RESEARCH ARTICLE

SCREENING OF INDIGENOUS *BACILLUS THURINGIENSIS* ISOLATES FOR NOVEL *cry1* CRYSTAL TOXIN GENE FOR USE AGAINST LEPIDOPTERAN SUGARCANE BORERS

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Abstract

Isolation and screening of indigenous isolate with Lepidopteran toxic *cry1* gene through polymerase chain reaction revealed the presence of *cry1* gene positive isolate in the collection. PCR results with specific primers for the *cry1* toxin gene families revealed that the isolate SBI-KK 27 possessed *cry1A, cry1C, cry1D, cry1E, cry1I, cry2A and vip3B* genes of which the partial sequence of *cry1D* and *cry1E* from this isolate was found to be novel holotype crystal toxin reported for the first time from India.

Key words : Sugarcane, borers, Lepidoptera, *Bacillus thuringiensis*, *cry1* gene, *vip* gene, holotypes

Introduction

Lepidopteran stem borers are among the major insect pests of sugarcane all over the world. Around 50 species of moth borers in the genera *Acigona, Chilo, Diatraea, Eldana, Emmalocera, Eoreuma, Scirpophaga, Sesamia* and *Tetramoera* attack sugarcane worldwide (Long and Hensley 1972; Goebel and Sallam 2011; Srikanth et al. 2016) often causing significant yield losses of nearly 25-30 percent (Kalunke et al. 2009). Moth borers are difficult to control with insecticides due to their inaccessibility and hidden nature of larval feeding inside the cane. Biological control has been the main component of their management in countries like India (Srikanth et al. 2016) despite limitations in providing efficient control of borers (Mukunthan et al. 2003). Insect resistant sources are not available in sugarcane germplasm collections of the world which limits the pursuit of insect resistance breeding program. In the above scenario, the advancements made in the biotechnology of the bacterium *Bacillus thuringiensis* (Bt) offer to develop insect resistant crop plants, including sugarcane through genetic engineering. Screening programs have identified thousands of Bt strains and insecticidal genes active against a wide range of insect orders, and several nematodes, mites and protozoans. To date, the International Committee on Bt Toxin Nomenclature (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) has classified 75 (Cry1 - Cry75) different types of Cry proteins (Crickmore et al. 2018). The Cry1, Cry2 and Cry9 groups exhibit strongest activity to lepidopteran insects; the Cry3, Cry7 and Cry8 groups are most toxic to coleopteran insects whereas Cry4 and Cry11 are most toxic to dipteran insects and host spectrum of other Cry toxins have well been reviewed (Van Frankenhuyzen 2013; Palma et al. 2014). Cry1 toxins are the largest and best known

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family used against lepidopteran pests world wide either as biological insecticides or for developing insect resistant transgenic crops (Sanchis 2011). ICAR-Sugarcane Breeding Institute carried out pioneering work on the development and evaluation of insect resistant transgenic sugarcane varieties with *cry1A* genes against borers in India (Christy et al. 2009; Arvinth et al. 2010). Despite the availability of several approaches in transgenic research (Srikanth et al. 2011), the fundamental requirement is the identification and use of novel genes that is free from any intellectual property issues.

Many research workers have prospected for novel Bt and crystal toxin gene from biodiversity rich hot spots like Nilgiri Bioshpere of western ghats in TamilNadu (Ramalakshmi and Udayasuriyan 2010), Western Ghats of Karnataka (Mahadeva Swamy et al. 2013), Andaman and Greater Nicobar island (Mahadeva Swamy et al. 2011; Asokan et al. 2013) other parts of Karnataka (Pooja et al 2013) and sugarcane ecosystem in Tamil Nadu (Singaravelu et al. 2013a; 2013b). Only three primary (*cry8Sa1*, *cry32Aa1* and *cry52Ca1)* and two secondary (*cry2Ai1* and *cry2Al1)* holotype Bt crystal toxin genes from India are recognized by the international committee on Bt toxin nomenclature. Of these holotypes, *cry8Sa1*, its sequence (NCBI Accession No.JQ740599) and the genome sequence of the isolate (Bt 62) carrying the gene were reported by us in recent studies (NCBI genome Accession No. SRP1275532 for chromosome and SRP 129858 for plasmid). Since no novel lepidopteran specific holotype *cry1* gene of Indian origin has been isolated yet, the study presented here was undertaken with aim of finding novel toxin genes by isolation and screening of indigenous *cry1* Bt isolates.

Materials and Methods

Soil samples collection

Soil samples were collected by scraping off surface soil with a spatula upto a depth of 5-10 cm. Samples were collected in plastic bags, transferred to the lab, labelled and stored at room temperature. Soil samples from diverse ecosystem including sugarcane fields from the states of Tamil Nadu, Karnataka, Maharashtra, Punjab, Haryana, Uttarakhand and Uttar Pradesh were included in the study.

Bt isolation

Bt isolation from soils was carried out with a slight modification to the method followed by Travers et al. (1987). Ten grams of soil sample was suspended in 100 ml of 0.85% NaCl solution. The samples were heated at 80°C for 15 min in water bath. One ml of the heat treated sample was inoculated in 50 ml of Luria Bertani (LB) broth and incubated overnight at 30°C with an orbital shaking of 250 rpm. From this broth, serial dilution (10^{-1} to 10^{-6}) was performed and each dilution was plated in Travers (T3) medium. The plates were then incubated for 48h at 30°C. After incubation, Bt like colonies were selected and streaked on T3 and incubated for 72h at 30°C. The single colonies were observed under phase contrast microscopy for identification of Bt. The isolates showing the presence of crystalline inclusions were selected as Bt and streaked on T3 agar medium for single colony purification. Broth culture (pH adjusted to 6.9) was made from the isolated single colonies of crystal positive Bt isolates. Glycerol stocks of Bt isolates were prepared by using equal amounts of 30% glycerol and 72 h old T3 broth culture and stored at -20°C for further studies.

PCR screening of *cry1* gene

Polymerase chain reaction (PCR) was used to identify *cry1* gene type Bt isolates. *cry1* gene positive reference Bt strain, *cry1* gene negative reference Bt strain and indigenous Bt isolates form various sugarcane ecosystem were streaked on Luria Agar (LA) plate and grown overnight at 30°C. Two swipes of the overnight grown cultures were suspended in 200 µl of sterile distilled water and the isolates were heated by placing them in boiling water for 10 min. The lysed cells were allowed to settle for 8-10 min at room temperature and the supernatant was taken as DNA template for PCR reaction. Universal primers (forward and reverse) of *cry1* gene were used to amplify a specific fragment as described by Bravo et al. (1998). All PCR reactions were carried out in 50µl reaction volumes.

Twenty µl of template DNA was mixed with reaction buffer containing 1.25 µl of 2.5mM deoxynucleoside triphosphate mix, 0.5µl of 10µM (direct and reverse) primers, 5 µl Taq buffer (10 X), and 1 U of Taq DNA polymerase. Amplifications were carried out in a DNA thermal cycler (Biorad S1000). The PCR conditions for screening the isolates were as follows: single denaturation step of 5 min at 94°C, a step cycle program set for 30 cycles (with a cycle consisting of denaturation at 94°C for 1 min, annealing at 50°C for 30 seconds and extension at 72°C for 45seconds), and an extra step of extension at 72°C for 7 min after completion of all the cycles. All PCR reactions were performed with the *cry1* gene positive reference strain *B. thuringiensis* serovar *kurstaki* (HD^{-1}) and *cry1* gene negative plasmid cured HD73 strain. Following the amplification, electrophoresis of each PCR sample was done on 1 % agarose-ethidium bromide gel.

Results and Discussion

Bt isolates were identified through phase contrast microscopy from the soil samples collected in sugarcane ecosystem. Bt isolates are generally identified by their crystal toxins which are easily visible under phase contrast microscope. In our study the Bt isolates were found to contain bipyramidal, spherical and rhomboidal shaped crystal toxin.

PCR screening of the isolates of Bt identified in this study with *cry1* gene universal primer revealed the presence of *cry1* gene Bt positive isolates which showed an amplicon of approximately 560 bp (Fig. 1). When the PCR amplicons of the *cry1* gene positive isolates were sequenced we found that the sequencing chromatogram yielded overlapping peaks for some isolates (Fig. 2). Since universal primers of *cry1* gene was used for the screening of the Bt isolates, the overlapping peaks observed in the sequencing chromatogram were due to the presence of multiple *cry1* subfamily genes.

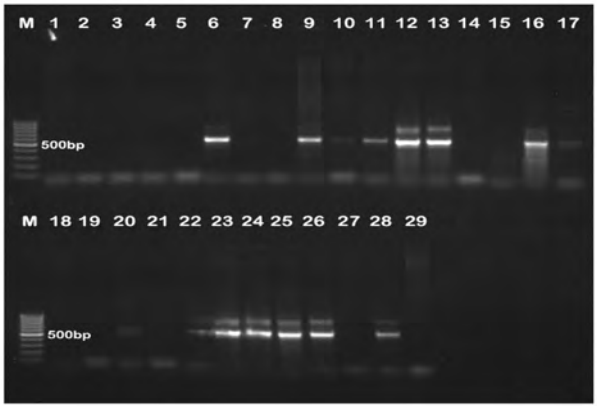

Fig. 1. PCR amplification of *cry1* gene of various indigenous Bt isolates. M; DNA marker; 1) $HD73^{-}$ 2) Bt62. 3) SBI-Bt6. 4) SBI-Bt8. 5) SBI-Bt50. 6) SBI-Bt24.7) SBI-Bt78. 8) SBI-Bt84. 9) SBI-NLK33 10) SBI-KK4. 11) SBI-KK6. 12) SBI-K17. 13) SBI-K18. 14) SBI-M1. 15) SBI-M6. 16) SBI-NLK30. 17) SBI-Bt481. 18) SBI-Bt93. 19) SBI-Bt482. 20) SBI-Bt 486. 21) SBI-Bt503. 22) SBI-Bt721. 23) SBI-Bt723. 24) SBI-KK27. 25) SBI-Bt1844. 26) SBI-Bt1850. 27) SBI-Bt62 (*cry8* positive reference) 28) Bt HD1 (*cry1* positive reference). 29 $HD73^{-}$ (*cry1* negative Bt reference).

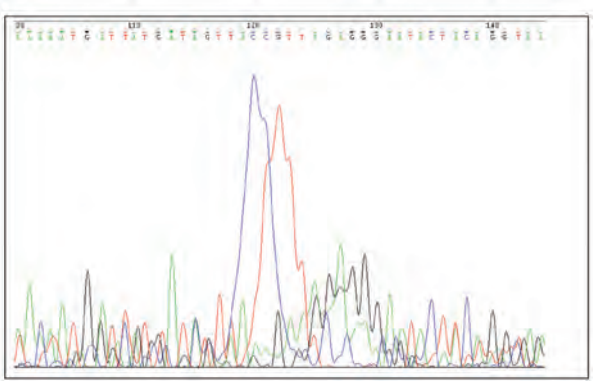

Fig. 2. Sequencing chromatogram of *cry1* gene amplicon of Bt isolate SBI-KK27 showing overlapping peaks of different *cry1*subfamily gene.

One of the Bt isolates, i.e. SBI-KK27 was taken up for further screening of its *cry1* gene content because its sequence chromatogram indicated the presence of more than one crystal toxin gene. PCR results with specific primers for the *cry1* toxin gene families revealed that the isolate possessed *cry1A, cry1C, cry1D, cry1E, cry1I, cry2A* and vip3B genes. Partial sequencing data from the individual amplicons of these crystal toxin genes of SBI-KK27 revealed that *cry1D* and *cry1E* from this isolate could be novel holotypes as per the definitions of the International Committee on Bt Toxin Nomenclature. As per the system established by the Bt toxin nomenclature committee, blastX results of the partial *cry1D* and *cry1E* from our Bt isolate revealed a pairwise amino acid identity of 91% and 86%, respectively with already identified *cry1D* and *cry1E* subfamily genes indicating the possibility of occurrence of putative holotype crystal toxin genes at the tertiary level.

Cloning and sequencing of the full coding sequence of these two putative holotype crystal toxin genes and expression of these novel putative crystal toxin proteins in sugarcane, after confirming their individual toxicity to various other borer pests, would strengthen the arsenal of crop protection technologies available for management of lepidopteran borers infesting sugarcane. Besides, the potential of these two genes for developing transgenics against important lepidopteran pests of other crops can also be explored.

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