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Identification and cloning of Cry1AB gene from *Bacillus Thuringiensis*

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Short Communication

ABSTRACT

Bacillus thuringiensis (Bt) is a bacterium that can be used as substitute for various chemical compounds especially insecticides. The insecticidal property of *B. thuringiensis* comes from the production of Crystal protein. The present study is focused on the cloning and characterization of Cry1AB gene which expresses to produce crystal protein. Cry1AB gene was amplified using specific primers which gave successful PCR results of 3.5 kb long gene amplification. The amplified gene was further verified by inserting it into the expression vector. Furthermore, a computational approach was used to understand evolutionary relationships among Cry1AB gene and to identify conserved protein domains in Cry1AB proteins. Use of insilico approach provided new insights of understanding functional characteristics of Cry1AB gene and its variants.

Keywords: *Bacillus thuringiensis,* Crystal protein, Cry1AB, PCR

INTRODUCTION

The emerging bio-rational Pesticide, *Bacillus thuringiensis* (Bt), is a gram positive bacterium that forms para-sporal crystal during the stationary phase of their life cycle. The bacterium was originally known as insect pathogenic bacterium as its property of forming para-sporal crystals contributed largely or completely (depends on insect type) against the insects. This analysis led to the formation of bioinsecticides built on *B. thuringiensis* insecticidal activity against various orders of insects such as; Coleoptera, Diptera and Lepidoptera (Schnepf *et al*., 1998). Many recent studies analyzed, experimented and reported insecticidal activity of *B. thuringiensis* against other insects order such as Mallophaga, orthopteran, Hymenoptera and Homoptera, and also extending their research to test the bacterium against mites, protozoa and nematodes (Feitelson *et al*., 1992; Feitelson, 1993). *B. thuringiensis* has already proved to be good and beneficial substitute for chemical insecticides in various fields which includes; mosquito control, forest management and commercial agriculture. Moreover, it is a major source of genes for transgenic expressions to develop resistance against insect in plants (Schnepf *et al*., 1998).

The production of insecticidal crystal protein (ICPs), also known as d-endotoxins, gives *B. thuringiensis* its insecticidal property. The ICPs consist of one or more crystal (Cry) proteins and Cytolitics (Cyt) toxins (Bravo *et al*., 2007). The cry genes have been characterized according the insect classes they are effective against (Crickmore, 2011). Strains of *B. thuringiensis* have different classes of cry genes (Bravo *et al*, 2011), and, thus, general toxicity of the strains usually depends on the expression of the specific cry gene, the relative concentration of many protoxins, solubility and proteolytic mechanisms of many proteases in the midgut of larva and toxin binding to receptor attached to epithelium of the vulnerable insect. Since the cloning of first cry gene encoding ICP from *B. thuringiensis* strain HD-1, the study for novel cry gene is a progressing effort globally with 560 cry gene discovered and placed in different families (Crickmore, 2011). The gene coding for insecticidal toxin protein in *B. thuringiensis* have been transferred in many crops and theses transgenic Bt crops have been commercially refined on large scale. However, with growing scale and

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lengthy production of Bt crops, the development of resistant in insects to the toxin in agriculture system has become the primary concern to sustainable future of Bt bioinsecticides and Bt crops (Tiewsiri & Wang, 2011).

Bt toxin resistance in insects in green house as well as in open field has already been studied in many species (Bravo *et al*, 2011). Moreover, several insect species with Bt toxin resistance have been studied in laboratories for the resistance mechanism (Bravo *et al*, 2011; Heckel *et al*, 2007). Insects have cadherin receptor proteins in their midgut region where cry protein binds through sequential interaction (Gomez *et al*, 2006) and it has been demonstrated that mutated cadherin receptor gene could interfere with receptor-protein binding and hence, create resistance in insects against cry protein (Ferre and Van, 2002; Morin *et al*, 2003; Xu *et al*, 2005). Many studies reported that the suppression of Bt cry protein resistance in insect could be accomplished by altering in cry gene in a way that they kill the insect without interacting the receptor proteins (Soberon *et al*, 2007; Pardo-Lopez *et al*, 2010). In case of bacterial cell, it has been reported that mutation caused even with single residue could alter the efficiency of cry proteins in regard to host range and toxicity levels (Saraswathy & Kumar, 2004; Nair & Dean, 2008). Furthermore, the toxins in the cry1A subfamily shows difference in specificity and toxicity due to small changes in amino acid (Tounsi *et al*, 1999). These observations suggest that the development of resistance in insects can be avoided or hindered if there are varieties of cry proteins, either naturally present or altered via genetic engineering (Gatehouse, 2008).

This study was focused on the identification and cloning of Cry1AB gene from *Bacillus thuringiensis* for better understanding of the Cry protein specificity and its insecticidal property.

MATERIALS AND METHODS

The objective of this research was to identify and clone the Cry1Ab gene isolated from *Bacillus thuringiensis.* The Studies were carried out to analyze and characterize the selected gene. Bioinformatics provides various tools and software's to analyze the highly expressed genes. The isolation and characterization of genes are useful for understanding their functions. The methods used are discussed below:

Culturing of Bacillus thuringiensis (Bt)

. *Bacillus thuringiensis* (Bt) strain was streaked using agar nutrient media. The cells were grown at 30^{o}C in shaking (260rpm) in 25ml Of LB media and harvested after 24 hrs of inoculation. The single colonies were cultured to isolate the plasmid and further verified by restriction digestion with *Hindi3* and EcoR1 and PCR amplification.

DNA extraction

The DNA extraction of *Bacillus thuringiensis* strains was carried out according to altered version of Jensen *et al.* (1995) stated in Stobdan *et al* (2004). The bacterial cultures were incubated at 30 ^{o}C in LB medium overnight along with constant shaking. DNA was extracted using DNA extraction kit (Macrogen)

Primer designing

Two sets of primers, forward and reverse, were designed and synthesized using Primer3 software tool. The primers used in the study were F: AGGCAAACGTGAAAAATTGG and R: AAAATAGCCGCATTGACACC.

PCR profile

Reaction mixture of 25ml (Table 1) was prepared for the amplification of Cry1AB gene. The DNA was initially denatured at 94 ºC for 4 min followed by 30 cycles consisting denaturation at 94 ºC for 30s, 58 ºC for 30s, 72 ºC for 3min and final extension at 72 ºC for 15min.

Agarose gel electrophoresis

Amplified PCR product of Cry1AB gene was separated on 1% agarose gel in 1X TAE buffer containing 20mg/mL ethidium bromide. 10 kb DNA ladder (Fermentas) was used as a molecular weight marker. The gels were viewed under UV light for the confirmation of amplification of the gene.

Table 1: Final concentration of PCR ingredients

Reagents	Concentration
$MgCl_2$	2.0 µl
Forward Primer (ITS-1F)	1.0 µl
Reverse Primer (ITS-4R)	1.0 µl
dNTPs	2.0 µl
Taq Polymerase	1.0 µl
Taq DNA Polymerase Buffer	5 µl
Template genomic DNA	3.0 µl
Water	5.0 µl

Results

Bacillus cultures were successfully streaked on Agar nutrient media (Fig 1).

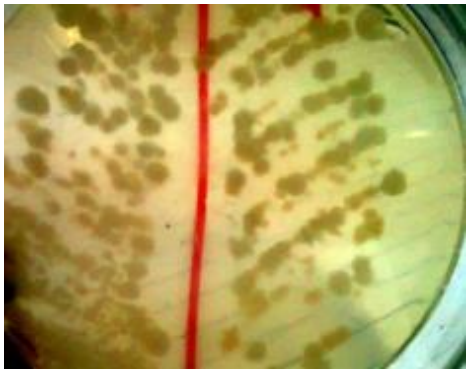

Fig. 1: Bacillus growth on Agar nutrient medium

Cloning and nucleotide sequencing

PCR products were cloned in expression vector derived from pJIT166 (pGE1) that contained GUS with intron under 2X35S promoter followed by CaMV terminator. Glycerol stock of the Cry1AB gene was streaked on LB agar plates having ampicilin. The single colonies were cultured to isolate the plasmid. The products were sequenced commercially by 1st base Malaysia sequencing. The recombinant DNA plasmid was digested using two restriction enzymes EcoR1 and HindIII, the sites of which was already present on the vector. The gene sequences were compared with others sequences in the Gene Bank databases and phylogenetic analysis of Cry1AB gene were performed using bioinformatics tools including CLUSTALW and CDD.

DNA extraction and Amplification

Genomic DNA of *B. thuringiensis* was extracted according to the method described previously (Jensen *et al.*, 1995). Cry1AB gene of size 3.5kb was amplified and sequenced by specific primers. The primers were designed and synthesized using Primer 3 software tool successfully amplified the gene region (Fig.2). The results of restriction digestion and PCR are shown in figure 2 below:

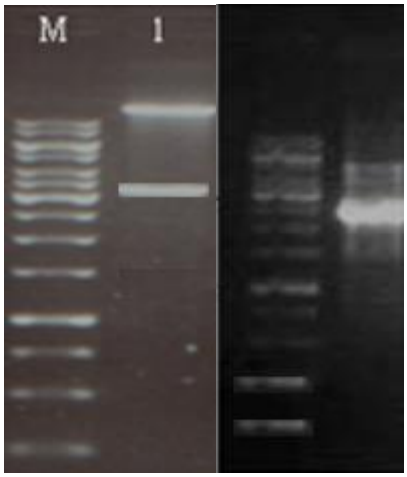

Fig. 2: Cloning of 3.5kb Cry1 AB gene in expression vector.

Phylogenetic Analysis

To study, evolutionary relationships among different species the best practice is to perform phylogenetic analysis. The genetic variants of Cry1 Ab were analyzed to study the evolutionary relationships among them. The genetic diversity of species varies differently. However, the ancestor matching by homology matching and score predictions with extent or degree of occurrence of any gene is useful in such studies. Further, the Cry1Ab gene was found distantly related with other compared genes. The analysis was performed by CLUSTAL W software. The results are shown in figure 3 below:

Fig. 3: Phylogenetic analysis of Cry1 AB gene with other Cry1 AB orthologues.

Protein Domain Analysis

Domain are conserved regions of proteins responsible of folding independently. The function of proteins can be conserved due to several reasons. The proteins of same gene can give rise to similar functions and can be best studied in evolutionary pathways. Three potential domains were found in Cry1Ab associated proteins. These

are listed in table 2 below. All of these belongs to super families. They were also found to be rich with toxins. Study of these domains can provide useful ways to predict function of gene. The analysis of these domains was performed by using CDD domain Analysis software. The results are shown in figure 4 below:

Table 2: Predicted protein domains in Cry1AB gene.

Name	Accession	Description	Internal	E-Value
Delta_ endotoxin	Cd04085	Associated with carbohydrates	463-606	5.72 e-57
Endotoxin_M	Pfam00555	Contains insecticidal toxins produced by bacillus species	259-461	2.10 e-51
Endotoxin_N superfaily	D04339	N-terminal domain. Contains insecticidal toxins	48-251	1.20 e-25

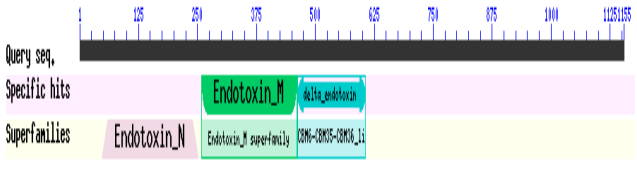

Fig. 4: Specific hits of three protein domains within super family.

Discussion and Conclusion

In recent years, the requirement of environmentally friendly pesticides has encouraged the replacement of chemicals usage with biological methods, which are much safer to the environment. Methods are being introduced to control a range of phyto-pathogenic agents such as development of transgenic plants of B. thuringiensis. This is useful for the production of insect-resistant Bt-transformed lines of any crops such as cotton, tobacco, corn, maize, potatoes tomatoes (Frutos *et al*. 1999).

Cry genes have been introduced in any plant related pathogens to enhance the efficacy and stability of crop which also helps to gain the maximum insect control on both subterranean and aerial surfaces of plants (Moar *et al*., 1994; Bora *et al.,* 1994; Downing *et al*., 2000).

Bacillus thuringiensis (Bt) is entomo-pathogenic bacterium that lives in soil. It is a Gram-positive bacteria and can be easily isolated from variety of sources (Azizoglu *et al*., 2016). It makes several proteins, which are toxic to some insects only. However, these proteins are not harmful for humans because they cannot activate them. Studies showed it had specific toxicity against target insects. Cry1 toxins are considered as one of the most important proteins with specific insecticidal activity against lepidopteran insects (Bergamasco *et al.,* 2013).

The cry proteins encoding 103-MDa plasmid was involved in regulatory production of crystal in *B. thuringiensis* subsp. *yunnanensis* (Srinivas *et al*., 1997) Moreover, one more *B. thuringiensis* strain with similar properties was isolated from soil samples of Africa soil samples (Bravo *et al*., 1998).

However, use of *B. thuringiensis* bioinsecticides has led to successful development of insect resistance to individual toxins (Zhao *et al.*, 2000; Akhurst *et al.*, 2003). Currently, the use of transgenic technology in agriculture biotechnology, highlights variety of pesticidal genes from *Bt*, variants. Furthermore, the improvement of broader spectrum biopesticides using novel *Bt* strains against target insects is also an important aspect for improving their long term persistence on plants. Several novel cry genes has been discovered with new spectra and higher toxicity for production of new products and the management of resistance.

The present study was performed to analyze and characterize Cry1 AB gene by using various bioinformatics software's. The computational analysis of Cry1 AB showed presence of three putative domains located within the entire region. All three domains were part of superfamily. The delta endotoxin, endotoxin M and endotoxin N all three were responsible for production of some insecticidal toxins. The identified gene was further cloned in general expression vector, PCR amplified to explore its more functions. Results showed, such genes can be used in future for understanding insecticidal properties.

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