สัญญาเลขที่BGL4580009

รายงานวิจัยฉบับสมบูรณ์

โครงการ การจำแนกชนิดของ *Bacillus thuringiensis* และศึกษาลำดับนิวคลีโอไทด์ของยีน *cry* ที่มีผลกระทบต่อ *Spodoptera exigua* ในประเทศไทย

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Executive summary

Thailand is the biggest area of tropical orchids, especially those in *Dendrobium* which earns the country foreign exchanges. Currently, orchids become the economically important cut flower but facing a problem of cut worms or beet armyworm (*Spodoptera exigua*) seriously. The worms destroy orchid stems, leaves and flowers and hence causing economical lose to this agroindustry. To help solving this problem, several works has been done in trying to transform plants of various kinds by introducing *Bacillus thuringiensis* (*Bt*) insecticidal crystal protein gene (*cry* gene) in order to produce biological insecticides against *Spodoptera*.

In this study, the objective is to identify *cry* gene isolated from *Bacillus thuringiensis* effecting to *Spodoptera exigua* in Thailand from the isolates that have been tested for their toxic to the *Spodoptera exigua*. After the *Bt cry* gene were identified, the promising isolate was cloned for nucleotide sequencing and submitted the sequence in the Genbank. The promising clone will be used as a target gene and the transform action of *Dendrobium* orchid will be carried out via biolistic bombardment transformation to mediate insect resistance for the next project

รหัสโครงการ: BGJ4580009

ชื่อโครงการ: โครงการ การจำแนกชนิดของ *Bacillus thuringiensis* และศึกษาลำดับนิวคลีโอไทด์ของยีน *cry* ที่มีผลกระทบต่อ *Spodoptera exigua* ในประเทศไทย

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บทคัดย่อ

Bacillus thuringiensis JC190 ที่ตรวจสอบแล้วว่าสร้างสารที่เป็นพิษต่อหนอนกระทู้หอม ได้นำมาใช้ศึกษาเพื่อหาชนิดของยีน *crylE(a)* โดยนำลำดับเบสบางส่วนที่โคลนได้จากยีน *cry* ของJC190 ไปเปรียบเทียบกับในฐานข้อมูลแล้วพบว่ามีความเหมือนกับยีน *crylE(a)* ดังนั้นในการทดลองนี้จึงสังเคราะห์ไพรเมอร์จากหัวและท้ายของยีนทั้งสอง การโคลนยีนด้วยเทคนิค PCR และหาลำดับเบสพบว่า ได้ชิ้นดีเอ็นเอขนาด 3,532 เบส และพบรหัสที่สมบูรณ์ของของยีนขนาด 3,516 เบส แปลเป็นกรดอะมิโน 1,171 เรซิดิว จากการเปรียบเทียบกรดอะมิโนที่โคลนได้กับกรดอะมิโนในฐานข้อมูลพบว่ามีความสัมพันธ์กับกรดอะมิโนจากยีน *crylE(a)* และมีความเหมือนกับโปรตีน CrylE(a) ของ B. thuringiensis อยู่ 98% ยีนที่โคลนได้นี้คาดว่าจะมีประโยชน์สำหรับการสร้างความต้านทานหนอนกระทู้หอมของไทยในอนาคต

Project Code : BGJ4580009

Project Title : Identification and nucleotide sequence of the *cry* gene of *Bacillus thuringiensis* that affecting to *Spodoptera exigua* in Thailand.

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Abstract

The endotoxin genes from 28 *Bacillus thuringiensis* strains which have been isolated and tested for their effecting to *Spodoptera exigua* in Thailand were identified for *cry1* type gene by PCR-RFLP. Two pairs of universal oligonucleotide primers were amplified cry-type gene fragments of the DNA template from *B.thuringiensis*. The RFLP patterns of PCR-amplified fragments from 28 *B. thuringiensis* Thai isolates revealed 8 possible distinct *cry*-type genes. These *cry*-type genes include *cry1A(a), cry1B, cry1C, cry1C(b), cry1D, cry1E* and *cry1F*. One of unidentified isolates from RFLP pattern, JC190, was selected for analysis partial DNA sequencing. The result showed that it contains 2 *cry*-type genes including *cry1C* and *cry1E*. The *cry1E* specific primer was amplified the 3.5 kb fragment, then cloned for analysis. The result showed that the DNA sequence of 3,516 bp encoded for one protein of 1,171 amino acid residues in length. The amino acid sequence has 98% homology with CrylE(a) protein of *B. thuringiensis* subsp. *kenyae* (accession number AAA22345.1) when compared its sequence with 13 other reported BT proteins in database. This JC190 isolate was submitted in the GenBank as *crylE(a)7* accession number AY894137. The cloned gene would be further used to improve Thai economic crops for insect resistance.

Keywords: endotoxin gene, *Bacillus thuringiensis*, *Spodoptera exigua*, *cry1E* and PCR-RFLP

Objectives

The objectives of this study are:

1. To identify *cry* gene isolated from *Bacillus thuringiensis* effecting to *Spodoptera exigua* in Thailand.
2. To clone and study the sequence of promising isolate.

Introduction

Bacillus thuringiensis is a gram positive endospore forming bacterium, which has been known to produce a variety of insecticidal crystal proteins or δ-endotoxins during its sporulation phase (Schnepf *et al.*, 1998). This bacterium was discovered by a Japanese biologist, Dr. S. Ishiwata in 1901, as a silkworm disease. The name *B. thuringiensis* was designated in 1911 after a German biologist, E. Berliner, introduced its insecticidal activity in insect larvae in Thuringen, Gemany (Lambert and Peferoen, 1992). The insecticidal crystal proteins have been used as biological pesticide for a range of lepidopteran, coleopteran and dipteran larvae for several decades. The toxin can also against to insect orders, Hymenoptera, Homoptera, Orthoptera and Mallophaga. Their activities are highly toxic to specific insect larvae and environment-friendly, therefore , these insecticidal proteins have been an alternative approach for pest control (Schnepf *et al.*, 1998). This soil bacterium has been isolated not only from soil (Chilcott and Wigley, 1993) but also in insects (Kaelin *et al.*, 1994) and stored products dust (Hongyu *et al.*, 2000). The crystal proteins are encoded by cry genes (Schnepf *et al.*, 1998). The *cry*-type genes have been classified more than 150 *cry*-type genes with 49 holotypes, *cry1 - cry49*, and *cyt1* to *cyt2* groups (http://www.lifesci,sussex,ac,uk/home/Neil_Crickmore/BT/index.html). The crystal protein of *cry1* genes are recognized about 130-140 kDa protoxin. An approximately 60-70 kDa active toxin is achieved by a gut protease in the high pH condition (about pH 9.5) of larvae gut. The activated toxin shows the pathological effect by binding to the specific receptor protein and creating pores in the larvae midgut epithelium cells and leads to larvae lethality (Schnepf *et al.*, 1998).

The purpose of this study is to identify the *cry*-type genes of 28 *B. thuringiensis* strains which have been collected and tested for their toxic against the *Lepidopteran* pest, *Spodoptera exigua*, with the facile method, PCR-RFLP. An interesting strain of these active toxins against the larvae was selected to cloned and determined for the

cry-type gene by DNA sequencing. The cloned gene would be constructed to be further introduced into *Dendrobium* orchid.

Materials and Methods

Bacterial strains and DNA Isolation

Bacillus thurinigiensis 28 strains which have been isolated and tested for their toxicity to Spodoptera exigua in Thailand were kindly supplied by Dr. Jariya Chanpaisaeng, faculty of Agricultural, Kasetsart University. A fresh colony of each isolate was grown in Luria-Bertani (LB) medium at 30°C with shaking at 250 rpms/ min for 16-18 hours. Cells were washed once time with GTE (50 mM Glucose, 25 mM Tris-HCL [pH 8.0], 10 mM EDTA).The pellet cells were lysed with 500 µl of lysis buffer (GTE containing 1 mg/ml lysozyme), incubated at 37°C for 15 min, then added 60 µl of 10 % SDS (Sodium Dodecyl Sulfate), incubated at 50°C for 15 min. Further DNA extraction was performed according to the method described by Sambrook *et al.*, 1989. The DNAs were electrophoresed through a 0.8 % agarose gel.

Polymerase chain reaction (PCR) procedures

Amplification of partial cry1 gene by PCR and RFLP pattern

The PCR technique was used to amplified the partial gene content of 28 *B. thuringiensis* strains by using two pairs of universal oligonucleotide primers, which were designed from three highly conserved regions of all *cry1*-type gene publications (Kuo and Chak, 1996). The first pair of oligonucleotide primers were K5un3- sense and K3un3- antisense. The second pair was K5un2-sense and K3un2-antisense. PCR reaction contained 1.0 µg DNA template, 2 µl of 10X PCR buffer, 0.4 µl of 10 mM dNTPs, 1.2 µl of 2.5 mM $MgCl_2$, 0.4 µl of each of 30 pmol/µl primer and 1 unit of Taq polymerase (Perkin Elmer, USA) with a 20 µl final volume. The reactions were carried through 30 cycles by using the following temperature sequence: 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, cycles were proceded by denaturation for 5 min at 95°C and a final extension cycle at 72°C for 15 min. PCR products were electrophoresed through a 0.8 % agarose gel. All the PCR amplifications were accomplished with Gene Amp PCR system 9600 (Perkin Elmer, USA). For RFLP

analysis, the appropriate restriction enzymes were digested the PCR products and compared the patterns as Kuo and Chak, 1996 described.

Full-length coding sequence amplification

In order to amplify the full length coding sequence of the putative *cry1Ea* gene by using the total DNA isolated from the interesting strain, JC190, the primers were designed based on sequences at the translation start and stop site that plublished in the GenBank. The 5′end specific primer of *cry1Ea* was derived from the Accession No M73252 (Feitelson 1991, unpublished). The 3′ end of gene was aligned and found the conserve sequence, therefore the 3′end of these *cry* genes were designed as the 3′universal primer. All oligonucleotide primers were flanked with *Sal*I restriction site. The reactions were carried through 30 cycles by using the following temperature sequence: 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. Cycles were preceded by denaturation for 5 min at 95°C and a final extension cycle at 72°C for 8 min. PCR products were electrophoresed through a 0.8 % agarose gel.

DNA Cloning

The PCR fragments were separated by gel electrophoresis and purified with Gene Clean II Kit (Bio 101. Vista., CA). The purified fragment of the partial *cry* genes were ligated to pGEM-T Easy vector (Promega, USA), and the full length coding fragment was ligated to pUCm-T vector (Biotechnology Department Bio Basic Inc., Canada). The ligation reactions were transformed into *E. coli* cells. The transformed cells were selected on the medium supplemented with ampicillin 50 μg/ml. Plasmid DNA extraction was achieved by alkaline lysis method as described by Sambrook *et al.*, 1989. *Escherichia coli* strain XL1- Blue and JM109, which were used for plasmid recombinant transformation, were grown at 37°C in LB medium supplemented with the proper antibiotic (Chang *et al.*, 2001).

DNA Sequencing and sequence analysis

The plasmid DNA was purified by Concert™ plasmid purification (LIFE TECHNOLOGIES Crop., USA). DNA sequencings were performed by using ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit with an ABI 373A Automatic DNA sequencer (Perkin Elmer, USA) according to the manufacturer's

protocol. The results were analyzed by using computer software and compared with another sequences in website http:// www.ncbi.nlm.nih.gov/ to identify the cry-type gene. The alignments were performed using the multiple alignment program, CLUSTALW (www.genebee.msu.su).

Results and discussions

PCR -RFLP screening of *B. thuringiensis* strains

The *B. thuringiensis* 28 strains were screened with two pairs of universal primers. The first pair (K5Un3 and K3Un3) amplified 1.4 kb PCR products. This amplified product is located in N-terminal or the 5′ end of full-length *cry* gene. RFLP pattern analysis after *Pst*I and *Eco*RI digestion devided *cry*-type genes into 2 groups as shown in Figure1. and Table 1. with the predicted fragment sizes. The first group probably contains 2 *cry*-type genes, one is *cry1Cb* and another is unable to identify *cry*-type genes. The second group is uncut pattern, it showed the *cry1C* type gene or maybe another *cry*-type genes that not correspond to these restriction patterns.

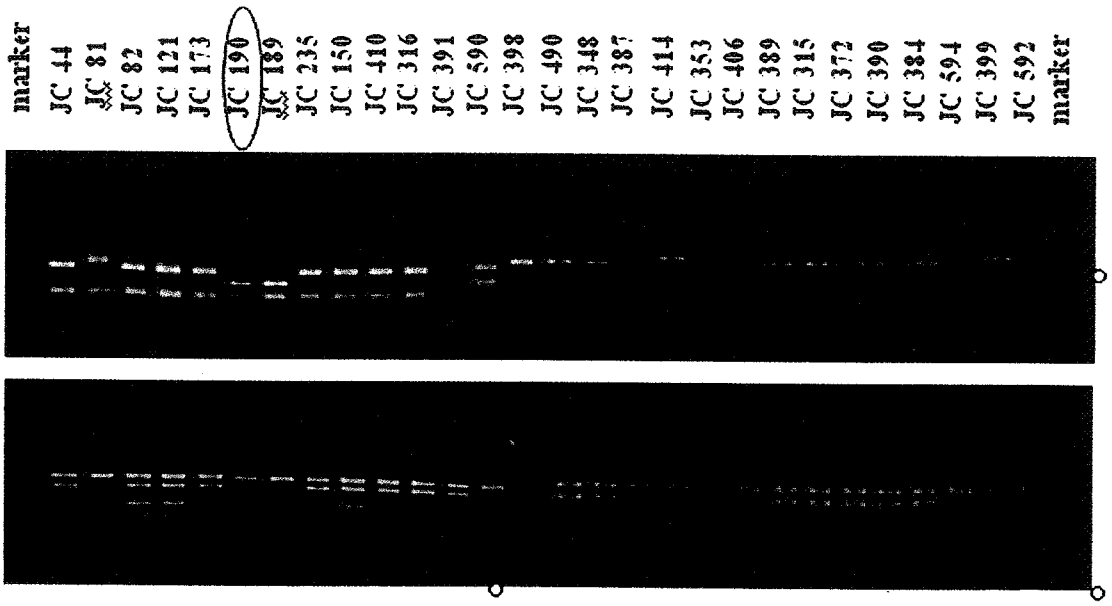

Figure 1. Upper: The 3′ end of *cry* gene fragments were digested with *Pst*1 and *Xba*1
Lower: The 5′ end of *cry* gene fragments were digested with *Pst*1 and *Eco*R1

For the second pair of primers, K5Un2 and K3Un2 were carried out 1.6 kb PCR product. This amplification is located in C-terminal or the 3′ end of full-length *cry* gene. Then, they were identified the *cry*-type genes with the RFLP analysis after restricted with *Pst*I and *Xba1*. The results showed 8 possible distinct groups as shown in Figure1. and Table 1.

From this PCR-RFLP analysis, this *B. thuringiensis* collection is classified to *cry1A(a), cry1B, cry1C, cry1C(b), cry1D, cry1E* and *cry1F*. It revealed that each *B. thuringiensis* strain contains more than one *cry*-type genes.

In this study, *B. thuringiensis* strains were tested for their toxic to *Spodoptera* species. Therefore, most cry-type genes contained in these strains have been revealed to related to those cry-type genes which have been published for their toxicities against *Spodoptera* species, *cry1Ab* and *cry1C*, especially in the *cry1C* gene that was reported as the most active to *Spodoptera* species (Strizhov *et al.*, 1996; Mazier *et al.*, 1997).

Table 1 The RFLP-PCR *cryI* profiles, H-serotyping, and toxicity bioassay of 31 *Bt* isolates affecting four lepidopterans.

	Cryl profile	5′ end	3′ end	[a]Subspecies of *Bt*	[b]#JC isolate affecting			
					HA	SE	OF	PX
1.	*cry1Aa, 1B, 1C*	*cry1C*	*cry1Aa, 1B*	unidentified subspecies: JC 281	-	-	-	-
2.	*cry1Aa, 1C*	*cry1C*	*cry1Aa*	*kurstaki*: JC 372, JC 592 *kenyae*: JC 81	81	81	-	-
3.	*cry1Aa, 1Cb, 1C*	*cry1C, 1Cb*	*cry1Aa*	unidentified subspecies: JC 276	-	-	-	-
4.	*cry1B, 1C*	*cry1C*	*cry1B*	*kurstaki*: JC 390, JC 398	-	398	-	-
5.	*cry1C, 1E*	*cry1C*	*cry1E*	*kenyae*: JC 189, JC 190	189,190	189	-	-
6.	*cry1C, 1Cb, 1E*	*cry1C*	*cry1E,*	*thailandensis*: JC 353	-	353	-	-
			cry1Cb/1D/1F	*kurstaki*:JC 590, JC 595	-	595	-	590
7.	*cry1C, 1Cb, 1D*	*cry1C, 1Cb*	*cry1D*	*galleriae*: JC 291 *kurstaki*: JC 389	389	-	291	-
8.	*cry1Cb, 1C*	*cry1C, 1Cb*	-	*kurstaki*: JC 397	-	-	-	-
9.	*cry1Cb, 1D*	*cry1Cb, 1D*	*cry1Cb/1D/1F*	*galleriae*: JC 44, JC 121, JC 150, JC 173, JC 235 *kurstaki*: JC 82, JC 315, JC 316	82 316	82	-	150,173
10.	*cry1Cb, 1F*	*cry1Cb, 1F*	*cry1Cb*	*kurstaki*: JC 384, JC 399, JC 406, JC 410, JC 414, JC 490 *neoleonensis*: JC 387	-	-	-	384
11.	*cry1Cb*	*cry1Cb*	-	*kurstaki*: JC 348	-	-	-	-

[a] *cryI* gene profiles were determined by RFLP-PCR patterns.
[b] Subspecies were determined by H-serotyping: *Bt. kurstaki* (3abc); *Bt. kenyae* (4ac); *Bt. galleriae* (5ab); *Bt. neoleonensi* (24); *Bt. thailandensis* (68).
[c] LC_{50} at 10^3–10^7 spores/ml was determined by probit analysis.
HA = *Helicoverpa armigera* Hubner; SE = *Spodoptera exigua* Hubner ; OF = *Ostrinia furnacalis*; PX = *Plutella xylostella*.

DNA Cloning

One interesting strain, JC190, which has not been determined the *cry*-type genes from these PCR-RFLP patterns and maybe contain 2 interesting *cry*-type genes was selected to identify the *cry* gene by DNA cloning. The partial fragments derived from each pair of primers, K5Un3 and K3Un3 amplified 1.4 kb fragment and K5Un2 and K3Un2 amplified 1.6 kb PCR product, were cloned into pGEM-T easy. The positive clone of each fragment was sequenced and analyzed with BLAST-N program of the NCBI database. Partial sequence analysis indicated that 1.6 kb fragment JC190 clone, it had 98% homology with *cry1Ea* of *B. thuringiensis* accession No. M73252 and U94323 (Barboza-Corona *et al.*, 1998).

For cloning the full-length coding *cry* genes of JC 190 strain, the specific primers for *cry1Ea* were designed. The PCR amplification of approximately 3.5 kb long was achieved only *cry1Ea* specific primers (Figure 2.), then cloned this fragment into pUCm-T vector. Only one putative clone derived from this transformation and was designated to pBT858.

Figure 2. The 3.5 Kb band from the JC 190 amplification with specific *cry1Ea* primer.

The full-length sequence of the JC190 *cry1Ea* gene analysis

The PCR amplified fragment with the specific *cry1Ea* gene primer from *B.thuringiensis* strain JC190 was about 3.5 kb long, then cloned into pUCm-T vector and the putative clone was designated as pBT858. The pBT858 was isolated and sequenced for the putative *cry1Ea* gene from JC190. The sequence was determined 3,532 bp in length (Figure 3.) and compared with the sequences available from the GenBank using Blastn program. This nucleotide sequence along the gene revealed that the putative *cry1Ea* of JC190 showed high similarity to *cry1Ea* gene with the homology of 99% to M73252.1 (Barboza-Corona *et al.*, 1998). The sequence of *cry1Ea* JC190 showed that it contained an open reading frame (ORF) of 3,516 bp . The ORF

>Cry IE(a) *B. thuringiensis* JC190, frame+1 , 1171 residues
MEIVNNQNQCVPYNCLNNPENEILDIERSNSTVATNIALEISRLLASATPIGGILLGLFDAIWGSIGPSQ
WDLFLEQIELLIDQKIEEFARNQAISRLEGISSLYGIYTEAFREWEADPTNPALKEEMRTQFNDMNSILV
TAIPLFSVQNYQVPFLSVYVQAANLHLSVLRDVSVFGQAWGFDIATINSRYNDLTRLIPIYTDYAVRWYN
TGLDRLPRTGGLRNWARFNQFRRELTISVLDIISFFRNYDSRLYPIPTSSQLTREVYTDPVINITDYRVG
PSFENIENSAIRSPHLMDFLNNLTIDTDLIRGVHYWAGHRVTSHFTGSSQVITTPQYGITANAEPRRTIA
PSTFPGLNLFYRTLSNPFFRRSENITPTLGINVVQGVGFIQPNNAEVLYRSRGTVDSLNELPIDGENSLV
GYSHRLSHVTLTRSLYNTNITSLPTFVWTHHSATNTNTINPDIITQIPLVKGFRLGGGTSVIKGPGFTGG
DILRRNTIGEFVSLQVNINSPITQRYRLRFRYASSRDARITVAIGGQIRVDMTLEKTMEIGESLTSRTFS
YTNFSNPFSFRANPDIIRIAEELPIRGGELYIDKIELILADATFEEEYDLERAQKAVNALFTSTNQLGLK
TDVTDYHIDQVSNLVECLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDPNFRGINRQPDRGWRGSTDIT
IQGGDDVFKENYVTLPGTFDECYPTYLYQKIDESKLKAYTRYELRGYIEDSQDLEIYLIRYNAKHETVNV
PGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPNLDCSCRDGEKCAHHSHHFSLDIDVGCTDLNEDLGVWV
IFKIKTQDGYARLGNLEFLEEKPLLGEALARVKRAEKKWRDKCEKLEWETNIVYKEAKESVDALFVNSQY
DRLQADTNIAMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGL
SCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIEDNTDEL
KFSNCVEEEVYPNNTVTCNNYTATQEEHEGTYTSRNRGYDEAYESNSSVHASVYEEKSYTDRRRENPCES
NRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE*

Figure 4. Show the amino acid sequences of CrylEa from the JC190 isolate.

The Figure 5. showed the phylogenetic tree of the representative Cry protein. The deduced amino acid sequence of Cry JC190 is extremely similar to that of the registered Cry1E proteins, showing high sequence identities with Cry1Ea, Cry1Ea4 and Cry1Ea1 (98%) as *B. thuringiensis* subsp. *kenyae* (Barboza-Corona *et al.*, 1998; Visser *et al.*, 1990) and Cry1Eb (78%) as *B. thuringiensis* subsp. *aizawai* at the deduced amino acid level. The Cry protein of JC190 was clustered into the group of the Cry1Ea protein, hence, the sequence of *cry1Ea* from *B. thuringiensis* strain JC190 has been deposited in the GenBank data base under the Accession No. AY894137.

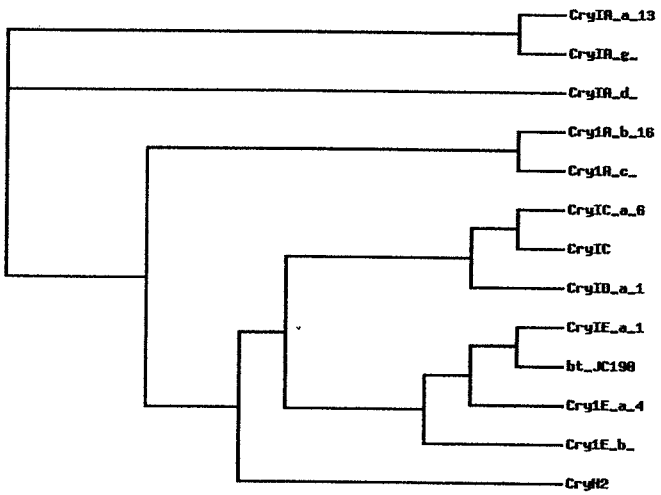

Figure 5. phylogenetic tree based on amino acid sequences encoded by Cry1Ea and other cry proteins collected from the Genbank database.

When using the NCBI Conserved Domain Search program for analysis the conserved domain of JC190 Cry protein, the results showed that this protein has higher homologies for the insecticidal crystal protein domains. The primary structure of the cry consisted of a N-terminal domain with five conserved blocks that is proposed to act the three conserved domains of Cryl protein (Figure 6.). The protein has elucidated to three conserved domains when aligned with the representative Cry domain in the database. The Domain I or endotoxin_N composed with 225 amino acid residues from residue 36 to 253. The Domain II or endotoxin composed with 203 amino acid residues from residue 258 to 453. The last conserved domain, Domain III composed with 138 amino acid residues from residue 468 to 601. The homology score of these domains showed 99.6 %, 99.5% and 97.1% respectively for each domain.

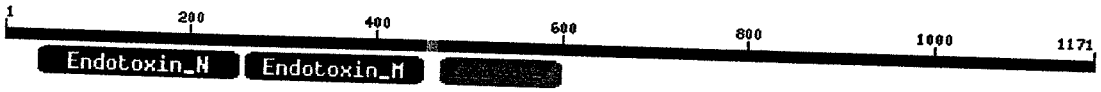

Figure 6. Show the related domain of Cry1Ea protein from JC190 with Conserved domain Search from NCBI database.

In this study the *cry1Ea* gene has been cloned from the *Bacillus thuringiensis* JC190. The nucleotide and amino acid sequences were submitted in the Genbank database, the accession number was AY894137 and the nomenclature name was cry1Ea7 from http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/BT/index.html, . This cry will be further benefit for the pest management by introduced into plant.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

ชื่อเรื่อง: Analysis of the insecticidal crystal gene type 1 of *Bacillus thuringiensis* isolates affecting lepidopterans.

ชื่อผู้แต่ง: Benjawan Lertwiriyawong, Krit Pinthong, Jariya Chanpaisaeng, Panapa Saksoong, Pattana Srifah Huehne

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