5 min, centrifuged at 12,000 × *g* for 5 min, and loaded onto a 12% SDS-polyacrylamide gel. *Escherichia coli* DH5α was used for common transformation. *E. coli* SCS110 was used to produce the nonmethylated recombinant plasmids that were used for Bt transformation. HD73$^-$, a Bt acrystalliferous strain, was used as the host strain for protein expression. The vector pHT-1A, which contains the pHT315 sequence, was used as the expression vector (27) linked with the promoter of *cry1Ac*. The *attP* site and *ccdB* gene were integrated into the plasmid for Gateway reaction and positive clone selection. All *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) medium. Bt strains were incubated at 30°C with shaking in 1/2 LB medium (0.5% NaCl, 0.5% tryptone, and 0.25% yeast extract). Erythromycin (Erm) and ampicillin (Amp) were used for bacterial selection at concentrations of 50 and 100 μg/ml, respectively. Erm was a selection marker for Bt strains, and Amp was a selection marker for *E. coli* strains.

Preparation of genomic DNA, DNA sequence, and computational analysis. Genomic DNA was extracted from Bt 1012 following the method described by Song et al. (28). The genome sequencing was performed on an Illumina HiSeq 2500 platform. The short sequence reads were assembled with SOAPdenovo (29). The open reading frames (ORFs) were predicted using GeneMark (30). A local Cry toxin database was established by available quaternary rank model amino acid sequences in the list of delta-endotoxins on the Bt toxin nomenclature website (15) and used for the Bt 1012 genome toxin gene annotation by the BLASTP program with a cutoff E-value of 10^{-6}. The phylogenetic tree was constructed by using the distance-based neighbor-joining method with MEGA 7.0 (31). SWISS-MODEL workspace was used to model the three-dimensional structure of Cry64Ba (32). Multiple alignments were performed by using Clustal W analysis (33). Protein structure comparison was performed by using the TM-align algorithm (34). The hydrophilic/hydrophobic amino acids were indicated by the Kyte-Doolittle method (35).

Transcriptional analysis. The promoter and terminator predictions were performed using BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) and FoldRNA (http://www.softberry.com/berry.phtml?topic=foldrna&group=programs&subgroup=rnastruct) on SoftBerry online programs. Total RNA was extracted from Bt 1012 after 48 h cultivation on LB agar plates using TRIzol following the manufacturer's instructions. DNase digestion was carried out to avoid contamination with genomic DNA, followed by the phenol-chloroform method to isolate the total RNA. cDNA was synthesized using PrimeScript RTase (TaKaRa, China) according to the manufacturer's instruction. Primers used to detect the transcription units are shown in Table 2. The thermal program of PCR was as follows: incubation at 95°C for 2 min and then 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min with a final extension at 72°C for 10 min.

Gene cloning of Bt toxins from strain 1012. The *cry64* genes were amplified from Bt 1012 genomic DNA using specific primers (Table 2) with an *attB1* site (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG

TABLE 2 Primers used for transcription analysis and gene cloning

Primer purpose and name	Sequence
Transcription analysis	
BaF	CGTTTAGCGTAAACATTGAGGGAAG
BaR	CCTTCTACCTGTACACCTCCCCCATTC
CaF	GATACTAGTGTTCCACAATCTATAAC
CaR	CAGTCCACGATCTATTGTTGTAGTCAG
Gene cloning	
Cry64BaF	AATAACGGCTTAAATATAATATACG
Cry64BaR	TTAAATATCAATAATAGATCCATCT
Cry64CaF	ATTTGCCTATAAAGTATTTTTTAAA
Cry64CaR	GTCTTACATTAACGATGCTGGTAT

CT-3′) and an *attB2* site (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3′) added to the 5′ end of the forward and reverse primers, respectively. The fragment containing both *cry64Ba* and *cry64Ca* was amplified with the Cry64BaF and Cry64CaR primers (Table 2). Gateway BP recombination reactions between *cry* gene fragments and pHT-1A were performed using BP Clonase enzyme mix (Thermo Fisher, USA) following the manufacturer's instruction. The recombinant products were transformed into *E. coli* DH5α. Positive clones were selected and verified by a 3730xl DNA sequencer (Applied Biosystems, USA).

Protein expression and purification. The recombinant plasmids were demethylated by transformation into *E. coli* strain SCS110. Purified plasmid from this strain was electroporated into Bt acrystalliferous strain HD73$^-$ for protein expression. Positive clones were selected by PCR. The method for protein extraction from recombinant strains was described by Zhou et al. (36). The recombinant strains were grown in 1/2 LB until 50% of the crystal was released. The spores and crystals were collected by centrifugation at 8,000 $\times$ *g* for 10 min at 4°C. After being washed with 1.0 mol/liter NaCl and distilled water, the mixture was resuspended in solubilization buffer incubated on ice (shaking at 100 rpm) for 1 h. The supernatant was collected by centrifugation at 12,000 $\times$ *g* for 10 min and then adjusted to pH 5.0 using 4.0 mol/liter sodium acetate-hydrogen acetate and kept at 4°C for 1 h. The pellet was harvested by centrifugation at 13,000 $\times$ *g* for 15 min, washed with distilled water twice, and dissolved in 50 mmol/liter Na_2CO_3 (pH 10). To purify the Cry64Ba/Cry64Ca proteins, an ion-exchange chromatography was conducted with HiTrap Q HP (GE Healthcare, USA). Target proteins were eluted with a linear ion strength gradient of 0 to 0.5 mol/liter NaCl included in the protein buffer, followed by gel filtration chromatography with HiPrep 26/60 Sephacryl S-100 HR columns (GE Healthcare). BSA was used as a molecular weight standard in gel filtration chromatography. Tris-HCl (20 mmol/liter; pH 8.0) was used as the mobile phase, with a flow rate of 0.5 ml/min. The protein concentration of the purified Cry64Ba/Cry64Ca complex was determined by ImageJ using BSA as a standard.

Bioassays. Liquid artificial diet for planthopper feeding was prepared according to Fu et al. (37). Based on the bioassay method described by Wang et al. (38), the container used in the experiment was improved in two points. First, we used a polyethylene terephthalate (PET) cylindrical bottle 7 cm in height and 4 cm in diameter with a screw cap. Two holes were opened in the bottle. The hole on the sidewall had a diameter of 6 mm and was covered with micropore tape pasted inside the bottle for ventilation; the other hole was on the cap to support the membrane-feeding package and had a diameter of 3 cm. Second, 1 ml 2% agarose gel was put on the bottom of the bottle to maintain a suitable humidity for rearing *L. striatellus*. Twenty *L. striatellus* nymphs (3rd instar) were put into the bottle. The liquid diet was replaced every 2 days. Mortality was recorded after 6 days of bioassay. The bioassay method for *S. furcifera* was same as above.

One-dose bioassay tests of the Cry64Ba/Cry64Ca protein (300 μg/g) against *O. furnacalis* (39), *P. xylostella* (40), and *C. bowringi* (41) were conducted as described. The newly hatched *O. furnacalis* moths were exposed to the semiartificial diet containing Cry64Ba/Cry64Ca toxin that was dispensed into 48-well trays. One neonate was added per well with 0.15 g testing medium and covered with a piece of wet paper under the lid. Seven days later, mortality was recorded. The leaves of cabbage (for *P. xylostella*) and rape (for *C. bowringi*) were cut into 6-cm disks, dipped into Cry64Ba/Cry64Ca solution for 10 s, air dried, and placed onto a plastic petri dish with a wet filter on the bottom to maintain humidity. Ten larvae were placed on each disk. Mortality was recorded at 48 h. All tested insects were reared at 27°C. Protein buffer (Tris-HCl at 20 mmol/liter; pH 8.0) was used as a negative control. Bioassays were repeated three times. The LC_{50} and 95% confidence limits (CL) were calculated by SPSS 21.0 with probit analysis.

Binding assay of Cry64Ba/Cry64Ca with BBMV. The whole bodies of 3rd instar *L. striatellus* nymphs were used for BBMV isolation by the differential precipitation method using $MgCl_2$ as reported (42). Quantification of total BBMV protein was done by the Bradford method (43). Cry64Ba/Cry64Ca protein (1 mg) was labeled with fluorescein DyLight 488 *N*-hydroxysuccinimide ester according to the manufacturer's instructions (Thermo Fisher, USA). Different concentrations (from 0 to 900 nmol/liter) of Cry64Ba/Cry64Ca were added to a mixture of 10 μg BBMV in binding buffer (0.1% BSA, 0.1% Tween 20 in phosphate-buffered saline [PBS]) in a 100-μl final volume and incubated for 1 h at room temperature. Elimination of unbound protein and detection of the amount of binding protein were done as reported by Jiang et al. (44). Briefly, after washing out the unbound protein by centrifugation, pellets containing the BBMV with the bound toxins were suspended in Laemmli sample buffer and analyzed by SDS-PAGE. The gel was scanned to collect green fluorescence signal (488 nm excitation [Ex]/518 nm emission [Em])

in a Typhoon 9410 scanner (GE Healthcare). The relative binding capacity is defined as the ratio of the fluorescence densitometry of binding protein to that of 50 ng labeled Cry64Ba/Cry64Ca. The fluorescence densitometry was read by ImageJ software (http://imagej.nih.gov/ij/) on the scanned image. The homologous competition binding assay was performed as follows: first 0 to 20,000 nmol/liter unlabeled Cry64Ba/Cry64Ca or trypsin-digested Cry1Ac protein was incubated with BBMV for 30 min as previously described, then 50 nmol/liter fluorescein-labeled Cry64Ba/Cry64Ca protein was added and incubated for 1 h. The sample was processed as described above. All of the binding experiments were repeated three times.

Histopathological examination of *L. striatellus* midgut. After 48, 72, and 96 h of toxin exposure of *L. striatellus* to Cry64Ba/Cry64Ca at a final concentration of 5 μg/ml, the midgut tissue was dissected and fixed in 2.5% glutaraldehyde in phosphate buffer (PB) (0.1 mol/liter, pH 7.2) at 4°C overnight. The fixed midguts were washed with PB three times and then postfixed with 1% osmium tetroxide for 1.5 h. Samples were washed and dehydrated in graded acetone and embedded in Spurr embedding resin. Samples were sectioned at a 100-nm thickness and stained with uranyl acetate and lead citrate. Finally, the sections were examined with an HT-7700 electron microscope (Hitachi, Japan).

Cytotoxicity assay. HepG2 cells were cultured and used to test the cytotoxicity of Cry64Ba/Cry64Ca by GenScript (Nanjing, China). Cytotoxicity assays were performed in 384-well microtest plates. Each well contained 40 μl cell suspension including 800 cells. Cry64Ba/Cry64Ca was added in 2-fold serial dilutions with a highest final concentration of 10 μg/ml. After 72 h of incubation, 30 μl CellTiter-Glo (Promega, USA) was added to each well, followed by further incubation for 2 to 3 min at room temperature. Finally, the number of relative light units (RLU) was measured on a PHERAstar microplate reader (BMG Labtech, Germany). Tris-HCl (20 mmol/liter, pH 8.0) was used as a negative control (NC). Doxorubicin (10 μmol/liter) was used as a positive control. Cell growth inhibition was calculated as follows: % of cell growth inhibition = $100\% \times (1 - RLU_{Sample}/RLU_{NC})$.

Hemolysis assays. Cry64Ba/Cry64Ca protein (from 6.25 to 100 μg/ml) dissolved in PBS was mixed with 50 μl of 2% sheep erythrocyte suspension in 0.9% NaCl, and the final volume was fixed to 200 μl. After 1 h of incubation at room temperature, the cells were centrifuged at 1,000 $\times$ *g* for 5 min, 150 μl of the supernatant was collected, and the absorbance (Ab) was measured at 540 nm in a FlexStation 3 microplate reader (Molecular Devices, USA). As the negative control (NC), 0.9% NaCl was used while the positive control (PC) corresponding to 100% hemolysis contained 1% Triton X-100. Percent hemolysis was calculated as follows: $(Ab_{sample} - Ab_{NC})/(Ab_{PC} - Ab_{NC}) \times 100\%$.

Accession number(s). Draft genome sequences of Bt 1012 have been deposited in GenBank under accession no. NTGL00000000. The nucleotide acid sequence data for *cry64Ba* and *cry64Ca* are available in GenBank under accession no. KC960014.

ACKNOWLEDGMENTS

This study was supported by the National Science and Technology Major Project of China (grant 2014ZX0800912B) and National Key R&D Program of China (grant 2017YFD0200400).

We declare no competing interests.

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