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Detection of a novel Cry2Ab toxin against *Etiella zinckenella* Treitschke from the *Bacillus thuringiensis* serovar *canadensis* SP142 strain

Ngoc Le Thu[1,2], Thanh Le Thi Minh[1,2], Ngoc Pham Bich[1,2], Ha Trinh Thi Thu[1], Quyen Dong Van[1,2], Binh Ngo Dinh[1], Ha Chu Hoang[1,2], Ha Hoang[1], Dong Nguyen Van[3]*

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam*
[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam*
[3]*Agricultural Genetics Institute, Hanoi, Vietnam*

**Corresponding author: minhthanh.ibt@gmail.com*

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Abstract: The soybean (*Glycine max*) is an important crop. The pod borer (*Etiella zinckenella*) is one of the most serious insects that attacks various *Leguminosae*. Common insecticidal controls are ineffective because of the insect's growth properties. Use of resistant crop varieties offers stabilisation of the yield and has benefits over the use of insecticides. *Bacillus thuringiensis* is widely used as a bioinsecticide for pest control and a genetic material for pest-resistant transgenic plants. However, the resistance evolution of target insects is emerging as a major threat to the long-term efficacy of these applications. Studies on the detection of novel highly host-specific pesticidal proteins have been in urgent demand. A search for the source of *Bt* Cry toxins against *E. zinckenella* in the Vietnamese *B. thuringiensis* strain collection has been performed. The *B. thuringiensis* serovar *canadensis* SP142 is one of strains that resulted in more than 80% mortality to this pod borer. Its genome was estimated about 7.1 Mb and revealed a putative novel *cry2Ab* gene. The sequence analysis of *cry2Ab* gene revealed an open reading frame of 1 899 bp encoding a 633-amino acid protein with a calculated molecular mass of 70 kDa and 99.05% to 99.21% homology to known *cry2Ab* genes in the GenBank. There are eighteen different nucleotide sites which lead to five amino acid changes in Domain I and II. This gene was expressed in *Escherichia coli* BL21(DE3) and the purified Cry2Ab was toxic to *E. zinckenella* larvae with an LC_{50} value of 1.74 μg/g diet. The novel Cry2Ab was designated as Cry2Ab39 by the Bacterial Pesticidal Protein Resource Center and its sequence was deposited in the GenBank (MN319700.1). This is a type of novel Cry2 toxin from *B. thuringiensis* against *E. zinckenella*, and it is important for breeding *E. zinckenella*-resistant soybeans.

Keywords: insecticidal control; Cry2Ab39 toxin; pesticidal protein

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The soybean (*Glycine max* L. Merr.) is one of the most globally important crops in tropical and subtropical areas that supply half of the global demand for protein and vegetable oil. *Etiella zinckenella* Treitschke have emerged as a major pod-borer during the pod stage and are considered as the most difficult soybean pests to be controlled due to the larval feeding behaviour. The percent pod damage of this insect is up to 30–40%, whereas the consequent yield loss can reach about 70%. To control the pod borer, farmers used chemical insecticides without considering the recommended economic threshold. However, common insecticidal controls are ineffective with *E. zinckenella* because it is almost impossible to reach the larvae inside the pods with pesticide sprays. Alternative control methods that are more effective and environmentally friendly are in demand. Cultural techniques, natural enemies, biopesticides and resistant varieties are recommended for sustainable crop management. The use of resistant varieties is not only the most efficient and economical strategy for pest control, but also environmentally friendly and compatible with other methods (Soheir & Eman 2020).

Bacillus thuringiensis (*Bt*) is widely used as a bioinsecticide for the control of many agricultural pests by biopesticides and genetic materials for the generation of pest-resistant transgenic plants (Betz et al. 2000; Chattopadhyay et al. 2004). It produces parasporal protein toxins called insecticidal crystal proteins (Cry and Cyt proteins) during the sporulation phase, and other non-crystalline toxins (Vip and Sip proteins) during the vegetative growth phase (Crickmore et al. 1998; Palma et al. 2014). These proteins are toxic to dipteran, lepidopteran, coleopteran, hymenopteran, homopteran, mallophagan, and nematode larvae (Bravo et al. 2007; Saleem & Shakoori 2017). The Cry toxin is most deeply and widely researched (Bravo et al. 2007; Heckel 2020). Up to now, 75 different Cry toxin groups (Cry1 to Cry75) consisting of more than 800 members have been reported (Crickmore et al. 1998; Azizoglu 2019). The Cry toxin comprised three distinct domains: seven α-helix clusters responsible for membrane insertion and pore formation (Domain I), three anti-parallel β-sheets connected by loops which plays an important role in receptor binding and the specificity determination (Domain II), and two antiparallel β-sheet to be related to the receptor recognition and membrane insertion (Domain III) (Xu et al. 2014). Individual Cry toxins have a defined spectrum of insecticidal activity, usually restricted to a few species within one particular order of insects. A few toxins have an activity spectrum that spans two or three insect orders – notably Cry2Ab which is toxic to dipteran as well as lepidopteran insects (Sevim et al. 2012).

The *cry2A*-type gene encodes the 60–70 kDa proteins, which are toxic to lepidopteran and dipteran larvae. Eleven *cry2A* genes (from *cry2Aa* to *cry2Al*) have been reported. Among these, 38 types of *cry2Ab* genes have been identified (www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).Currently, Cry2A toxins have been demonstrated to be alternative candidates for use in the production of second-generation transgenic plants to control insect pests instead of Cry1-type toxins. Cry2A proteins are lethal to some lepidopteran pests that are unaffected by Cry1 toxins (Fujimoto et al. 1993), these toxins exhibited a low level of cross-resistance in Cry1-resisitant insects (Gouffon et al. 2011; Elleuch et al. 2016), and the genes encoding the Cry2A toxins are thought to have greater advantages in the transformation as well as expression in plants due to their smaller molecular size than the *cry1* genes (Hire et al. 2009). The investigation for novel toxins with a wide-spectrum and high toxicity is also considered as one of the potential approaches not only for transgenic plant development to control crop pests, but also for the management of insect resistance evolution (Ye et al. 2012). In 2019, 190.4 million hectares of biotech crops were grown in 29 countries, contributing significantly to the food security, sustainability, climate change mitigation, and upliftment in the lives of up to 17 million farmers and their families worldwide. Four major biotech crops – soybeans, maize, cotton, and canola – are the most adopted biotech crops. Soybeans lead the way at 91.9 million hectares, which is 48% of the global biotech area. Today, there are six events for *Bt* insect resistance in 42 genetically modified (GM) soybean events approved in the world. Cry1Ac, Cry1Ab2, Cry1A.105, cry1F and Cry2Ab from *Bt* strains are pest-resistant transgenic soybeans. They are resistant against some lepidopteran larvae, but are not resistant to *E. zinckenella* larvae. In Vietnam, there is one event for resistance to lepidopteran insects of eight events approved in the GM soybean of the Monsanto Company (Cry1Ac delta-endotoxin) (www.isaaa.org).

Further studies are needed to reveal the proteins from *Bt* responsible for the bio-insecticidal activ-

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ity against the pod borer. In this study, we characterised a novel *cry2Ab39* gene of the *Bt* serovar *canadensis* SP142 strain with insecticidal activity against *E. zinckenella* larvae. This gene could be used as a gene source for the varietal improvement of the transgenic soybean resistance against the pod borer.

MATERIAL AND METHODS

Bacterial strains, plasmids, insect larvae and reagent. The *B. thuringiensis* serovar *canadensis* SP142 strain was isolated from a bioinsecticide free soil sample collected from Lao Cai province of northern Vietnam, and previously identified based on biochemical and H-serotyping (Binh et al. 2000). This strain has been preserved at the Vietnam *Bt* collection (B10002) of the Institute of Biotechnology, the Vietnam Academy of Science and Technology, Vietnam. *E. coli* DH5α and *E. coli* BL21 (DE3) were utilised for the cloning and expression of the *cry* gene. The pJET1.2/blunt Cloning Vector (Thermo Fisher Scientific™, USA) was used for the routine cloning of the polymerase chain reaction (PCR) products and the expression vector pET-32a(+) (SnapGene) was designed for high-level recombinant protein production. Second-instar larvae of *E. zinckenella* Treitschke provided by the Plant Protection Research Institute, Vietnam were used for the insect bioassays. The 6x-His Tag monoclonal antibody (Invitrogen, USA) was utilised by following the manufacturer's recommendations for the detection of the recombinant proteins.

Genomic DNA preparation, genome sequencing and computational analysis. The total DNA from the *Bt* SP142 strain was extracted following the method described by Mayjonade et al. (2016). The genomic DNA quality was evaluated using Qubit fluorometer and Qubit High Sensitivity (HS) DNA assay reagents (Thermo Fisher Scientific™, USA) and the input length was accessed using an Agilent 2100 Bioanalyzer system (Agilent, USA). The library was constructed using SMRTbell Template Prep Kits (PacBio, USA) and then sequenced on a PacBio SEQUEL system (PacBio, USA) according to the manufacturer's instructions. The sequencing signals were processed, evaluated and converted into a raw read by the Primary Analysis Computer server. All the data were automatically transferred to the Secondary Analysis Server system via the intranet. The data were assembled with HGAP4 software, and the quality of the assembly was evaluated using QUAST (version 4.6.3) (Gurevich et al. 2013) and BUSCO (version 3.0) (Simão et al. 2015; Waterhouse et al. 2018). Afterwards, the genome sequence was then annotated with the PATRIC server (Wattam et al. 2018).

Prediction of candidate *Bt* toxins. The gene sequences encoding the *Bt* toxins were predicted with three modules (BLAST, HMM and SVM) incorporated into the computational pipeline named Bt-Toxin_scanner constructed by Ye et al. (2012) available at http://bcam.hzau.edu.cn/BtToxin_scanner/. Based on the different types of input sequences, the corresponding modules would be called to convert them into protein sequences. Any sequence that passed either of the prediction modules were considered as candidate Cry proteins, whereas the candidate Vip and Cyt proteins were predicted only with the HMM module.

Amplification and cloning of the *cry2Ab39* gene. To isolate the potential novel *cry2Ab* gene in its full length from the *Bt* SP142 genome, a conventional PCR was conducted with the primer pair designed based on the sequences of the conserved regions of the *cry2Ab* genes. The following primers were used to amplify the *cry2Ab* gene: forward, 5'-CGCGGATCCATGAATAGTGTATTGAATAGCGG-3'; reverse, 5'-CCGCTCGAGATAAAGTGGTGAAATATTAGTTGG-3' (the underlined sequences indicate the *Bam*HI and *Xho*I restriction sites, respectively). The PCR reaction mixture (in a final volume of 50 μL) containing a 5× reaction buffer, 0.2 mM dNTPs, 500 nM of each primer, 0.5 IU proofreading Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific™, USA) and 100 ng of the total DNA was run on an Applied BioSystems 2720 thermal cycler (Thermo Fisher Scientific™, USA) following the cycling conditions: 30 s initial denaturation at 98 °C, 30 amplification cycles (10 s denaturation at 98 °C, 15 s annealing at 60 °C and 1.5 min extension at 72 °C) with a final hold for an extra 5 min at 72 °C. The 1.9 kb blunt-ended PCR product was analysed by electrophoresis in a 0.8% agarose gel and then purified using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific™, USA) before being ligated into the pJET1.2/blunt Cloning Vector to form the plasmid pJET-*cry2Ab* according to the manufacturer's instructions. The ligation product was subsequently cloned into *E. coli* DH5α using the heat-shock method and the transformants

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were analysed by colony-PCR using the pJET1.2 sequencing primer pair available in the CloneJET PCR Cloning Kit. The plasmid pJET-*cry2Ab* was isolated and purified from the correct recombinant colony and finally confirmed by Sanger sequencing with an automatic Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific™, USA). The nucleotide sequences were analysed using BLAST from the National Center for Biotechnology Information (NCBI). The protein alignment was performed using ClustalX and annotated with the characteristics of Cry-conserved blocks.

Construction of expression vector pET32a. In order to investigate the expression of the *cry2Ab39* gene, an expression vector was constructed as follows: a pJET vector carrying the *cry2Ab39* gene digested with *Bam*HI and *Xho*I restriction enzymes and ligated into *Bam*HI/*Xho*I-linearized pET-32a(+) yielding the expression vector pET32a-*cry2Ab39*. This ligation mixture was then transformed into *E. coli* BL21 and the recombinant plasmid extracted from the colonies on the selection agar plate was confirmed by restriction analysis and subsequently sequenced to verify the correct N-terminal fusion of the target peptide Cry2Ab39 with the Trx-His-Stag construct encoded in pET-32a(+).

Expression of *cry2Ab39* gene in *E. coli* BL21. A single colony of *E. coli* BL21 strain harbouring the expression vector pET32a-*cry2Ab39* was inoculated in a 5 mL lysogeny broth containing 100 µg/mL ampicillin and grown overnight at 37 °C under continuous shaking condition. This pre-culture was diluted 1/100 in a fresh lysogeny broth medium supplemented with 100 µg/mL ampicillin and further cultivated at 37 °C with vigorous agitation until the optical density (OD_{600}) reached about 0.4 to 0.6. The production of the fusion protein Trx-His-Stag-Cry2Ab39 was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich®, USA) to a final concentration of 0.1 mM and incubated for 4 h at 37 °C. The cultivation with no inducer added was proceeded in parallel as a negative control. The samples were then harvested by centrifugation for 10 min at 4 °C, and the cell pellets were stored at –80 °C until further processing. After being subjected to freeze-thaw cycles, the cell pellets were suspended in pre-cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and followed by sonication (15 cycles of 10 s) on ice using Vibra Ultrasonic Liquid Processors (Sonics and Materials Inc., USA). The sonicated lysates were centrifuged for 20 min to separate the soluble fraction and the pellet. The presence of Trx-His-Stag-Cry2Ab39 in the total cellular lysate as well as in the supernatant and insoluble fraction was analysed by being subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). A western blot assay using an anti-6xHis serum was also performed to confirm the expression of the target product. In order to improve the solubility of the recombinant protein, various lower induction temperatures and incubation times were investigated, including 28 °C for 6 h, 15 °C for 16 h, and 4 °C for 72 h. The target protein solubility enhancement was assessed through the SDS-PAGE analysis, and the most proper condition was employed in the large-scale production of Trx-His-Stag-Cry2Ab39 for the subsequent purification experiment.

Purification of Cry2Ab39 protein. The Trx-His-Stag-Cry2Ab39 recombinant protein was purified by an NTA-Ni column (Cube Biotech, Germany). The induced cell pellets were re-suspended in a binding buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 8.0), sonicated on ice and then centrifuged at 10 000 × *g* for 20 min to clarify the cell lysate. The supernatant was subsequently incubated with an Ni-NTA resin and mixed gently at 4 °C for an hour to allow for the binding of Trx-His-Stag-Cry2Ab39. The protein/resin complex was applied to a miniature column and washed with a washing buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 40 mM imidazole, pH 8.0) to remove the unbound non-specific proteins. Finally, the Trx-His-Stag-Cry2Ab39 was released from the column using an elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 500 mM imidazole, pH 8.0). One-millilitre fractions were collected separately in microcentrifuge tubes and an SDS-PAGE assay was carried out to analyse the purity of the protein samples. The fractions containing the purified protein were then pooled for dialysis against PBS to remove the imidazole and finally concentrated using a 3K MWCO protein concentrator column (Thermo Fisher Scientific™, USA). The protein concentration was determined using the Bradford assay (Bradford 1976) with bovine serum albumin (Sigma-Aldrich®, USA) as a standard. The purified fusion protein Cry2Ab39 was preserved in glycerol (50%, v/v) at –20 °C and was then used for the toxicity bioassays.

SDS-PAGE and western blot. A glycine SDS-PAGE, for protein analysis, was followed by the

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description of Laemmli (Laemmli 1970). In brief, the samples, after being treated with a dissociation buffer (50 mM Tris-HCl pH 6.8, 1% SDS, 50 mM dithiothreitol, 8 mM ethylenediaminetetraacetic acid, 0.01% bromophenol blue) and heated for 5 min at 95 °C, were loaded on a 5% stacking gel and further separated on a 10% resolving gel under reducing conditions. The resolved proteins on the gel were stained for 30 min with 0.2% (w/v) Coomassie Brilliant Blue R-250 (Merck, Germany) in a mixture of methanol/acetic acid (40:10, v/v) and then de-stained with this mixture until a transparent background was achieved. The PageRuler Prestained Protein Ladder 10–180 kDa (Thermo Fither Scientific™, USA) was used as the molecular weight standard.

The western blot procedure was performed according to the protocol described by (Pham et al. 2019). After being subjected to SDS-PAGE, the separated proteins on the gel were transferred onto a nitrocellulose membrane by an electroblot apparatus (Bio-Rad, USA). The membrane was blocked for 1 h in blocking buffers [5% (w/v) non-fat dry milk in a PBS buffer] and then incubated with the 6x-His Tag monoclonal antibody (Invitrogen, USA) as the primary antibody at 4 °C for 3 hours. After washing three times with PBS-Tween, a secondary antibody (HRP-conjugated goat anti-mouse IgG; GE Healthcare, UK) was added and further incubated for 1 h at room temperature. Specific signals were detected by incubating the membrane with 3,3-diaminobenzidine (Thermo Scientific Pierce, Thermo Fither Scientific™, USA) dissolved in 0.05 M Tris-HCl and 0.04% hydrogen peroxide for 10 min in the dark.

Insect bioassay. Bioassays were made with three different proteins: crystal and spore suspensions harvested from *Bt* SP142 strain, a recombinant protein suspension (pET32a-Cry2Ab39) from *E. coli* BL21(DE3), and a purified recombinant protein.

Preparing the artificial food for bioassay: (1) weigh 200 g of artificial food, add 625 mL of distilled water, and into a blender; (2) weigh 20 g of agar, add 375 mL of distilled water, and heat the agar; (3) mix (1) into (2), and mix well in a blender; (4) pour 20 g of food into a plastic box (9 cm × 6 cm × 3.5 cm) to cool the food and cut it into small pieces (2 × 2 mm). Ingredients of the artificial food: soybean meal (125 g/L), corn flour (20 g/L), ascorbic acid (4 g/L), yeast extract (40 g/L), barley germ (4 g/L), acetic acid (2 g/L), choline chloride (2 g/L), casein (25 g/L), sorbic acid (1.5 g/L), multivitamins (5 mL/L), dohyfral (0.5 g/L).

The *B. thuringiensis* SP14.2 strain was grown in 100 mL CYS medium in 250 mL flasks at 30 °C by rotary shaking for 72 h. The crystal and spore suspensions were harvested by centrifugation at 10 000 × *g* for 10 min and responded in 10 mL of water. An aliquot (2.0 mL) of the crystal and spore suspension was mixed with 20 g of artificial food in a plastic cup containing ten second-instar larvae of *E. zinckenella*. In the control experiment, sterile water was used with the food. The larvae were allowed to feed on the artificial food mixture for five to seven days at 28 °C, and the end of feeding, the larval mortality was recorded. The experiments were repeated three times. The percent mortality was corrected according to Abbott's formula (Abbott 1925). The sonicated lysates of the recombinant protein suspension were designed as the bioassay of SP142 strain. The purified recombinant protein, which determined the concentration of the toxic protein by the Bradford method using bovine serum albumin as a standard protein, using the same procedure, was repeated for the final toxin concentration of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4 µg/g diet. The median lethal concentration (LC_{50} value) and its fiducial limits were determined with a Probit analysis using IBM SPSS Statistics software (version 20). Cell-free extracts of the culture of *E. coli* host BL21 containing no pET-Cry2Ab39 and the PBS buffer alone prepared in a similar way as the test samples were used as controls.

RESULTS

Genome sequence of *B. thuringiensis* strain SP142 and prediction of candidate Bt toxins. The SMRTbell library was constructed from the total genomic DNA, 214 contigs were produced with a total length of 7 100 191 bp and an N50 value at 70 282 bp. The genome size of the SP142 strain was estimated at about 7.1 Mb with an average guanine-cytosine (GC) content of 35%. The genome functional annotation revealed 9 806 protein-coding and 108 RNA genes including 79 tRNA and 29 rRNA clusters. To predict the sequences encoding the Bt toxins, the generated sequence data was analysed with a BtToxin_scanner, and 22 candidates were identified, comprised of 17 candidates homologous to the Cry toxins, four candidates homologous

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to the Vip proteins, and one candidate homologous to the Cyt proteins. Among the 17 candidate *cry* sequences, the *cry1* types accounted for the majority of the sequences (11 sequences) followed by the *cry2* types (six sequences). Despite that numerous candidate Bt toxins were predicted in the SP142 genome, all of their amino acid sequences showed high identity with those of the known Bt toxins, ranging from 99.05% to 100%. Among these putative *cry* sequences, the novel *cry2Ab* gene sequence was chosen to cloning, expression and assessment of insecticidal activity to the insect of interest, *E. zinckenella*.

Cloning and molecular characterisation of the novel *cry2Ab* gene. The full-length *cry2Ab* gene was amplified from the SP142 genomic DNA by a conventional PCR. A single PCR product of approximately 2 kb was obtained (Figure 1A) and cloned into the pJET1.2/blunt cloning vector. The Sanger sequencing result confirmed the coding sequence of the predicted *cry2Ab* gene consisting of 1 899 nucleotide coding for a 633-amino-acid polypeptide with an estimated molecular mass of 70 kDa. The nucleotide and translated amino acid sequences of the isolated gene were found to be 99.05% and 99.21%, respectively, identical to those of the *cry2Ab3* gene in the GenBank (AF164666.1). Like other members belonging to the three-domain Cry toxin family, the predicted Cry2Ab protein also had three typical conserved domains, including endotoxin_N (pfam03945), endotoxin_mid (pfam09131) and endotoxin_C (pfam03944). The sequence alignment of the *cry2Ab3* and predicted *cry2Ab* genes revealed 18 different nucleotide sites, but only six of them corresponded to the amino acid changes in the codons 232 (K232R), 317 (S317T), 318 (N318Q), 329 (S329T), and 346 (I346V) (Figure 2). Except for the change in codon 232 located in Domain I responsible for the membrane insertion and pore formation, all the amino acid substitutions lie in Domain II, which is involved in insect-specific receptor recognition and binding. The nucleotide and translated amino acid sequences of the novel *cry2Ab* gene were deposited in the GenBank with accession numbers MN319700.1 and QIQ19560.1, respectively, and this Cry toxin was received as Cry2Ab39 by the *Bt* delta-endotoxin nomenclature committee (Bacterial Pesticidal Protein Resource Center – BPPRC).

Construction, expression and purification of recombinant Trx-His-Stag-Cry2Ab39. The full coding sequence of *cry2Ab39* was inserted into the *Bam*HI/*Xho*I sites of pET-32a(+), yielding the *E. coli* expression vector pET32a-*cry2Ab39*, which expresses the chimeric fusion protein Trx-His-Stag-Cry2Ab39 under the regulation of the T7*lac* promoter (Figure 1C). The result of the double-enzymatic digestion confirmed the correct insertion of *cry2Ab39* in the recombinant plasmid pET32a-*cry2Ab39* (Figure 1B). This vector was further transformed into the host cell *E. coli* BL21 (DE3) to investigate the expression of the target

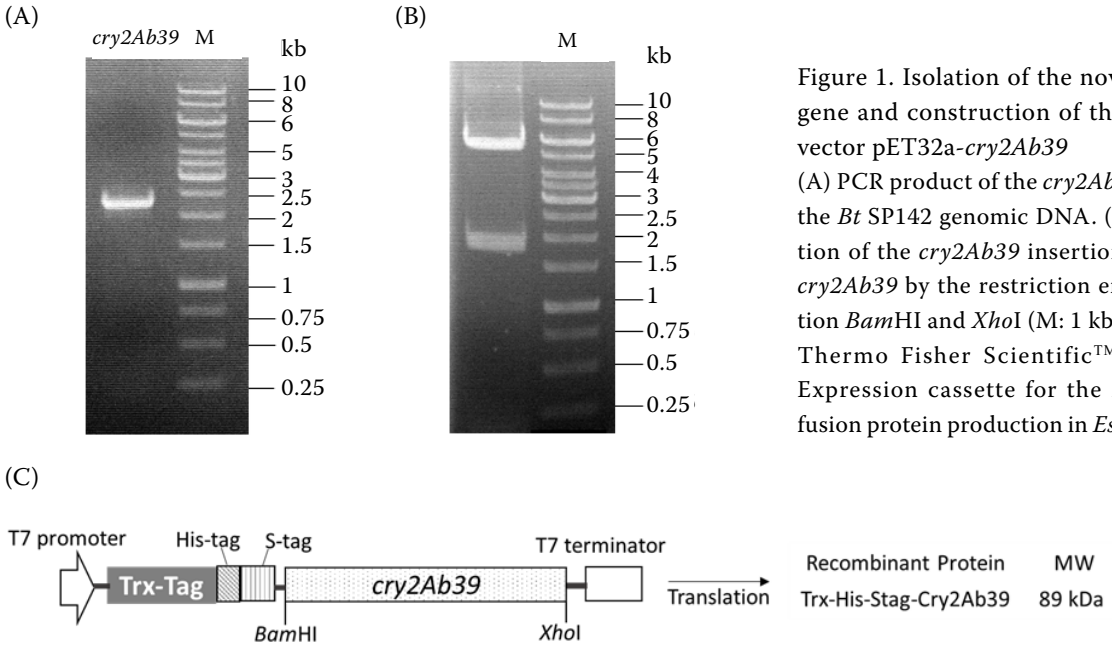

Figure 1. Isolation of the novel *cry2Ab39* gene and construction of the expression vector pET32a-*cry2Ab39*

(A) PCR product of the *cry2Ab39* gene from the *Bt* SP142 genomic DNA. (B) Identification of the *cry2Ab39* insertion in pET32a-*cry2Ab39* by the restriction enzyme digestion *Bam*HI and *Xho*I (M: 1 kb DNA ladder; Thermo Fisher Scientific™, USA). (C) Expression cassette for the recombinant fusion protein production in *Escherichia coli*

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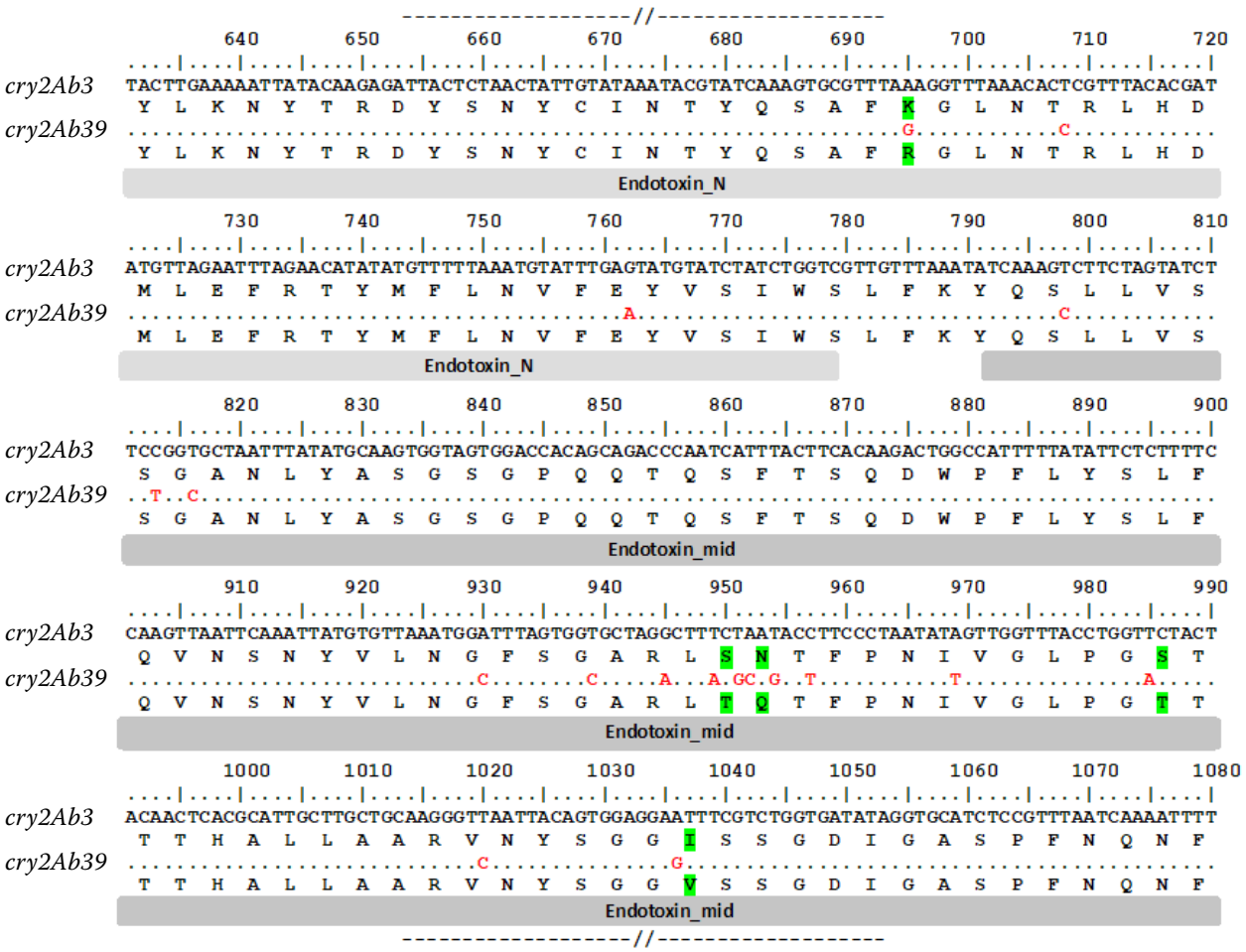

Figure 2. Nucleotide and translated amino acid sequence alignment of *cry2Ab3* and *cry2Ab39*

The eighteen different nucleotide sites between the two genes are in red, and five amino acid substitution positions are highlighted in green. Absolute identity in the coding sequence between the two genes is shortened and represented by dashed lines (---//---)

protein. Under the control of the T7*lac* promoter induced by 0.1 mM IPTG at 37 °C for 4 h, the protein of Trx-His-Stag-Cry2Ab39 was over-expressed, comprising about 45% of the total cellular proteins according to quantification by the ImageJ software (version 1.53a). The molecular weight of the fusion protein was shown to be in the expected size (approximately 89 kDa, containing 70.5 kDa of Cry2Ab and 18.5 kDa of the three fusion tags) (Figure 3A). The expressed chimeric protein was also confirmed by western blot using an anti-6xHis Tag serum, whereas no signal was detected in the negative control (Figure 3B).

However, the solubility analysis revealed that the Trx-His-Stag-Cry2Ab39 protein was produced and accumulated mostly in the form of insoluble aggregates (or inclusion bodies) (Figure 3C, lane 2). Only a small amount of Cry2Ab39 was observed in the soluble fraction (Figure 3C, lane 1). Therefore, the Cry2Ab39 protein was expressed at lower temperatures to improve the protein solubility. No significant change in the mass of the soluble target protein was noted at induction temperatures of 28 °C and 15 °C (data not shown). The amount of Trx-His-Stag-Cry2Ab39 in the supernatant was significantly increased (Figure 3C) when the fermentation temperature was lowered to 4 °C for 72 h. Although over 50% of the expressed protein remained as inclusion bodies (Figure 3C, lane 4), 4 °C was found to be a more suitable incubation temperature for the expression of the fusion protein Cry2Ab39 in *E. coli* BL21.

The lysis supernatant fraction containing a soluble form of Trx-His-Stag-Cry2Ab39 was used

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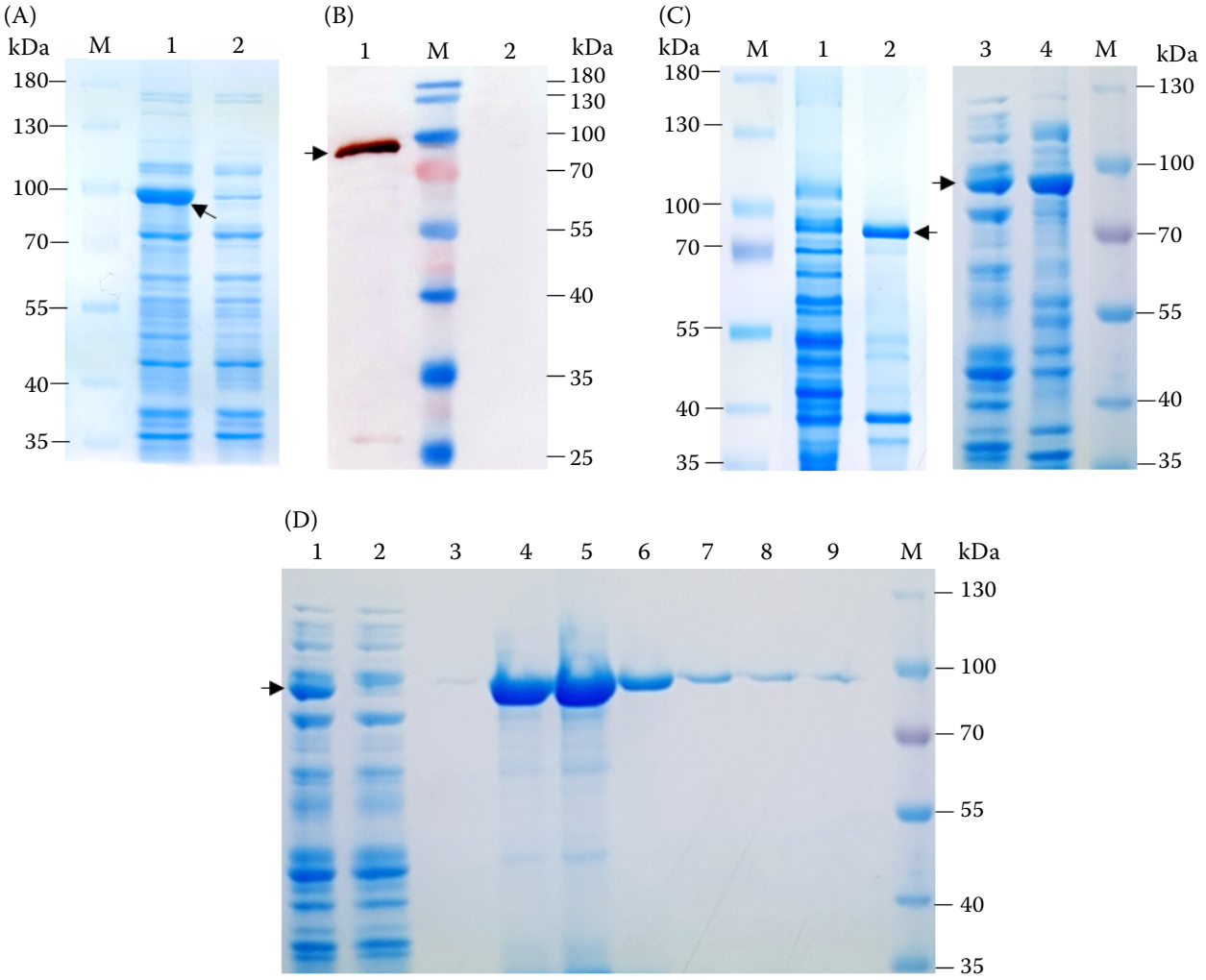

Figure 3. Expression and purification of the Cry2Ab39 expression in *Escherichia coli* BL21
(A) and (B) analysis of the Cry2Ab39 expression by SDS-PAGE and western blot, respectively; total cellular proteins induced by 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 37 °C for 4 h (lane 1), un-induced control (lane 2). (C) solubility analysis of Cry2Ab39 by SDS-PAGE: total cellular proteins induced by 0.1 mM IPTG at 37 °C for 4 h in supernatant (lane 1), precipitate fractions (lane 2), total cellular proteins induced by 0.1 mM IPTG at 4 °C for 72 h in supernatant (lane 1), precipitate fractions (lane 2). (D) SDS-PAGE analysis of native purification procedure of Cry2Ab39: total soluble proteins (lane 1), flow-through (lane 2), elution fractions (lane 3 to 9). Molecular weight marker (Thermo Scientific, 10–180 kDa) (lane M). Black arrows indicate the target fusion protein Cry2Ab39

for the purification by affinity chromatography with an Ni-NTA column. The purified recombinant protein was obtained with a purity of greater than 90%, and the purification yield was estimated to be about 3.2 mg/100 mL of the bacterial fermentation (Figure 3D).

Toxicity assay. The crystal and spore suspensions harvested from the SP142 strain and the sonicated lysates of recombinant protein suspension showed high toxicity against the second-instar larvae of *E. zinckenella* with 93.33% and 96.67% mortality after seven days of treatment, respectively. The purified recombinant protein Trx-His-Stag-Cry-2Ab39 was toxic to the *E. zinckenella* with an LC_{50} value of 1.74 µg/g diet. A small amount of larvae (10% to 20%) stopped feeding and died within 24 h at high toxin concentrations (3.5 and 4.0 µg/g diet), whereas the insect mortality started later (after 48 h) at lower treatments. The toxin concentration-total mortality responses of Trx-His-Stag-Cry-2Ab39 for the *E. zinckenella* larvae were recorded after 96 h and subjected to a fit with a Probit regression line (Figure 4, Table 1). The regression parameters demonstrated a well-fitting Probit model

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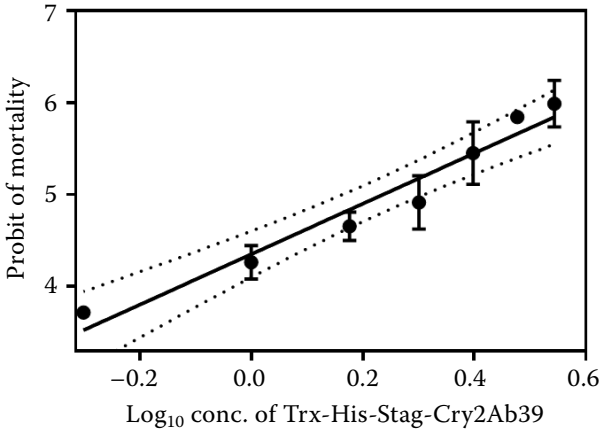

Figure 4. Probit regression of the concentration-mortality curve to estimate the LC_{50} of Trx-His-Stag-Cry2Ab39 against *Etiella zinckenella*

The data are reported as the mean ± SE (standard error) from three replicates and analysed in IBM SPSS Statistics software (version 20). The dotted lines represent 95% confidence intervals of the regression line

(chi-square test: 2.37, 6), and the relatively narrow confidence limits of the LC_{50} value (1.33–2.16 µg/g) indicated highly reliable estimation results.

DISCUSSION

As a result, from the broad-spectrum insecticidal activity of Cry toxins, a *Bt*-based insecticide as well as transgenic *Bt* crops have been developed and applied for biological pest controls on a global scale. However, the resistance evolution by target insects is emerging as a worldwide threat to the durable effect of these applications (Tabashnik et al. 2009; Wu 2014). Consequently, a study on the detection of novel highly host-specific pesticidal proteins is now considered an urgent necessity (Sayyed et al. 2000; Palma et al. 2014). In the past, PCR-based investigations were the main approaches utilised for predicting and isolating new *cry* genes in uncharacterised *Bt* strains (Beron et al. 2005), but there are numerous limitations to these methods, including being less effective in finding novel genes and in the difficulty in obtaining full-length-sequence genes (Noguera & Ibarra 2010; Ye et al. 2012). Recently, next-generation sequencing (NGS) technologies, which enable rapid whole-genome sequencing with profitable cost-effective ratios, have become powerful tools for the discovery of potentially novel *Bt* toxin genes (Ye et al. 2012; Palma et al. 2014; Sajid et al. 2018; Lazarte et al. 2021; Pacheco et al. 2021; Naveenarani et al. 2022). In the present work, the entire genome of the *Bt* serovar *canadensis* strain SP142 was sequenced using a single-molecule real-time (SMRT) sequencing technology developed by Pacific Biosciences (Pac-Bio). The complete genome data of this strain exhibited typical features similar to other *Bt* strains, including the genome size, GC content and number of predicted coding DNA sequences. The prediction results of the insecticidal genes that were performed using the Bt-Toxin_scanner, a web-based high-throughput bioinformatics tool for mining toxin protein genes from *Bt*, revealed a putative novel *cry2Ab* gene. The subsequent results of the isolation, cloning and Sanger sequencing once again proved the accuracy of the previous prediction. The putative *cry2Ab* gene displays 99.05% similarity with the nucleotide sequence of the *cry2Ab3* gene in the GenBank (AF164666.1), whereas their translated amino acid sequences share 99.21% homology with six different sites. The nucleotide and translated amino acid sequences of the novel *cry2Ab* gene has been deposited in the GenBank with accession numbers MN319700.1 and QIQ19560.1, respectively. Interestingly, most of the amino acid substitutions are located in the Endotoxin_mid domain responsible for insect-specific receptor recognition and binding. This may suggest the possibility of a different spectrum as well as the target insect specificity of the newly discovered toxin. This identified novel *Bt* toxin was assigned as Cry2Ab39 by the *Bt* delta-endotoxin nomenclature committee (Bt Toxin Nomenclature or the Bacterial Pesticidal Protein Resource Center).

Currently, Cry2 proteins have gained much attention and are considered as alternatives to Cry1 proteins for the development of insect-resistant

Table 1. Toxicity of Trx-His-Stag-Cry2Ab39 against *E. zinckenella* and the parameters of the Probit regression

LC_{50} (µg/g) (95% CI)	Regression line		Goodness-of-fit test		
	slope ± SE	intercept ± SE	χ^2	df	*P**
1.74 (1.33–2.16)	2.74 ± 0.27	4.35 ± 0.09	2.37	6	0.88

**P*-value of chi-square test for the Probit model

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transgenic crops (Hire et al. 2009). To date, many Cry2Ab-type toxins from different *Bt* isolates have been found and characterised. Most of them displayed toxicity against Lepidopteran pests while a minority were toxic to Diptera. The Cry2Ab expressed in *E. coli* EG1344 had toxicity against *Lymantria dispar*, *Heliothis virescens* and *Trichoplusia ni* with LD_{50} values of 45, 124 and 143 μg/mL, respectively (Dankocsik et al. 1990). The Cry2Ab-type toxin from a Brazilian *Bt* strain expressed in insect cells (BTI-Tn5B1-4) exhibited toxicity against second-instar *Spodoptera frugiperda* larvae with an LC_{50} of 3.45 μg/mL (Lima et al. 2008). Cry2Ab25 expressed in BL21(DE3) exhibited high toxicity against *Malacosoma neustria* and *Rhagoletis cerasi* with 73% and 75% mortality after five days of treatment, respectively (Sevim et al. 2012). Cry2Ab30 toxin had high insecticidal activity against *Plutella xylostella* and the LC_{50} value was 0.010 3 μg/mL (Zhizhen et al. 2014). In this study, the novel Cry2Ab39 toxin derived from the *Bt* serovar *canadensis* strain SP142 in Vietnam was characterised in molecular details and assessed for biological activity. A simple, but efficient, approach for improving the solubility of recombinant Cry toxin expressed in *E. coli* was performed. The purified recombinant protein Cry2Ab39 was toxic to *E. zinckenella* with an LC_{50} value of 1.74 μg/g diet. In addition, the host range of *Bt canadensis* has been further identified for insect pests of soybeans (Lepidoptera), which was previously only known to be toxic to mosquito larvae (Diptera) (Ragni et al. 1996; Balaraman 2005). Besides, the soybean is a major crop and the most adopted biotech crops, which covered 48% of the global biotech area in 2019. Cry1Ac, Cry1Ab2, Cry1A.105, cry1F and Cry2Ab2 from the strains *Bt* subsp. *kumamotoensis*, *Bt* var. *aizawai* and *Bt* subsp. *kurstaki* HD73 are six events approved for the Bt insect resistance of GM soybeans in the world. In Vietnam, there is an event (Cry1Ac delta-endotoxin from *Bt* subsp. *kurstaki* strain HD73) approved in the GM soybean. They were resistant against some lepidopteran larvae (*Ostrinia nubilalis*, *Diatraea grandiosella*, *D. crambidoides*, *Helicoverpa zea*, *S. frugiperda*, *Papaipema nebris*, and *D. saccharalis*), but not resistant to *E. zinckenella* larvae (https://www.isaaa.org). Therefore, further studies are needed to reveal bioinsecticides, such as crystal insecticidal proteins derived from *B. thuringiensis*, that will be effective against this pod borer. Previously, two toxins Cry1Aa4 and Cry1Ia34 of the *Bt* TH19 strain were revealed to have toxicity to *E. zinckenella* larvae (Hang et al. 2021). This study is demonstrated a type of novel Cry2 toxin (Cry2Ab39) from the *Bt* serovar *canadensis* SP142 strain that is capable of killing *E. zinckenella* larvae, which is a harmful insect attacking leguminous crops and is difficult to control by chemical insecticides. It is necessary to fully assess the insecticidal spectrum as well as host-specificity of this Cry2Ab39 protein. This novel Cry toxin could be used as a source of genes for the varietal improvement of transgenic soybean resistance against the pod borer in the future. Currently, a patent application of the *Bt canadensis* SP142 strain carrying the *cry2Ab39* gene is under consideration in Vietnam (Application No. 1-2020-06575, filed on December 18, 2020), and further studies for transgenic varieties in soybeans are being performed.

CONCLUSION

In this study, a new Cry2Ab39 toxin against the larvae of the soybean pod bore *E. zinckenella* from the *Bt* serovar *canadensis* strain SP142 in Vietnam has been detected. It could not only be a precious gene for the generation of insect-resistant transgenic plants, but also a promising candidate for new bioinsecticides in pest-crop controls in the future. This study also reported a simple, but efficient, approach for the discovery of potentially novel toxin genes in uncharacterised *Bt* strains. However, further studies should be conducted to give a clearer insight into the insecticidal spectrum as well as the host-specificity of this toxin.

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