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Regular Article

High-throughput screening for novel *Bacillus thuringiensis* insecticidal proteins revealed evidence that the bacterium exchanges Domain III to enhance its insecticidal activity

Ruth Cong, Jing-Tong Hou and Takashi Yamamoto*

Bacillus Tech LLC, Dublin California 94568, USA

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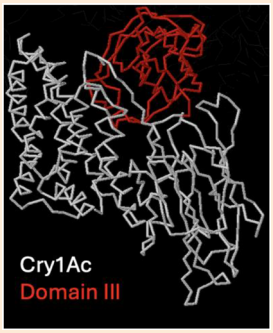
Supplementary material

Approximately 3000 *Bacillus thuringiensis* (Bt) isolates were screened to discover novel three-domain (3D) Cry proteins active against *Helicoverpa zea* (corn earworm). From 400 active isolates found during the primary screening, Cry1Ac and Cry2A, which are known to be active against *H. zea*, were removed using multiplex-primer PCR and high-throughput column chromatography. This process reduced the number of active cultures to 48. DNA segments encoding Domain III of these 48 cultures were amplified by PCR and sequenced. Sequencing revealed two novel Cry1B-type Domain IIIs. Further sequencing of the flanking regions of these domains revealed that one was part of Cry1Bj (GenBank: KT952325). However, the other Domain III lacked Domains I and II. Instead, this Domain III was associated with two open reading frames, ORF1 and ORF2. ORF1 was identified as an ATP-binding protein, and ORF2 as an ATPase, suggesting that Bt exchanges Domain III among homologous Cry proteins.



Keywords: *Bacillus thuringiensis*, Bt, Domain III, high-throughput screening, Cry1Bj, *Helicoverpa zea*.

Introduction

Bacillus thuringiensis (Bt), a rod-shaped bacterium that forms spores, was initially identified in Japan as a highly infectious pathogen of *Bombyx mori* (silkworm).[1] Later, the bacterium was found again in a German town named Thuringia leading to its current name, *B. thuringiensis*.[2] Bt is known for producing a variety of proteins that are highly toxic to certain insects. These insect-toxic proteins are referred to as Cry proteins, such as Cry1A, Cry1B, Cry1C, and so on. These proteins are produced in large quantities during the sporulation stage of Bt and crystallize within the bacterium's cytoplasm. Once the spore matures, the Bt cells lyse, releasing free spores and crystals into the surrounding medium or environment. When insects ingest these released spores and crystals, the crystals are dissolved in the insect's alkaline digestive fluid, releasing soluble Cry proteins. The Cry proteins, which are usually protoxins of 135 kDa, are then activated by proteases in the insect gut fluid to become 65 kDa mature toxins. Some Bt strains produce 65 kDa preactivated Cry proteins such as Cry2 and Cry3. The X-ray structure of the 65 kDa activated Cry proteins (mature toxins) revealed three distinctive domains: Domain I, Domain II, and Domain III.[3] As a result, these Cry proteins are referred to as three-domain (3D) proteins.

Bt Cry proteins bind to insect receptors as a critical step in their insecticidal action. Recent research has identified an ABC transporter as the receptor for the Bt Cry1Aa protein.[4] Specifically, Domain II of the three-dimensional Cry protein attaches to the extracellular loops of the ABC transporter.[5] Although the role of Domain III is not fully understood, it is believed to be involved in binding to a secondary receptor in certain insects, such as *Heliothis virescens* (tobacco budworm).[6] Bt Cry proteins are highly specific to insect species. For example, Cry1Aa is particularly lethal to *B. mori* but relatively ineffective against *H. virescens*, whereas Cry1Ac shows the opposite pattern of toxicity.

* To whom correspondence should be addressed.
E-mail: contact@bacillustechllc.com
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The epithelial cells of the midgut in Bt susceptible insects have various ABC transporters.[7] These transporters, even those sharing the same name such as ABCC2, exhibit diverse amino acid sequences across different insect species. Consequently, transporters are designated by species, such as BmABCC2 for the ABCC2 transporter of *B. mori*. The binding affinity of a Cry protein to a specific ABC transporter is a determining factor for its insect specificity. For instance, Cry1Aa binds with high affinity to BmABCC2, while other Cry1A proteins, like Cry1Ab, show lower affinity for the same transporter.[4] The binding affinity of Cry proteins to ABC transporters within the same family, such as ABCC2, varies among insect species.

Since Bt is highly toxic but specific to only limited insect species (*i.e.*, inactive against non-target organisms such as humans), it has been used in sprayable insecticide formulations as an environmentally friendly agent. Additionally, Bt has been utilized in transgenic (GMO, Genetically Modified Organism) crops such as Bt-cotton, Bt-corn, and Bt-soybean to confer resistance to insects.[8] In 1990, the first transgenic Bt-cotton was produced with Cry1Ac due to its high toxicity to heliothine cotton pests.[9] However, the widespread use of Bt has led to the emergence of Bt-resistant insects, with the first case reported in 1990.[10] Recently, certain populations of *Spodoptera frugiperda* (fall armyworm) have become resistant to Bt-corn expressing Cry1Fa. This resistance is attributed to mutations and deletions in the SfABCC2 transporter to which Cry1Fa binds.[11] Researchers are working to address this resistance problem by identifying a new Bt Cry protein(s) that does not interact (*i.e.*, binds to a different receptor) with the Cry proteins currently used in Bt crops. To discover a new protein, Bt's large-size plasmids were sequenced using the Next Generation Sequencer (NGS).[12] It was established as early as 1981 that Bt *cry* genes are located on the large-size plasmids.[13] With the advent of NGS, as many as 1000 novel sequences encoding Cry-like proteins have been discovered. Nevertheless, it is necessary to determine the insecticidal activities of proteins encoded by those genes. The process of cloning those *cry*-like genes in an expression system individually, isolating the proteins, and assaying them against insects is very time-consuming. Moreover, these proteins need to be tested against a variety of insect species due to the specific insect spectrum of each Cry protein. It appears that only a few of these numerous *cry*-like genes discovered by NGS have shown beneficial activity against commercially significant insect pests. Therefore, we have established a high-throughput screening method to purify and determine the insecticidal activity of the Cry proteins produced by naturally occurring Bt. Using this approach, we screened 3000 Bt isolates and achieved some promising results, as detailed in this report.

In conjunction with the discovery of new Cry proteins, the industry has been working on engineering existing proteins to improve their effectiveness against specific target insects. A review of these engineering projects[14] indicates that one method, domain swapping of 3D Cry proteins, particularly Domain IIIs, has produced commercially significant results. The first successful attempt was made in the mid-1990s by de Maagd *et al.*,[15] in collaboration with Sandoz Agro. This domain modification improved the activity of Cry1Ab against the *Spodoptera* complex, including *Spodoptera exigua* (beet armyworm). Building on this initial success, the industry embraced this approach and developed new proteins such as Cry1A.105,[16] Cry1B.2,[17] and so on. The diverse domain compositions of naturally occurring Bt 3D Cry proteins suggest that Bt is involved in domain exchange, particularly with Domain III. Our research in this report provides evidence supporting Bt's domain exchanges with Domain III of Cry1B proteins. Cry1B binds to ABCB1, which differs from the receptors of Cry1Ab and Cry1Fa (ABCC2) currently used in Bt-corn. The insect specificity of Cry1Bs for *Spodoptera* and *Heliothinae* appears to rely on their Domain III.

Materials and methods

1. Domain phylogenetic trees

The amino acid sequences of Bt Cry proteins were obtained from the list published by the *Bacillus thuringiensis* Cry Protein Nomenclature Committee.[18] The domain junction was determined based on the X-ray structure of Cry3Aa.[3] The alignments of Cry domains were generated using Geneious Prime Sequence Alignment selecting the "global alignment with free end gaps" option and the BLOSUM62 (BLOcks SUbstitution Matrix) cost matrix. The phylogenetic trees were created using Geneious Phylogenetics Tree Builder with the Jukes-Cantor Genetic Distance Model and the neighbor-joining method without an outgroup.

2. High-throughput production of Bt Cry proteins

Bt isolates were grown on LB-agar in 96 multi-well plates until sporulation. These plates were referred to as seed plates. For protein (crystals/spores) production, 96-deep-well plates (2 mL each well) called production plates were prepared with 1 mL of CYS medium and a 3 mm stainless steel (SS) ball in each well. The recipe for CYS medium was previously published.[19] The SS ball facilitates vigorous aeration for Bt during its growth. Bt cultures from the seed plates were transferred to the production plates using 96-pin inoculators. The plates were sealed with Qiagen AirPore Tape and incubated in a New Brunswick Innova 44R at 30°C, 350 rpm for 72 hr until Bt cells sporulated and lysed. Free spores and crystals were collected by centrifugation in an Eppendorf 5810R plate centrifuge at 3200×*g* for 1 hr. The centrifuged spores and crystals were suspended in 100 μL water on a plate shaker and chilled on ice. Crystals were then solubilized by adding ice-cold 100 μL of 4% 2-mercaptoethanol with its pH adjusted to pH 10 using NaOH. The SS balls were removed using a magnetic plate, and then the production plates were centrifuged in an Eppendorf 5810R plate centrifuge at 3200×*g* at 4°C for 1 hr. The supernatant containing solubilized Cry proteins was aspirated using 96-channel pipettes such as the Agilent Bravo or Integra Mini 96 liquid handler and desalted with Sephadex G25 column chromatography in 2 mL 96-deep-well filter plates. For chromatography, the filter plates

with polypropylene or glass-fiber filter such as Whatman Unifilter (cat# 7720-7236) were filled with a 1.8 mL bed volume of super-fine Sephadex G25 suspended in 25 mM Tris-HCl, pH 8 buffer, and 200 μL of Cry protein solution were loaded on the top of the Sephadex gel columns using 96-channel pipettes. The plates were centrifuged at 100×*g* for 30 sec to load the samples, and then, the proteins were eluted by centrifugation at 1000×*g* for 1 min into 96-well receiver plates placed under the filter plates. The final Cry protein samples in 25 mM Tris-HCl, pH 8 were analyzed for purity and concentration using the Caliper LabChip GX protein analyzer. After the protein analysis, the concentrations of Cry protein samples were normalized to 1 mg/mL and assayed against *H. zea* in 96-well plates. If any protein concentration was not high enough to reach 1 mg/mL for bioassay, 10 kDa molecular weight cutoff filters such as Amicon Ultra Centrifugal Filter 10 kDa MWCO (cat# UFC5010) were used to concentrate the proteins. To prepare higher amounts of Bt Cry proteins, multiple copies of the production plates were used and combined during the centrifugation for spore and crystal samples. This process of high-throughput Cry protein purification is illustrated in Supplemental Fig. S1.

To produce Cry proteins from isolated genes (*e.g.*, *cry1Bj*), the gene was cloned into a *cry* gene-negative Bt strain (*i.e.*, all plasmids containing *cry* genes were cured) using a *Bacillus-E. coli* shuttle vector as previously described by us[20] to create a Bt culture expressing the cloned *cry* gene. The protein was then isolated individually using a method similar to high-throughput production.

3. Removing Cry1Ac and Cry2As from the screening samples

Cry1Ac is known for its high activity against *H. zea*. The *cry*-gene contents of the 400 Bt cultures that showed activity against *H. zea* during the primary screening were analyzed using multiplex-primer PCR following the method described by Kalman *et al.*[21] Bt isolates containing the *cry1Ac* gene were removed from the 400 Bt cultures for the subsequent round of screening. These *cry1Ac*-free isolates were then re-arrayed in a 96-well plate to create a new seed plate from which Cry proteins were prepared.

Cry2A is also active against *H. zea*. Studies have indicated that Cry2A specifically binds to ABCA2 to kill this insect.[22] Therefore, the Cry2A protein was removed from the Cry protein samples prepared from the new seed plate through DEAE-agarose column chromatography for the next screening round. This ion-exchange chromatography was conducted in 96-well filter plates using a method similar to the one described for Sephadex G25. DEAE-agarose was suspended in 10 mM Tris-HCl, pH 8, and packed in the filter plates up to a 500 μL bed volume. The Cry proteins, which had been solubilized in an alkaline solution followed by neutralizing their pH, were loaded onto the DEAE agarose columns. Since Cry2A is an alkaline protein,[23] it passed through the DEAE agarose at pH 8. The Cry1 proteins retained in the DEAE agarose were eluted with 0.1 N NaOH and neutralized with a small amount of 1 M Tris-HCl, pH 7. These Cry2A-free samples were then assayed against *H. zea*.

4. High-throughput insect bioassay

The insecticidal activity of Cry protein samples was determined in 96-well plates through a feeding assay as described by Cong *et al.*[20] The assay was conducted with 10 μL of protein samples mixed with 40 μL of artificial insect diet prepared with Southland Premix formulated for Lepidopteran insects (Southland Product Inc., Lake Village, Arkansas, U.S.A.). The diet was made of agarose with a low gelling temperature to ensure that Cry proteins were not exposed to high heat during mixing. The diet was kept molten in a 50°C reservoir mounted on the deck of a liquid-handling robot such as the Agilent Bravo or Hamilton Microlab Nimbus. Assay plates (96-shallow-well plates) with 10 μL Cry protein sample pre-filled in each well were placed on the shaker platforms of the liquid handling robot. Immediately after pipetting the diet into the assay plate, the shaker was activated to evenly mix the proteins into the diet before it solidified. By uniformly mixing the protein samples into the diet, the insects were exposed to evenly concentrated sample proteins. Several neonate insect larvae were placed in each well with a soft brush, and the assay plates were sealed with clear film using the Agilent PlateLoc Thermal Microplate Sealer. After punching a few pinholes through the sealing film for air exchange, the plates were incubated at 28°C for 4 days.

The response of insects to the proteins was determined using a numerical scoring system ranging from 0 to 3 based on the size and mortality of the larvae in each well. A score of 0 was given if there was no response (*i.e.*, normal growth). A score of 1 was assigned when insect growth was slightly stunted. A score of 2 indicated that the larvae were severely stunted (close to neonate size) but still alive. A score of 3 was given when all or most of the larvae in the well were dead. The percent response for each treatment was calculated by dividing the total score of replicating wells, usually 6, for each treatment by the maximum possible scores for 6 replications. For example, if one treatment (*i.e.*, one sample, one dose) had 6 replicating wells, the total highest possible score (*i.e.*, 100% response) would be 3×6=18.

5. PCR amplification of Domain III

DNA fragments encoding Domain III were amplified using PCR from 48 Bt cultures that were identified as active against *H. zea* during the secondary screening of the 96 Bt cultures. The PCR primers were designed based on sequences of two homologous regions found in all known Cry1-type Cry proteins as shown in Supplemental Fig. S2 and listed in Table 1. These homologous regions were identified at the amino and carboxyl boundaries of Domain III to Domain II and the protoxin region, respectively.

For PCR, DNA samples were extracted from Bt cells collected in the early stage of growth in LB broth (2 to 3 hr at 30°C after inoculation), and DNA was isolated from the cells using the Qiagen Plasmid Kit with a slight modification. Since Bt *cry* genes are located on large plasmids, they may be denatured in the alkaline lysis buffer provided in the Qiagen kit. Therefore, the lysis buffer was substituted with 1% SDS without NaOH. For PCR, an annealing temperature as low as 37°C was used for

Table 1. List of primers designed for PCR amplification of DNA fragments encoding Cry1 Domain III

5 Forward Primers	4 Reverse Primer (Reverse/complement)
5′-GGACCAGGATTTACAGGAGG	5′-GTAAACAGCTCATTCACC
5′-GGCCCAGGATTTACAGGAGG	5′-GTAAACAGCGCATTCACC
5′-GGTCCTGGATTTACAGGCGG	5′-GTAAACAGAGCATTCACC
5′-GGGCCCGGGTTTACGGGAGG	5′-GTAAACAGGGCATTCACC
5′-GGCCCAGGATTTACTGGTGG	

The primers were obtained from two homologous regions of all known *cry1* genes that delineate Domain III.

2 min to increase the likelihood of amplifying novel *cry* genes. Otherwise, a standard denaturation step of 1 min at 94°C and an extension step at 72°C for 3 min were employed. The PCR products were analyzed using agarose gel electrophoresis. DNA bands amplified by PCR, which are expected to encode Domain III, were extracted from the gel, and cloned into the pCR-TOPO vector using the TOPO TA Cloning Kit from Thermo Fisher Scientific. Nucleotide sequencing of 48 TOPO clones from each Bt isolate was conducted by DNA sequencing service providers such as Elim Biopharm (Hayward, California, U.S.A.). Each DNA sample was sequenced in both directions.

6. *Sequencing flanking regions of DNA fragments encoding Domain III*

The DNA sequences flanking the PCR-amplified DNA fragment encoding Domain III were determined using the Takara Bio (Clontech) GenomeWalker Kit following the manufacturer's instructions. Initially, DNA extracted from Bt was digested with restriction enzymes and then ligated to the GenomeWalker adapter to create GenomeWalker libraries. Subsequently, the DNA in these libraries was amplified through PCR using two primers, one for the GenomeWalker adapter and the other for a sequence within the Domain III coding region. The PCR-amplified DNA fragments were then separated by agarose gel electrophoresis and sequenced. Finally, the sequences of the PCR-amplified DNA fragments from the GenomeWalker libraries were assembled to generate the sequence of the region encoding Domain III and its flanking regions.

7. *Searching for sequences homologous to Domain III and its flanking sequences*

The sequences obtained through GenomeWalker sequencing were searched for homologous sequences in the National Library of Medicine (NIH) nucleotide sequence database using a BLASTn tool provided by NIH (https://blast.ncbi.nlm.nih.gov). The alignment of homologous sequences was performed using Clustal Omega Multiple Sequence Alignment provided by the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (https://www.ebi.ac.uk/Tools/msa/clustalo).

Results

1. *Domain compositions of Cry1As and Cry1Bs*

Phylogenetic trees of Cry1A domains show diverse compositions (Fig. 1). These trees display approximately four clusters in both Domain I and II each with constituents that differ between these domains. For example, the Domain I sequences of Cry1Aa, Cry1Ab, and Cry1Ac are highly homologous, but the sequence of Cry1Aa Domain II differs from those of Cry1Ab and Cry1Ac. Additionally, Cry1Ac does not cluster with Cry1Aa and Cry1Ab in the Domain III phylogenetic tree due to substantial differences in their sequences. The diverse domain structure of Cry1As suggests that Bt may exchange its domains. Cry1Ac is substantially more active against *H. virescens* and *H. zea* than Cry1Ab[24] even though they have highly homologous Domain IIs. It has been reported that Cry1Ab and Cry1Ac Domain IIs bind to the same receptor (ABCC2) as they have similar sequences,[4,25] and Cry1Ac Domain III binds to *H. virescens* BBMV.[6] These reports suggest that there may be two receptors in heliothine insects for Cry1Ac, the primary receptor for Domain II and the secondary receptor for Domain III, which contributes to this Cry protein's high activity against *H. zea*.

Cry1Bs also have diverse domain compositions as shown in Fig. 2 suggesting that Bt exchanges Cry1B domain IIIs with other Cry proteins such as Cry1Ac and Cry1Cb to alter insect specificity. Cry1B is a promising candidate to combat insect resistance to current Bt crops. Domain II plays a crucial role in determining the insect specificity of Cry proteins. Cry1B's Domain IIs differ significantly from those of Cry1Ab and Cry1Ac used in GMO Bt-corn and Bt-cotton. The difference is illustrated in the phylogenetic tree (Fig. S11) of a review article[14] published

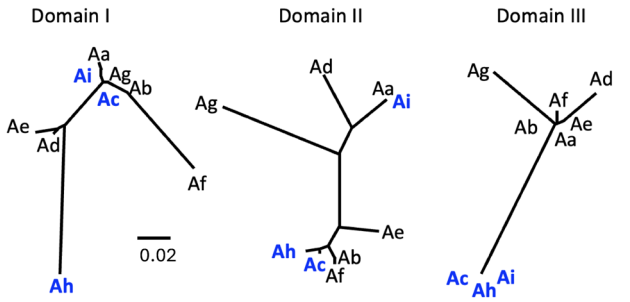


Fig. 1. Phylogenetic trees of Cry1A domains. Cry proteins are labeled without the Cry1 prefix, for example, "Aa" indicates Cry1Aa. Blue Cry proteins are those with the Cry1Ac-type Domain III. The bar indicates the unit (average substitutions per amino acid residue) of tree branch lengths. The purpose of Fig. 1 and Fig. 2 is to identify clusters and their components. Some long branches (> 0.07) were shortened to fit in the figures.

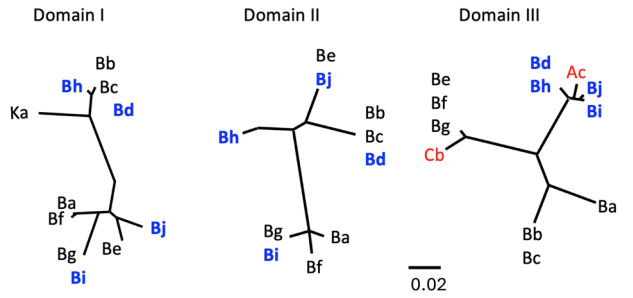


Fig. 2. Phylogenetic Trees of Cry1B domains. Cry proteins are labeled without the Cry1 prefix, for example, "Ba" indicates Cry1Ba. Blue Cry proteins contain the Cry1Ac-type Domain III and are active against *H. zea*. Red Cry1 proteins, Cry1Ac(Ac) and Cry1Cb (Cb) are known to be effective against *H. zea* and *S. exigua*, respectively.

in this journal in 2022. The receptor for Cry1B in *H. zea*, which binds to Cry1B Domain II, is likely different from that of Cry1Ab and Cry1Ac. ABCB1 is known to be the receptor for Cry1B in *S. frugiperda*,[25] while the receptor for Cry1Ab and Cry1Ac is ABCC2. In *H. zea*, ABCB1 is likely the receptor for Cry1B, but further research is required for confirmation.

As shown in Fig. 2, Cry1Bd, Cry1Bh, and Cry1Bi have a Domain III similar to that of Cry1Ac. Interestingly, these Cry1B proteins are active against *H. zea*. When we assayed Cry1Bd against this insect, we observed 20% mortality at 200 ppm, a significant level for the Cry1Bs, which are typically inactive against this insect. Cry1Bh and Cry1Bi have weak but certain activity against *H. zea*. Cry1Bh's activity was described in the literature as "3% mortality" at 9 μg/cm^2 (diet surface treatment).[26] In our study, Cry1Bi caused growth inhibition but no mortality at 200 ppm (diet mixing). These results suggest that the Domain IIIs of these Cry1Bs contribute to the activity against *H. zea*.

The Cry proteins used in transgenic Bt crops have very high activity against target insects with LC_{50} levels typically below 10 ppm as per our unpublished observation. The activity against *H. zea* of Cry1Bs with a Cry1Ac-type Domain III was not sufficient for commercial application. Therefore, we are interested in finding new Cry1B proteins that are more effective against this insect.

2. High-throughput screening of Bt Cry proteins

Previously, we successfully attempted to enhance the activity of Cry1Ca against *S. exigua* through DNA shuffling.[20] In that study, we created thousands of Cry1Ca mutants and assessed their insecticidal properties using high-throughput insect assay with 96-well plates. This time, we utilized a similar high-throughput approach to screen Cry proteins from 3000 naturally occurring Bt isolates for their activity against *H. zea*. The protein samples were standardized to a final concentration of 1 mg/mL and then diluted in the insect diet five times to achieve a concentration of 200 μg/mL (200 ppm). Through this primary screening, we identified approximately 400 Bt isolates that exhibited significant activity defined as obtaining at least a 50% response at the Cry protein concentration of 200 ppm.

These 400 active Bt cultures were re-arrayed into four 96-well seed plates, and their *cry* gene contents were analyzed by PCR to identify Bt isolates containing the *cry1Ac* gene, which makes the Cry protein samples active against *H. zea*. The PCR analysis revealed that approximately 300 out of the 400 cultures contained the *cry1Ac* gene. The remaining 100 cultures without *cry1Ac* were then re-arrayed into one 96-well seed plate, and Cry proteins were produced in four production plates (4 mL CYS broth per isolate). The Cry proteins from those 96 isolates were analyzed by SDS-PAGE. Many isolates exhibited both 135 kDa Cry1 and 65 kDa Cry2 proteins as shown in Fig. 3. Since Cry2 is known for its activity against *H. zea*, these Cry2 proteins were removed from the Cry protein samples using DEAE agarose column chromatography for the next round of bioassay. The separation process was monitored by SDS-PAGE, which displayed clean separation between Cry1 and Cry2 proteins as shown in Fig. 3. The 135 kDa Cry1 proteins eluted from DEAE agarose were then assayed against *H. zea* at 200 ppm with 6 replications for each isolate. The assay was repeated 3 times, and 48 cultures with total scores above 17 out of a possible 54 were selected as shown in Fig. 4. DNA samples were extracted from these 48 finalists, and DNA fragments encoding Domain III were amplified using PCR.

A flowchart summarizing the entire screening process of 3000 Bt isolates to identify novel *H. zea*-active Bt Cry proteins is provided in Supplemental Fig. S3.

3. PCR amplification of cry gene fragments encoding Domain III

PCR amplification of the DNA segments encoding Domain III was conducted using multiple primers for each direction (Table

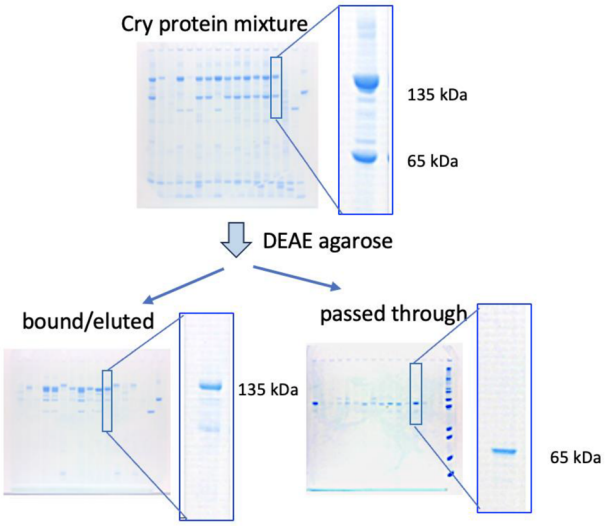


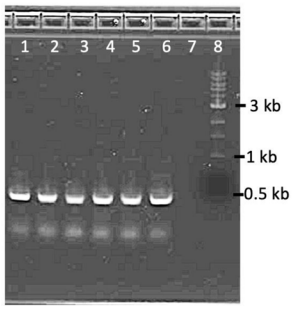
Fig. 3. Separation of Cry1 from Cry2 using high throughput DEAE agarose column chromatography. The acidic Cry1 proteins (135 kDa) that bound to the DEAE agarose were eluted with 0.1 N NaOH, while the basic Cry2 proteins (65 kDa) that passed through the ion-exchange gel were discarded. Only one plate is shown for each step in this figure, while SDS-PAGE required six plates to analyze the 96 samples.

Cry1 (separated) activity

	1	2	3	4	5	6	7	8	9	10	11	12
A	22	17	22	14	15	15	15	27	31	18	2	17
B	8	30	15	40	16	11	26	29	39	6	27	13
C	16	18	27	22	25	12	11	22	22	8	21	23
D	21	29	29	34	14	13	21	39	17	8	29	15
E	25	14	30	37	14	7	22	9	6	10	14	18
F	7	18	35	12	31	22	40	13	8	11	12	15
G	16	10	25	10	27	25	35	31	17	21	8	18
H	19	6	9	10	7	9	33	15	19	5	32	11

Fig. 4. The activity against *H. zea* of 96 Cry1 protein samples purified by DEAE agarose column chromatography. This assay results were obtained from six treatments for each sample with a maximum possible score of 18. The assay was repeated three times resulting in a total maximum score of 54. The scores were ranked by color, >30 (orange) 20–30 (yellow), and 17–19 (green).

1), obtained from known Bt *cry1* genes. All of these Cry1 proteins have five highly homologous regions,[18] including one at the junction between Domains II and III, and another at the beginning of the protoxin region (Supplemental Fig. S2). PCR selectively amplified the region encoding Domain III using primers that anneal to these Domain III boundaries, producing a 400 bp band for each isolate as observed by gel electrophoresis (Fig. 5A). The 400 bp bands were extracted from the gel, cloned into the pCR-TOPO vector and then sequenced. The sequencing revealed 42 *cry1* Domain III sequences three of which were new (*i.e.*, not a 100% match with known *cry* genes) (Fig. 5B). Our goal is to find *cry1B*-type genes that are effective against *H. zea*. As shown in Fig. 3, many Bt isolates contained various *cry* genes including those potentially active against *H. zea*. The new genes we are seeking may be obscured by these established *H. zea*-specific genes. Consequently, we did not attempt to assess the specific roles of these 42 *cry* genes in the activity of each Bt



B

	Found	Novel
Cry1Ab	13	0
Cry1B	8	0
Cry1Bx	4	2
Cry1Da	2	0
Cry1Fb	1	0
Cry1Hb	5	0
Cry1Jx	6	1
Cry1Ka	3	0
Total	42	3

Fig. 5. PCR amplification of Domain III of Cry1s from the final 48 Bt isolates that are active against *H. zea*. The PCR products were analyzed using agarose gel electrophoresis as shown in (A) displaying 400-bp bands in Lanes 1 to 6 representing the first 6 samples out of the total 48 samples that produced identical 400-bp bands. The 400-bp bands were extracted from the gel, mixed, cloned into pCR-TOPO, and then plated. Lane 7 was left empty, and a size marker (Promega 1kb ladder) was placed in Lane 8. Forty-eight colonies of the TOPO clone were picked and sequenced in both directions. The sequencing revealed 42 *cry1* Domain III sequences with 3 of them being new as shown in (B). Cry1Bx and Cry1Jx indicate novel sequences meaning there were some sequence differences from the known Cry1B and Cry1J sequences.

isolate against *H. zea*.

As shown in Fig. 5B, four Cry1B-type sequences (Cry1Bx) were found among the 42 sequences, with three of the four being identical, and one being different. In addition to Cry1Bs, a novel Cry1j-type sequence was found, but only the Domain III sequence was characterized, as our primary focus was on novel Cry1B sequences. These two Cry1B sequences were tentatively named Cry1B Type-A and Type-B Domain IIIs as shown in Fig. 6. The flanking sequences of these Domain III sequences obtained using the Takara Bio GenomeWalker kit revealed that Type-A was the Domain III of Cry1Bj (GenBank accession number: KT952325). The *cry1Bj* gene was discovered by our colleagues as described in the GenBank description shortly before we determined the full-length sequence of Type-A. We cloned the full-length gene encoding Cry1Bj into a *Bacillus-E. coli* shuttle vector to produce the protein in *cry*-minus Bt. When assaying the Cry1Bj protein against *H. zea*, we observed a relatively high activity level with an EC_{50} of 105 ppm. As shown in the phylogenetic trees of Cry1Bs (Fig. 2), Cry1Bd, Cry1Bh, Cry1Bi, and Cry1Bj having Cry1Ac-type Domain IIIs were active against *H. zea*. Among these Cry1Bs, Cry1Bj appeared to have the highest activity level against this insect. However, the activity level of Cry1Bj is not sufficient for commercial application in transgenic corn to control *H. zea*. Therefore, we engineered Cry1Bj to enhance its activity against *H. zea* as described by Yamamoto in his review article published earlier in this journal.[14]

The flanking regions of Cry1B Type-B Domain III were also sequenced. Surprisingly, no sequence encoding Domains I and II was found in the upstream region of the Type-B Domain III coding region. Instead, two open reading frames, referred to as ORF1 and ORF2, were identified. These ORF sequences are listed in Supplemental Fig. S4A and S4B. A BLAST search of these ORFs against the NIH protein database revealed that ORF1 was an ATP-binding protein, and ORF2 was an ATPase. The N-terminal sequence immediately upstream of the Type-B Domain III beyond the forward primer used to amplify this domain contained an amino acid sequence representing the Domain

Cry1B-Type A

PGFTGGDLVRLNNSGNNIQNRGYLEVPIQFI
STSTRYRVRVRYASVTPIQLSVNWGNSNIFS
SIVPATATSLDNLQSRDFGYFESTNAFTSAT
GNVVGVRNFSENAGVIIDRFEFIPVTATFEA
EYDLERAQEAVNALF

Cry1B-Type B

PGFTGGDLVRLNRNNGNIQNRGYIEVPIQFS
STSTRYRVRVRYASVTPIELNIVVGNGVIFS
NTVPGTAASLDNLQSGDFGDFEVTNTFTSAS
GNIVGLRNFSANADVIIDRFEFIPVTATFEA
ESDLEGARKAVNALF

Fig. 6. Deduced amino acid sequences of novel Cry1B-type Domain III fragments. The carboxyl end of Domain III is denoted by the red-letter "R" (Arg), and PCR primer sequences are highlighted in yellow.

III β14 sequence. Further upstream of β14, there was a stretch of 29 amino acid (AA) residues (Supplemental Fig. S5). There was no sequence homologous to this 29-AA sequence according to a BLAST search. On the other hand, at the carboxyl side of the Type-B Domain III, a typical sequence of the protoxin region was found. The DNA structure of this Type-B Domain III including its flanking regions is illustrated in Fig. 7A. While we believe that this Type-B protein does not have any insecticidal activity, no assay was conducted to confirm this belief.

Identical DNA structures containing ORF1, ORF2, Type-B Domain III, and its protoxin were found in the Bt genomic sequence database as depicted in Fig. 7B and 7C. These nucleotide sequences were discovered on the chromosomes of Bt HD600 and G25-93 strains. Additionally, the Type-B Domain III sequence lacking Domains I and II existed in a plasmid (pGR157) of Bt GR007 strain as shown in Fig. 7D. In the case of the pGR157 plasmid, the upstream sequence of this GR007 Type-B contained only one open reading frame whose sequence differed from the ORFs associated with the chromosomal Type-B Domain III. The sequence of this plasmid ORF is also listed in Supplemental Fig. S4C. A BLAST search of this GR007 ORF revealed no homologous sequences in the NIH protein database.

Discussion

In 1994, Bosch *et al.* first demonstrated the benefits of artificially swapping Domain III in Cry proteins.[27] They discovered that the insecticidal activity was not directly related to the high affinity of Cry1Ab to the midgut BBMV (Brush Border Membrane Vesicles) of *S. exigua*. Cry1Ca and Cry1Ea exhibit high insecticidal activity against *S. exigua*, prompting the replacement of Domain III of Cry1Ab with those from Cry1Ca and Cry1Ea. This resulted in chimeric Cry1Ab proteins with significantly enhanced activity against *S. exigua*. Sandoz Agro then adopted this concept and used a method developed by Kalman *et al.*[28] to insert these chimeric genes into the chromosome of their commercial Bt strain. Field trials confirmed that these recombinant Bt strains effectively controlled *S. exigua*,[29] thus validating the commercial potential of domain swapping. This approach of artificial domain swapping has since been widely used in the industry to produce new *cry* genes for transgenic Bt crops such as Cry1A.105 (Cry1Ab Dm 1/II-1Ac Dm III), Cry1A.2 (Cry1Ac-1Ca), Cry1B.2 (Cry1Be-1Ka),[17] and eCry3.1Ab (Cry1Ah-3A).[30] Among these Domain III-swapped chimeric proteins, Cry1Bs are of particular interest due to their potential to overcome insect resistance to current Bt crops.

The first Cry1B-type protein identified in nature was Cry-1Ba,[31] which exhibits dual insect specificity by being active against both Lepidoptera and Coleoptera insects. Interestingly, it appears that the insect specificity of Cry1B can be altered by swapping Domain III. Among the Cry1Bs with artificially swapped Domain III, Cry1B.2 possesses the Domain III of Cry1Ka. In the phylogenetic tree of Cry1 Domain IIIs shown in Fig. S12 of the review article,[14] Cry1Ka aligns closely with Cry1Fa and Cry1Ig. As reported by Chen *et al.*,[17] Cry1B.2 is active against *Spodoptera* species but inactive against *Heliothinae* species, as predicted from its Domain III. Cry1Fa is highly ac-

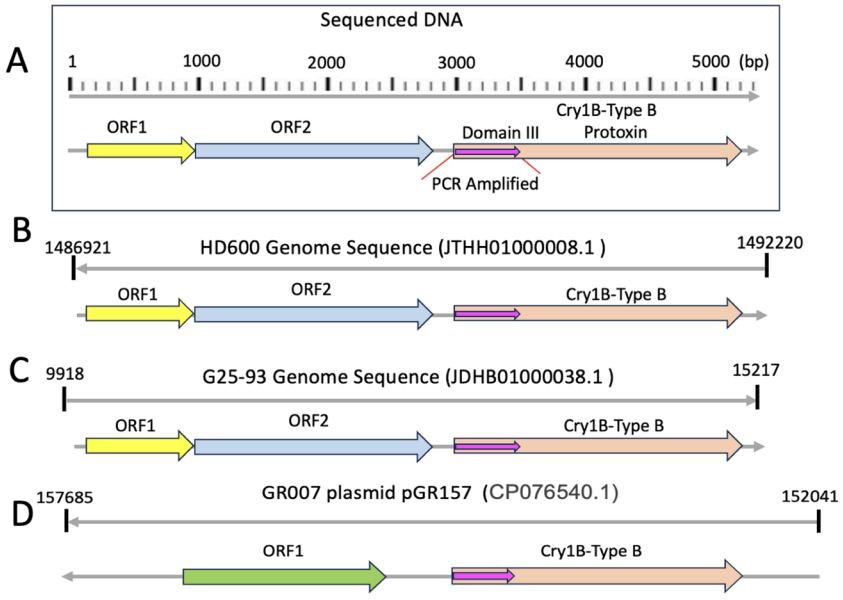


Fig. 7. DNA structures of the flanking regions of Cry1B Type-B Domain III that were identified during screening as shown in (A). The Domain III-protoxin complex depicted as a single protein is indicated by an orange arrow of approx. 2.2 kb, with Domain III shown in pink. Upstream of Domain III, two open reading frames, ORF1 (yellow) and ORF2 (blue) were discovered instead of Domains I and II. A BLAST search revealed that DNA sequences identical to that in (A) were found in the chromosomes of Bt strains HD600 (B) and G25-93 (C). Additionally, a similar Domain III sequence without Domains I and II was found in a plasmid of GR007 (D). In this case, there was only one ORF (green) which differed from the chromosomal ORFs. These Bt genome and plasmid sequences can be accessed in the NIH database at the following URLs. (B) https://www.ncbi.nlm.nih.gov/nuccore/JTHH00000008. (C) https://www.ncbi.nlm.nih.gov/nuccore/LDHB01000038. (D) https://www.ncbi.nlm.nih.gov/nuccore/CP076540.1.

tive against *Spodoptera* species. Another domain-swapped Cry1B.868, which contains Cry1Be Domains I and II and Cry1Ca Domain III, has been found to bind to *S. frugiperda* ABCB1.[25] Notably, Cry1B.868 is effective against *S. frugiperda*, as anticipated from its Cry1Ca Domain III. On the other hand, the naturally occurring Cry1Bs with a Cry1Ac-type Domain III are active against *Heliothinae* species such as *H. zea*. It is fascinating to observe how Domain III of chimeric Cry1Bs determines insect specificity between *Heliothinae* and *Spodoptera* species.

Four Cry1B proteins with Cry1Ac-like Domain III exist naturally. They are Cry1Bd, Cry1Bh, Cry1Bi, and Cry1Bj, as depicted in Fig. 2. This study found that Cry1Bj exhibits the most potent activity against *H. zea* among these Cry1Bs. These proteins have slight variations in their amino acid sequences within Domain III. The sequence alignment, as shown in Supplemental Fig. S5, reveals a difference in the junction between β15 and β16. Cry1Bd and Cry1Bh have RNNG while the corresponding sequence for Cry1Bi and Cry1Bj is SSGN. It has been reported that the β15–β16 region of the Cry1Ac Domain III containing SSGN binds to *N*-acetylgalactosamine (GalNAc).[32] Further studies are necessary to assess the differences in the GalNAc binding and activity against *H. zea*, if any, between SSGN and RNNG in the β15–β16 loops.

The phylogenetic trees (Figs. 1 and 2) show significant variation within Domain III among certain Cry1A and Cry1B proteins suggesting that these proteins undergo domain exchanges through homologous recombination. As illustrated in Fig. 7, a gene encoding a standalone Cry1Ac-type Domain III without Domain I and II was found on both the chromosome and a plasmid of Bt. This independent Domain III is believed to be involved in recombination events with pre-existing Bt genes. Interestingly, the β15–β16 loop sequence within this stand-alone Domain III aligns with that of the Cry1Bd-type (RNNG) rather than the Cry1Ac-type (SSGN), as shown in the alignment provided in Supplemental Fig. S5. This alignment suggests that the Cry1Bd and Cry1Bh proteins may have originated from this stand-alone Domain III, or this domain may have been expelled from these Cry1B proteins.

Bt has been artificially modified to enhance its insecticidal activity by inserting the *cry1Ca* gene into a specific location on the Bt chromosome utilizing Bt's own capability of homologous recombination.[28] Recent advancements in genome editing, such as the method described by Altenbuchner[33] using CRISPR-Cas9 in *Bacillus subtilis*, suggest the potential application of CRISPR-Cas9 to Bt. This includes the possibility of swapping Domain III of Cry proteins to target specific insect species. While swapping Domain III may enhance the effectiveness of Bt Cry proteins against certain insects, the exact role of Domain III at the molecular level is not yet fully understood. Interactions between the Cry1Ac Domain III and GalNAc have been observed,[32,34,35] but studies on Cry1Ca and Cry1Fa (Cry1Ka) Domain IIIs have not been reported. It is uncertain whether the binding of the Cry1Ac Domain III to GalNAc is a secondary event after Domain II binds to the primary receptor such as the ABC transporter. BmABCC2 has been identified as a receptor for Cry1Aa Domain II through mutation studies in live insects[36] and subsequent expression in cell lines like Sf9 and HEK-293 for toxicity assessments.[37] The expression of an insect receptor for Bt in HEK-293 is particularly interesting as this mammalian cell line should lack any insect receptor for Bt. Conversely, Sf9, an insect-derived cell line is sensitive to certain Cry proteins without any external Bt receptor gene cloned and expressed.[25,38] It could be worthwhile to express two receptors such as an ABC transporter for Domain II and aminopeptidase (GalNAc) for Domain III in HEK-293 to evaluate the efficacy of Cry proteins with and without the Cry1Ac Domain III.

Bt Cry proteins, unlike most chemical insecticides, are stomach poisons and must be ingested by insects to be lethal. To evaluate the insecticidal activity of these proteins, we incorporate them into the diet of the test insects for oral consumption. In the literature, it's common to see Bt Cry proteins applied to the surface of the diet or plant leaf disks, a method referred to as "surface treatment." While this might be suitable for spray applications, we argue that it does not accurately represent Bt crops when an artificial insect diet is used. In Bt crops, Cry proteins are internally expressed within the plant tissue, not merely on the surface. Furthermore, we contend that surface treatment of an artificial diet is not the best way to assess the activity differences between engineered Cry proteins and their parent protein or among different engineered proteins. When Bt Cry proteins are applied to the diet's surface, they diffuse into the diet, creating a concentration gradient. The protein concentration is highest on or near the surface and gradually decreases towards the bottom of the diet. Since Bt Cry protein is a potent feeding deterrent, certain insect species, like *H. zea*, tend to burrow into the diet to avoid surface feeding. To address this issue, the Cry protein must be evenly mixed into the diet for accurate activity assessment. Mixing a small volume of samples into the diet in 96-well plates consistently across all 96 wells is particularly challenging. Therefore, we employ a liquid-handling robot to mix Cry proteins into the molten insect diet at a temperature low enough to avoid the denaturation of the protein samples. Our years of experience engineering Bt Cry proteins, as reviewed by Yamamoto,[14] have shown us the importance of this diet mixing method for accurate and reproducible assay results.

To evaluate the activity of a vast array of 3000 Bt isolates, it is crucial to determine the accurate concentration of sample proteins. Ensuring uniform protein concentrations across all assay samples is essential for obtaining dependable screening results. In our study, Bt proteins prepared for bioassay were analyzed and quantified using the Caliper LabChip GX protein analyzer to meet this requirement. According to our tests, the analyzer showed a linear relationship with high fitness between signal output and protein concentration, effective up to 10 mg/mL. Additionally, the analyzer is equipped to accommodate 96-well plates, streamlining the handling of multiple samples. It was particularly useful for analyzing thousands of Cry protein samples

prepared for high-throughput screening.

To assess the effectiveness of Bt Cry proteins, we have created a scoring method to assess both feeding inhibition (growth deterrence) and lethality. This method generates an EC_{50} value indicating the Effective Concentration for a 50% response in the target insect population, rather than an LC_{50} value, which represents a Lethal Concentration. Rapid mortality is crucial when applying Bt-based insecticides to crops, as Bt's efficacy in the field is short-lived. If insects survive without feeding until Bt loses its efficacy, they can recover from the initial Bt intoxication and resume damaging crops. Conversely, with transgenic Bt crops, immediate insect mortality is less important, as the plants continuously produce Bt Cry proteins providing ongoing crop protection through feeding inhibition. Since our primary goal is to find new Bt Cry proteins for genetically modified crops, we prefer using this scoring system. Additionally, this system is preferred for its ability to provide a wide dynamic range in measuring insect responses with a limited number of treatments. Unlike a mortality-based score, which is binary (alive or dead), our system ranges from 0 to 3, allowing for more nuanced assessments. This is particularly beneficial for high-throughput screening, which cannot accommodate a large number of treatments for each sample. Our extensive experience in engineering Bt Cry proteins for increased activity requires the rapid and accurate analysis of thousands of samples.[14] By screening a large variety of samples, our chances of identifying valuable Bt Cry protein variants are increased.

In this research, we employed high-throughput methods for protein preparation and insect assay developed in our laboratory to conduct the study. We enhanced our approach by incorporating gene analysis and protein separation procedures. To find novel Cry proteins active against *H. zea*, Cry1Ac and Cry2A proteins were excluded from our screening samples due to their known activity against this insect species. We used multiplex-primer PCR to identify and remove Bt isolates containing the *cry1Ac* gene. Additionally, high-throughput column chromatography was applied to remove Cry2A proteins from our samples. These methods, in conjunction with the insect assay, streamlined the screening process of Bt isolates, allowing us to identify potential novel genes through DNA sequencing, reducing the initial pool from 3000 to just 48 isolates.

DNA fragments encoding Domain III of the Bt Cry protein were successfully amplified by PCR, utilizing a set of mixed primers for each direction. The design of these primers was based on conserved regions found in all known *cry1* genes. This PCR technique was adapted from the work of Dr. Takebe's team at Kinki University, who had identified novel Cry32 proteins using a similar method.[39] Employing this strategy led to the discovery of a novel Cry1B protein, designated as Cry1Bj, which showed significant effectiveness against *H. zea*, making it a prime candidate for further enhancement in our engineering efforts. Concurrently, our PCR process also revealed a separate, stand-alone Domain III fragment, indicating that Bt may exchange Domain III through homologous recombination.

Conclusion

We have developed a high-throughput approach for efficiently and accurately screening Bt Cry proteins from a collection of 3000 Bt isolates. This led to the successful discovery of a new Cry1B-type protein, Cry1Bj, which demonstrated the highest activity against *H. zea* among naturally occurring Cry1Bs. Cry-1Bj was further engineered to enhance its effectiveness for commercial use as discussed in a review article.[14] Additionally, our screening process revealed evidence that Bt undergoes homologous recombination to exchange Domain III of its Cry proteins, which enhances insecticidal activity and alters host insect specificity. We have discussed how the swapping of Domain III affects Bt's specificity towards different insect hosts.

Conflicts of interest and acknowledgments

The experiments for this study were conducted while we were employed at Verdia Inc. Redwood City, California U.S.A. Verdia was later acquired by Pioneer Hi-Bred International, which approved the publication of the results under our subsequenct affiliation. There are no other conflicts of interest to disclose. RC contributed to protein production and sequenced the flanking regions of PCR-amplified Domain III. JH conducted bioinformatic analyses throughout the project. TY conceived the idea for the study, led the team in developing and conducting the high-throughput screening of Bt Cry proteins, and performed PCR amplification, cloning, and sequencing of DNA fragments encoding Domain III. We would like to acknowledge the technical assistance provided by David Cerf and his insect bioassay team. Additionally, we thank Dr. Michi Izumi Willcoxon and her team for producing the Cry1Bj protein for bioassay. Dr. Hideo Ohkawa, Professor Emeritus of Kobe University, encouraged us to publish this paper in the Journal of Pesticide Science. We appreciate his support and for reviewing the manuscript.

Electronic supplementary materials

The online version of this article contains supplementary materials (Fig. S1–S5) which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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