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Screening of Novel Cry Genes Active Against Nematodes from *Bacillus thuringiensis* Thai Isolates

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Abstract

Cry proteins of *Bacillus thuringiensis* have been successfully used as biopesticides and transgenic crops throughout the world. However, resources against the most serious agricultural pathogens, plant root-knot nematodes, are limited. Nematicidal Cry proteins produced by *Bacillus thuringiensis* (Bt) are used for effiecient pest control. Cry 5, Cry 6, Cry 21, and Cry 55 were known to have nematicidal activity. In the present study, 80 Bt isolates from diverse locations in Thailand have been investigated for their nemeticidal *cry* gene content. Bt isolates were screened through Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Polymerase Chain Reaction (PCR), ligated into the cloning vector PJET 1.2/blunt, transformed into *Escherichia coli* DH5α, and diagnostic restriction enzyme digest for the presence of nematode-active *cry* genes. Of 80 Bt Thai isolates, 24 isolates (30%) have 54-kDa protein which is similar with the molecular weight of Cry 6 protein and 5 isolates (6%) have ±45 kDa protein which is similar with the molecular weight of Cry 55protein. Only one Bt isolate (Bt 32) was suspected to be positive for the presence of *cry 6* gene.

Key Words: *Bacillus thuringiensis, Nematicidal Cry Genes, SDS-PAGE, PCR, Transformation, Restriction Digest.*

INTRODUCTION

Bacillus thuringiensis (Bt) is a gram-positive and sporulating bacterium that produces crystal proteins (δ-endotoxins) as parasporal crystalline inclusions during the stationary phase of growth (Bravo *et al*., 2011). Many Bt strains that show activity towards Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga insect orders have been reported. In addition, Bt strains active against nematodes have also been isolated (deMaagd *et al*., 2001). Cry proteins act by binding to receptors and subsequent insertion into the brush border membrane in the midgut of susceptible insects, leading to disruption of osmotic balance, cell lysis and eventually death (Bravo *et al*., 2005). The success of these toxins is caused by their high toxicity toward insects but no/low toxicity toward other animals. Bt toxins have an excellent track record in >100 years of use by organic and conventional farmers and very effective when expressed directly in transgenic plants (Wei *et al*., 2003).

The agricultural economic damage caused by more than 4100 species of plant-parasitic nematodes has been estimated to cost $US80 billion per year (Nicol *et al*., 2011). A large proportion is due to the serious damage caused by the plant-parasitic nematode *Meloidogyne incognita* (Mukhtar *et al*., 2013). The most successful commercially available biopesticide for agricultural pest biocontrol is Bt. In support of this, several families of Cry proteins (Cry5, Cry6, Cry21, and Cry55) produced bt Bt were shown to be toxic to a number of free-living and parasitic nematodes (Guo *et al*., 2008), but the full spectrum of nematicidal Cry toxins and their host targets is still far from completion. Although many Cry toxins are known to target various insects, only a few nematicidal crystal protein genes, have been found and utilized, of which only *cry5Ba* and *cry6Aa* have been applied (Li *et* al., 2008). To overcome the scarcity of nematicidal proteins used as biopesticides, the identification of more novel nematicidal crystal proteins is needed.

The present study was conducted to screen National Center for Genetic Engineering and Biotechnology (BIOTEC) collection of native Bt isolates for the presence of nematode-active cry genes that can be used to control plant-parasitic nematodes and decrease agricultural damage caused by plant-parasitic nematodes in a safe way because this toxic pollution does not harm the environment.

MATERIALS AND METHODS

Bacterial Isolates

Eighty Bt isolates recovered from diverse locations in Thailand were used in this study. Bt isolates were stored at -80° ultra low temperature

freezer in National Center for Genetic Engineering and Biotechnology, Thailand.

Growth Media for Bacterial Isolates and Strain

Luria Bertani Agar (LA), Double-strength Schaeffer's–Glucose (*2XSG*), and Luria Bertani Broth (LB) were used for the growth of Bt isolates and strains.

SDS-PAGE

Bt isolates were cultured in LA for 1 day at 37°C incubator and moved to 2XSG sporulation media for 3 days at 37°C incubator. Bt spores were collected 15 mL tube and were centrifuged at 8500 rpm for 10 min in a centrifuge and washed twice with distilled water. The supernatant was discarded and the pellet was resuspended in distilled water. Samples for SDS PAGE consist of 12 µL protein and 8 µL 5X sample buffer. Samples were centrifuged at 14000 rpm for 2 min and boiled for 10 min. Samples were stored at -4°C until further use. The gel was divided into two parts, separating gel (30% Acrylamide, 1.5 M Tris-HCl buffer with pH 8.8, 10% SDS, ddH_2O, 10% APS, Temed) and stacking gel (30% Acrylamide, 1.0 M Tris-HCl buffer with pH 6.8, 10% SDS, ddH_2O, 10% APS, Temed). About 15 µL of denatured crystal-spore mixture for each Bt strain were put in each well, as well as, standard protein marker. The gel was run for 2 hours at 120 Volt. The Standard Protein Molecular Weight Marker ranged from 14.4 – 116.0 KDa from FERMENTAS was used. Proteins were visualized by staining with Coomassie blue R-250. The protein profiles of the tested Bt strains were scanned.

Genomic DNA Extraction

Genomic DNA was extracted from Bt isolates by using Qiagen Plasmid Mini kit (Qiagen, Germany). A single colony from a freshly streaked plate was moved into 2 mL LB medium and incubated at 37°C overnight with shaking at 200 rpm. The cells were harvested by centrifuging at 8500 rpm for 3 min and the supernatant was carefully taken out. Method as described in the supplier's manual was followed, with the modification in time of centrifuging the suspension to avoid DNA shearing. Genomic DNA was size fractionated on 0.8% agarose gel along with 1 kb DNA ladder (MBI Fermentas, Germany). DNA bands were observed under UV in the gel documentation system (Alpha gel imager).

PCR Analysis

PCR reaction was carried out for amplification of *cry 6* gene using set of primers: *primer* 1: 5'-ATGATTATTGATAGTAAAACGACTTTACC-3' (Cry6A&BfFr) and *primer* 2: 5'-ACAACAAATCCTAACAATGGTCCTAA-3' (Cry6A&BmRv). For amplification of *cry 55* using set of primers: *primer* 1: 5'-ATGAACAAAAAATCTATTACTCATGAAGA-3' (Cry55AflFr) and *primer* 2: 5'-TTATGAAAATCTAGGATTAAAATTTACCTG -3' (Cry55AflRv). PCR was carried out in a reaction mixture of 20 µL. PCR buffer with $(NH_4)_2SO_4$ and $MgSO_4$ (10X) 2 µl; deoxy ribonucleotide triphosphate (dNTPs) (2mM) 2 µl; primers (10 µM) 2 µl each; Pfu DNA polymerase 0.5 µl and sterile nuclease free water in thermal cycler. Amplification was performed in a thermal cycler using a single denaturation step (30 seconds at 95ºC), followed by a 35-cycle program, with each cycle consisting of a denaturation step of 95ºC for 20 seconds; annealing step of 42°C for 20 seconds, and an extension step of 72ºC for 3 minutes. A final extension step of 72ºC for 10 minutes was also included. Twenty µL samples from the PCR mixtures were electrophoresed on agarose gel and stained with ethidium bromide (0.5 ml/ml).

PCR Purification from Agarose Gel

PCR products were fractionated by electrophoresis on agarose gels. After electrophoresis, the gel was placed onto the transilluminator and longitudinally sliced only the part of the gel containing the reference DNA. DNA was recovered from the agarose gel by the use of the QIAquick gel extraction kit (Qieagen, Germany) and the amount of purified DNA was quantified using nanodrop.

Ligation and Transformation

Ligation was carried out in a reaction mixture of 20 µL consist of 10X Reaction Buffer 2 µL; purified PCR product 16 µL; pJET 1.2/blunt Cloning Vector 1 µL and T4 DNA Ligase 1 µL at 4°C. The ligation mixture was briefly mixed by vortexing and centrifuged for 3 seconds. It was kept at -20°C for further use.One aliquot of *Escherichia coli (E. coli)* DH5α was thawed on ice and 20 µl of ligation product were transferred. Following 30 minutes on ice, a heat shock step was performed for 2 minutes in a 42°C water. The cells were then immediately incubated on ice for 2

minutes. Following this incubation, 800 µl of LB medium were added, and the mixture was transferred to a 1,5 mL Eppendorf tube. The cells were then agitated for 60 minutes at 37 °C and 200 rpm in the rotary imcubator, and subsequently 200 µl of the *E. coli* were plated on LA plate supplemented with 100 µg/ml ampicillin. The plate was incubated over night at 37°C in the incubator. After 12h, the colonies were counted.

Plasmid Extraction

Isolates were inoculated on LB supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C. The QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions with some modification in time of centrifuging the suspension to get higher concentration. Two µL samples from the extracted plasmid were electrophoresed on agarose gel and stained with ethidium bromide (0.5 ml/ml).

Diagnostic Restriction Enzyme Digest

The plasmid was digested using restriction enzymes NdeI and BglII to confirm the presence of the vector and insert DNA in an appropriate specific reaction buffer depens on the enzymes. Digestion was performed in incubator at 37°C overnight. Twenty µL of digested plasmid were electrophoresed on agarose gel and stained with ethidium bromide (0.5 ml/ml).

RESULTS AND DISCUSSION

Protein Production from Bt Isolates

SDS-PAGE analysis of their sporecrystal suspensions revealed that there were eighty different protein profiles, which some strains had protein bands with the same molecular weights as Cry 6 (54 kDa) and Cry55 (45 kDa), c*ry55Aa1* encoded a 45-kDa protein, *cry6Aa2* encoded a 54-kDa protein [7]. All the results showed that there were 24 Bt strains (number 4, 7, 12, 13, 14, 16, 17, 19, 20, 21. 23, 26, 29, 30, 48, 54, 57, 58, 60, 61, 66, 73, 74, and 78) have 54-kDa protein which is similar with the molecular weight of Cry 6 proteinand 5 strains (number 21, 41, 42, 56, and 59) have 45-kDa protein which is similar with the molecular weight of Cry 55 protein (Fig. 1). Although some isolates have the same molecular weight as the protein target, further analyses is required to ensure that the protein is encoded by the targeted gene. There are substantial differences even among those crystal proteins having the same molecular weight (Marroquin *et al*., 2000).

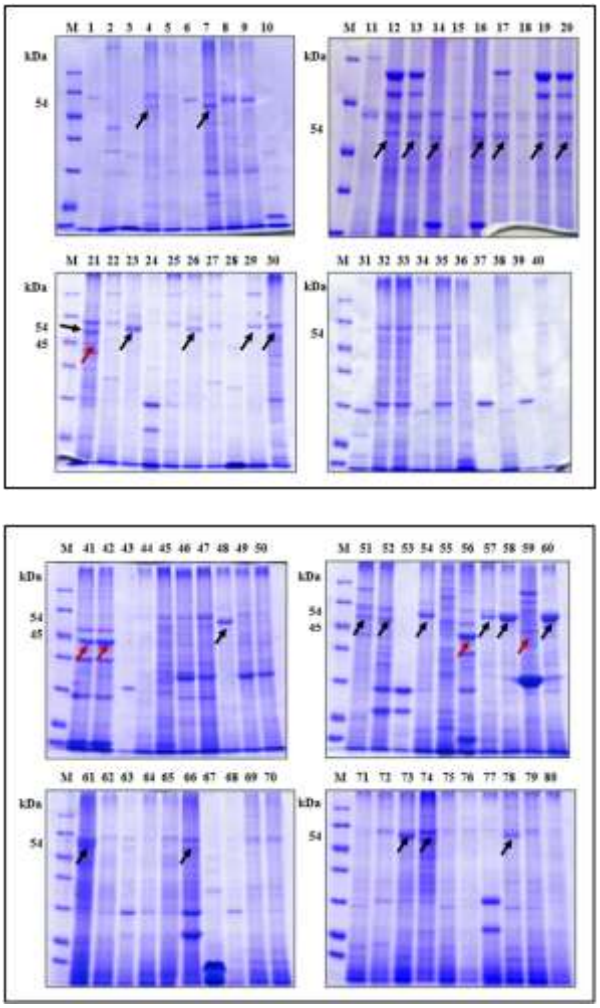

Figure 1. SDS-PAGE result from 80 Bt isolates. M : Unstained Protein Molecular Weight Marker Fermentas; lanes 1-80 : number of Bt isolates; black arrows show ±54 kDa proteins; red arrows show ±45 kDa proteins

Detection of cry6-type genes

Twentyfour Bt isolates were subjected to PCR amplification using Cry6A&BfFr and Cry6A-fulR primer set to detect various genes of *cry 6* gene family, also using Cry55AflFr and Cry55AflRv primer set to detect various genes of *cry 55* gene family. Expected size of the PCR product is about 1.472 kb with varying intensity corresponding to the highly conserved region of *cry 6* gene family was generated in 1 Bt isolate (number 21: Bt 32) but there is no size of the PCR product that matches with the size of the *cry 55* gene. One representative gel picture is shown here (Fig. 2). The presence of non-specific bands is most likely due to annealing temperatures used are too low or the primers are not ideal (Shenghe *et al*., 2016), primer pairs should be "unique", so they won't bind to other locations in the genome

except the intended gene or DNA fragment (Baghaee *et al*., 2015). PCR purification by gel extraction is needed to obtain only the targeted gene.

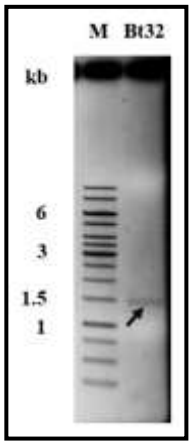

Fig. 2. PCR screening of *cry 6* genes in Bt. M: 1kb DNA Ladder; Bt 32: name of Bt isolate; arrow shows 1.472 kb band

PCR Purification

PCR product from Bt 32 is purified to obtain only the targeted gene. One representative gel picture is shown here (Fig. 3). Based on this result, the size of the gene can be confirmed around 1.472 kb, which is corresponds to the size of *cry 6* gene. The purified PCR product then used as a component for ligation and transformation. PCR-based systems are the most widely used for the identification of novel *cry* genes (Noguera and Ibarra, 2010), but there are several limitations to these systems.

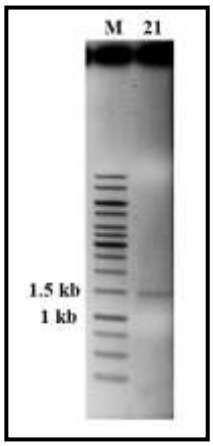

Fig. 3. Purified PCR product for *cry6* gene. M : 1 kb DNA Ladder ; Bt 32: name of Bt isolate; arrow shows 1.472 kb band

Plasmid Extraction

The result of plasmid extraction showed that there are three different size of the plasmids, about ±2.5 kb (number 65), ±2.9 kb (number 1, 2, 3, 4, 5, 8, 10, 13, 24, 25, and 37), and ±4 kb (number 14 and 29). Three representative gel pictures are shown here (Fig. 4). To ensure that the plasmid is correct (pJET) and it contains the gene target (*cry 6*), all of the plasmids were checked using some restriction enzymes.

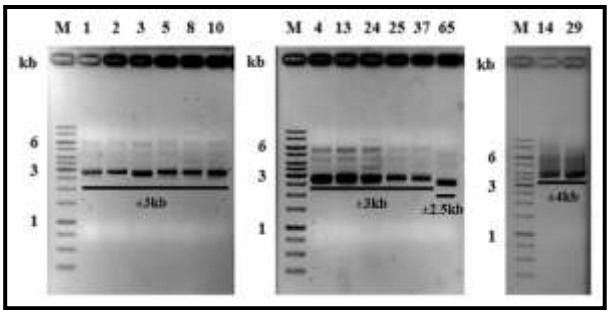

Fig. 4. Representative picture of extracted plasmid. M : 1 kb DNA Ladder; lanes after M: number of plasmid

Diagnostic Restriction Enzyme Digest

Restriction digest takes advantage of the fact that restriction enzymes cleave DNA at specific sequences called restrictions sites. Digestion with enzyme *NdeI* will linearize the 4.446 kb plasmid (1.472 kb insert + 2.974 kb backbone) into one single 4.446 kb fragment because according to the GenBank sequence database, *NdeI* has one restriction site in the insert, at 341 bp. Also, digestion with *BglII* will result in two bands; the 1.472 kb insert and the 2.974 kb backbone because *BglII* have two restriction sites in the vector, at 337 and 383 bp. *Bgl*II is a type II restriction endonuclease of 223 amino acids that targets the sequence AGATCT by encircling DNA from the major groove side (Lukacs *et al*., 2001). The restriction digest results are shown in Figure 5 and 6.

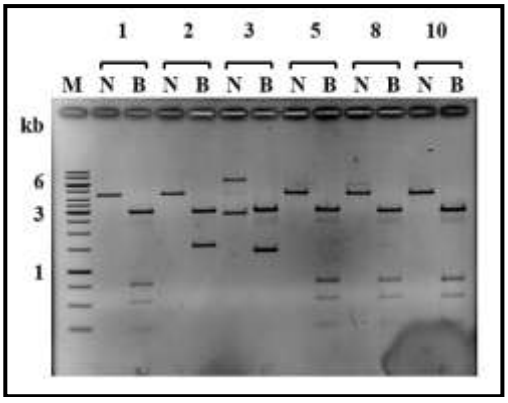

Fig. 5. Diagnostic restriction digest result by *NdeI* (N) and *BglII* (B). M: 1 kb DNA Ladder; 1,2,3,5,8,10: number of plasmid

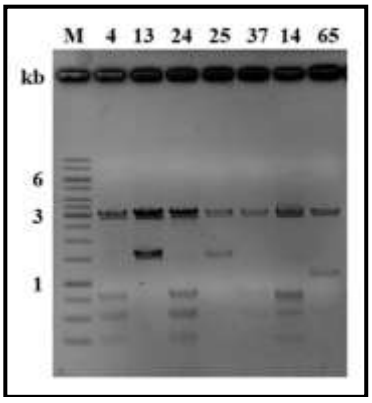

Fig. 6. Diagnostic restriction digest result by *BglII*. M : 1 kb DNA Ladder; lanes after M: number of plasmid

The plasmid has a total size of 4.446 kb, inclusing 1.472 kb insert. The result show that digestion using *NdeI* detected single band of ±4.5 kb in samples number 1, 2, 5, 8, and 10 representing the full size of the plasmid, proves that the targeted gene was integrated into the pJET cloning vector. Different from the result of other samples, there are 2 bands in sample number 3 at ±6 kb and ±3 kb which not correspond to the size of the plasmid, maybe the insert is different from the others and having two *NdeI* restriction sites, but can be clarified by digestion using *BglII* enzyme. Digestion result using *BglII* enzyme showed positive result for all samples, produces 2 bands at ±3 kb and ±1.5 kb, matching the backbone and insert, respectively. The result proves that the size of the insert is correspond to *cry 6* gene.

This may be very useful preliminary study of strain characterization for cry gene content in their genome before isolation of specific full length genes. The suspected positive isolate which must be confirmed by DNA sequencing in this study may be further used for expression into *E.coli* and nematode bioassay for checking the toxicity of Cry 6 protein against nematodes. Sequencing is especially important when cloning a PCR-amplified product since there is a higher risk of introducing unwanted mutations into the insert due to the fidelity of the polymerase. Sequencing can verify the sequence of a plasmid and detecting even single-point mutations (Czar *et al*., 2009). Although many toxins have been found in *Bt* strains, only a few of them are effective to control nematode pests. Moreover, some pests have developed resistance against some *Bt* toxins (Song *et al*., 2003). In order to solve these problems, isolation of new strains and toxins is crucial.

CONCLUSION

In this study, of 80 Bt isolates that have been screened through SDS-PAGE, 24 isolates (30%) have 54-kDa protein which is similar with the molecular weight of Cry 6 protein and 5 isolates (6%) have ±45 kDa protein which is similar with the molecular weight of Cry 55protein. After screened through PCR, followed by transformation into *E. coli* and verified by diasnostic restriction digest, only one Bt isolate (number 21: Bt 32) was suspected to be positive for the presence of *cry 6* gene.

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