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Cloning of partial *cry1Ac* gene from an indigenous isolate of *Bacillus thuringiensis*

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The discoveries of novel *cry* genes of *Bacillus thuringiensis* (Bt) with higher toxicity are important for the development of new products. The *cry1* family genes are more toxic to the lepidopteran insects according to the previous reports. In the present study, nine indigenous isolates of Bt were used for screening of *cry1* genes by PCR using degenerate primers specific to *cry1* family genes. Two of the nine new isolates of Bt showed amplification with *cry1* family primers. Partial *cry1*gene(s) was cloned from one of the *cry1* positive isolate of Bt, T133. Nucleotide sequence data generated for partial *cry1* gene of T133 showed 98% homology with 1420 bp of the partial *cry1Ac1* gene. Deduced amino acid sequence of the partial *cry1Ac* of Bt strain, T133 showed two per cent variation in comparison to Cry1Ac1 by differing at 5 positions; one deletion at 442th position and five substitutions at the following positions, 296, 367, 440 and 563.

Key words: *Bacillus thuringiensis, cry1* genes, degenerate primer, cloning.

INTRODUCTION

The control of agriculture pest populations is achieved mainly by the application of chemical insecticides; the continuous use of synthetic pesticides led to serious problems like environmental degradation and development of resistance in insect pest (Shelton et al., 2002). Recently, there has been a renewed interest in the development of biological alternatives to chemical pesticides. The *Bacillus thuringiensis* (Bt) Berliner is considered as one of the most versatile microbial insecticides. It is a gram-positive spore-forming soil bacterium. The insecticidal activity is based on the ability of the bacterium to produce large quantity of larvicidal proteins known as delta-endotoxins (Cry proteins). The Cry toxins constitute a family of related proteins that can kill insects belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera and Mallophaga, as well as other invertebrates (Schnepf et al., 1998; Feitelson et al., 1999).

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Abbreviations: Bt, *Bacillus thuringiensis*; **PCR,** polymerase chain reaction; **IPM,** integrated pest management; **IPTG,** isopropyl thiogalaactoside; **LB,** Luria-Bertani; **cry,** crystal protein.

Table 1. Primers used for screening and amplification of *cry1* gene fragments.

Primer name	Sequence (5' to 3')	Amplicon size (bp)
JF	MDATYTCTAKRTCTTGACTA	~1500 to 1600
JR	TRACRHTDDBDGTATTAGAT	

B = C, G or T; D = A, G or T; H = A, C or T; K = G or T; M = A or C; R = A or G and Y = T or C. Source: (Juarez-Perez et al., 1997).

The advancement in molecular biology led to the cloning of Bt crystal protein (*cry*) gene for the first time in 1981 (Schnepf and Whiteley, 1981). So far more than 445 *cry* genes have been successfully cloned and characterized for their insecticidal potential.

Cry1A toxins are very important because of their high toxicity to lepidopteran pests and widespread distribution among Bt strains (Li et al., 1995; Bravo et al., 1998; Uribe et al., 2003). Variation in toxicity and specificity exist among different Cry1A toxins due to minor amino acid substitutions (Tounsi et al., 1999). About 69 *cry1A* genes are classified into *cry1Aa* to *cry1Ai* sub-types (http:// www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). It is still essential to search for novel Bt strains that may lead to the discovery of additional insecticidal proteins with higher toxicity against wider pest range. The PCR method is proven to be a powerful tool which allows quick, simultaneous screening of many Bt samples, identification of specific insecticidal genes carried by different Bt strains, classification of *cry* genes and subsequent prediction of their insecticidal activities (Carozzi et al., 1991; Ben-Dov et al., 1997; Juarez et al., 1997). The amplification, subsequent cloning and sequence analysis of the *cry* genes from new isolates of Bt are important because they may provide new gene sequences encoding more active toxins which could be used for developing better versions of transgenic crop plants. So, the present study was undertaken for cloning and sequencing of *cry* gene from indigenous isolates of Bt.

MATERIALS AND METHODS

Bacterial strains and plasmids

Nine indigenous Bt strains (T17, T21, T50, T75, T81, T133, T134, T142 and T147) and a reference strain, Bt (indigenous strain, 14r1) are from the collection of Bt strains maintained by the corresponding author in the Department of Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India. *Escherichia coli* vector pTZ57R\T obtained from Fermentas INC.

Amplification of Bt DNA by PCR

Total genomic DNA from *B. thuringiensis* strains, T17, T21, T50, T75, T81, T133, T134, T142 and T147 were extracted as described earlier by Kalman et al. (1993) and used as the template for the PCR screening. The genomic DNA of a reference strain of Bt, 14r1 was used as positive control. A set of degenerate primers (JF and JR) described by Juarez-Perez et al. (1997) were used to screen the new isolates of Bt for the presence of *cry1* family genes (Table 1). These primers are expected to amplify fragments of ~ 1.5 kb from the *cry1* family genes.

The PCR was accomplished using an Eppendorf thermal cycler in 25 µl reaction volume containing total genomic DNA of Bt (30 ng), 2.5 µl of 10X PCR buffer (10 mM Tris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 0.5 Units of *Taq* DNA polymerase. The PCR was performed for 30 cycles as follows: 94°C for 1 min, 40°C for 45 s, 72°C for 2 min and the final extension was performed for 7 min at 72°C.

Cloning of partial *cry1* fragments from Bt isolate, T133

The gel eluted PCR products of partial *cry1* gene (~1.5 kb) from Bt isolate, T133 was ligated into T/A vector (pTZ57R\T Fermentas INC) as per the manufacturer's instruction. The ligated mixture was transformed into *E. coli* as per the standard procedure (Sambrook et al., 1989). The transformed colonies of *E. coli* were screened by colony PCR with M13F and M13R primers for checking the presence of insert (partial *cry1* fragment of Bt isolate, T133).

Nucleotide sequencing of recombinant plasmids

The plasmid DNA was isolated from the *E. coli* transformants containing partial *cry1* gene of Bt isolate, T133 and nucleotide sequence of recombinant plasmids was carried out by automated sequencing (Ist Base, Singapore). The sequence data was subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/Blast). The deduced amino acid sequence was generated by BioEdit (Hall, 1999).

RESULTS

Screening of new isolates of Bt for *cry1* genes

Total genomic DNA isolated from indigenous isolates of Bt was used as template for screening of *cry1* genes by PCR using the degenerate primers specific to *cry1* family genes. Amplification of expected size (~ 1.5 kb) of PCR products were obtained from two new Bt isolates T17 and T133, whereas amplification was not observed in the remaining seven isolates (Figure 1).

Cloning and sequence analysis of partial *cry1* gene from Bt isolate, T133

The gel eluted PCR product (~1.5 kb) of partial *cry1* gene

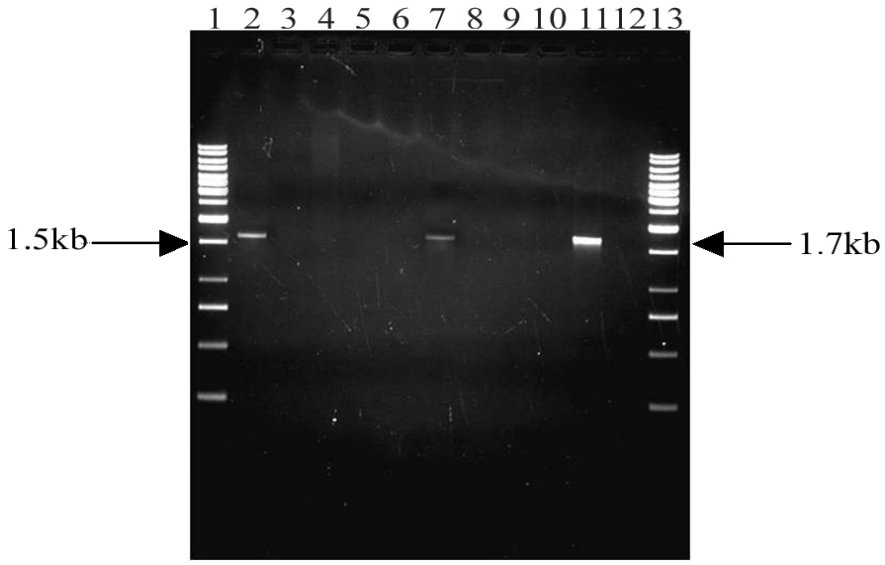

Figure 1. Screening of Bt strains for the presence of *cry1* gene by PCR. **Lanes 1 and 13:** 1 kb marker; **lanes 2 and 7:** new isolates of Bt positive for *cry1* gene (T17 and T133); **lanes 3, 4, 5, 6, 8, 9 and 10:** new isolates of Bt negative for *cry1* gene (T21, T75, T81, T134, T142, T147 and T50); **lane 11:** positive control (14r1 genomic DNA) and **lane 12:** negative control.

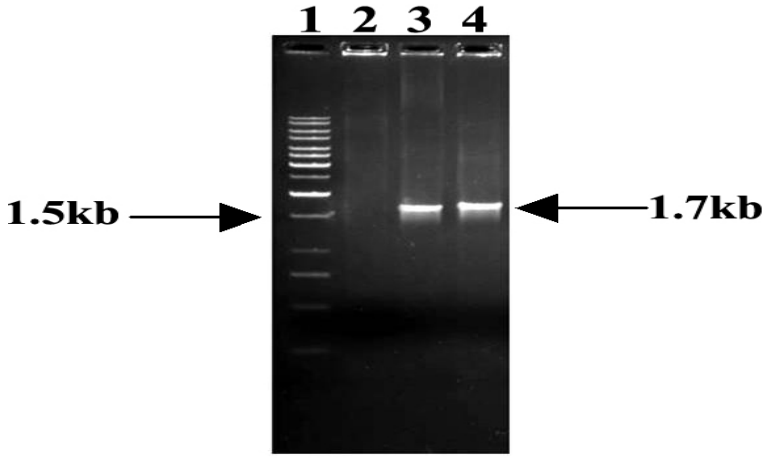

Figure 2. Screening of recombinant *E. coli* colonies by PCR for presence of partial *cry1* gene. **Lane 1:** 1 kb marker; **lane 2:** negative control; **lane 3:** partial *cry1* gene fragment from 14r1 (positive control) and **lane 4:** partial *cry1* gene from new isolates of Bt, T133.

fragment from Bt isolate, T133 was cloned into pTZ57R/T vector (T/A vector). The recombinant clones (white colonies) were selected on LB agar containing X-gal, IPTG and ampicillin. Presence of insert was confirmed in recombinant *E. coli* colonies, by colony PCR with M13 forward and M13 reverse primers. Agarose gel electrophoresis of DNA amplified from the transformants of *E. coli* showed expected size of ~1.7 kb corresponding to the sum of insert DNA of 1.5 kb and vector sequence of about 200 bp (Figure 2). Recombinant plasmid isolated from three of the *E. coli* clones were used to determine nucleotide sequence of the partial *cry1* gene of Bt strain,

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Query  1    RSPHSMDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIV  60
            RSPH MDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIV
Sbjct  292  RSPHLMDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIV  351

Query  61   AQLGQGVYRTLSSTFYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLD  120
            AQLGQGVYRTLSST YRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLD
Sbjct  352  AQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLD  411

Query  121  EIPPQNNNVPPRQGFSHRLSHVSMFRSG-SSSSVSIIRAPMFSWIHRSAEFNNIIASDSI  179
            EIPPQNNNVPPRQGFSHRLSHVSMFRSG S+SSVSIIRAPMFSWIHRSAEFNNIIASDSI
Sbjct  412  EIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSI  471

Query  180  TQIPAVKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRARV  239
            TQIPAVKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYR RV
Sbjct  472  TQIPAVKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRV  531

Query  240  RYASVTPIHLNVNWGNSSIFSNTVPATATSLNNLQSSDFGYFESANAFTSSLGNIVGVRN  299
            RYASVTPIHLNVNWGNSSIFSNTVPATATSL+NLQSSDFGYFESANAFTSSLGNIVGVRN
Sbjct  532  RYASVTPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGNIVGVRN  591

Query  300  FSGTAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSN  359
            FSGTAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSN
Sbjct  592  FSGTAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSN  651

Query  360  LVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQG  419
            LVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQG
Sbjct  652  LVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQG  711

Query  420  GDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEI  472
            GDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEI
Sbjct  712  GDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEI  764
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Figure 3. Homology between the deduced amino acid sequence of partial *cry1Ac* gene of T133 and holotype, Cry1Ac1. **Query:** *cry1Ac* amino acid sequence of Bt strain, T133; **Subject:** *cry1Ac1* amino acid sequence. Boldface letters indicate the six amino acid differences between Cry1Ac of Bt strain, T133 and Cry1Ac1 (holotype).

T133 by automated DNA sequencing. Nucleotide sequence data (Acc. No. FJ794964) revealed 98% homology with 1420 bp bases in the 5' region of holotype, *cry1Ac1*. Deduced amino acid sequence of partial Cry1Ac gene showed one deletion at 442th position and five substitutions at the following positions, 296,367, 440 and 563 of Cry1Ac1 holotype sequence (Figure 3).

DISCUSSION

Indiscriminate use of broad-spectrum chemical insecticides has caused adverse effects to human health, other non-target organisms and has led to the build-up of chemical resistance in insect pests (Waage, 1997). Therefore, the urgent need for environmentally safe pest control is required to maintain sustainability of the environment. Deployment of integrated pest management (IPM) strategies to minimize crop production losses incurred due to insect pests can make significant contribution to food security in the developing countries.

The diversity of Bt strains facilitates isolation of new types of *cry* genes. PCR is a useful technique for quick and simultaneous screening of Bt strains for classification and prediction of insecticidal activities. Several screening projects of Bt collections from different parts of the world have been described (Juarez-Perez et al., 1997; Bravo et al., 1998; Ben-Dov et al., 1997; Thammasittirong and Attathom, 2008). In the present study, nine indigenous isolates of Bt were screened for the presence of *cry1* genes through PCR with the *cry1* family primers described by Juarez-Perez et al. (1997). Among the nine new isolates, only two showed amplification of expected size as in the case of the reference strain of Bt, 14R1 which is known to harbor for *cry1* genes.

Most of the commercial Bt formulations used for the

control of lepidopteran pests, contain toxins of Cry1A family, especially Cry1Aa, Cry1Ab and Cry1Ac proteins (Hofte and Whiteley, 1989). Cloning of the first *cry* gene, namely *cry1A(a),* was reported by Schnepf and Whiteley (1981). Recently, Xue et al. (2008) reported cloning of novel *cry1Ah* gene and its protein was more toxic to lepidopteran Asian corn borer. Swiecicka et al. (2008) reported that the novel Cry1Ab21 that produces a quasicuboidal crystal protein which is toxic to larvae of *Trichoplusia ni.*

In the present study, the partial *cry1* gene fragments amplified from genomic DNA of Bt strain, T133 are cloned in T/A vector. Nucleotide sequence of partial *cry1Ac* gene showed 98% similarity to the already reported *cry1Ac1* holotype sequence. The deduced amino acid sequences of partial Cry1Ac of Bt isolate, T133 showed difference at six positions in comparison to that of Cry1Ac1.

Result for the present study revealed the presence of new variant of *cry1Ac* in Bt isolate, T133. Variation of a single amino acid can significantly influence the level of toxicity in Cry proteins (Udayasuriyan et al., 1994; Liao et al., 2002). Therefore, further studies on expression of complete ORF of the novel *cry1Ac* cloned from the new isolate of Bt, T133 will be useful to know the insecticidal potency of its proteins.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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