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Screening of *Bacillus thuringiensis* Isolates Recovered from Diverse Habitats in India for the Presence of *cry*1A-type Genes and Cloning of a *cry*1Ac33 Gene Toxic to *Helicoverpa armigera* (American Bollworm)

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ABSTRACT

Helicoverpa armigera (American bollworm) is a severe pest of many economically important crops such as cotton, pigeon pea, chickpea and tomato in the Indian subcontinent. Insecticidal *cry* genes from the bacterium *Bacillus thuringiensis* (Bt) have been used for developing transgenic crops. Transgenic cotton expressing *cry*1Ac gene has shown good level of protection from *H. armigera*. However, there is a threat of eventual development of resistance in insects upon large-scale cultivation of transgenic crops. Therefore, prospecting of Bt strains for new types of *cry* genes is desirable. This study was undertaken for screening of native Bt isolates for the presence of *cry*1Aa,b,c-type genes with the objective of cloning, sequence analysis, expression and evaluation of toxicity. Sixty three native Bt isolates recovered from different soil and grain samples from diverse agricultural and non-agricultural locations in India, along with 10 known Bt strains used as reference, were screened for the presence of full length *cry*1A-type genes by Polymerase Chain Recation (PCR) using a set of primer. The full length gene was obtained in the native Bt isolate SK-711 recovered from Red gram field, Lam, Guntur Andhra Pradesh and in 3 Bt strains. The gene from the native Bt isolate was cloned into an *E. coli* expression vector. The sequence of the cloned gene (GenBank accession No. GQ866913) was analyzed by comparison with previously known *cry*1A-type genes and was assigned the name *cry*1Ac33 by the *Bacillus thuringiensis* Nomenclature Committee. The gene was expressed in *E. coli* and evaluated for toxicity towards *H. armigera*. The *cry*1Ac33 gene, cloned from a native Bt isolate, has been found to be more toxic towards *H. armigera* than the holotype *cry*1Ac1 used as a control, based on LC_{50} toxicity analysis.

Key words: *Bacillus thuringiensis*, *cry* genes, *Helicoverpa armigera*, India, PCR

INTRODUCTION

Bacillus thuringiensis (Bt) is an aerobic, gram-positive, spore-forming bacterium producing crystal proteins (Cry) which are selectively toxic to target insects (Feitelson *et al*., 1992; (Karim *et al*., 1999; Garcia-Robles *et al*., 2001; Amjad *et al*., 2001; Zia-ur-Rehman *et al*., 2002;

Zaied *et al.*, 2003; De Escudero *et al.*, 2006; Halima *et al.*, 2006; Ali *et al.*, 2010). Cry proteins act by insertion into the microvillar brush-border membranes in the midgut of susceptible insects, leading to disruption of osmotic balance, lysis of epithelial cells and eventually death of insect (Schnepf *et al.*, 1998). Insect resistant Bt transgenic crops were first developed using native *cry*1Ac gene (Barton *et al.*, 1987; Anwar *et al.*, 1999; Khan *et al.*, 2001). The total acreage of transgenic crops has been steadily increasing with countries including 10 developed and 19 developing countries having adopted commercial cultivation of transgenic crops on 140 million hectares in 2010 (Gatehouse, 2008; James, 2010). The use of Bt crops has resulted in increased yields and significant reductions of insecticide application, thus providing economic and environmental benefits (Brookes and Barfoot, 2008; Carpenter, 2010).

The bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) is a polyphagous pest of worldwide occurrence that inflicts an annual crop damage in India worth US$ 1 billion (Subramanian and Mohunkumar, 2006). It is a major pest of several economically important crops such as cotton, pigeonpea, chickpea, tomato, sorghum, okra and blackgram. Transgenic cotton expressing *cry*1Ac gene, released for commercial cultivation in India and China, has shown good level of protection from *H. armigera* (Wu *et al.*, 2003; Choudhary and Gaur, 2010).

However, there is a threat of eventual development of resistance in *H. armigera* exposed to Cry1Ac protein season-long in all plant parts upon large-scale cultivation of Bt cotton (Shelton *et al.*, 2002; Kaur, 2004). Development of second-generation Bt transgenic crops requires new insecticidal genes for stacking or pyramiding of genes wherein more than one insecticidal genes are used in combination as a resistance management strategy (Zhao *et al.*, 2003; Kaur, 2006). Two *cry* genes (out of *cry*1Ab, *cry*1Ac, *cry*2A, *cry*1C) pyramided rice lines and their hybrids exhibited excellent efficacy against stem borers and leaf folders in field evaluation (Yang *et al.*, 2011). The rapidity of development of resistance necessitates isolation of new genes for transgenic development. A significant exponential increase in the frequency of alleles conferring *cry2Ab* resistance in Australian field populations of *H. punctigera* since the adoption of a second generation Bt cotton expressing Cry2Ab protein was observed (Downes *et al.*, 2010). Therefore, the isolation of novel *cry*-type genes from Bt isolates is a useful approach to tackle the development of resistance against currently deployed limited number of *cry*-type gene(s).

Bt isolates are being screened the world over in search of new types of insecticidal genes. Bt strains have been isolated worldwide from diverse habitats, including soil (Bernhard *et al.*, 1997; Kaur and Singh, 2000a; Wang *et al.*, 2003), stored grains (Meadows *et al.*, 1992) phyllospheres (Hansen *et al.*, 1998; Kaur and Singh, 2000b; Jara *et al.*, 2006) and other miscellaneous habitats (Uribe *et al.*, 2003; Apaydin *et al.*, 2005; Stobdan *et al.*, 2005; Martinez and Caballero, 2002). Over 200 *cry* genes grouped into 40 families with insecticidal spectrum extending over several invertebrate orders have been isolated from Bt strains worldwide (Crickmore *et al.*, 1998; Wang *et al.*, 2003; Stobdan *et al.*, 2004; Swiecicka *et al.*, 2008). The identification of *cry* genes by Polymerase Chain Reaction (PCR) has proven to be a very useful method for characterization of *cry* genes present in Bt strains due to its rapidity and reproducibility (Porcar and Juarez-Perez, 2003; Beron *et al.*, 2005; Kaur, 2006).

Native Bt isolates from diverse habitats in India have been isolated and screened for the presence of different *cry* genes in our laboratory (Kaur and Singh 2000a, b; Stobdan *et al.*, 2005). A novel *cry*1Ab type gene has been cloned by PCR amplification with specifically designed primers from a native Bt isolate from cold desert soil of Ladakh region (GenBank Accession No. AY 319967) and named as *cry*1Ab18 by the *Bacillus thuringiensis* Nomenclature Committee

(http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/) (Stobdan *et al.*, 2004). A novel *cry* gene (GenBank accession number DQ023296) was cloned from a native Bt isolate (SK-729) recovered from stored cotton seeds and designated as *cry*1Ac18 by the *Bacillus thuringiensis* Nomenclature Committee (Kaur and Allam, 2006). In this study, sixty three native Bt isolates obtained from different soil and grain samples from diverse locations in India have been screened for the presence of *cry*1Aa,b,c-type genes with specially designed primers and the amplified gene has been cloned, sequenced and expressed in *E. coli* for analysis of toxicity towards *H. armigera*.

MATERIALS AND METHODS

Materials

Bacterial isolates and strains: Sixty three native Bt isolates recovered from different soil and grain samples from diverse agricultural and non-agricultural locations in India in the corresponding author Dr. S. Kaur's laboratory were used in this study (Table 1). Bt strains and the recombinant strain ECE 53 (*E. coli* clone with *cry1Ac1* gene) used as reference in this study were kindly provided by Dr. D.R. Ziegler, Director, Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA to Dr. S. Kaur (Table 2).

Table 1: Native Bt isolates recovered from diverse habitats in India used in this study

Bt isolates	Source
SK-1	Chickpea field, Rohtak, HR
SK-4	Chickpea field, Rohtak, HR
SK-9	Cotton field, Agroha, UP
SK-13	Cotton field, Malout, PB
SK-20	Cotton field, Malout, PB
SK-28	Soil near spring, HP
SK-82	Grain dust, Bhareri, HP
SK-84	Soil, Baramulla, J and K
SK-88	Chickpea and mustard field, Dungarpur, RJ
SK-110	Chickpea field, HP
SK-217	Phyllosphere, IARI, ND
SK-219	Phyllosphere, IARI, ND
SK-222	Phyllosphere, IARI, ND
SK-223	Phyllosphere, IARI, ND
SK-232	Phyllosphere, IARI, ND
SK-301	Nematode infested Field, IARI, ND
SK-304	Nematode infested Field, IARI, ND
SK-305	Nematode infested Field, IARI, ND
SK-405	Chilli field black soil, Vemboor, TN
SK-449	Chilli field black soil, Vemboor, TN
SK-463	Chilli field black soil, Vemboor, TN
SK-617	Cattle shed, Vemboor, TN
SK-629	Cattle shed, Vemboor, TN
SK-677	Kitchen garden, Alahabad
SK-678	Kitchen garden, Alahabad
SK-711	Red gram field, Lam, Guntur, AP
SK-721	Soil from cotton field, Lam, Guntur, AP
SK-722	Soil from cotton field, Lam, Guntur, AP
SK-741	Cotton seeds Var: LK-389, Lam, Guntur, AP

Table 1: Continued

Bt Isolates	Source
SK-753	Chickpea seeds, Lam, Guntur, AP
SK-754	Chickpea, Lam, Guntur, AP
SK-792	Chilly seeds Warehouse Nallapuda, Guntur, AP
SK-794	Chilly seeds Warehouse Nallapuda, Guntur, AP
SK-851	Wheat field, Burdwan, WB
SK-921	Desert soil, Sriganganagar, RJ
SK-922	Desert soil, Sriganganagar, RJ
SK-930	Desert soil, Sriganganagar, RJ
SK-935	Desert soil, Sriganganagar, RJ
SK-942	Desert soil, Sriganganagar, RJ
SK-944	Desert soil, Sriganganagar, RJ
SK-952	Cotton field, Sriganganagar, RJ
SK-953	Cotton field, Sriganganagar, RJ
SK-956	Cotton field, Sriganganagar, RJ
SK-957	Cotton field, Sriganganagar, RJ
SK-958	Cotton field, Sriganganagar, RJ
SK-959	Desert soil, Sriganganagar, RJ
SK-960	Desert soil, Sriganganagar, RJ
SK-962	Desert soil, Sriganganagar, RJ
SK-973	Cotton field, Sriganganagar, RJ
SK-977	Cotton field, Sriganganagar, RJ
SK-980	Cotton field, Sriganganagar, RJ
SK-995	Cotton field, Sriganganagar, RJ
SK-996	Grain dust, Sriganganagar, RJ
SK-1007	Insect infested wheat grain, FCI godown, RJ
SK-1008	Insect infested wheat grain, FCI godown, RJ
SK-1009	Insect infested wheat grain, FCI godown, RJ
SK-1025	Barren land, RJ
SK-1026	Barren land, RJ
SK-1027	Barren land, RJ
SK-1028	Barren land, RJ
SK-1034	Soil, Beminar, J&K
SK-1035	Soil, Beminar, J&K
SK-1036	Soil, Beminar, J&K

AP: Andhra Pradesh, RJ: Rajasthan, WB: West Bengal, ND: New Delhi, J&K: Jammu and Kashmir, HP: Himachal Pradesh, TN: Tamil Nadu, PB: Punjab, HR: Haryana

Table 2: Bt strains used as reference in this study

Bt strains	BGSC code	Original code
B. thuringiensis subsp. *taumanoffi*	4N1	HD 201(B-30-2)
B. thuringiensis subsp. *thuringiensis*	4A6	1715
B. thuringiensis subsp. *aizawai*	4J2	HD137 (HDB-24)
B. thuringiensis subsp. *israelensis*	4Q5	4Q2-72
B. thuringiensis subsp. *darmstadiensis*	4M2	HD199 (102)
B. thuringiensis subsp. *aizawai*	4J4	HD11
B. thuringiensis subsp. *kurstaki*	4D1	HD1
B. thuringiensis subsp. *sotto*	4E3	*sotto*
B. thuringiensis subsp. *kenyae*	4F1	HD136
B. thuringiensis subsp. *alesti*	4C3	HD4 (*B. alesti* 143)

Table 3: Characteristics of specific primers designed as per Beard *et al.* (2001) for amplification of full length *cry*1A-type genes

Primer pair	Sequence 5'-3'	Gene(s) recognized	Product size (kb)
Forward *cry*1Aa,b,c	TCATAATGAATTGGTATCTT-	*cry*1Aa, *cry*1Ab,	3.6
Reverse *cry*1Aa,b,c	GTAATTCCACGCTGTC	*cry*1Ac	

Growth media for bacterial strains: Luria Bertani Agar (LA) and Luria Bertani Broth (LB) were used for the growth of *E. coli* and Bt strains.

Oligonucleotide PCR primers: A set of primers designed as per Beard *et al.* (2001) was used for screening of Bt isolates for the presence of full length *cry*1Aa,b,c-type genes (Table 3).

Methods

Genomic DNA extraction: Genomic DNA was extracted from Bt isolates by the method modified from Kalman *et al.* (1993). A single colony from a freshly streaked plate was incubated into 5 mL Luria Bertani (LB) medium containing penicillin (10 μg mL^{-1}) and incubated at 30°C overnight with shaking at 150 rpm. This starter culture was added to 50 mL LB medium and incubated at 30^0C with shaking at 150 rpm to an optical density of 0.8 at 600 nm. The cells were harvested by centrifuging at 7000 rpm for 10 min (Beckman JA 20 rotor) at 4°C. The cell pellet was washed in 5 mL TES buffer (10 mM tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) and suspended in 5 mL of resuspension solution (25% sucrose, 25 mM Tris-HCl, 25 mM EDTA, pH 8.0) containing 1 mg mL^{-1} Lysozyme. The cell suspension was incubated at 37°C for 1 h. 10% SDS was added to the suspension to a final concentration of 2% and the suspension was incubated at 50^0C for 15 min and then at 4°C overnight. The suspension was centrifuged at 10,000 rpm (JA 20 rotor, Beckman) for 15 min and the supernatant was carefully taken out. DNA in the supernatant was precipitated with 2 volumes of ethanol. DNA was resuspended in 10 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 M NaCl, 10 μg mL^{-1} RNase and 0.6 mg mL^{-1} Proteinase K and incubated at 30°C for 30 min. The mixture was extracted with phenol-chloroform (1:1) and DNA was precipitated with ethanol. DNA pellet was washed once with 70% ethanol, air-dried and dissolved in 300 μL of TE buffer.

Plasmid DNA extraction: Plasmid DNA was isolated from Bt isolates and reference strains by using Qiagen Plasmid Midi kit (Qiagen, Germany). A single colony from a freshly streaked plate was inoculated into 10 mL LB medium containing penicillin (10 μg mL^{-1}) and incubated at 30°C with shaking at 150 rpm. Overnight grown cultures were pelleted by centrifugation at 7000 rpm (Beckman JA20 rotor) for 10 min at 4°C. Method as described in the supplier's manual was followed, with the modification of preheating of elution buffer to 50°C for isolation of large plasmids. DNA pellet was dissolved in 200 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Plasmid DNA was size fractionated on 0.8% agarose gel along with 1 kb DNA ladder (MBI Fermentas, Germany) used as molecular weight marker. DNA bands were observed under UV in the gel documentation system (Alpha gel imager).

PCR amplification of *cry*1Aa,b,c-type genes: PCR was carried out in a reaction mixture of 25 μL containing DNA template 50 ng; PCR buffer with $(NH_4)_2SO_4$ and $MgCl_2$ (10X) 2.5 μL; deoxy ribonucleotide triphosphate (dNTPs) (2 mM) 2.5 μL; primers (1 μM) 1 μL each, Taq DNA polymerase 1.0 U and sterile distilled water in thermal cycler (BioRad). Amplification was carried

out for 30 cycles of denaturation at 94°C for 1 min, primer annealing at 42°C for 1 min and extension at 72°C for 4 min. Denaturation in the first cycle was performed for 2 min and extension was performed for 15 min in the last cycle. PCR products were visualized on 0.8% agarose gel using 1 kb DNA ladder marker. PCR product was analyzed on 0.8% agarose gel by electrophoresis using 1 kb DNA ladder marker (MBI Fermentas).

Cloning of full-length *cry*1Aa,b,c-type gene: The 3.6 kb PCR product amplified with primers designed as per Beard *et al.* (2001) from native Bt isolate SK-711 was eluted from the gel using QIA Mini Elute gel extraction kit (Qiagen) and cloned into pQE-30 UA expression cloning vector (Qiagen, Germany) as per manufacturer's instructions. This vector is specially designed for direct cloning of PCR product as it provided with "U" overhangs at each 3' end, which hybridizes with, "A" overhang at each 3' end of the PCR product amplified using Taq DNA polymerase and is also suitable for expression in *E. coli*. The 3.6 kb gel purified PCR product of native Bt isolate SK-711 was ligated with vector and the recombinant vector was transformed into competent *Escherichia coli* strain XL-1 Blue following the manufacturer's protocol with some modifications. Recombinant clones were analyzed for the presence of insert DNA by restriction and PCR amplification of plasmid DNA of the clones. Plasmid DNA was isolated from the clones using Plasmid Mini kit (Qiagen) and digested with restriction enzymes *Bam*HI to linearize the plasmid and with *Bam*HI and *Sal* I (Fermentas) to take out the insert at 37^{0}C for 4 h. Restricted DNA was analyzed on 0.8% agarose gel by electrophoresis using 1 kb DNA ladder marker. PCR analysis of plasmid DNA of positive clones was carried out using primer pair *cry*1AabcF and *cry*1AabcR as described.

Nucleotide sequencing and analysis: Sequence determination of the clone pRKM-711 carrying 3.5 kb *cry* gene in the pQE-30 UA expression cloning vector was performed by service provider. Homology search was done using BLAST programme of NCBI (National Centre for Biotechnology Information) web site: http://www.ncbi.nlm.nih.gov and Multalin version 5.4.1 for multiple alignments.

Expression of cloned *cry*1Ac33 gene into *E. coli*: The recombinant clone pRKM-711 was grown in LB containing ampicillin (100 μg mL^{-1}) at 37°C with shaking at 150 rpm and was induced to express *cry1Ac33* gene with IPTG (isopropyl-β-D-galactopyranoside) at a final concentration of 1 mM for 4 h. The cells were harvested by centrifuging at 7000 rpm for 10 min (Beckman JA 20 rotor) at 4°C. SDS-PAGE analysis of protein purified from induced recombinant clone was carried out by a method modified from Lee *et al.* (1992) and analysed on 12% SDS-PAGE.

Solubilised toxin preparation from recombinant clone carrying *cry* gene in expression vector: The solubilised protein was prepared from the recombinant *E. coli* clone carrying *cry1Ac33* gene in pQE30 UA expression vector. The recombinant clone was induced to express *cry* gene with IPTG at a final concentration of 1 mM for 4 h. The cells were harvested by centrifugation and after sonication, the pellet was washed thrice with wash solution 1 (0.5 M NaCl and 2% triton X-100), thrice with wash solution 2 (0.5 M NaCl) and finally thrice with double distilled water. The toxin was solubilised by incubating pellet in 2 mL of solubilisation buffer (50 mM sodium carbonate containing dithiothreitol 10 mM, pH 10.5). This toxin was used for insect bioassays after protein

Table 4: Protein concentration of solubilized protein preparation of recombinant *E. coli* clones

Protein Code	Source	Protein content (μg)*
pRKM-711	*E. coli* clone carrying *cry1Ac33* gene	7.0
ECE-53	*E. coli* clone carrying *cry1Ac1* gene	11.3

*Protein content is expressed as μg of total protein per μL of soluble protein solution

quantification by Invitrogen™ Qubit flourometer (Table 4). The solubilised protein preparation of the recombinant strain ECE 53 (*E. coli* clone with *cry1Ac1* gene) was also prepared for use as the control in insect bioassays.

***H. armigera* rearing for evaluation of toxicity of Cry protein:** Susceptibility of *H. armigera* collected from cotton and chickpea field from IARI location was tested by exposing neonates of this insect to different concentration of toxin mixed with artificial diet. The larvae were allowed to grow until pupation on the artificial diet. The artificial diet prepared was essentially gram based diet. Pupae were collected and kept in cage for adult emergence. The adults were paired and then kept in a cage and were given 10% honey solution for egg laying which was done on Markin cloth. The eggs, thus collected, were separated day wise and allowed to hatch. The neonates were then transferred to the artificial diet. The insects were reared at 27°C and 60-70% RH. The pupae were separated and then kept in a jar at 27°C until adult emergence. The adults were then allowed to mate and lay eggs.

Insect bioassay: Bioassays were carried out on *H. armigera* by diet incorporation method using 3 replicates having 10 insects taken with appropriate control. An aliquot of toxin solution was mixed with 10 g diet at different concentrations. The estimates of toxins were based up on their protein content. The treated diet was then divided into three replicates and fed to neonates separately. The insects were allowed to feed freely. The mortality of larvae was observed each day until 4 and 7 days. The mortality data were used for estimation of LC_{50} in terms of μg g^{-1} diet as per maximum likelihood programme. The solubilised protein preparation of recombinant strain ECE 53 (*E. coli* clone with *cry1Ac1* gene) was used as control in bioassays.

RESULTS

Screening of Bt isolates for the presence of full length *cry*1Aa,b,c-type genes: Sixty three native Bt isolates and 10 Bt reference strains were subjected to PCR amplification using specially designed primer set as per Beard *et al.* (2001) for the amplification of the full length *cry*1Aa,b,c-type genes. The prominent band of expected size of 3.6 kb was observed in native Bt isolate SK-711. Three Bt reference strains namely, *B. thuringiensis* subsp. *taumanoffi* (4N1), *B. thuringiensis* subsp. *aizawai* (4J2 and 4J4) were also found to be positive. These Bt strains are known to have full length *cry*1A-type genes. *B. thuringiensis* subsp. *israelensis* (4Q5) was used as a negative control as it doesn't have full length *cry*1A-type genes. In addition, to band of expected size, some bands of small size were also observed in some isolates (Fig. 1a-c). Most of the remaining Bt isolates and Bt strains showed the variant bands of smaller size than expected and some didn't showed any band.

Cloning of PCR amplified full length *cry*1A-type gene from native Bt isolate: PCR product of native Bt isolate SK-711 amplified using the set of primers designed as per Beard *et al.* (2002)

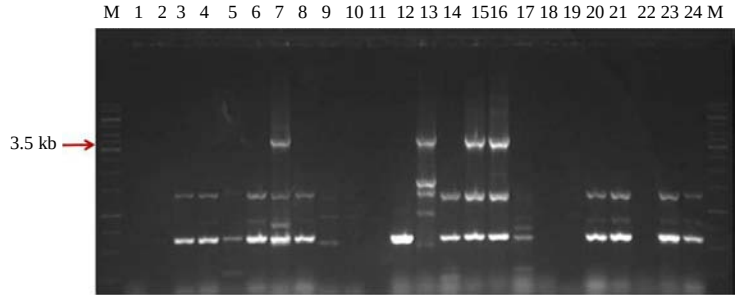

Fig. 1a: PCR amplification of native Bt isolates and Bt strains used as reference with primers designed as per Beard *et al*. (2001). M: 1kb DNA Ladder; Lane 1: SK-930, Lane 2: SK-629, Lane 3: SK-722, Lane 4: SK-922, Lane 5: SK-851, Lane 6: SK-958, Lane 7: SK-711, Lane 8: SK-20, Lane 9: SK-305, Lane 10: SK-222, Lane 11: SK-1, Lane 12: SK-13, Lane 13: 4N1, Lane 14: 4A6, Lane 15: 4J2, Lane 16: 4J4, M: 1kb DNA Ladder, Lane 17: 4Q5, Lane 18: 4C3,Lane 19: 4M2, Lane 20: SK-977, Lane 21: SK-980, Lane 22: SK-995, Lane 23: SK-935 and Lane 24: SK-959

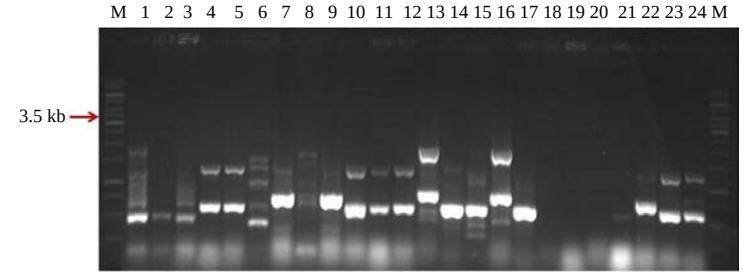

Fig. 1b: PCR amplification of native Bt isolates with primers designed as per Beard *et al*. (2001). M: 1kb DNA Ladder, Lane 1: SK-88, Lane 2: SK-4, Lane 3: SK-28, Lane 4: SK-232, Lane 5: SK-463, Lane 6: SK-957, Lane 7: SK-678, Lane 8: SK-754, Lane 9: SK-677, Lane 10: SK-921, Lane 11: SK-960, Lane 12: SK-942, Lane 13: SK-944, Lane 14: SK-304, Lane 15: SK-996, Lane 16: SK-9 M: 1kb DNA Ladder, Lane 17: SK-219, Lane 18: SK-617, Lane 19: SK-741, Lane 20: SK-953, Lane 21: SK-753, Lane 22: SK-449, Lane 23: SK-82 and Lane 24: SK-110

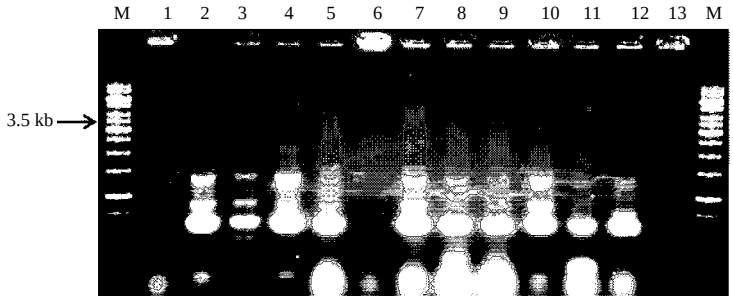

Fig. 1c: PCR amplification of native Bt isolates using primers designed as per Beard *et al*. (2001). M: 1kb DNA Ladder, Lane 1: SK-792, Lane 2: SK-952, Lane 3: SK-962, Lane 4: SK-1007, Lane 5: SK-1008, Lane 6: SK-1009, Lane 7: SK-1025, Lane 8: SK-1026, Lane 9: SK-1027, Lane 10: SK-1028, Lane 11: SK-1034, Lane 12: SK-1036 and M: 1kb DNA Ladder

was purified from the agarose gel matrix using Qiagen Mini Elute gel extraction kit (Fig. 2). It was cloned into pQE-30 UA expression cloning vector, which is 3.5 kb in size. Seven colonies were observed on LA plates containing ampicillin (100 μg mL^{-1}). Molecular confirmation of recombinant clones was carried out by restriction analysis and PCR amplification. Plasmid DNA of each colony was isolated and examined for their size by agarose gel electrophoresis (Fig. 3). One recombinant clone (711-D) showed a band of about 8.0 kb while the rest showed a band of about 2.5 kb. Recombinant plasmid DNA of clone 711-D was digested with *Bam*H1 restriction enzyme and as expected, a single band of size 7.1 kb corresponding to linearized vector was observed. The recombinant clone was further confirmed by

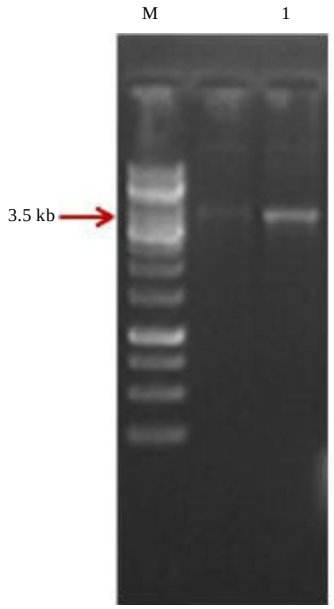

Fig. 2: Gel purification of PCR product of native Bt isolate SK-711. M: 1 kb DNA Ladder, Lane 1: Purified PCR product

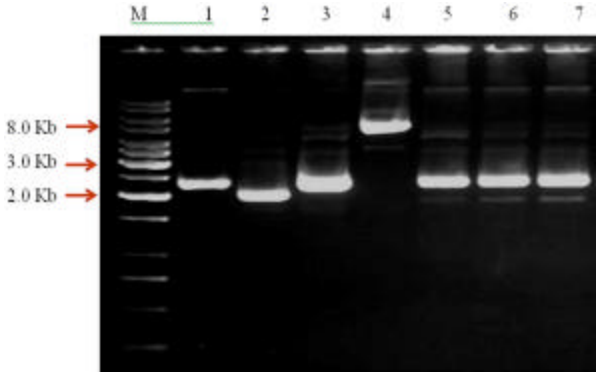

Fig. 3: Gel electrophoresis of uncut recombinant plasmid DNA. M: 1 kb DNA Ladder, Lane 1: 711-A, Lane 2: 711-B, Lane 3: 711-C, Lane 4: 711-D, Lane 5: 711-E, Lane 6: 711-F, Lane 7: 711-G

double digestion using *Bam*H1 and *Sal*1 restriction enzymes for excising the insert of size 3.6 kb. Two bands of size 3.5 and 3.6 kb corresponding to vector and insert, respectively were observed (Fig. 4). PCR amplification of recombinant plasmid DNA of clone 711-D and of plasmid DNA of native Bt isolate SK-711 was carried out, using a set of primers designed as per Beard *et al*. (2001) to confirm the presence of desired insert in recombinant vector DNA. All the reaction conditions were kept same as in the previous PCR reaction through which Bt isolates were screened by this set of primers. The prominent band of 3.6 kb as expected was observed with both the native Bt isolate SK-711 as well as the recombinant clone 711-D (Fig. 5). The recombinant clone was named as pRKM-711.

Sequence determination and analysis: The sequence of the *cry* gene cloned in the recombinant clone pRKM 711 was determined by primer walking. Homology search and analysis was done by using BLAST programme of NCBI (National Centre for Biotechnology Information) web site: http://www.ncbi.nlm.nih.gov.). The sequence of the cloned gene was deposited in the NCBI GenBank (accession No. GQ866913). The sequence was analyzed by comparison with previously known *cry*1A-type genes and was assigned the name *cry*1Ac33 by the *Bacillus thuringiensis* Nomenclature Committee (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/. The sequence was further analyzed for comparison with all the previously reported *cry*1Ac-type genes downloaded from the site http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/. using Multalin (version 5.4.1) web-based tool for multiple alignments. The gene sequence showed high level of homology with previously reported *cry*1Ac genes.

Expression of recombinant clone pRKM-711 in *E. coli*: The gene was expressed in *E. coli* under IPTG induction. The expected band of 130 kDa was observed in SDS-PAGE with Coomassie blue staining (Fig. 6).

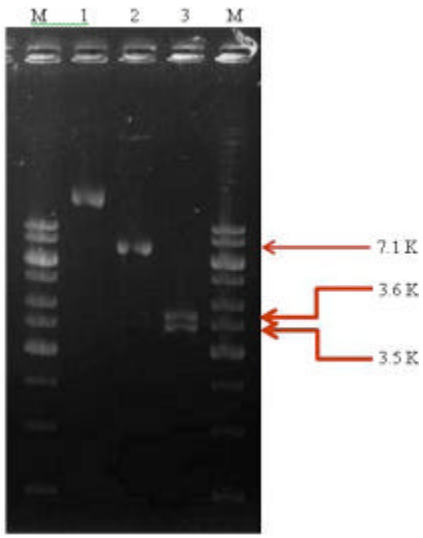

Fig. 4: Restriction analysis of recombinant clone pRKM-711. M: 1 kb DNA Ladder, Lane 1: Uncut plasmid, Lane 2: Single digestion with *Bam*H1, Lane 3: Double digestion with *Bam*H1+*Sal*1, M: 1 kb DNA Ladder

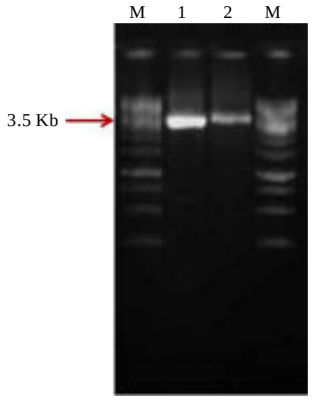

Fig. 5: Confirmation of recombinant clone pRKM-711 by PCR amplification by PCR amplification with primers designed as per Beard *et al.* (2001). M: 1 kb DNA Ladder, Lane 1: PCR product with pRKM-711 as template, Lane 2: PCR product with native Bt isolate SK 711 as template, M: 1 kb DNA Ladder

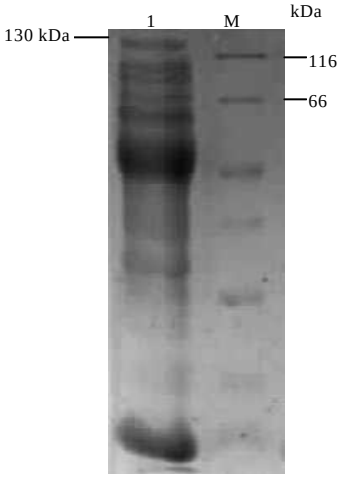

Fig. 6: SDS-PAGE analysis of recombinant clone pRKM-711 with a *cry*1Ac-type gene in pQE-30 UA vector expressed in *E. coli*. Lane 1: Recombinant clone pRKM-711; M: Protein Marker.

Evaluation for toxicity towards *H. armigera*: The solubilised protein preparation was evaluated for toxicity towards H. *armigera* (Table 4). It was observed that the *cry*1Ac33 gene was more toxic towards *H. armigera* than the holotype *cry*1Ac1 used as a control, based on LC_{50} toxicity analysis (Table 5, 6).

DISCUSSION

Several molecular techniques have been employed for detection of the *cry* genes out of which the identification of Bt *cry* genes by Polymerase Chain Reaction (PCR) has proven to be a very useful and rapid method for strain characterization and selection (Porcar and Juarez-Perez, 2003;

Table 5: Evaluation of toxicity of solubilized liquid Cry1Ac33 protein (code: NRCPB RKM) against *H. armigera**

		Percent corrected Mortality		
Days of treatment	Dose (ppm)	pRKM-711	ECE-53 (*cry*1Ac1)	Water
4 days	1	37.93	27.58	0
	5	48.27	37.93	-
7 days	1	49.99	57.14	0
	5	78.57	71.42	-

* 30 insects were used in three replicates for each assay

Table 6: Toxicity of *Cry* toxins to the neonates of *H. armigera*

Toxins	LC_{50} (96 hrs) µg g^{-1} diet	Fiducial limits 95%	Slope ±SE	χ^2	df
RKM-711	10.3891	5.1476-68.924	0.76±0.273	0.257	2
ECE 53 C	20.3480	8.723-380.320	0.68±0.280	0.432	2

Kaur, 2006). PCR and its variants have been widely used for detection of *cry* genes in collection of native Bt isolates since last decade, worldwide (Bravo *et al*., 1998; Beard *et al*., 2001; Choi *et al*., 2007; Thaphan *et al*., 2008; Thammasittirong and Attathom, 2008; Zhu *et al*., 2009). The genes of *cry*1 family are specifically toxic to insects of order Lepidoptera which include some major devastating pests of economically important crops and are responsible for most of the crop losses.

PCR screening of native Bt isolates for the presence of full length *cry*1Aa,b,c-type genes: Sixty three native Bt isolates and 10 Bt strains used as reference were screened using specially designed primers for the amplification of full length *cry*1Aa,b,c-type gene(s). Cry1A toxins are very important because of their high toxicity to lepidopteran pests and widespread distribution among Bt strains (Uribe *et al*., 2003; Wang *et al*., 2003; Stobdan *et al*., 2004; Beron *et al*., 2005; Kaur, 2006).

The *cry*1Aa, *cry*1Ab and *cry*1Ac genes are the most frequently found in Bt strains and native isolates (Ben-Dov *et al*., 1997; Bravo *et al*., 1998; Uribe *et al*., 2003; Stobdan *et al*., 2004; Armengol *et al*., 2007; Ramalakshmi and Udayasuriyan, 2010; Shankar *et al*., 2010). In order to obtain whole open reading frame of *cry*1A-type gene(s) for practical utility, amplification and cloning of full length *cry*1A-type genes was attempted by screening of 63 native Bt isolates with 10 reference Bt strains using specially designed primer sets as per Beard *et al*. (2001) in PCR amplification. These primer sets are specifically designed for the amplification of full length *cry*1A-type genes through the analysis of conserved regions by multiple alignments of all nucleotide sequences of *cry*1A-type genes available in databases. The prominent band of expected size 3.6 kb was observed in the native Bt isolate SK-711 recovered from Red gram field, Lam, Guntur Andhra Pradesh. In addition, to this native Bt isolate, 3 Bt strains namely, *B. thuringiensis* subsp. *taumanoffi* (4N1), *B. thuringiensis* subsp. *aizawai* (4J2 and 4J4) used as reference, were also found to be positive with the band of expected size along with some variant bands of small size. These three Bt strains are known to harbour *cry*1A-type genes on their plasmids (http://bacillus.biosci.ohio-state.edu/). The appearance of unexpected bands may imply that the primers may have recognized related *cry* genes sequences. Some of these bands may also be due to favoured amplification of shorter region of homology. It is also possible that some of the Bt isolates may harbour the *cry*1A-type genes in the truncated form such that primer recognition region is not

present. Such a situation may seem conceivable due to frequent recombination events in *cry* genes helped by flanking transposons (Lereclus and Arantes, 1992).

Cloning of full length gene amplified by primer set designed as per Beard *et al.* (2001): The fragment of 3.6 kb amplified from native Bt isolate SK-711 by using primer set designed as per Beard *et al.* (2001) was cloned into pQE-30 UA expression cloning vector and transformed into competent Escherichia coli strain XL-1 Blue cells. All the colonies grown on LA plates containing ampicillin were selected as the vector lacks any other selection marker apart from the ampicillin resistance for the selection of colonies having recombinant vector. One recombinant clone (pRKM-711-D) was selected out of seven on the basis of plasmid DNA profile and was further confirmed by restriction analysis as well as by PCR amplification of recombinant plasmid DNA using the same set of primer designed as per Beard *et al.* (2001) as used for amplification of plasmid DNA of Bt isolate SK-711.

Sequence determination and analysis of cloned full length gene: Sequence similarity of the cloned *cry*1Ac33 gene with previously reported *cry*1Ac genes was observed. The nucleotide sequence of the cloned gene was almost 99% identical to all other *cry*1Ac genes except some differences of gap region and substitution of a particular nucleotide at specific positions. The nucleotide sequence corresponding to C-terminal of the Cry protein showed more than 50% homology with all the previously reported *cry*1Ac-type genes. The C-terminus portion of the protoxin is cleaved during proteolysis in the midgut of insects by proteases. The *cry*1A-type genes typically range from 3.1 to 3.6 kb. Variation in toxicity and specificity due to minor amino acid substitutions exist among different Cry1A toxins (Tounsi *et al.*, 1999). Sequences of 62 *cry*1A genes, classified into *cry*1Aa to *cry*1Ai types, have been published (Crickmore *et al.*, 1998).

The sequence of the cloned *cry*1Ac33 gene was also found to be almost 99% identical to the previously isolated *cry*1Ac18 gene (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/, GenBank accession no. DQ023296) from the native Bt isolate SK-729 recovered from cotton seed, var.: LK-861, Lam, Guntur Andhra Pradesh in our laboratory (Kaur and Allam, 2006). The sequence was also 95% identical to the previously isolated *cry*1Ab18 gene (http://www.lifesci.sussex.ac.uk/ home/Neil_Crickmore/Bt/, GenBank accession No. AY319967) from the Bt isolate recovered from Ladakh and from native Bt isolate SK-222 (GenBank accession No. DQ023297) recovered from Pea phyllosphere in our laboratory (Stobdan *et al.*, 2004). These genes had been cloned from native Bt isolates using two sets of specially designed ORF primers corresponding to full-length *cry*1Aa,b,c-type genes (Stobdan *et al.*, 2004). In this study, the set of primers designed as per Beard *et al.* (2001) was employed.

Expression in *E. coli* and evaluation for toxicity towards *H. armigera*: The development of resistance in target insects towards Cry toxins could diminish the long-term efficacy of Bt crops (Tabashnik, 2008). To delay the onset of resistance in insects towards the known genes, one or more different types of *cry* genes can be used in gene pyramiding strategy. Therefore, the search for the *cry* genes by screening of native Bt isolates is of much interest for deployment of these genes in transgenic crops.

The *cry*1Aa, *cry*1Ab and *cry*1Ac genes have been reported to be toxic to lepidopteran pests, particularly, *H. armigera*, a severe and polyphagous pest of several economically important crops

such as cotton, pigeon pea, chickpea and tomato (Zafar *et al*., 2000; Khalique, 2003; Gupta *et al*., 2007). Of all the *cry* genes, the *cry*1Ac genes are most toxic to *H. armigera* (Padidam, 1992; Kumar *et al*., 2004).

The *cry*1Ac33 gene was expressed in *E. coli* and evaluated for toxicity towards *H. armigera* and has been found to be more toxic towards *H. armigera* than the holotype *cry*1Ac1 used as a control, based on LC_{50} toxicity analysis. The differences in nucleotide and derived amino acid sequence of *cry*1Ac33 gene from that of *cry*1Ac1 gene may be involved in observed higher toxicity of this gene. To tackle the imminent problem of development of resistance in target insect pests towards *cry* genes currently deployed in transgenic plants, novel insecticidal genes are required (Kaur, 2007). This gene can, thus, be useful for deployment for insect control.

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