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PCR-Based Detection of *cry* Genes in Local *Bacillus thuringiensis* DOR Bt-1 Isolate

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ABSTRACT

A sporulating culture of *Bacillus thuringiensis* ssp. *kurstaki* strain DOR Bt-1 was isolated from castor semilooper (*Achaea janata* L.) cadavers from a castor bean (*Ricinus communis* L.) field at Kothakota mandal of Mahaboobnagar district, Andhra Pradesh, India. This strain has shown high toxicity against many insect pests and the wettable power formulation of this strain obtained through solid-state fermentation has been registered with the Central Insecticides Board (CIB), India under section 9 (3b). Based on the bioassay results and the efficacy of the formulation on different Lepidopteran insects, it was concluded that the strain must be carrying diverse *cry* genes. Therefore, to understand the *cry* gene profile of this strain, a PCR-based assay was carried out. Genomic PCR analysis of the DOR Bt-1 isolate with universal primers specific to *cry* gene classes indicated the presence of both *cry1* and *cry2* class-specific genes. Further, PCR analysis with *cry* gene-specific primers revealed the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab* genes. Thus, DOR Bt-1 isolate harbors a combination of different *cry* genes, such as Lepidoptera-active *cry1Aa*, *cry1Ab* and *cry1Ac*, and Lepidoptera-Diptera-active *cry2Aa* and *cry2Ab* genes, indicating that this isolate has the potential to be effective against both Lepidopteran and Dipteran insect pests.

Keywords: bioassay, Central Insecticides Board, insect pests, PCR, primers

INTRODUCTION

Bacillus thuringiensis (*Bt*) is a Gram-positive, aerobic bacteria that is characterized by the production of insecticidal proteins (*cry* proteins or δ-endotoxins that are encoded by *cry* genes) during sporulation (Schnepf *et al*. 1998). *Bt* has been used as a successful biological insecticide for more than 100 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests (Nester *et al*. 2002; Musser and Shetton 2003; Carrier *et al*. 2003; Quid *et al*. 2003; Romeis *et al*. 2006; Ali *et al*. 2010; He *et al*. 2011). Many of the crystal proteins are toxic to Lepidopteran larvae and are predominantly synthesized in the form of crystalline protoxins, crystal proteins toxic to other classes of insect pests like Coleopterans and Dipterans have also been identified and explained (Simeon *et al*. 2005; Bravo *et al*. 2011). These protoxins are solubilized in the alkaline midgut of susceptible larvae and cleaved by trypsin like gut proteases to produce a mature toxin that specifically binds to a receptor on the membrane leading to pore formation, cell lysis and eventually insect death (van *et al*. 1990; Vonterch *et al*. 1994; Schnepf *et al*. 1998; Bravo *et al*. 2011).

Bio-insecticides derived from *Bt* are widely used for the control of insects belonging to Lepridoptera, Diptera and Coleopteran orders and can be used to control insects in the order Hymenoptera as well as other types of pests including Nematodes and Mites (Gore *et al*. 2002; Ruiz *et al*. 2006; Ali *et al*. 2010; He *et al*. 2011). The efficacy and host range of the strain is dependent on the crystal protein genes the strain carries (Chang *et al*. 1998; Schnepf *et al*. 1998; Aly 2007). Usually the *Bt* strains carry a combination of *cry* genes and therefore become effective against different insect pests. Therefore, identifying the *cry* genes carried by a strain provides a clue regarding the utility of the strain against different groups of insects.

Since the first *cry* gene was cloned from *Bt* ssp. *kurstaki* HD-1 in 1981 (Schnepf and Whiteley 1981) the search for new *cry* gene is an ongoing effort worldwide and so far 560 *cry* genes have been characterized (Crickmore *et al*. 2011). It is emphasized that discovery of novel *cry* genes with new or broad activity spectra or higher toxicity is important for the development of new products and the management of insect resistance (Xue *et al*. 2008; Darsi *et al*. 2010). Such genes could be sourced from the *Bt* strains that show wider host range as well as better efficiency in terms of killing the target insect(s) (Darsi *et al*. 2010).

Among the techniques used in profiling the *cry* genes carried by a strain, PCR based one has distinct advantages such as sensitivity in detection levels, robustness, minimum lab facilities required, practicability and rapidity. Therefore, different PCR based methods have been proposed to identify the *cry* genes in *Bt* strains (Porcar and Juarez-Perez 2003; Corina *et al*. 2005; Thammasittirong and Attathom 2008; Goncalves *et al*. 2009; Aboussaid *et al*. 2011). Multiplex PCR method was established to rapidly identify and classify *Bt* strains containing *cry* genes toxic specifically to Lepidopteran, Coleopteran and Dipteran insects (Carozzi *et al*. 1991; Ben-Dov *et al*. 1997; Goncalves *et al*. 2009). As primers specific to different class of genes could be used in the same reaction, multiplex PCR increases the robustness of this approach and permits identification of several genes. Simultaneously, thereby decreasing cost and optimizing time.

DOR Bt-1 is a *Bt* isolate obtained from castor semilooper (*Achaea janata* L.) cadavers from castor bean (*Ricinus communis* L.) fields of south India. Bioassays have showed that this strain is highly toxic to *Achaea janata, Helicoverpa armigera* and *Spodoptera litura,* three important insect pests of castor in India and the wettable powder formulation of this strain obtained through solid-state fermentation has been registered with the Central Insecticides Board (CIB), India under section 9 (3b) (Vimala-Devi and Sudhakar 2006). Based on the trials carried out with this

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Table 1 Details of the primers for *cry1* (Un1) and *cry2* (Un2), universal and gene specific primers for five *cry* genes *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab*.

Primer pair[a]	Sequence of the primer (3′-5′)	*cry* genes	Position[b]	Product size (bp)
Un1 (F)	CATGATTACTGCGGCAGATAA	*cry1*		
Un1 (R)	TTGTGACACTTCTGCTTCCCAT	*cry1*	-	277
Un2 (F)	GTTATTCTTAATGCAGATGAAT	*cry2*		
Un2 (R)	CGGATAAAATAATCTGGGAAATA	*cry2*	-	689 to 701
Lep1A (F)	CCGGTGCTGGATTTGTGTTA	*cry1Aa/cry1Ab/cry1Ac*		
Lep1B (R)	AATCCCGTATTGTACCAGCG	*cry1Aa/cry1Ab/cry1Ac*	-	490
Lep2A (F)	CCGAAAAGTCAAACATGCG	*cry1Aa/cry1Ac*	-	986
Lep2B (R)	TACATGCCCTTTCACGTTCC	*cry1Ab*	-	908
DOR1 (F)	TTCCCTTTATTTGGGAATGC	*cry1Aa*	1001-1021	
DOR1 (R)	CCATTCCGGAACAACAAGGAC	*cry1Aa*	3015-3036	2035
DOR2 (F)	CGGATGCTCATAGAGGAGAA	*cry1Ab*	920-940	
DOR2 (R)	CTCCTTGTACGCTGTGACAC	*cry1Ab*	3016-3036	2116
DOR3 (F)	GGAAACTTTCTTTTTAATGG	*cry1Ac*	1586-1606	
DOR3 (R)	TTATTAAAATCACCATTTTTA	*cry1Ac*	3171-3192	1606
DOR4 (F)	GTTATTCTTAATGCAGATGAA	*cry2Aa*	571-592	
DOR4 (R)	GAGATTAGTCGCCCCTATGAG	*cry2Aa*	1048-1069	498
DOR5 (F)	GTTATTCTTAATGCAGATGAA	*cry2Ab*	699-1566	
DOR5 (R)	TGGCGTTAACAATGGGGGGAG	*cry2Ab*	1224-1245	546

[a] F and R – referred to forward and reverse primers, respectively.
[b] Primer position of the sequence of respective *cry* gene in the GenBank database.

strain (Kaur *et al*. 2008; Kandibane *et al*. 2010) it was concluded that the strain must be carrying different *cry* genes. Therefore, to understand the *cry* gene profile of this strain, PCR based methods were carried out and the results obtained have been reported here.

MATERIALS AND METHODS

Bacterial strains

Bt ssp. *kurstaki* (DOR Bt-1) isolated from fields of castor crops where there was no previous history of *Bt* spray formulations or of being planted with transgenic crops was obtained from the Directorate of Oilseeds Research (Madhusudhan *et al*. 2008). In the present study, along with DOR Bt-1 isolate, two commercial *Bt* strains Delfin, Halt and three standard *Bt* isolates D2, D20 and D21 (all belonging to *Bt* ssp. *kurstaki*) were also included for the PCR analysis.

Oligonucleotide PCR primers

Preliminary insect bioassay was carried out with DOR Bt-1 isolate using the 2nd and 3rd instar larvae of *Achaea janata*, *Helicoverpa armigera* and *Spodoptera litura*. Positive results obtained with this indicated that DOR Bt-1 isolate could be carrying *cry1* and/ or *cry2* genes that are known to be effective against Lepidopteran pests. Universal primers (Ben-Dov *et al*. 1997) specific to *cry1* and *cry2* classes of genes were used to assess whether DOR Bt-1 strain carried *cry1* and/or *cry2* genes. Subsequently, PCR was carried out with Lep1 and Lep2 primers (Carozzi *et al*. 1991) to identify the type of *cry1A* genes. To further confirm the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab* genes, forward and reverse primers for these genes were designed from the GenBank database according to Pinto and Fiuza (2003). The sequence of the designed primers is presented in **Table 1**.

Bacterial genomic DNA isolation

Genomic DNA was isolated by following the standard method (Ausubel *et al*. 1999). In short, 6 ml of overnight grown bacterial culture was used for isolation. The culture was grown until saturation (16-18 h). The culture was centrifuged in 1.5 ml Eppendorf tube for 2 min at 4500 rpm. The pellet was resuspended in 600 µl of TE buffer by repeated pipetting. To this resuspension 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added, mixed well and incubated at 37°C for 1 h followed by addition of 100 µl of 5 M NaCl and thoroughly mixed. To this, 80 µl of CTAB/ NaCl solution was added, mixed well and incubated at 65°C for 10 min. Equal volume of phenol/chloroform/isoamyl alcohol was added, mixed well slowly until it became monophasic and centrifuged at 8000 rpm for 7 min. The upper phase was taken to a fresh Eppendorf tube, and an equal volume of chloroform/isoamylalcohol was added, mixed well and centrifuged at 8000 rpm for 5 min. This step was repeated twice. Finally, the supernatant was taken into a fresh Eppendorf tube and the DNA was precipitated with 0.6 vol of isopropanol. After incubation at room temperature for 30 min, it was centrifuged at 8000 rpm for 7 min to pellet the DNA. The supernatant was decanted. The pellet was washed with 70% alcohol twice, air-dried and dissolved in TE [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)].

PCR analysis of *Bt* isolates

PCR was carried out with the universal primers for both *cry1* and *cry2* genes included in the same reaction while the PCRs with Lep1 and Lep2 primers as well as with gene specific primers were carried out independently. Each PCR was repeated at least twice to confirm the results. Total genomic DNA isolated from *Bt* isolates was used as template for PCR amplification. 20-50 ng DNA was used as template in 20 ul of reaction mixture containing 0.5 uM of each primer, 0.2 mM of each of the four dNTPs, (1X) *Taq* DNA polymerase buffer, 2.5 U *Taq* DNA polymerase (NEB, USA). PCR cycling profile was 1 cycle at 94°C for 4 min, 30 cycles of 94°C for 1 min, 54-60°C for 1 min and 72°C for 2 min followed by a final extension step at 72° C for 10 min. PCR products were separated using agarose gel electrophoresis in 1% TAE buffer and stained with 0.2 mg/ml ethidium bromide according to Sambrook *et al* (2001). PCR products were visualized under UV transilluminator and the sizes of the amplicons were estimated based on 100 bp and 1 kb DNA ladders loaded as size markers.

RESULTS AND DISCUSSION

Importance of the isolate

A local isolate of *Bt* (DOR Bt-1) strain had shown high toxicity against larvae of *Achaea janta*, *Helicoverpa armigera* and *Spodaptora litura*, three important Lepidopteran pests (Vimala-Devi and Sudhakar 2006; Madhusudhan *et al*. 2008). *Bt* DOR Bt-1 isolate formulation obtained through solid-state fermentation has been registered with the Central Insecticides Board (CIB), India under section 9(3b) (Vimala-Devi and Sudhakar 2006) and the strain has been used as effective biopesticide against insect pests in different crops (Vimala-Devi *et al*. 2005; Vimala-Devi and Sudhakar 2006; Kaur *et al*. 2008; Kandibane *et al*. 2010). It is well known that, the overall toxicity profile of a *Bt* isolate depends on the combination of *cry* genes carried by it, regulation and expression of individual *cry* genes, the relative amounts of the various protoxins included in the crystal,

solubility and their proteolytic processing of crystal proteins by different proteases within larval midgut and binding of the activated toxins to the receptor present on the midgut of epithelium of the susceptible insect (Madhusudhan *et al*. 2008) and presence of different *cry* genes in the same *Bt* strain has been reported by several authors (Ben-Dov *et al*. 1997; Aly 2007; Aly *et al*. 2009; Patel *et al*. 2009; Aly *et al*. 2011; Ammouneh *et al*. 2011). Therefore, profiling of the *cry* genes present in a strain is an important step to predict or confirm the insecticidal potential of that strain.

The identification of known *cry* genes in the *Bt* strains is important, since the specificity of action is known for many of the Cry toxins. This fact allows the possibility of selecting native strains that could be used in the control of some target insect pests and in selecting strains with the highest activity. The PCR screening is a rapid method for detecting and differentiating *Bt* local isolates by their PCR product profiles and for predicting their insecticidal activity in order to direct them for subsequent toxicity assays against Lepidoptera, Coleopteran and Dipteran insect pests (Aly 2007; Xue *et al*. 2008; Aly *et al*. 2009, 2011).

Identification of *cry* genes in *B. thuringensis* DOR Bt-1 isolate

Initial rep-PCR based analysis of DOR Bt-1 isolate indicated that it belonged to *Bt ssp. kurstaki* (data not shown). Preliminary insect bioassay carried out at the Directorate of Oilseeds Research, Rajendranagar, Hyderabad, India with DOR Bt-1 isolate indicated that the strain was effective against three major insect pests *Helicoverpa armigera, Spodoptera litura* and *Achaea janata* (data not shown). It is well established that Lepidopteran pest tolerance is imparted by *cry1* and /or *cry2* genes (Tailor *et al*. 1992; Ranjekar *et al*. 2003; Wang *et al*. 2003; Patel *et al*. 2009). Therefore, it was conjectured that this isolate would be carrying *cry1* and *cry2* genes. PCR is a tool that has been widely used for enumeration of *cry* genes carried by local *Bt* collections (Porcar and Juarez-Perez 2003; Aly 2007; Aly *et al*. 2009; Aboussaid *et al*. 2011; Aly *et al*. 2011). To analyze the *cry* genes carried by DOR Bt-1, PCR analysis was carried out in two phases; initially by screening using universal primers (Ben-Dev *et al*. 1997) specific for different classes of *cry* genes and then identifying the *cry* genes in local isolate by using gene specific primers.

Genomic PCR of DOR Bt-1 and standard isolates, with universal primers for *cry*1 genes yielded the expected 277 bp fragment and with universal primers for *cry*2 genes, yielded the expected 689-701 bp fragment indicating that DOR Bt-1 carried both *cry*1 and *cry*2 genes (**Fig. 1A**). PCR-based strategy has also been adopted for identifying strains that carry *cry* genes belonging to different classes. 134 local *Bt* isolates from Thailand were analyzed to identify the strains that possessed *cry1*, *cry2* and *cry9* class genes using PCR based approach by Thammasittirong and Attathom (2008) and they inferred the potential of the strains with respect to their ability to control Lepidopteran insects. In a similar study, 25 *Bt* isolates collected from different regions in Syria were analysed for the presence of different *cry* genes using PCR strategy with a set of general primers that recognized *cry1* and *cry2* class genes Ammouneh *et al*. (2011).

To identify the specific *cry1A* gene(s) carried by the isolate, the two sets of *cry1A* class specific primers, Lep1A, Lep1B and Lep2A, Lep2B (Carozzi *et al*. 1991) were employed. PCR amplification with these primers gave 490 bp (which indicated the presence of *cry1Aa*/*cry1Ab*/*cry1Ac*), 908 bp (which indicated the presence of *cry1Ab*) and 986 bp (which indicated the presence of *cry1Aa*/*cry1Ac*) (**Fig. 1B**). To further confirm the presence of different *cry1A* and cry*2A* genes, genomic PCR was carried out with DOR Bt-1 isolate along with standards using these *cry1A* and cry*2A* specific primers (**Table 1**). PCR with *cry1Aa* gene specific primer pair yielded 2035 bp fragment, with *cry1Ab* specific primer pair a 2116 bp fragment was obtained, with *cry1Ac* specific primer pair 1606 bp fragment was amplified, with

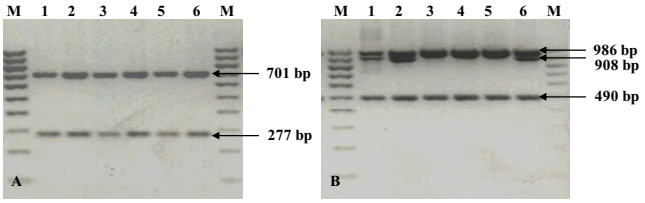

Fig. 1 PCR profiling with Universal primers to determine the *cry1* genes carried by DOR Bt-1 isolate. (**A**) Amplification with universal primers for *cry1* and *cry2* class of genes. PCR was carried out with the universal primers for both *cry1* and *cry2* genes included in the same reaction. (**B**) Amplification with *cry1A* specific primers. PCR carried out with Lep 1 and Lep 2 primers. PCRs were carried out separately with each set of primers and then the reaction mix was loaded together for electrophoresis. Three known *Bacillus thuringiensis ssp. kurstaki* strains, two commercial strains as well as unidentified local isolate DOR Bt-1 were analyzed. Lane-1 HD-1 (*Bt ssp. kurstaki*), lane-2 Halt (commercial strain), lane-3 Delfin (commercial strain), lane-4 D2 (*Bt ssp. kurstaki*), lanes-5 D20, lane-6 DOR Bt-1 (local isolate), lane-M 100 bp marker.

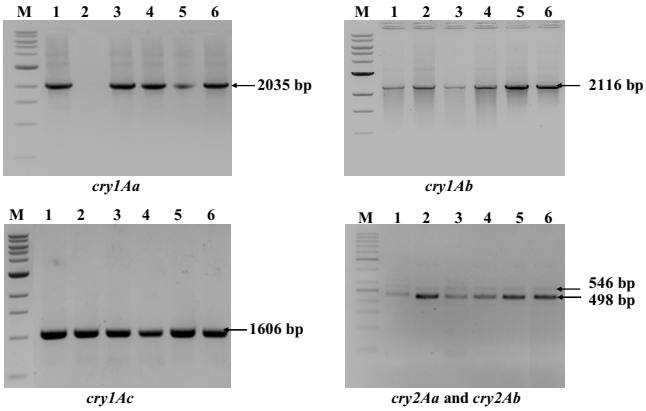

Fig. 2 PCR amplification of local *Bt* isolate DOR Bt-1 with primers specific to *cry* genes. Primers specific to *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab* genes were used for amplification with the genomic DNA. In case of *cry2Aa* and *cry2Ab*, a multiplex PCR was carried out by including both sets of primers in the reaction. Three known *Bacillus thuringiensis ssp. kurstaki* strains, two commercial strains as well as unidentified local isolate DOR Bt-1 were analyzed. Lane-1 HD-1 (*Bt ssp. kurstaki*), lane-2 Halt (commercial strain), lane-3 Delfin (commercial strain), lane-4 D2 (*Bt* ssp. *kurstaki*), lanes-5 D20, lane-6 DOR Bt- 1 (local isolate), lane-M 100 bp/1 Kb DNA marker. Gene fragments amplified and their sizes are indicated.

cry2Aa specific primer pair a 498 bp fragment was obtained and with *cry2Ab* specific primer pair 546 bp fragment was obtained (**Fig. 2**). However, there was no amplification with *cry1Ad*, *cry1Ae* and *cry2c* gene specific primers indicating the absence of these genes in DOR Bt-1. In these studies, standard *Bt kurstaki* strains HD-1, D2 and D20 along with commercial strains Halt, and Delfin were included as reference strains because these strains are known to harbour *cry1Aa*, *cry1Ac*, *cry1Ab*, *cry1Ia*, and *cry2* genes. Taken together, amplification with different *cry1A* and *cry2A* gene specific primers revealed the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa* and *cry2Ab* genes in this strain. In a similar study, Li *et al*. (2002) reported that the PCR analysis with a promising *Bt* isolate K1 from Korea contained *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2a* and *cry1E* type genes along with a *cry1F* type gene. The presence of different *cry* genes in the same *Bt* strain has been reported by other workers also (Ben-Dov *et al*. 1997; Aly 2007; Aly *et al*. 2009; Patel *et al*. 2009; Ammouneh *et al*. 2011).

Thus the PCR analyses with different sets of primers have indicated that DOR Bt-1 isolate belonged to *Bt* ssp. *kurstaki* and carried both *cry1* and *cry2* genes. Among the *cry1* class of genes, the strain carried *cry1Aa*, *cry1Ab* and *cry1Ac* genes while among the *cry2* class of genes, *cry2Aa*

and *cry2Ab* genes were present in the strain. From the results obtained we infer that the wide range of target insect pests displayed by DOR Bt-1 could be due to the wider *cry* gene profile carried by this isolate.

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