JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2010. Vol. 4(2), p. 545-549

Screening and Cloning of Partial *cry1Ac* Gene Fragment from an Indigenous Isolate of *Bacillus thuringiensis*

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(Received: 02 April 2010; accepted: 14 May 2010)

PCR is a useful technique for quick and simultaneous screening of *Bacillus thuringiensis* strains for classification and prediction of insecticidal activities. This experiment has been contacted by screening of *cry1Ac* gene from new isolates Bt. Two indigenous isolates of Bt were used as a template for screening of *cry1Ac* genes by mixtures of degenerate primer and specific primer of c*ry1Ac* genes. Two new isolates of Bt, T17, 14r1 showed amplification with its *cry1Ac* gene. But among these two strains, 14r1 strain was showed variation during their amplification (~925bp). The partial *cry1Ac* gene fragment amplified from Bt isolates, 14r1 and cloned in *E. coli* vector. The recombinants clones of *cry1Ac* gene (~925bp) were also showed variation in their amplification.

Key words: *Bacillus thuringiensis, cry1Ac* gene, degenerate primers, cloning.

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 and Feitelson *et al.,* 1999).

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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 and 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. Novel toxins are also important for providing alternatives for coping with the emergence of resistant insect populations (Nester *et al*., 2002). 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 and 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 (*cry1Ac*) from indigenous isolates of Bt.

MATERIAL AND METHOD

Bacterial strains and plasmids

The *B. thuringiensis* strains were used in this study were obtained from the Bt Biotechnology laboratory, Centre for Plant Molecular Biology. Tamil Nadu Agricultural University, Coimbatore, India. *E. coli* vector pTZ57R\T obtained from Fermentas INC.

Screening of *cry1* gene from indigenous isolates of Bt

Total genomic DNA from *B. thuringiensis* strains were extracted as described earlier by Kalman *et al.* (1995) and used as the template for the PCR screening. A set of degenerate family primers (JF and JR) and type primer (J1Ac) described by Juarez *et al*., (1997) were used to screen the new isolates of Bt, 14r1 and T17 for the presence of *cry1A* family genes and *cry1Ac* gene (Table 1). These primers are expected to amplify a family fragment of ~ 1.5 kb and type fragment about ~ 925bp from the *cry1A* family genes.

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

Primers name	Sequence (5' 3')	Amplicon size (bp)
JF	MDATYTCTAKRTCTTGACTA	~1500 to 1600
JR	TRACRHTDDBDGTATTAGAT	
1Ac	GGAAACTTTCTTTTTAATGG	~825

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 PCR was accomplished using an Eppendorf thermal cycler in 25 µl reaction volume containing total genomic DNA of Bt (30ng), 2.5µl of 10X PCR buffer (10 mMTris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) 75iM each of dNTPs, 50ng 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 1min, 43°C for 45s, 72 °C for 2min and the final extension was performed for 7min at 72 °C.

Cloning of partial *cry1* fragments from Bt isolate, 14r1

The amplified and gel eluted PCR products of partial subfamily *cry1Ac* gene (~925 kb) from Bt isolate**,** 14r1 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 the presence of insert (partial *cry1Ac* fragment of Bt isolate, 14r1).

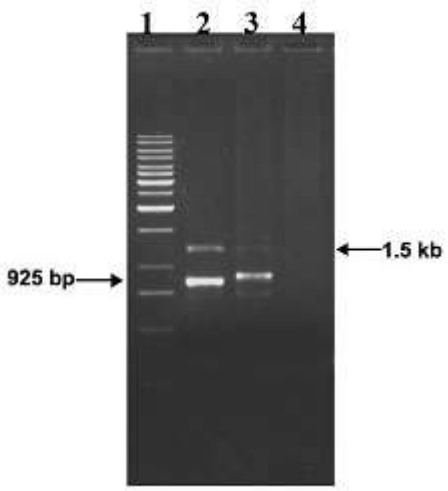
RESULTS

Screening of new isolates of Bt for *cry1Ac* gene

Total genomic DNA isolated from two indigenous isolates of Bt, T17 and 14r1 were used as template for screening of *cry1Ac* genes by using combination of family primers and sub family primers (*cry1Ac*). Two amplicons were obtained in both isolates one is family gene (*cry1*) and another one is subfamily gene (*cry1Ac*). Among these strains the amplified *cry1Ac* gene of Bt, 14r1 showed variation in their amplification (~ 925 kb) of PCR products (Fig 1).

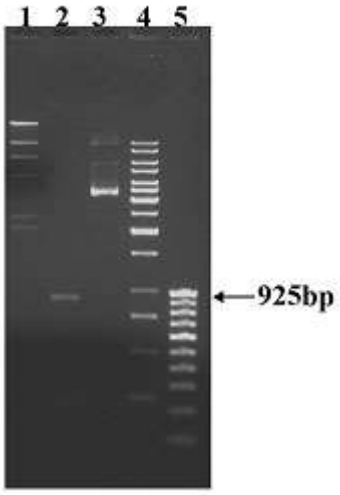
Amplification and cloning of partial *cry1Ac* fragments from new isolates of Bt 14r1

The partial *cry1Ac* gene fragment of ~925kb was amplified by PCR, using a set of degenerate family primers and specific primer (JF,



Lane 1: 1kb marker
Lane 2: Bt isolate, T17
Lane 3: Bt isolate, 14r1
Lane 4: Negative control

Fig. 1. Screening of Bt strains for the presence of *cry1Ac* gene by PCR

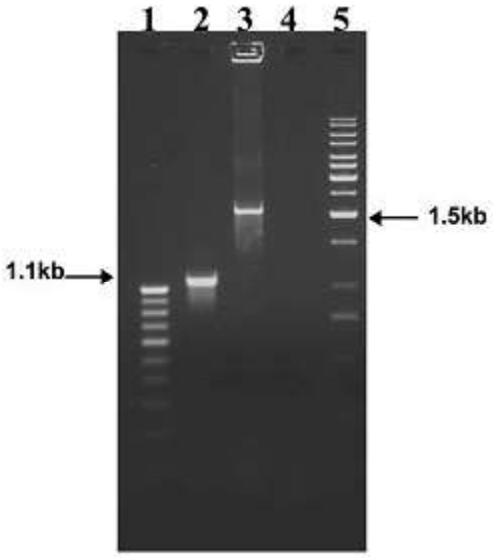


Lane 1: λ/*Hind*III
Lane 2: Bt isolate, 14r1
Lane 3: Positive control (HD73)
Lane 4: 1kb marker
Lane 5: 100bp marker

Fig. 2. Amplification of *cry1Ac* gene from new isolates of Bt, 14r1

Lane1: λ/*Hind*III
Lane2: Plasmid from white colony
Lane3: λ/*Hind*III

Fig. 3. Agarose gel electrophoresis of plasmid DNA isolated form transformants of *E. coli*



Lane1: 100bp marker
Lane2: Recombinant plasmid of *cry1Ac* from Bt,14r1
Lane3: Positive control
Lane4: Negative control

Fig. 4. Screening of plasmid isolated from *E. coli* transformant by M13F and M13R primers

JR and 1Ac). An intact band of ~ 925kb was observed without any nonspecific amplification (Fig. 2). The *cry1Ac* gene fragment (~925kb) was eluted from agarose gel. The eluted product also showed intact band in agarose gel. The DNA fragment of ~ 925kb as ligated with T/A vector (pTZ57R/T). The ligated mixture was used for transformation of the *E. coli,* DH5á competent cells. 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. Plasmid DNA was isolated from one of the PCR positive colonies of *E. coli* grown in LB broth containing ampicillin. Quantification of plasmid was made in reference with known concentration of ë/*Hind*III marker DNA (Fig. 3). The plasmid was checked for the presence of insert DNA with M13F and M13R primers. Agarose gel electrophoresis of PCR products showed (~ 1.1 kb including of vector sequence) amplification of expected size of DNA (Fig. 4).

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, and Thammasittirong and Attathom, 2008). In present study, two indigenous isolates of Bt were screened for the presence of *cry1Ac* genes through E- PCR with the *cry1Ac* subfamily primers and family primers described by Juarez-Perez *et al*., (1997). Among the two new isolates, both showed amplification of expected size as but in the case of Bt, 14r1 showed variation in amplification. Porcar *et al.* (2002) suggested that strains yielding unusual PCR products could be selected for further analyses leading to the identification and characterization of hypothetical novel *cry* genes. From this result 14r1 was selected for further cloning and the cloned genes were selected and send into the automated DNA sequencing.

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. Therefore, further studies on complete sequencing of *cry1Ac* gene from new isolates of Bt, 14r1 will be useful to find out the novelty of the gene.

ACKNOWLEDGEMENTS

The authors thank Dr. V. Udayasuriyan for critical reading of the manuscript and many valuable suggestions and Dr. A. Ramalakshmi for assistance in preparing the manuscript. This research was supported by Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Tamilnadu, India.

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