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Cloning, characterization and expression of a novel haplotype *cry2A*-type gene from *Bacillus thuringiensis* strain SWK1, native to Himalayan valley Kashmir

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ABSTRACT

Bacillus thuringiensis (*Bt*) is a gram positive bacterium which is effectively being used in pest management strategies as an eco-friendly bioinsecticide. In the present study a new *cry2A* gene was cloned from a promising indigenous *B. thuringiensis* SWK1 strain previously characterized for its toxicity against *Spodoptera litura* and *Helicoverpa armigera* larvae. The nucleotide sequence of the cloned *cry2A* gene pointed out that the open reading frame has 1902 bases encoding a polypeptide of 634 amino acid residues with a probable molecular weight of 70 kDa. Homology comparisons showed that the deduced amino acid sequence of Cry2A had a similarity of 94% compared to that of the known Cry2Aa protein in the NCBI database and this gene has been named as *cry2Al1* by the *B. thuringiensis* δ-endotoxin Nomenclature Committee. *cry2Al1* was ligated into pET 22b vector and expressed in *Escherichia coli* BL21 (DE3) pLysS under the control of T7 promoter induced by isopropyl-beta-D-thiogalactopyranoside (IPTG). SDS-PAGE analysis confirmed the expression of *cry2Al1 as* ~65 kDa protein. Insect pest bioassays with neonate larvae of *S. litura* and *H. armigera* showed that the purified Cry2Al1 are toxic to *S. litura* and *H. armigera* with LC_{50} 2.448 μg/ml and *H. armigera* with 3.374 μg/ml respectively.

1. Introduction

Bacillus thuringiensis (*Bt*), is a species of Gram positive, crystalliferous, sporulating soil bacterium of the family Bacillaceae (Saadaoui et al., 2010). The parasporal crystal (Cry and Cyt) proteins of *Bt* show specific insecticidal activity against insects belonging to the orders Coleoptera, Diptera, Lepidoptera, Homoptera, Hymenoptera and Mallophaga (as well as some invertebrates) but are benevolent to the environment (Schnepf et al., 1998; Li and Bouwer, 2012; Elleuch et al., 2015; Neethu et al., 2015). Commercial *Bt* based bioinsecticides used worldwide are applied at 10–50 g/acre or about 10^{20} molecules/acre, while chemical pesticides such as organophosphates and pyrethroids are applied about 8×10^{24} molecules/acre and 3×10^{22} molecules/acre respectively. Thus, molecular potency of these toxins is 80,000 times better than organophosphates and 300 times greater than synthetic pyrethroids (Feitelson et al., 1992). Ever since the cloning of first *cry* gene (*cry1Aa*) from *B. thuringiensis* spp. *kurstaki* HD-1 (Schnepf and Whiteley, 1981), 304 such *cry* holotypes protein genes have been reported (Crickmore et al., 2016; 15th December 2016, http://www.btnomenclature.info/). These insecticidal crystal protein genes are the chief resources for production of insect-resistant transgenic plants (Romeis et al., 2006). Genetically engineered crops that produce insecticidal proteins of *Bt* for the control of pests have been planted on greater than 560 million hectares worldwide since 1996 (James, 2013). However there have been some tribulations with this approach, for instance narrow insecticidal range and evolution of insect resistance (Sumerford et al., 2012; Pardo-López et al., 2013; Gassmann et al., 2014). A latest study of 24 cases, with each case relating to responses of single pest species in one country to an individual *Bt* toxin, demonstrated that the practical influence of field-evolved resistance can differ from none to severe, based on the magnitude, incidence and spatial distribution of resistance (Bravo et al., 2011; Tabashnik et al., 2013, 2014). Hence, isolation of new *Bt* strains with novel toxins is of significance for providing alternatives to these problems.

The *cry2A*-type genes encode 60–75 kDa proteins and are potential candidates for insect resistance management as these exhibit a wide spectrum of toxicity, different modes of action compared to other Cry proteins and small size of toxin (Schnepf et al., 1998; Sasaki et al., 1997; Alcantara et al., 2004).

Valley Kashmir that is often referred as *Terrestrial Paradise on Earth* is situated at northern western tip of Himalayan biodiversity

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hotspot (Mittermeier et al., 2005). It is located roughly between 32°.15′ and 37°.05′ North latitude and 72°.35′ to 83°.20′ East longitude, with complicated geomorphologic characteristics (i.e. snow clad mountains, vast meadows full of flowers, thick forests, small mountains, valley lakes and numerous serpentine rivers). These distinctive features associated with variety of animal forms ranging from higher groups such as vertebrates, including mammals, birds, reptiles, amphibians to lower groups like invertebrates including insects and even unicellular micro-organisms provide the opportunity to isolate novel *Bt* strains harbouring new *cry* genes. In this study, we isolated a lepidopteran active *Bt* strain SWK1 from Himalayan valley Kashmir. The present report describes the characterization of the *Bt* isolate SWK1, including the cloning and sequence analysis of a novel haplotype crystal protein gene, *cry2Al1*. In addition, the *cry2Al1* gene was successfully expressed in *Escherichia coli* BL21 (DE3) pLysS cells. The expressed protein Cry2Al1 was found to be toxic to *Spodoptera litura* and *Helicoverpa armigera*.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. thuringiensis strain SWK1 was isolated from a spider web collected at a cattle shed in Badran Magam, Kashmir, a Himalayan valley. It was found effective against two major lepidopteran insect pests *S. litura* (LC_{50} 1.978 μg/ml) and *H. armigera* (LC_{50} 2.199 μg/ml). The *E. coli* DH5α strain was exploited for retaining the cloned gene and *E. coli* BL21 (DE3) pLysS was employed for expression of our *cry* gene. The plasmid vector pTZ57R/T (Fermentas life science, Mumbai, India) was used for DNA cloning and sequencing while pET22b vector (Novagen, Madison, WI) for the expression studies of cloned *cry* gene. *E. coli* DH5α harboring pTZ57R/T-*cry2A* and *E. coli* BL21 (DE3) pLysS with pET22b-*cry2Al1* were grown on Luria Bertani (LB) Ampicillin (50 μg/ml) medium.

2.2. Scanning electron microscopy

A single colony of *B. thuringiensis* strain SWK1 was grown in 5 ml T3 broth and incubated at 30 °C, 200 rpm, for approximately 72 h till more than 90% of sporulation. The spore crystal mixture was then collected by centrifugation at 10, 000 rpm for 5 min at 4 °C. The pellet was washed with 1 M NaCl and distilled water. The sample was smeared on a small piece of adhesive carbon tape which was fixed on a brass stub and then subjected to gold coating using sputtering unit (model: JFC1600 JEOL, Tokyo, Japan) for 10 sec at 10 mA of current. The gold coated sample was placed in chamber of SEM (Jeol, JSM 6390LA, Tokyo, Japan) and secondary electron/Back Scattered electron images were recorded. The scanning electron microscopy was performed at Sophisticated Analytical Instrument Facility (SAIF), Cochin University, Kerala.

2.3. SDS PAGE analysis of spore crystal mixture

B. thuringiensis strain SWK1 was grown in LB broth (25 ml) in a shaking incubator (Hasthas Scientific Instruments, India) maintained at 30 °C and 200 rpm, until more than 90% of cells had lysed (~72 h). The spore crystal mixture was harvested by centrifugation and pellet was washed once with 5 ml of ice-cold Tris–EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)] and one time with 5 ml of ice-cold 0.5 M NaCl followed by two additional washes with 5 ml of Tris–EDTA buffer with 0.5 mM PMSF. All the centrifugations were performed at 10,000 rpm for 5 min at 4 °C (Superspin R-V/FM Plasto Crafts, Plasto Craft Scientific Pvt. Ltd, Mumbai, India). Finally, the spore-crystal pellet was suspended in 100 μl of sterile distilled water containing 1 mM PMSF and stored in −20 °C. Aliquots of spore–crystal mixture of *Bt* strain SWK1 was analyzed by SDS-PAGE (Laemmli, 1970) using 10% running and 4% stacking gel. The molecular mass of proteins was determined using higher range protein molecular weight marker (myosin rabbit muscle 205 kDa, phosphorylase b 97.4 kDa, bovine serum albumin 65 kDa, ovalbumin 43 kDa and carbonic anhydrase 29 kDa) obtained from GeNei™, Bangalore, India.

2.4. Full length cloning and sequencing of the cry2A type gene

Genomic DNA was extracted from *B. thuringiensis* strain SWK1 using HiPurA™ Bacterial and Yeast Genomic DNA Purification Spin Kit (HiMedia, India). PCR with *cry2Aa* specific primers (Ben-Dov et al., 1997) was performed. To determine the full length sequence of the *cry2A* type gene, a DNA fragment of approximately 1.9 kb was amplified using genomic DNA as template and primer 2ARF (5′-ATGGTACCATGAATAA TGTATTGAATAGTGGAA-3′) and 2ARS (5′-GTTCTAGACTCAAACCTTAATAAAGTGG TG-3′) synthesized at Xcelris Labs Ltd, Gujarat, India. These primers could amplify from +1 to +1902 of *cry2A* gene. For polymerase chain reaction (PCR), 0.1 μg of total DNA and 1 μM of each primer were mixed with 10 μl of 2× PCR Master Mix (GeNei™, Bengaluru, India) consisting of dNTPs, Taq DNA polymerase and PCR buffer. The final volume was made up to 20 μl with double distilled water. PCR amplification was performed in an thermal cycler (cyber cycler-P series PCR peltier model p96+ USA) using the program: a 2 min denaturation step at 94 °C, 30 amplification cycles of 40 sec at 94 °C, 40 sec at 60 °C, and 1 min at 72 °C, with a final extension step of 7 min at 72 °C. The amplified PCR product was purified using GeneJET™ PCR purification kit (Fermentas life science, Mumbai, India). InsTAclone™ PCR Cloning Kit (Fermentas Life Science, Mumbai, India) was used for cloning of purified PCR product. The PCR amplified *cry2A* product was ligated in pTZ57R/T vector as per instructions in user manual. The recombinant vector pTZ57R/T-*cry2A* was transformed in *E. coli* DH5α. Putative positive clones were identified by blue white screening. The recombinant plasmid DNA was extracted from the putative clones and the existence of the cloned gene was confirmed by PCR and restriction digestions. The nucleotide sequences of positive clones each from the pTZ57R/T-*cry2A* constructs were further confirmed by complete sequencing using an automated DNA sequencer (ABI 3730xl Genetic, Thermo Fisher Scientific, US) at Xcelris Labs Ltd, Gujarat, India. In order to confirm the middle non readable sequence segment of the full length sequence, two internal primers (F1-1238-5′-ATCTGGGAAATAGTTTGAATTTC-3′; R1-705 5′-ACACCCGTTTA CACGATATGTTAG-3′) were designed. The forward and reverse sequences were edited using Bioedit program (Hall, 1999) and BLAST was performed using BLASTN. Open reading frame obtained was further analyzed for the restriction endonuclease pattern. The cutting and non cutting endonucleases were identified by *in silico* digestion of SWK1 *cry2A* sequence with Webcutter 2.0. Comparative analysis of the derivative amino acid sequence was carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). We submitted sequence of our *cry* gene to the *B. thuringiensis* delta-endotoxin nomenclature committee which assigned name to the isolated sequence from *B. thuringiensis* strain SWK1 (Crickmore et al., 2016; http://www.btnomenclature.info). The theoretical molecular weight and PI value of the Cry protein were calculated by EXPASY. The three dimensional structure of the Cry2Al1 protein was predicted using SWISS-MODEL automated modeling server from deduced amino acid sequence. The 3D structure templates were validated using automated program present in the server. Sequence was submitted in GenBank and accession number was obtained.