Biotechnol Lett (2010) 32:1311–1315
DOI 10.1007/s10529-010-0301-1

ORIGINAL RESEARCH PAPER

# Cloning and characterization of truncated *cry1Ab* gene from a new indigenous isolate of *Bacillus thuringiensis*

**Sandhya Darsi · G. Divya Prakash · V. Udayasuriyan**

Received: 15 February 2010 / Accepted: 6 May 2010 / Published online: 18 May 2010


**Abstract** The insecticidal crystal protein(s) encoded by *cry* gene(s) of *Bacillus thuringiensis* (Bt) have been used for insect control both as biopesticides and in transgenic plants. A new 3′-truncated *cry1Ab* gene was cloned from an indigenous isolate of Bt, A19-31. Nucleotide sequencing and homology search revealed that the deduced amino acid sequence of Cry1Ab toxin of Bt strain A19-31 had a variation of two amino acid residues with the holotype sequence, Cry1Ab1. Expression of the 3′-truncated *cry1Ab* gene was studied in an acrystalliferous strain of Bt (4Q7). SDS-PAGE and immunostrip analysis of spore-crystal mixture revealed a low level expression of the 3′-truncated *cry1Ab* gene. Insecticidal activity assay showed that the recombinant 3′-truncated *cry1Ab* gene product was toxic to larvae of both *Helicoverpa armigera* and *Spodoptera litura*.

**Keywords** *Bacillus thuringiensis* · *cry1Ab* · Cloning · Expression · Insecticidal activity

S. Darsi · G. Divya Prakash · V. Udayasuriyan (✉)
Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore 641003, Tamil Nadu, India
e-mail: udayvar@yahoo.com

## Introduction

*Bacillus thuringiensis* (*Bt*) is a Gram-positive, aerobic, sporulating bacterium which synthesizes crystalline proteins during sporulation. These crystalline proteins are highly insecticidal at very low concentrations. The mode of action of insecticidal crystal proteins (ICP) involves a cascade of events including solubilization of the crystal, activation of the toxins by gut proteases and recognition of a binding site on the midgut brush border membrane followed by pore formation and cell lysis leading ultimately to insect death. Majority of the Cry proteins are generally are of two lengths: either 130–140 kDa or approximately 70 kDa. Three domains required for toxicity are present in the *N*-terminal half of the larger proteins, whereas the *C*-terminal half is constituted in a protoxin domain and is not found in smaller proteins. Therefore, 3′-truncated *cry* genes of larger proteins (such as *cry1*) encode for proteins with insecticidal activity (Schnepf et al. 1998). The Cry proteins have been classified as belonging to Cry 1–50 families on the basis of amino acid sequence homology (www.biols.susx.ac.uk/home/NeilCrickmore/Bt).

The most wide-spread *cry1* genes encode the 130–138 kDa delta-endotoxins that form ICPs active against lepidopteran larvae. Difference in the level of toxicity and specificity exist among different Cry1A toxins due to minor variations in amino acid residues (Udayasuriyan et al. 1994; Rajamohan et al. 1996). Continuous exposure to a single kind of Bt

toxin can lead to resistance development in insects. Discovery of new insecticidal protein genes is of importance for delaying the development of resistance in target insects. In the present work, we have cloned, sequenced and expressed a 3′-truncated *cry1Ab* gene exhibiting sequence variation from the known genes within the Cry1Ab group.

## Materials and methods

### Bacterial strains and plasmids

*Bacillus thuringiensis* (Bt) strain, A19-31 was from Prof. V. Udayasuriyan, TNAU, Coimbatore (Ramalakshmi and Udayasuriyan 2010). The Bt strain 4Q7 (acrystalliferous) and the *E. coli-Bt* shuttle vector pHT3101 (Lereclus et al. 1989) were originally obtained from Bacillus genetic stock centre, Ohio University, Columbus, Ohio, USA. *E. coli* strain DH5α was used for maintaining plasmid constructs. Plasmid pTZ57R/T (Fermentas INC) was used for DNA cloning and pHT3101 (*E. coli-Bt* shuttle vector) was used for gene expression in recombinant Bt strain.

### Cloning and DNA sequencing of truncated *cry1Ab* gene from a new indigenous isolate of Bt, A19-31

Total genomic DNA was isolated as described by Kalman et al. (1993) from an indigenous isolate of Bt, A19-31, and used as template for PCR amplification. Four oligonucleotide primers specific for the 3′-truncated *cry1Ab* gene were designed based on the published sequence of *cry1Ab1* gene. One primer pair (1AbFS1; 5′-GGAACCTCCTCAAATTTGCCATCC GC-3′, 1AbRS1; 5′-TCATTGCCTGAATTGAAGAC ATGAGC-3′) for screening and another pair (1AbF1; 5′-C**CCCGGG**CCTGGGTCAAAAATTGATATTT AG-3′, 1AbR2; 5′G**CTGCAG**TGCTCTTTCTAAAT CATATTCTGCC-3′) were used for cloning. Sites for *Xma*I (CCCGGG), and *Pst*I (CTGCAG) are in bold letters. For the amplification of the 3′-truncated *cry1Ab* gene, 50 ng total DNA was used as template in 25 µl reaction mixture containing 2.5 µl 10× PCR buffer (10 mM Tris/HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 100 ng each of forward and reverse primers and 0.5 units of *Taq* DNA polymerase. PCR cycling profiles were 1 cycle at 94°C for 2 min, 30 cycles of 94°C for 40 s, 60°C for 45 s and 72°C for 2 min, followed by a final extension step at 72°C for 7 min. The PCR product was ligated with pTZ57R/T and then transformed into *E. coli* strain DH5α. The resulting construct is designated as pT1Ab. The plasmid carrying *cry1Ab* gene was sequenced. Sequences alignment was performed in BLAST web (http://www.ncbi.nlm.nih.Gov/BLAST/) or using ClustalX.

### Expression of truncated *cry1Ab* gene in acrystalliferous Bt strain, 4Q7

The recombinant T/A plasmids carrying 3′-truncated *cry1Ab* gene were digested by *Xma*I and *Pst*I restriction enzymes to release the cloned DNA fragment. Simultaneously the shuttle vector pHT3101 was also digested using the same enzymes to linearize it. Both were ligated and ligation mixture was transformed into competent cells of *E. coli* DH5α and recombinant *E. coli* clones were selected on LB plates with ampicillin. The recombinant pHT plasmid carrying the truncated *cry1Ab* gene was isolated from recombinant *E. coli* clone and transformed into the acrystalliferous Bt strain 4Q7 by electroporation (Mahillon et al. 1989). Transformed Bt colonies were selected on LB agar plate containing erythromycin. Spore-crystal mixtures were prepared from recombinant and non-recombinant Bt strains 4Q7 as described by Lenin et al. (2001). Isolated spore-crystal mixtures were subjected to SDS-PAGE analysis.

### Immunostrip analysis of spore-crystal mixture from recombinant Bt strain

The Bt spore-crystal mixtures isolated from 4Q7 and recombinant 4Q7 Bt strains were tested by Cry1A immunostrips (Envirologix, USA) as per the manufacturer's instruction.

### Susceptibility of *H. armigera* to the cloned Cry1Ab toxin

For bioassay, laboratory cultures of *H. armigera* and *S. litura* (originally initiated from field-collected larvae) were reared on a semi synthetic diet. Insect bioassays were conducted using neonate larvae of *H. armigera* and *S. litura* by surface-diet contamination method. Each treatment was replicated three

times in ten units. Larval mortality was recorded periodically for 7 days.

## Results and discussion

### Cloning of truncated *cry1Ab* gene from new indigenous isolate of Bt, A19-31

Young colonies of *Bacillus thuringiensis* (Bt) strains A19-31 and A22-73 were screened by colony PCR with primer pair 1AbFS1 and 1AbRS1 specific to *cry1Ab* gene. Amplicons of expected size (~600 bp) were obtained in the case of A19-31. This indicated the presence of *cry1Ab* gene in A19-31 indigenous isolate whereas A22-73 used as negative control failed to produce any amplification. The 1AbF1 and 1AbR1 are corresponding to 2,100 bp DNA fragment containing 149 bp upstream region (promoter) and 1,860 bp 5′-region of *cry1Ab* gene (for 620 amino acids). An intact DNA fragment of 2.1 kb was amplified using 1AbF1 and 1AbR1 primers from genomic DNA of indigenous strain of Bt, A19-31. The purified PCR product was cloned into pTZ57R/T vector (T/A cloning vector). The resulting construct was named as pT1Ab. Restriction digestion of its recombinant plasmids (pT1Ab) with *Xma*I and *Pst*I released fragments of expected size (Fig. 1). The recombinant clone carrying 3′-truncated *cry1Ab* gene was sequenced and the gene sequence was deposited in NCBI GenBank under the accession number EU357806. Alignment of 3′-truncated Cry1Ab with known Cry1Ab proteins revealed residue changes which were as follows:

- when was compared to Cry1Ab1, CryAb3, Cry1Ab4, Cry1Ab5, Cry1Ab9, Cry1Ab10, Cry1Ab12, Cry1Ab13, Cry1Ab14, Cry1Ab15, Cry1Ab18, Cry1Ab20, two residues were different: $Val^{433}Ala$ and $Trp^{455}Gly$;
- when was compared to Cry1Ab2, 45 residues were different: Aligned sequences are given in Fig. 2;
- when was compared to Cry1Ab6, five residues were different: $Val^{433}Ala$, $Trp^{455}Gly$, $Asn^{461}Glu$, $His^{542}Asp$ and $His^{569}Thr$;
- when was compared to Cry1Ab7, six residues were different: $Val^{433}Ala$, $Pro^{450}Ala$, $Trp^{455}Gly$, $Leu^{537}Phe$, $Ile^{545}Pro$ and $Ile^{568}Thr$;

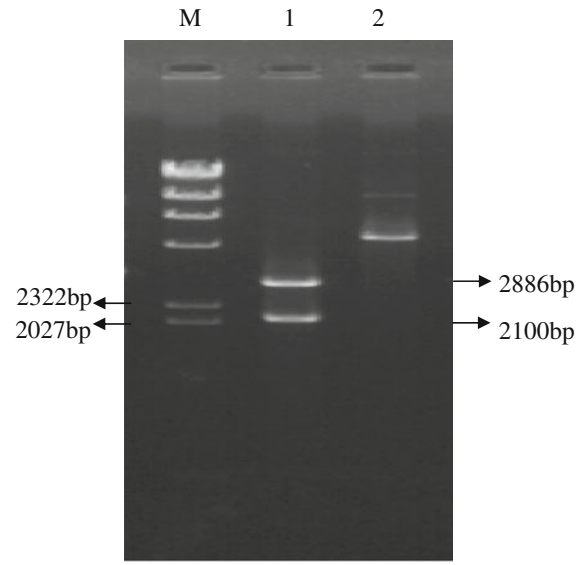
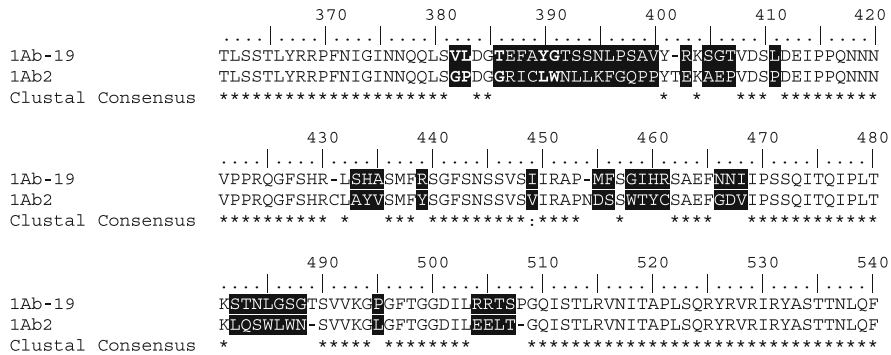



**Fig. 1** Restriction analysis of recombinant plasmid pT1Ab. *M* molecular mass marker *λ/Hin*dIII, *1* pTAb digested with *Xma*I and *Pst*I

- when was compared to Cry1Ab8, four residues were different: $Ala^{282}Gly$, $Leu^{283}Ser$, $Val^{433}Ala$ and $Trp^{455}Gly$;
- when was compared to Cry1Ab16, four residues were different: $Val^{165}Ala$, $Leu^{176}Ser$, $Val^{433}Ala$ and $Trp^{455}Gly$;
- when was compared to Cry1Ab17, four residues were different: $Pro^{170}$ Ser, $Val^{433}Ala$, $Gly^{450}Arg$ and $Trp^{455}Gly$;
- when was compared to Cry1Ab19, three residues were different: $Glu^{385}Gly$, $Val^{433}Ala$ and $Trp^{455}Gly$;
- when was compared to Cry1Ab21, three residues were different: $Pro^{262}Gln$, $Val^{433}Ala$ and $Trp^{455}Gly$;
- when was compared to Cry1Ab22, three residues were different: $Leu^{176}Ser$, $Val^{433}Ala$ and $Trp^{455}Gly$.

### Expression of truncated *cry1Ab* gene of Bt strain A19-31 in an acrystalliferous Bt strain 4Q7

The 3′-truncated *cry1Ab* gene cloned in the pTZ57R/T vector was released by *Xma*I and *Pst*I digestion and ligated to *E. coli*-Bt shuttle vector, pHT3101 in the same sites. Recombinant plasmids were selected based on the restriction digestion with *Xma*I and *Pst*I



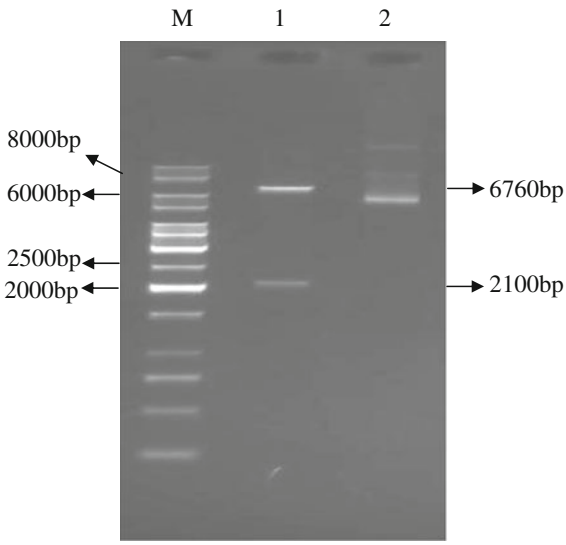




**Fig. 3** Restriction analysis of recombinant plasmid pHT1Ab. *M* molecular mass marker 1 kb, *1* pHT1Ab digested with *Xma*I and *Pst*I

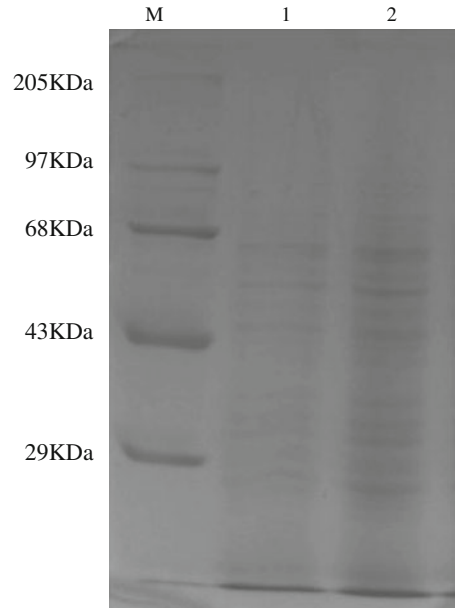



**Fig. 4** SDS-PAGE analysis of spore crystal mixture isolated from 4Q7 and recombinant (Cry1Ab transformant) Bt strains. *M* molecular mass marker, *1* non recombinant Bt strain 4Q7, *2* recombinant Bt strain 4Q7

that releases 2.1 kb *cry1Ab* gene and 6.7 kb pHT3101 vector (Fig. 3). The recombinant pHT3101 containing the 3′-truncated *cry1Ab* gene was named as pHT1Ab. The plasmid construct was electroporated into acrystalliferous Bt strain 4Q7. Spore-crystal mixtures isolated from the both recombinant 4Q7 Bt strain and non-recombinant 4Q7 Bt strain were subjected to SDS-PAGE analysis. Expression of Cry1Ab toxin was not obvious as a distinct band of expected size (~65 kDa) in the protein profile of recombinant 4Q7 strain (Fig. 4).

However, the Cry1A immunostrip had a positive reaction to the spore crystal mixture isolated from recombinant Bt strain of 4Q7. But the spore crystal mixture of Bt strain 4Q7 did not react with the Cry1A immunostrips (Fig. 5). Thus it can be suggested that the level of expression of truncated *cry1Ab* in transformants of Bt is low.

## Toxicity analysis

Minor variations in the Cry toxins can lead to significant changes on the level of toxicity and insecticidal activities of the protein (Sasaki et al. 1997). Bioassay by artificial diet contamination method with spore-crystal mixture isolated from the Cry1Ab transformant of Bt strain 4Q7 (pHT1Ab) showed 70 and 20% mortality in *H. armigera* and *S. litura* neonate, respectively. Whereas all the larvae on control diet


**Fig. 5** Immunostrip analysis of spore crystal mixture isolated from 4Q7 and recombinant (Cry1Ab transformant) Bt strains. *1* Non recombinant Bt strain 4Q7, *2* recombinant Bt strain 4Q7

coated with Bt strain 4Q7 were alive until the 7 days after treatment. Higher level of expression of the truncated *cry1Ab* or whole *cry1Ab* gene of the new Bt strain A19-31 in transgenic microbes or plants could result in improved level of toxicity.

**Acknowledgements** The authors are thankful to Dr. V. Balasubramani and Mrs. Annakodi for their help in the maintenance of *H. armigera* culture. S.D. acknowledges Department of Biotechnology, Government of India, New Delhi, for the Junior Research Fellowship.

## References

Kalman S, Kiehne K, Libs J, Yamamoto T (1993) Cloning of a novel *cryIC*-type gene from a strain of *B. thuringiensis* subsp. *galleriae*. Appl Environ Microbiol 59:1131–1137

Lenin K, Asia Mariam M, Udayasuriyan V (2001) Expression of *cry2Aa* gene in an acrystalliferous *B. thuringiensis* strain and toxicity of Cry2Aa against *H. armigera*. World J Microbiol Biotechnol 1:273–278

Lereclus D, Arantes O, Chaufaux J, Lacadet M (1989) Transformation and expression of a cloned delta-endotoxin gene in *B. thuringiensis*. FEMS Microbiol Lett 60:211–218

Mahillon J, Chungjatopornchai W, Decock J, Dierickx S, Michiels F, Peferoen M, Joos H (1989) Transformation of *Bacillus thuringiensis* by electroporation. FEMS Microbiol Lett 60:205–210

Rajamohan F, Alzate O, Cotrill JA, Curtiss A, Dean DH (1996) Protein engineering of *Bacillus thuringiensis* delta-endotoxin: mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae. Proc Natl Acad Sci USA 93:14338–14343

Ramalakshmi A, Udayasuriyan V (2010) Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamil Nadu State, India. Curr microbiol. doi:10.1007/s00284-009-9569-6

Sasaki J, Asano S, Hashimoto N, Lay W, Hastowo S, Jizuka T (1997) Characterization of a *cry2A* gene cloned from an isolate of *B. thuringiensis* serovar *sotto*. Curr Microbiol 35:1–8

Schnepf E, Crickmore N, Van Rie J, Lerecurs D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *B. thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Shin BS, Park SH, Choi SK, Koo BT, Lee ST, Kim JI (1995) Distribution of *cryV-type* insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *entomocidus*. Appl Environ Microbiol 61:2402–2407

Udayasuriyan V, Nakamura A, Mori A, Masaki H, Uozumi T (1994) Cloning of a new *crylA*(a), gene from *B. thuringiensis* strain FU-2-7 and analysis of chimeric *cry1A* (a) proteins of toxicity. Biosci Biotechnol Biochem 58:830–835