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Screening, expression and characterization of scarabcidal crystal protein from a strain (MHB11.3) of *Bacillus thuringiensis* serovar *galleriae* in Vietnam

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Bacillus thuringiensis **strains were isolated from sugarcane farming soil in three provinces of northern Vietnam. Of these, 24 strains were identified as serotype H5a, 5b, *B. thuringiensis* serovar *galleriae*. A strain, namely MHB11.3 (deposited in GenBank: FN796428) belonging to the serovar *galleriae*, was highly toxic to larvae of the scarab beetles, *Anomala cuprea* and *Cotinis nitida* (Coleoptera: Scarabaeidae). A 2-kb DNA fragment, obtained by PCR from the strain MHB11.3 using *cry8* specific primers, namely *cry8Dat* (truncated *cry8Da* gene), was cloned and expressed in *Escherichia coli*. The sequence of the *cry8Dat* from *B. thuringiensis* serovar *galleriae* MHB11.3 showed almost complete identity (99%) to that of *B. thuringiensis* serovar *galleriae* SDS502 (GenBank: AB089299), differs only by two nucleotides (positions A26G; A1493T), leading to 2 amino acid changes (Y9C and N498I). The recombinant protein pET32a(+)-Cry8Dat of 96 kDa molecular weight was purified by $Ni^{(2+)}$ affinity chromatography. This polypeptide was tested to be toxic to *A. cuprea* and *C. nitida*. The amino acid change (Y9C) is located in a region responsible for the host specificity of the Cry8Da toxin. The strain *B. thuringiensis* serovar *galleriae* MHB11.3 harboring *cry8Da* gene as characterized in this study is a promising candidate as a biopesticide and suggested as an important genetic material for gene transfer into sugarcane for scarab beetle control.**

Key words: *Bacillus thuringiensis* serovar *galleriae*, Coleoptera, *cry8Da gene*, *Anomala cuprea*, *Cotinis nitida.*

INTRODUCTION

One of the most important findings in *Bacillus thuringiensis* research, after their establishment as alternative for control of insect pests in agriculture (Burges, 1981; Roh et al., 2007) was the discovery of Cry proteins with Coleopteran specificity. Krieg et al. (1983) first cloned a novel gene from *B. thuringiensis* serovar *tenebrionis* encoding the Cry3A protein that kills Colorado potato beetle. Soon after, Herrnstadt et al. (1986) isolated a similar strain, *B. thuringiensis* serovar *morrisoni san diego*, and its insecticidal crystal protein (ICP) gene was sequenced. Among *B. thuringiensis* ICPs, Cry3, Cry7, Cry8, Cry18, Cry34, Cry35 and Cry43 are known to be active against coleopteran species. Of these, Cry8Ca is reported to be active against scarab beetles (Ohba et al., 1992; Shu et al., 2007, 2009; Yamaguchi et al., 2010). Asano et al. (2003) reported a new *B. thuringiensis* strain, called serovar *galleriae* SDS-502, that showed high insecticidal activity against *Anomala cuprea, Anomala*

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orientalis and *Popillia japonica*, which are species of scarab beetles known as the Japanese beetles. The ICP gene responsible for the highly activity of SDS-502 against Japanese beetles has been isolated and was designated *cry8Da*. The *cry8Da* gene was transferred into turf grass, which showed strong resistance against feeding attack by the Japanese beetle (Asano et al., 2005).

In Vietnam, sugarcane is an important crop for the sugar industry. Today, the sugarcane growing area is approximately 300,000 ha, with productivity of about 110 tons/ha, and a total sugar yield of approximately one million tons per year. Recently, insect activity has harmed the sugarcane areas in Vietnam. Outbreaks of black scarabs and long-horned beetles cause heavy damage to the sugarcane crop every year, and have reduced sugar productivity by 35% (Binh et al., 2005, 2007). Measures to prevent these outbreaks, such as chemical insecticides and mechanical methods, were unsuccessful. Moreover, the chemical insecticides caused environmental pollution and are harmful to humans. Consequently, an alternative agent to control the above mentioned coleopteran in sugarcane farming areas is needed. No research had yet focused on *cry8* gene and its insecticidal toxins application in agriculture in Vietnam. Therefore, in this study we examined soil from sugarcane planting areas of northern Vietnam for the presence of *B. thuringiensis* strains and characterize their coleopteran insecticidal Cry8D proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

B. thuringiensis strains used in this study were isolated from sugarcane farming soil in Vietnam. *Escherichia coli* DH5α was used to maintain the cloned gene and *E. coli* BL21(DE3)pLysS was used for expression of the gene. The plasmids pGEM-T Easy (Promega) and pET32a(+) (Novagen) were used for cloning and expression, respectively. *E. coli* and *B. thuringiensis* were grown as batch cultures in Luria Bertani (LB) broth: peptone 20, yeast extract 10 and NaCl 5 (g/l) with shaking at 37 and 30°C, respectively. *E. coli* harboring pGEM-T Easy and pET32a(+), and their derivatives, were grown in the presence of ampicillin (50 µg/ml).

Isolation of *B. thuringiensis*

Forty-seven soil samples were collected from sugarcane farming areas of three provinces in northern Vietnam (Hung Yen, Hoa Binh and Ha Noi). Sampling fields had not previously been sprayed with any commercial *B. thuringiensis* biopesticides. The isolation of *B. thuringiensis* was carried out according to the method of Ohba and Aizawa (1986), Yasutake et al. (2006). Briefly, one gram of soil was suspended in 9 ml of sterile phosphate-buffered saline (PBS) and shaken for 5 min. The upper layer of soil suspension was transferred into a test tube and heated at 65°C for 30 min to kill heat-sensitive organisms. Ten-fold serial dilutions were made in PBS and plated on nutrient agar (pH 7.6) consisting of 1% meat extract (w/v), 1% polypeptone (w/v), 0.2% NaCl (w/v) and 2% agar (w/v). The isolates were incubated at 27°C for 3 to 4 days. Colonies with morphological features characteristic of *Bacillus cereus* group were examined under a phase microscope, and those that formed parasporal inclusions (crystals) were assigned to *B. thuringiensis*. *B. thuringiensis* were maintained on nutrient agar for serological and biological tests.

Flagellar (H) serotyping

H-serotyping of *B. thuringiensis* strains was performed by the slide agglutination reaction (Ohba and Aizawa, 1978). H antisera were obtained from rabbits against the reference of *B. thuringiensis* H-serotypes 1–60 (Lecadet et al., 1999). The reference *B. thuringiensis* H-serotypes were kindly provided by Prof. D. Dean and Dr. R. Zeigler from the *Bacillus* Genetic Stock Center, Ohio State University, USA. The slide agglutination reaction was performed by mixing 1 µl of the flagellated broth culture of bacteria with 1 µl of 20- to 50-fold dilutions of H antiserum on a glass slide.

Preparation of crystals and scanning electron microscopy (SEM) observation

B. thuringiensis strains were precultured in 20 ml of LB broth at 30°C by rotary shaking at 220 rpm overnight, 1.0 ml of the preculture was transferred into 50 ml of CYS medium (Yamamoto, 1990) in 250 ml flasks, then cultured at 30°C by rotary shaking at 220 rpm for 6 days until sporulation and lysis. The fully matured crystals and lysed cells were harvested and washed with sterilized water by centrifugation at 12,000 g for 10 min. The crystals and spore mixture were incubated in 0.01% Triton X-100 and 1.0 M NaCl solution at 4°C overnight. Crystals of *B. thuringiensis* were separated from spores and cellular debris by a 30 to 70% NaBr gradient centrifugation at 17,000 g for 120 min. The crystal layer (upper) was harvested with Pasteur pipette, dissolved in water and washed twice by centrifugation. Then the crystal pellets in water were stored at -20°C.

B. thuringiensis crystals were examined with a JSM 5410 LV scanning electron microscope (Jeol, Japan) according to the methods of Iizuka et al. (1982) and Lee et al. (2004). Crystal suspensions of *B. thuringiensis* strains were prefixed in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS, pH7.3) for 20 min at 4°C and fixed with 1.33% osmium tetroxide in the same buffer for 10 min at 4°C. They were dehydrated with 50 to 100% ethanol. Samples were air dried on a collodion grid, coated with gold and were examined in SEM.

Bioassays

The three insect species used in this study were *Anomala cuprea*, *Cotinis nitida* (Coleoptera: Scarabaeidae), and the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). The three larvae were supplied by the Vietnam National Institute of Plant Protection. Bioassays were made with three different proteins as the crystal and spore suspensions harvested from Vietnam *B. thuringiensis* strains, extracted crystal protein from MHB11.3 strain and purified recombinant protein (rCry8Dat) from *E. coli* BL21(DE3)pLysS.

B. thuringiensis strains were grown in 50 ml CYS medium in 250ml flasks at 30°C by rotary shaking at 220 rpm for 72 h. The crystal and spore suspensions were harvested by centrifugation at 12,000 g for 10 min and responded in 10 ml water. An aliquot (1.0 ml) of the crystal and spore suspension was mixed with 5 g of sterile compost in a plastic cup containing five second-instar larvae of *A. cuprea* or *C. nitida*. In the control experiment, sterile water was used with the compost. Four set of control group and of each isolate were designed. The larvae were allowed to feed on the compost mixture for 7 days at 28°C, and the end of feeding, larval

Table 1. Isolation of *B. thuringiensis* serovar *galleriae* strains in soils from three sugarcane planting areas of northern Vietnam.

Soil sampling area	Number of soil samples tested	Number of samples with Bt (%)	Number of Bt isolates	Strains of spherical crystals	*B. thuringiensis* serovar *galleriae*
Soc Son[a] (Ha Noi)	18	6 (33.3)	27	10	8
Van Lam[a] (Hung Yen)	14	4 (28.5)	23	11	11
Luong son[a] (Hoa Binh)	15	6 (40.0)	28	18	5
Total	47	16 (34.0)	78	39	24

[a]Sugarcane farming areas belong to Hanoi, Hung Yen and Hoa Binh provinces, respectively.

mortality was recorded. The experiments were repeated five times in order to get best average values. The same protein and spore suspensions were assayed against third-instar larvae of *P. xylostella*. In this case, the crystal suspension was placed on cabbage leaves, and insects were allowed to feed for 5 days at 28°C, and the end of feeding, larval mortality was recorded.

Bioassays of extracted crystal protein from MHB11.3 strain and purified recombinant protein (rCry8Dat) from *E. coli* BL21, which determined concentration of toxic protein by the Bradford method using bovine serum albumin as standard protein, were designed as bioassay of *B. thuringiensis* strains. Same procedure was repeated for the different concentrations of protein from 0.625 to 10 µg/ml. By the procedure of bioassay, LC50 was calculated and associated with 95% confidence intervals with the Probit analysis programme (SPSS 16.0 software - International Business Machines Corp. USA).

PCR, cloning and DNA sequencing of *cry8Dat* gene

A 2.0-kb fragment of *cry8Dat* was amplified by PCR using total DNA from 24 *B. thuringiensis* serovar *galleriae* strains as templates. The sequence of the forward *cry8D*-specific primer was 5'-CGGATCCATGAGTCCAAATAATCAAAATG-3' (contained a *Bam*HI site, is underlined), and the reverse 5'-GAGCTCTCACACATCTAGGTCTTCTTCT-3' (contained *Sac*I site, is underlined). PCR application conditions were as described in a previous report (Asano et al., 2005). DNA from *B. thuringiensis* strains was prepared from 1 ml CYS medium cultures shaken overnight. Then cultures were centrifuged and washed three times with distilled water, the pellet was suspended in 100 µl distilled water and boiled for 10 min to lyse the cells (Birnboim and Doly, 1979). Lysed suspensions were centrifuged, and 2 µl of supernatant was used as a template for PCR. Following amplification, the PCR product was analyzed by 0.8% agarose gel electrophoresis.

DNA cloning was performed according to the standard methods described by Sambrook and Russell (2001). In brief, the 2-kb DNA fragment amplified by PCR was ligated into pGEM-T Easy and transformed into competent *E. coli* DH5α. The recombinant plasmid was named pGEM-*cry8Dat.* The transformed *E. coli* DH5α were screened by colony hybridization. Recombinant plasmids were isolated and purified from transformed *E. coli* DH5α, and examined by restriction digestion with *Sac*I and *Bam*HI. The 2-kb cry8Dat fragment was sequenced using an ABI-3100 Avant automated DNA sequencer (Applied Biosystems, USA) available at the Institute of Biotechnology (Vietnam). Nucleotide sequence was analyzed using BLAST in NCBI. Protein alignment was performed using ClustalX and annotated with the characteristics of Cry-conserved blocks.

Expression and purification of recombinant protein Cry8Dat

The 2.0-kb *cry8Dat* fragment was digested from recombinant plasmid pGEM-*cry8Dat* using *Bam*HI and *Sac*I, and then ligated into pET32a(+) that was linearized with the same enzymes. Recombinant plasmid pET32a-*cry8Dat* was cloned into *E. coli* BL21(DE3)pLysS for expression. An overnight culture of the recombinant *E. coli* was diluted 1:50 in fresh LB broth containing 50 µg/ml ampicillin and grown at 37°C to OD_{600} = 0.5 to 0.7. Isopropyl β-thio-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation was continued for a further 4 h. Cells were then lysed and expression was monitored by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

The recombinant protein Cry8Dat was purified by affinity column chromatography using Probond Nickel-Chelating Resin (Invitrogen) under denaturing conditions. Cells were collected by centrifugation following growth and IPTG induction. Cell pellets were suspended in lysis buffer (6 M guanidine hydrochloride; 20 mM sodium phosphate, pH 7; 500 mM NaCl) and ultrasonicated, and then the suspension was separated by centrifugation at 12,000 g for 15 min. The protein-containing supernatant was applied to an affinity chromatography column, and purified recombinant protein was recovered with elution buffer and stored at 4°C. The protein concentration was determined by the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

RESULTS

Isolation and distribution of *B. thuringiensis* serovar *galleriae* strains in sugarcane farming soil samples

To isolate for *B. thuringiensis* strains that produce ICPs against coleopteran insects, and especially the sugarcane-damaging scarab beetles, soil samples were collected from the sugarcane farming areas of three provinces in northern Vietnam, namely Soc Son (Ha Noi), Van Lam (Hung Yen) and Luong Son (Hoa Binh).

As shown in Table 1, *B. thuringiensis* was isolated from all three provinces. Of the 47 soil samples examined, 16 (34%) contained *B. thuringiensis*, with 78 strains producing parasporal inclusions.

The isolates fell into four categories based on crystalliferous morphology, that is, bipyramidal, spherical, cuboidal and heterogeneous shape. Of the 78 *B. thuringiensis* strains, 39 (50%) produced spherical crystals whose sizes varied markedly. Serological tests of the 39 strains with spherical crystals indicated that 24 strains belonged to *B. thuringiensis* serovar *galleriae* (H5a, 5b) (Table 1). The 24 strains (belong to serovar *galleriae),* were screened against *A. cuprea, C. nitida* and *P. xylostella*, of which one of the highest activity was designated as MBH11.3 and chosen for further study. The MHB11.3 appeared to be a typical *B. thuringiensis* producing a spherical crystal (Figure 1). The crystal mor-

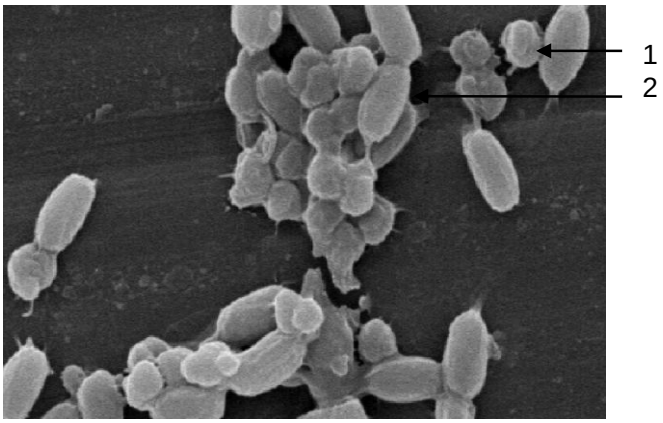

Figure 1. Scanning electron micrograph of morphology of crystals from *B. thuringiensis* serovar *galleriae* MHB11.3 (1 - crystal, 2 - spore).

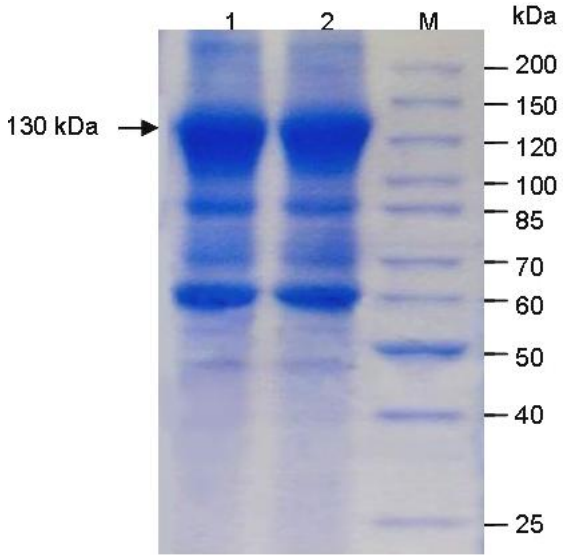

Figure 2. Crystal protein of MHB11.3 strain on the SDS-PAGE 12.5%. Lane 1 and 2: crystal protein of MHB11.3 strain; M: protein size marker (Fermentas).

phology of MHB11.3 was similar to that of the reference strain *B. thuringiensis* serovar *galleriae* (H5a, 5b).

Insecticidal activity of *B. thuringiensis* serovar *galleriae* MHB11.3

Preparation of crystals protein from MHB11.3 strain was performed as seen in the materials and methods section. Quantity of crystal protein was 54.4 µg per 1 ml of culture (Figure 2).

The insecticidal activities of crystal proteins produced by the MHB11.3 strain were evaluated by using *A. cuprea*, *C. nitida* and *P. xylostella* larvae. At different concentrations of protein (from 0.625 to 10 µg/ml), results of the periodic determination of the LC_{50} are given in the tabular forms that are calculated by the Probit analysis programme (Table 2 and Figure 5). It was found that LC_{50} (2.476 µg) of Cry8Da crystal protein from MHB11.3 strain with *A. cuprea* larvae is bit less than LC_{50} (2.857 µg) with *C. nitida* larvae, while the crystal protein did not have toxicity against *P. xylostella*, because it's mortality was very low (from 1 to 3%) (Table 2). It is indicated that MHB11.3 has high specificity to coleopteran insects.

Cloning, expression and purification of recombinant protein Cry8Dat

A single product of 2 kb *cry8Dat* fragment was amplified using genomic DNA as template from all 24 *B. thuringiensis* serovar *galleriae* strains (Figure 3A and B).

The PCR-amplified fragment from *B. thuringiensis* serovar *galleriae* MHB11.3 was first cloned into pGEM-T Easy and then subcloned into pET32a(+). *E. coli* strain BL21 (DE3)pLysS was transformed with the recombinant plasmid pET32-*cry8Dat.* Total protein was analyzed on 12.5% SDS polyacrylamide gels. IPTG-induced cultures contained high levels of a protein of approximately 96 kDa, which was absent in the control sample (Figure 4A). This protein was purified using a His-tag column chromatography, and protein quantification obtained was 45 µg per 1 ml of culture (Figure 4B).

The test for toxicity of the purified recombinant protein Cry8Dat against two coleopteran and one lepidopteran insect species showed that this protein killed *A. cuprea* larvae at LC_{50} = 2.391 µg and *C. nitida* larvae at LC_{50} = 2.710 µg in 7 days, while no toxicity was observed against *P. xylostella* (Table 2 and Figure 5). As results, the recombinant Cry8Dat protein exhibited toxicity as the same level against *A. cuprea* and *C. nitida* larvae as the Cry8Da crystal protein extracted from MHB11.3 strain. The recombinant protein was a biologically active insecticidal crystal protein and as the MHB11.3 strain has substantial activity against Scarabaeidae insects.

Sequence analysis

Both strands of the *cry8Dat* fragment in pGEM-*cry8Dat* were sequenced (Figure 6) and deposited in GenBank (accession number FN796428.1). Nucleotide sequence analysis using PCGENE software revealed an open reading frame of 2031 nucleotides coding for 677 amino acids with an estimated molecular mass of 77 kDa. A homology search revealed that this gene has very highly similarity (99%) with other reported a scarabcidal cry gene, including *cry8Da* of *B. thuringiensis* serovar *galleriae* SDS-502 (GenBank accession number AB089299), (Asano et al., 2003). The translated amino acid sequence of *Cry8Dat* from *B. thuringiensis* serovar *galleriae* MHB11.3 was named Cry8Da and the gene was

Table 2. Insecticidal activity of Cry8Da crystal protein from MHB11.3 strain and Cry8Dat recombinant protein (rCry8Dat).

Insect	Protein	LC_{50} (µg)*	LC_{50} 95% confidence limits	
			Lowest	Highest
Anomala cuprea	Crystal protein of MHB11.3	2.476	1.767	3.458
	rCry8Dat	2.391	1.688	3.355
Cotinis nitida	Crystal protein of MHB11.3	2.857	1.977	4.238
	rCry8Dat	2.710	1.839	4.066
Plutella xylostella	Crystal protein of MHB11.3	ND	ND	ND
	rCry8Dat	ND	ND	ND

*P-values < 0.05 were considered to be statistically significant. ND: not done, due to its mortality was very low.

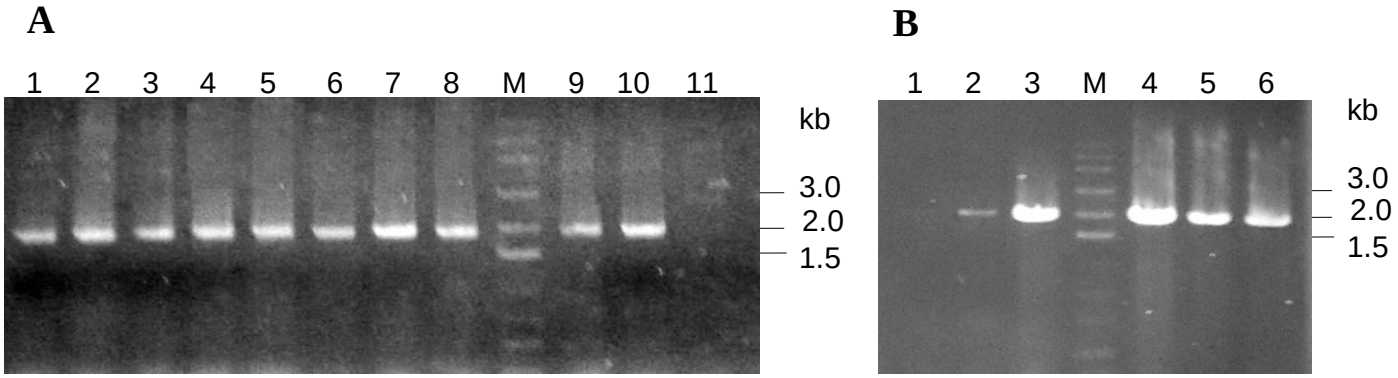

Figure 3. Agarose gel (0.8%) electrophoresis analysis of PCR products generated with *cry8D*-specific primers. (A) Lanes 1–8: strains MSS1.2, MSS1.3, MSS1.4, MSS2.3, MSS2.4, MSS2.7, MHY1.1, MHY1.3; lane M: Marker (10,000 bp); lane 9: MHY1.10; lane 10: MHY3.8; lane 11: Negative control (Btk). (B) Lane 1: Negative control (Btk); lane 2: MHB10.6; lane 3: MHB11.5; lane M: Marker (10,000 bp); lanes 4-6: MHB11.3, MHB9.3, MHB5.2.

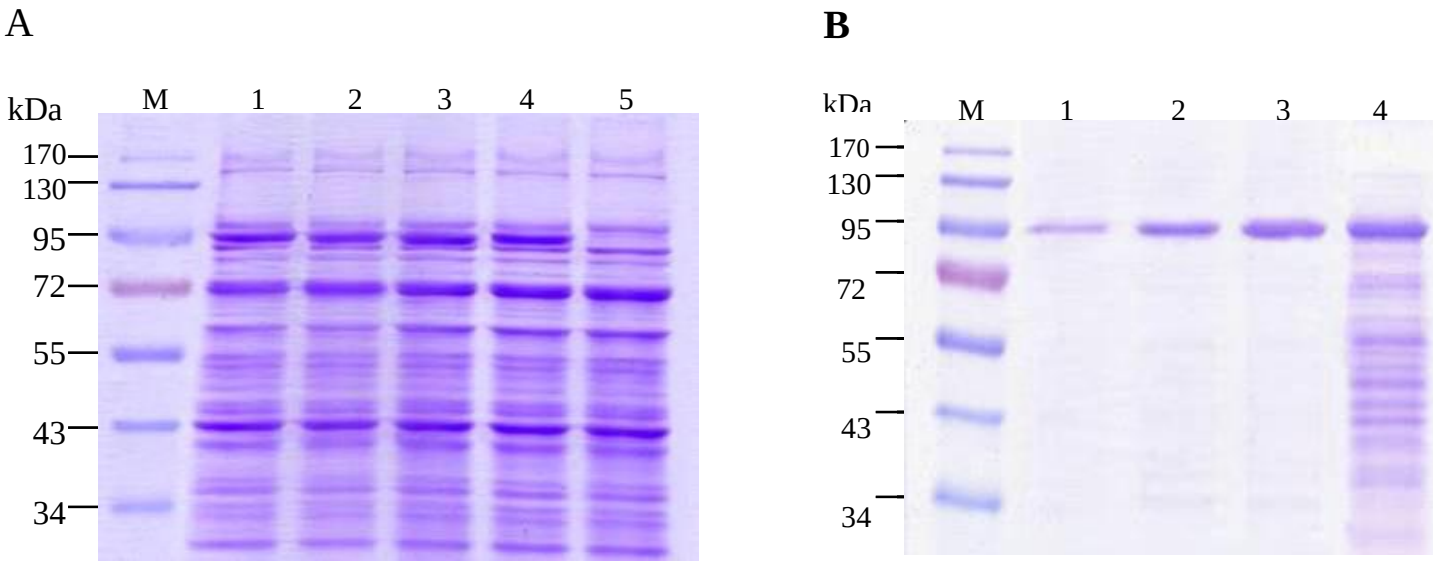

Figure 4. (A) Expression of Cry8Da protein in *E. coli* BL21(DE3)pLysS on 12.5% polyacrylamide gel. M: Protein size marker (Fermentas); lanes 1–4: colonies containing the *cry8Da* gene with IPTG induction; lane 5: no induction control. (B) Purification of Cry8Da recombinant protein using a His-tag column. M: protein size marker; lanes 1 to 3: Cry8Da following purification; lane 4: total protein from BL21(DE3) pLysS expressing Cry8Da.

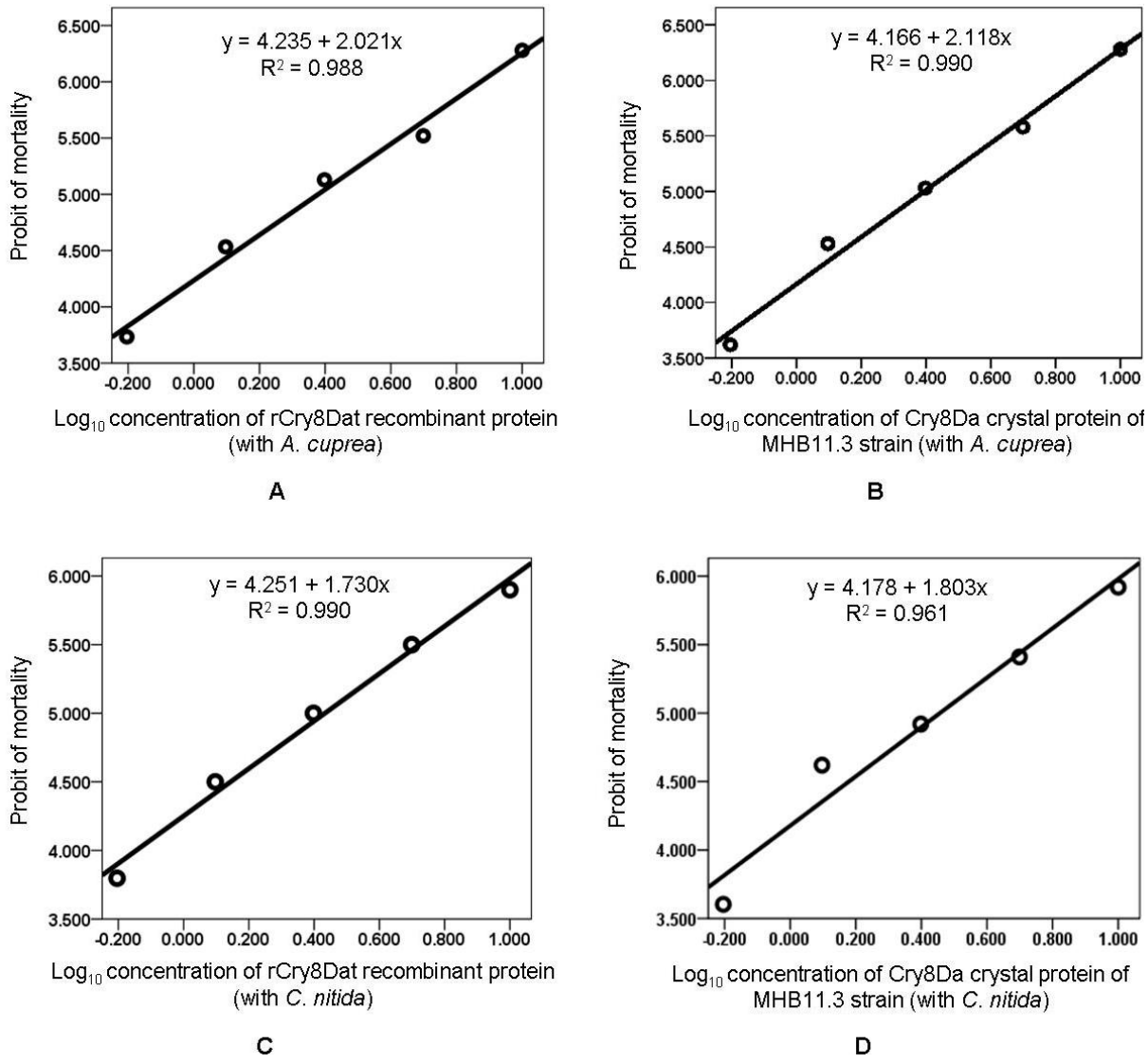

Figure 5. Toxicity of Cry8Da crystal protein from MHB11.3 strain and recombinant (rCry8Dat) protein from *E. coli* BL21(DE3)pLysS against *A. cuprea* (A, B) and *C. nitida* (C, D).

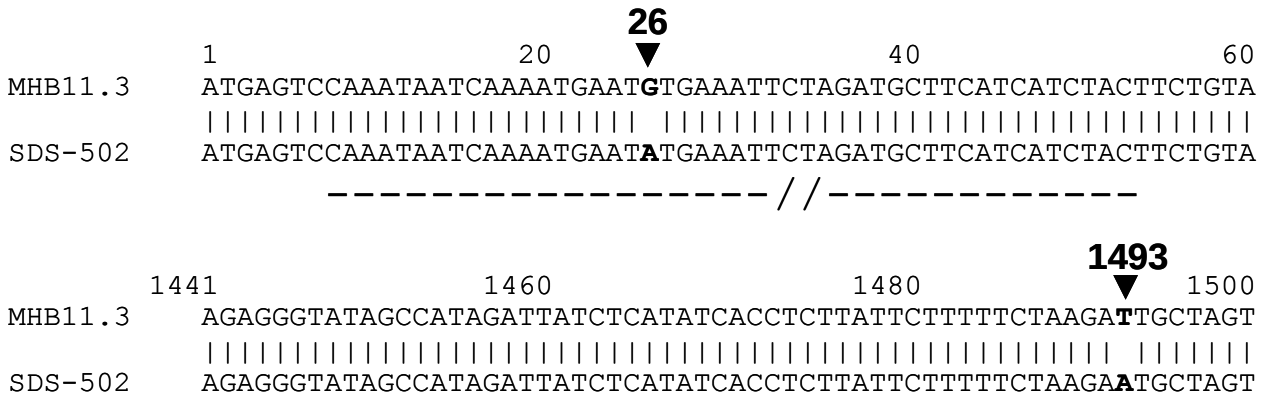

Figure 6. Alignment of a portion of *cry8Da* nucleotide sequence from the MHB11.3 (upper line) and SDS-502 (under line) strains and two nucleotide difference (positions 26 and 1493) between the two strains is shown by arrows. Complete identity in the coding sequence between the two strains is shortened and indicated by dashes (---//---).

reclassified as *cry8Da* of SDS-502. Alignment of the nucleotide sequence of *cry8Da*-SDS502 with our sequence (*cry8Da* of MHB11.3) revealed two nucleotide differences at positions 26 A26G and A1493T (Figure 6), which correspond to amino acid change in codons 9 (Y9C) and 498 (N498I), respectively. The substitution at amino acid position 9 is in the host specificity determining region (presumably forming Domain I), as defined in Cry8Da toxin (Asano et al., 2003).

DISCUSSION

In this study, soil samples collected from three provinces of sugarcane planting areas contained many *B. thuringiensis* isolates, supporting our previous data, that *B. thuringiensis* is widely distributed in Vietnam (Martin and Travers, 1989; Binh et al., 2005). The frequency of *B. thuringiensis* was 34.0% among the tested soil samples, which is comparable with results from soil from southern Vietnam that was analyzed by Martin and Travers (1989), soil samples from New Zealand (Chilcott and Wigley, 1993), soil samples from Korea (Lee et al., 1995) and soil samples from Japan (Ohba and Aizawa, 1986).

Of the 39 isolates with spherical crystal proteins, 24 (61%) reacted with H5a, 5b, serovar *galleriae* antiserum, while the average frequency of *B. thuringiensis* serovar *galleriae* is only 1.5% in the Vietnam *B. thuringiensis* Collection (Binh et al., 2007). The reason for this is that the high frequency of *B. thuringiensis* serovar *galleriae* in the current study may be due to the location of soil sampling, specifically sugarcane planting areas.

One of our striking results is that all 24 strains (100%) of *B. thuringiensis* serovar *galleriae* contained *cry8Da* genes. In the present study, we cloned the *cry8Dat* gene from *B. thuringiensis* serovar *galleriae* MHB11.3, determined its sequence, and expressed in *E. coli* as a tag-fused Cry protein. This *cry8Dat* gene is 2031 base pairs long and has 99% sequence homology with *cry8Da* gene, a scarabcidal Cry toxin from *B. thuringiensis* serovar *galleriae* SDS-502. Previous studies indicated that the protein produced by *cry8Da*-type gene has toxicity against *A. cuprea, A. orientalis* and *P. japonica* and this Cry8Da toxin also kills the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Bauer and John, 2011), and thus this toxic protein could serve as an active ingredient in a noxious organism-controlling agent for EAB. Moreover, in our toxicity bioassay, both the natural Cry8Da from *B. thuringiensis* serovar *galleriae* MHB11.3 and the recombinant protein showed high toxicity to two scarab beetles of our subject such as for *A. cuprea* and to an additional species, *C. nitida* (Scarabaeidae). Both species practically cause considerable damage to sugarcane, coconut, tobacco, acacia and ginger crops, and thus have a significant economic impact on farmers in Vietnam.

Regarding the structure of the *cry8Da* gene, it encodes a 130-kDa protein in SDS-502 strain, which has three domains. Domain I (from residues 1–310) and a part of Domain II were more conserved than the residues in the remaining toxic region (up to residue 680), while the core of Domain II (residues 390 and 470) was highly divergent. Several sections of conserved sequence were observed in the region between residues 480–660, and the protoxin region of Cry8Da, presumably starting at residue 681, was very similar to the corresponding region of Cry8Ca (Asano et al., 2003). Alignment of the nucleotide sequences of *cry8Da* from MHB11.3 and SDS-502 revealed only two nucleotides and deduced amino acid differences. Of these, the change at nucleotide position 26 is particularly interesting. This variant, located in the host specificity-determining region (Domain I), as defined in Cry8Da toxin, changed a highly conserved tyrosine at position 9 to cysteine (Y9C). The other change, at nucleotide position 1493, causes an N498I substitution in Domain II. A role for both of these variants in modulating the toxicity of this protein for different insect larvae cannot be ruled out, and further molecular and structural investigations may provide an answer to this question.

The possibility of the comparative bioassay between the insecticidal activity of protein from the SDS 502 and the MBH11.3 strain has not been conducted, due to the patent of the SDS 502 strain. The SDS 502 strain, according to these patents, has insecticidal activity against four insect species, that is, *A. orientalis, P. japonica, A. cuprea* and *Agrilus planipennis.* In our case, we have had experiments against one of these four species, *A. cuprea* and an additional experiment to test insecticidal assay against *Cotinis nitida* (Coleoptera: Scarabaeidae) commonly found in local plants, including sugarcane, tobacco, acacia and ginger crops.

The *B. thuringiensis* serovar *galleriae* MHB11.3 strain isolated and characterized in our study is confirmed to be the first and novel Bt strain in Vietnam. Both natural and recombinant Cry8Da proteins from this strain exhibited high toxicity to two species of scarab beetles, *A. cuprea* and *C. nitida*– the two common insects causing enormous damage to a range of field crops including sugarcane. Up to date, no publication on insecticidal activity on *C. nitida* species as the novel property of MHB11.3 strain assayed in this study is available.

In conclusion, the *B. thuringiensis* serovar *galleriae* MHB11.3 isolated in Vietnam harbor *cry8Da* gene and have highly insecticidal activities against coleopteran insects such as *A. cuprea* and *C. nitida,* both of which are common insects causing enormous damage to a range of field crops including sugarcane in Vietnam. These results suggest that *B. thuringiensis* serovar *galleriae* MHB11.3 is a viable alternative for the control of insect pests in agriculture, and the possibility of gene transfer of *cry8Da* isolated from this strain to generate the coleopteran-resistant sugarcane plants.

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