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RESEARCH

Cloning, Characterization, and Expression of a New *cry1Ab* Gene from DOR Bt-1, an Indigenous Isolate of *Bacillus thuringiensis*

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Abstract A new *cry1Ab* gene was cloned from the promising local isolate, DOR Bt-1, a *Bacillus thuringiensis* strain isolated from castor semilooper (*Achaea janata* L.) cadavers from castor bean (*Ricinus communis* L.) field. The nucleotide sequence of the cloned *cry1Ab* gene indicated that the open reading frame consisted of 3,465 bases encoding a protein of 1,155 amino acid residues with an estimated molecular weight of 130 kDa. Homology comparisons revealed that the deduced amino acid sequence of *cry1Ab* had a variation of seven amino acid residues compared to those of the known Cry1Ab proteins in the NCBI database and this gene has been designated as *cry1Ab26* by the *B. thuringiensis* δ-endotoxin Nomenclature Committee. *cry1Ab26* was cloned into pET 29a(+) vector and expressed in *E. coli* strain BL21 (DE3) under the control of T7 promoter with IPTG induction. ELISA, SDS-PAGE, and Western blot analysis confirmed the expression of 130-kDa protein. Insect bioassays with neonate larvae of *Helicoverpa armigera* showed that the partially purified Cry1Ab26 caused 97 % mortality within 5 days of feeding.

Keywords *Bacillus thuringiensis* · Bioassay · Cloning · Protein expression · *Cry1ab26* · ELISA · SDS-PAGE

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Introduction

Bacillus thuringiensis (Bt) is a gram-positive, aerobic bacterium that has been used as a successful biologic insecticide. The insecticidal property of Bt is due to the production of insecticidal crystal proteins (ICPs) also called δ-endotoxins, consisting of one or more crystal (Cry) proteins and Cytolitic (Cyt) toxins [1]. The *cry* genes have been categorized according to the class of insects they are effective against [2]. Bt strains carry different classes of *cry* genes [3], and therefore the overall toxicity profile of the isolate depends on regulation of expression of individual *cry* genes, the relative amounts of the various protoxins included, solubility and their proteolytic processing by different proteases within larval midgut and binding of activated toxin to the receptors present on the midgut of epithelium of the susceptible insect. Since the first *cry* gene encoding crystal protein was cloned from Bt strain HD-1, the search for new *cry* genes is an ongoing effort worldwide with 560 *cry* genes discovered and classified into different families [2]. Bt genes coding for insecticidal

toxins have been deployed in several crops and these transgenics (Bt crops) have been commercially cultivated on a large scale. But, with increasing scale and prolonged planting of Bt crops, evolution of insect resistance to Bt toxins in agricultural systems has become the foremost threat to the long-term future of Bt biopesticide and Bt crops [4].

Already, insect resistance to Bt toxins in open fields and green houses has been reported in six species [3]. Also, through laboratory selections many insect lines with resistance to Bt toxins have been realized with various resistance mechanisms [3, 5]. Cry proteins bind to cadherin receptor proteins in the midgut region of the insects through sequential interaction [6] and it has been shown that mutations in the cadherin receptor gene could block this binding and thus confer "mode 1" type of resistance to insects against crystal proteins [7–9]. There are clear studies, which suggest that suppression of resistance in insects to Bt *cry* toxins could be achieved by modifying the *cry* gene in such a way that they can kill the insect without binding to the receptor [10, 11]. On the bacterial side, it has been documented that even single residue changes in Cry proteins alter their efficiency with respect to host range as well as toxicity levels [12, 13] and the toxins within the Cry1A subfamily exhibit variation in toxicity and specificity due to minor amino acid substitutions [14]. Based on these observations, it has been opined that the development of resistance in insects might be delayed if there are variants of crystal proteins, either naturally occurring or modified through genetic engineering [15].

Most of the commercial Bt formulations used for the control of lepidopteran pests contain toxins of Cry1A family, especially Cry1Aa, Cry1Ab, and Cry1Ac proteins [16]. Among the different *cry* genes deployed in transgenic plants, *cry1Ab* is an important one with transgenic plants developed in several crops [17]. Bt *cry1Ab* gene is known to be effective against different insects including *Helicoverpa armigera*, an important polyphagous pest of worldwide concurrence that inflicts an annual crop damage of worth US $ 1 billion in India [18, 19]. A variant of *cry1Ab* with increased toxicity up to 40-fold against larvae of gypsy moth has been identified [20]. Modified or engineered *cry* genes have been used successfully to increase the host range of Cry toxins [21]. Therefore, discovery of *cry* gene variants could increase the scope of identifying the *cry* gene forms with broader host range activity or higher toxicity for the development of new products and is also considered as an important strategy in reducing the onset of insect resistance [22, 23].

B. thuringiensis DOR Bt-1 is an isolate obtained from a cadaver of castor semilooper (*Achaea janata* L.) larva collected from a castor bean field at Kothakota Mandal of Mahaboobnagar district, Andhra Pradesh, India. Bioassays have demonstrated that this strain is highly toxic to *A. janata*, an important pest 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, India under section 9(3b) [24]. This formulation has shown promise against other insect pests such as rice leaf folder and showed efficacy similar to that of chemical control [25, 26]. Preliminary *cry* gene profiling of DOR Bt-1 indicated that the strain carried a copy of *cry1Ab* gene along with other *cry1A* genes. Considering the importance of *cry1Ab* gene in terms of its effectiveness against many lepidopteran pests, full-length *cry1Ab* gene was cloned from DOR Bt-1 strain and characterized. We report here the cloning and expression of *cry1Ab* from DOR Bt-1 in *E. coli*, BL21 (DE3) cells, and the insect bioassays carried out with the partially purified protein against neonate larvae of *H. armigera*.

Materials and Methods

Bacterial Strains and Plasmids

DOR Bt-1 isolate being maintained at the Directorate of Oilseeds Research, Rajendranagar, Hyderabad, was used in this study. The *E. coli* strain Top10 cells were used for maintaining the cloned gene and *E. coli* BL21 (DE3) was used for expression of the gene. The plasmid vectors pGEM-T vector system (Promega) was used for DNA cloning and sequencing, pET29a(+) vector (Novagen) for expressing the cloned *cry* gene from DOR Bt-1 strain. *E. coli* and *B. thuringiensis* were grown as batch cultures in LB broth with shaking at 37 and 30 °C, respectively. *E. coli* harboring pGEM-T were grown in the presence of Ampicillin (100 mg/l) and *E. coli* BL21 (DE3) with pET29a(+) and it's derivatives were grown in the presence of Kanamycin (50 mg/l).

Preliminary Work with DOR Bt-1 Isolate

Preliminary insect bioassay was carried out with ICP from DOR Bt-1 isolate using neonate larvae of *H. armigera* and *A. janata*. Positive results obtained with this indicated that DOR Bt-1 isolate could be carrying *cry1* and/or *cry2* genes which are known to be effective against lepidopteran pests. Therefore, PCR analysis was carried out with the universal primers specific to *cry1* and *cry2* classes of genes [27] to assess whether DOR Bt-1 strain carried *cry1* and *cry2* genes. To identify the specific *cry1* genes present in the isolate, two sets of specific primers, Lep1A, Lep1B and Lep2A, Lep2B [28] were used in the PCR reaction. As Lep primers could not distinguish *cry1Aa* and *cry1Ac*, specific reverse primers for *cry1Aa* and *cry1Ac* were used in

conjunction with Un1 forward primer to detect the presence of *cry1Aa* and *cry1Ac*. Along with DOR Bt-1 isolate, two commercial Bt strains Delfin and Halt and three standard Bt isolates D2, D20, and D21 (all belonging to *B. thuringiensis ssp. kurstaki*) were included as controls for the PCR analyses.

Cloning and Confirmation of the *cry1Ab* Gene

Total genomic DNA was isolated as described by Kalman et al. [29] from DOR Bt-1 and used as template for PCR amplification. To synthesize primers specific to *cry1Ab* gene, multiple sequences of *cry1Ab* genes were aligned and two primers, one upstream of 5′ and the other downstream of 3′ end of the aligned sequences were designed and synthesized. The primer sequences used were Forward: 5′-TT**CATATG** GATAACAATCCGAACATCATT-3′ (the first two bases were the protection bases, the boldface indicate *Nde*I site) and Reverse: 5′-TT**CTCGAG**TTATTCCTCCATAAGAA GTAAT-3′ (the first two bases were the protection bases, the boldface indicate *Xho*I site). These primers were used for amplifying the full-length *cry1Ab* gene. PCR reaction mix of 20 µl included 0.5 µM of each primer, 0.2 mM each of the four dNTPs, (1×) Taq DNA polymerase buffer, 2.5 U Taq DNA polymerase (Long Amp Taq NEB, USA), and 20–50 ng genomic DNA as template. PCR cycling profile included 1 cycle at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 3.5 min followed by a final extension step at 72 °C for 20 min. Pilot PCR gave the expected 3.5-kb fragment. To clone the *cry1Ab* gene, PCR was carried out in a 80-µl reaction mix under same conditions as was for pilot amplification and the obtained product was purified using QIAquick DNA purification kit (Qiagen), ligated with pGEMT-Easy vector (Promega) to obtain the recombinant pGEMT—*cry1Ab*, and transformed into *E. coli* Top10 cells by standard protocol [30]. The recombinant plasmid DNA was extracted from the putative clones and the presence of the cloned fragment was confirmed using restriction digestions and PCR with gene specific as well as with universal T7 and SP6 primers (present in the vector). These analyses confirmed the cloning of 3.5-Kb fragment. The recombinant plasmids were sequenced and the BLAST analysis confirmed that the cloned gene corresponded to *cry1Ab*. Comparative analysis of the derived amino acid sequence was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) which revealed differences between the cloned *cry1Ab* sequence and the already reported *cry1Ab* sequences. The confirmed nucleotide sequence was submitted to the *B. thuringiensis* delta-endotoxin nomenclature committee which assigned *cry1Ab26* to the isolated gene from DORBt-1 strain [2]. The theoretic molecular weight and PI value of the gene were calculated by EXPASY. With the deduced amino acid sequence, the three dimensional structure of the Cry1Ab26 protein was predicted using PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2) software.

Cloning of *cry1Ab26* Gene in pET29a(+) Vector

Recombinant plasmid pGEM-T—*cry1Ab26* and pET29a(+) vector were digested with *Nde*1 and *Xho*1. Using Qiagen DNA purification kit, an approximate 3.5-kb DNA fragment of *cry1Ab26* gene and 5.4-kb fragment of pET29a(+) vector were purified after agarose gel electrophoresis and ligated to get recombinant pET29a(+)—*cry1Ab*26 expression construct. *E. coli* BL21 (DE3) was transformed with the ligation mix and the positive clones obtained on the selection plate were cultured overnight at 37 °C in LB medium with 50 mg/l kanamycin and the plasmid DNA was isolated from the positive clones according to Sambrook et al. [30] and confirmed by restriction analysis as well as PCR.

Expression of *cry1Ab26* in *E. coli* BL21 (DE3) Cells

Overnight grown culture of a positive clone of pET29a(+)—*cry1Ab*26 was subcultured into fresh medium (1:100) for 4 h and then induced with 2 mM IPTG for 4–8 h. Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4 °C and resuspended in sonication buffer (pH 8.0) 50 mM Tris–Cl, 5 mM EDTA, 5 mg phenyl methane sulfonyl fluoride (PMSF) lysed on ice by ultrasonication (Sonic-Vibra Cell^{TM}) at 100 W three times of 1 min duration each [31]. The pellets were washed with 0.5 M NaCl plus 2 % Triton X twice, followed by washing with 0.5 M NaCl five times, and with double distilled water twice. After centrifugation at 12,000 rpm for 10 min, the pellets were solubilized in 50 mM sodium carbonate buffer (pH 10.2) containing 10 mM DTT. The un-solubilized proteins were removed by centrifugation at 12,000 rpm for 10 min at 4 °C. Expression of Cry1Ab26 in the positive clone was confirmed by qualitative ELISA using the Cry1Ab antibody (supplied by Amar Immunologicals, Hyderabad). To quantify the expressed protein, ELISA was carried out using Quantiplate^{TM} (supplied by Amar Immunologicals, Hyderabad) and quantified using ELISA reader (Thermo Multiskan Spectrum). The expressed protein was separated on 10 % SDS-PAGE and stained with Coomassie brilliant blue [32].

Western Blot Analysis

25 µg of *cry1Ab26* protein solubilized in 50 mM sodium carbonate buffer (pH 10.2) was separated by 10 % SDS–polyacrylamide gel electrophoresis (PAGE) carried out at 50 V overnight and was transferred onto PVDF nylon membrane (Hybond^{++}, GE Healthcare, India). The membrane was blocked in PBST solution (155 mM NaCl, 1 mM

KH_2PO_4, 3 mM $K_2HPO_4 \cdot 7H_2O$, pH 7.4, 0.25 % Tween 20) plus 5 % skimmed milk for 1 h at room temperature and then incubated with primary antibody (monoclonal rabbit anti-Cry1Ab antibody, 1:1,000, Amar Immunologicals, Hyderabad, India) in PBST solution overnight at 4 °C. The membrane was then washed three times with PBST solution, followed by incubation with anti-rabbit IgG alkaline phosphatase conjugate (1:1,000, Amar Immunologicals, Hyderabad, India) secondary antibody in PBST solution. The membrane was washed three times (15 min each) and the color was developed using 5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphatase substrate.

Insect Bioassay with Cry1Ab26

IPTG-induced *E. coli* BL21 (DE3) cells harboring pET29a(+)—*cry1Ab*26 vector were disrupted by sonication and Cry1Ab26 was extracted by alkaline solubilization [33, 34]. Protoxin was dissolved in 50 mM Na_2CO_3 (pH 10.2) containing 50 mM EDTA and 3 % 2-mercaptoethanol for 2 h at 40 °C and the solution was centrifuged at 12,000 rpm for 10 min to remove any insoluble debris [22]. Further, the protoxin was precipitated by adding 4 M NaAc-HAc (pH 4.5) until the pH of the solution reached 5.0, followed by incubating at 37 °C for 4 h. In a preliminary experiment, the insecticide activity against the laboratory cultures of *H. armigera* maintained at 27 °C temperature and 70 % relative humidity with photoperiod of 12 h light and 12 h dark was assayed by exposing neonate larvae to an artificial diet (that consisted of nutrient agar 6.5 g, yeast extract 10 g, ascorbic acid 1.3 g, sorbic acid 250 mg, methyl parahydroxy benzoate 1 g, casein 5 g, cholesterol 55 mg, streptomycin sulfate 100 mg, formaldehyde 0.5 ml, vitamin-E 400 mg, multivitamin 400 mg, and bengal gram powder 55 g. Nutrient agar was molten with 260 ml water and added to the above mixture and the surface was smeared with different concentrations (0.46, 0.93, 1.39, 1.85, and 2.32 μg toxin/cm^2 of diet) of the partially purified Cry1Ab26 in 12-well plates. Partially purified Cry1Ab obtained from the standard strain, HD1, acted as standard check. As the mortality (number of dead insects/total number of insects used) of the larvae was comparable between 1.85 and 2.32 μg toxin/cm^2 of diet, further insect bioassay was carried out with 1.85 μg toxin/cm^2 of diet. Twelve larvae were used per treatment and each treatment was triplicated and mortality was recorded periodically up to 5 days. The cumulative mortality of the larvae was recorded for 5 days after the exposure [23].

Results and Discussion

Initial rep-PCR-based analysis of DOR Bt-1 isolate indicated that the strain belonged to *B. thuringiensis ssp. kurstaki* (data not shown). Wettable powder formulation of this isolate was effective against different insect pests of castor and rice [24, 26] and preliminary insect bioassay carried out in vitro with crude protein extracted from DOR Bt-1 isolate had indicated that the strain was effective against three major lepidopteran insect pests *H. armigera, A. janata*, and *Spodoptera litura* (data not shown). It is well established that lepidopteran pest tolerance is imparted by *cry1* and/or *cry2* genes [35–37]. Therefore, it was conjectured that DOR Bt-1 isolate would be carrying *cry1* and/or *cry2* genes. Genomic PCR of DOR Bt-1 isolate with universal primers for *cry*I class of genes amplified a-277 bp fragment while primers for *cry2* class genes amplified 689–701-bp fragments [27] indicating that DOR Bt-1 carried both *cry1* and *cry2* genes (Supplementary Fig. 1A). PCR with Lep1A, Lep1B primer pairs [28] amplified 490-bp fragment indicating that the strain carried either *cry1Aa/cry1Ab/cry1Ac* genes. Subsequent PCR with Lep2A, Lep2B primer pairs yielded 986 bp which indicated the presence of *cry1Aa/cry1Ac* in the strain and 908-bp fragment which confirmed the presence of *cry1Ab* gene (Supplementary Fig. 1B). Therefore, to further confirm whether the strain carried *cry1Aa* and/or *cry1Ac*, specific primers for *cry1Aa* and *cry1Ac* were used and the amplification of specific, expected size products with these confirmed the presence of *cry1Aa and cry1Ac* genes in the strain (data not shown). Among the different *cry* genes deployed in transgenic plants, *cry1Ab* is an important one with transgenic plants developed in several crops such as corn, tomato, tobacco, and cotton have already been commercialized [17] and in crops such as potato, rice, and brinjal the developed transgenics have been field tested [38]. Cry1Ab is known to show synergistic interaction with other crystal proteins such as Cry1Ac in insecticidal activity against different insects [39–41]. Also, Bt *cry1Ab* gene is known to be effective against different insects including *H. armigera*, an important polyphagous pest [18, 42]. Considering the importance of *cry1Ab* gene in terms of its effectiveness against many lepidopteran pests and especially against *H. armigera*, full-length *cry1Ab* gene was cloned from DOR Bt-1 strain and characterized.

Cloning and Sequencing of the *cry1Ab* from DOR Bt-1

The entire coding region of the *cry1Ab* gene was amplified by PCR with *cry1Ab* gene specific primers. The PCR amplified 3.5-Kb full-length gene was purified and cloned into pGEM-T vector. The recombinant construct pGEM-T–*cry1Ab* was confirmed for the presence of full-length *cry1Ab* gene using restriction digestions (Fig. 1) and sequenced. BLAST analysis of the sequence confirmed that the isolated gene corresponded to *cry1Ab* gene, but showed variations compared to the previously reported *cry1Ab*

genes. The gene consisted of 3,465 bases coding for a protein of 1,155 amino acid residues with an estimated molecular weight of 130 kDa and a pI value of 5.02. This sequence was designated *cry1Ab26* by the *B. thuringiensis* delta-endotoxin nomenclature committee, and thus the isolated gene became a new member of Bt toxins. The sequence of *cry1Ab26* is deposited in genetic sequence database at the National Center for Biotechnical Information (NCBI) (GenBank ID: HQ847729).

The deduced amino acid sequence of Cry1Ab26 was compared with all known Cry1Ab. Cry1Ab26 has seven amino acid residues (His_{180}, Ser_{333}, Ala_{491}, Asp_{762}, Glu_{908}, Pro_{1031}, and Tyr_{1035}) different from those of the known Cry1Ab (supplementary Fig. 2A). Among these variations, His_{180}, Ser_{333}, and Ala_{491}, were found within the three active domains of the protein, while the remaining four were in the carboxyl terminal part of the protein which gets cleaved by protease activity and is not part of the activated peptide of the crystal protein. Alignment of Cry1Ab26 with known Cry1Ab revealed differential amino acid residue changes which were as follows: In comparison to Cry1Ab1 (M13898), Cry1Ab3 (M15271), Cry1Ab4 (D00117), Cry1Ab5 (X54939), Cry1Ab9 (X54939), Cry1Ab11 (I12419), Cry1Ab12 (AF0596 70), Cry1Ab13 (AF254640), Cry1Ab15 (AF358861), and Cry1Ab20 (DQ241675) seven residues, viz., His_{180}, Ser_{333}, Ala_{491}, Asp_{762}, Glu_{908}, Pro_{1031}, and Tyr_{1035} were different in Cry1Ab26. When compared with Cry1Ab2 (M12661) and Cry1Ab18 (AY319967), 62 and 54 amino acid residues were different (supplementary Fig. 2B). Also, Cry1Ab26 differed with respect to 17, 13, 10, 10, 11, 13, 11, 11, 8, and 10 amino acid residues when compared with Cry1Ab6 (M37263), Cry1Ab7 (X13233), Cry1Ab8 (M16463), Cry1Ab10 (A29125), Cry1Ab14 (U94191), Cry1Ab16 (AF375608), Cry1Ab17 (AY646166), Cry1Ab19 (AY847289), Cry1Ab21 (EF683163), and Cry1Ab22 (EU220269) sequences, respectively. Similar comparisons of the derived amino acid sequences with the existing database of crystal proteins have been carried out by others [23]. It is important to note that several mutation studies with Cry1Ab have indicated that even a few amino acid changes, especially at critical positions, have altered the binding or toxicity of the protein [12, 13, 43]. The variations observed in Cry1Ab26 are not the previously characterized mutations, and so the effect of these variations with respect to toxicity against insects could not be predicted and needs to be empirically determined.

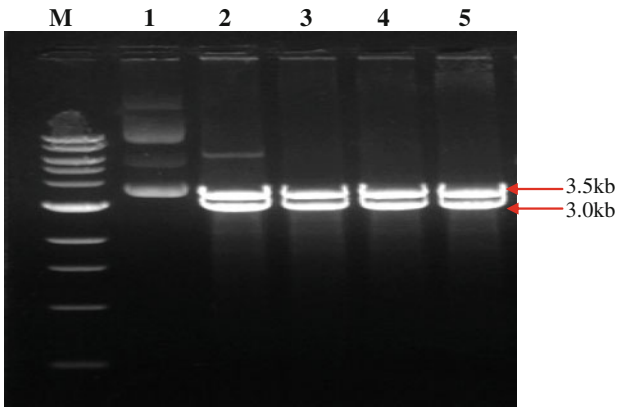

Fig. 1 Confirmation of the recombinant plasmid pGEM-T—*cry1Ab26* using restriction analysis. *Lane M*: 1-Kb DNA marker; *lane 1*: Uncut recombinant plasmid; *lane 2–5*: pGEM-T—*cry1Ab26* recombinant plasmid digested with *Nde*I and *Xho*I

Three dimensional structure of the crystal protein is demonstrated to be important for its interaction with the receptor proteins for getting inserted into the membrane and for pore formation in the gut region of insects [44]. So far, three dimensional protein structures have been determined through X-ray crystallography for seven different Bt toxins and for five crystal proteins by homology modeling [45]. The three dimensional structure of Cry1Ab26 was predicted by means of the PHYRE^2 program. As Cry1Ab26 showed 87 % identity with Cry1Aa, the 3-D structure of Cry1Ab26 was predicted based on the structural alignment with that of Cry1Aa. Similarly, the three dimensional structures of two other Cyr1Aa proteins (Cry1Aa17 and Cry1Aa 22) have been predicted by homology modeling [45, 46]. Based on the predicted structure, domain I of Cry1Ab26 consisted of 221 (Tyr_{33} to Arg_{253}) residues in 7 α-helices with an estimated molecular weight of 25.6 kDa and the modified residue Q180H was embedded in the loop between helices α5 and α6, domain II consisted of 198 residues (Arg_{265} to Phe_{462}) in three antiparallel β-sheets folded into three loops with a calculated molecular weight of 21.9 kDa and the altered F333S embedded in loop of strands β3–β4 and domain III consisted of 147 residues (Asn_{464} to Thr_{610}) in sandwich of two antiparallel β-sheets with a predicted molecular weight of 15.8 kDa and the variant residue G491A was embedded in loop between strands β14 and β15 (supplementary Fig. 3). The N- and C-termini of activated Cry1Ab26 based on the predicted cleavage sites of protease reaction appeared to be Ile_{29} and Thr_{610}.

Expression of *cry1Ab26* and Western Blot Analysis

Full-length *cry1Ab26* was cloned from pGEMT—*cry1Ab26* into pET29(a) vector and the recombinant clones were confirmed for the presence of full-length *cry1Ab26* gene using restriction digestions (Fig. 2) and PCRs. The confirmed clone, pET29(a)—*cry1Ab26,* was completely sequenced and transferred into *E. coli* BL21 (DE3) for expressing the gene under induction conditions. The SDS-PAGE analysis with the protein extracted after induction with IPTG showed a protein band of 130 kDa which was absent in the protein isolated from the strain transformed with empty vector

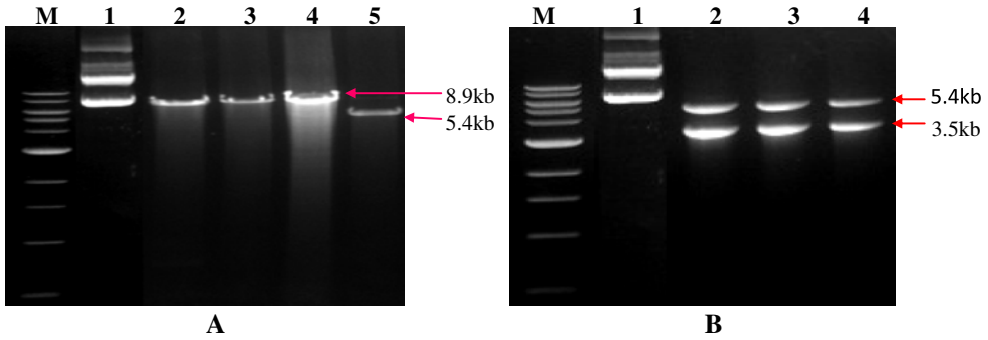

Fig. 2 Confirmation of the recombinant plasmid pET29a(+)—*cry1Ab26* using restriction analysis. **a** *Lane M*: 1 Kb DNA marker; *lane 1*: Uncut recombinant plasmid; *lane 2–4*: pET29a(+)—*cry1Ab26* recombinant plasmid digested with *Nde*I; *lane 5*: pET29a(+) vector digested with *Nde*I. **b** *Lane M*: 1 Kb marker; *lane 1*: Uncut recombinant plasmid; *lane 2–4*: Restriction digestion of pET29a (+)—*cry1Ab26* with *Nde*I and *Xho*I. Restriction released the 3.5-kb fragment of *cry1Ab26*

[pET29(a)], and corresponded to the estimated size for Cry1Ab26 based on the predicted amino acid sequence of the protein (Fig. 3). Qualitative enzyme-linked immunosorbent assay (ELISA) results confirmed the expression of Cry1Ab26 (Supplementary Fig. 4) and the quantification using Quantiplate™ indicated that the protein concentrations ranged between 6 and 10 ppb after 4–8 h of induction. Western blot analysis of protein extracts with the antibody raised against Cry1Ab26 showed a clear binding of the antibody to the protein band of 130 kDa observed on SDS-PAGE (Fig. 3). This showed that the cloned *cry1Ab26* gene expressed stably to yield full-length protein. Similarly, other workers have expressed the isolated *cry* genes in *E. coli* to confirm the stable expression of the crystal proteins [23, 47, 48].

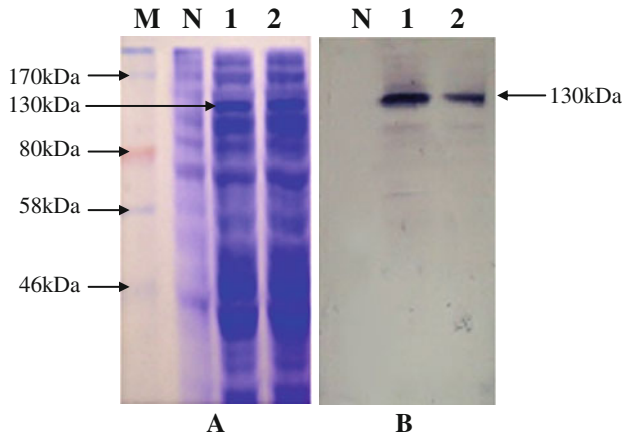

Fig. 3 **a** Confirmation of the expression of Cry1Ab26 in the *E. coli* BL21 (DE3):pET29a(+)—*cry1Ab26* through SDS-PAGE analysis of the total protein. *Lane M*: Multicolored Protein marker; *lane N*: Negative control (*E. coli* BL21 (DE3):pET29a(+)); *lane 1*: Total protein from the recombinant clone of Cry1Ab26; *Lane 2*: Total protein from the clone of Cry1Ab of standard Bt HD1. **b** Western blot analysis of Cry1Ab26 using monoclonal rabbit anti-Cry1Ab antibody. *Lane N*: Negative control; *lane1*: Cry1Ab26 band of DOR Bt-1; *lane 2*: Cry1Ab band of Bt HD1

Insect Bioassay

The polyphagous noctuid insect pest *H. armigera* feeds and breeds on 181 host species belonging to 45 plant families in India [49] inflicting huge economic losses [19] and the insect is susceptible to Cry1Ab [18, 23, 47]. Therefore, bioassay was carried out with the partially purified Cry1Ab26 using neonate larvae of *H. armigera*. Partially purified Cry1Ab from the standard strain HD1 was used as a check to assess the relative efficiency. Preliminary insect bioassay with different concentrations indicated that Cry1Ab26 at 1.85 μg toxin/cm^2 of diet was as effective as 2.32 μg toxin/cm^2 of diet. Therefore, further insect bioassay was carried out at 1.85 μg toxin/cm^2 of diet. Results of this bioassay showed

Table 1 Insecticidal assay results carried out with the neonate larvae of *Helicoverpa armigera* with the partially purified Cry1Ab26 protoxin

Treatment[a]	Mortality (%)[b] of *H. armigera* larvae
Partially purified Cry1Ab26 protein	97
Partially purified Cry1Ab from Bt HD-1	86
Protein from control *E. coli* cells	0
Control	0

[a] Partially purified Cry1Ab26 protein refers to the protein isolated from recombinant *E. coli* BL21 transformed with pET-29a(+)—*cry1Ab26* after induction with IPTG; Partially purified Cry1Ab from Bt HD-1 refers to the protein isolated from recombinant *E. coli* BL21 transformed with pET-29a(+)—*cry1Ab* from Bt HD-1 strain and this was used as the standard check; Protein from control *E. coli* cells refers to protein isolated from *E. coli* BL21 transformed with the basal pET 29a(+) vector. Control refers to the diet surface smeared with the buffer used for suspending the crystal proteins

[b] Mortality as % of larvae dead after 5 days of feeding on diet surface smeared with crystal protein at 1.85 μg toxin/cm^2 of diet. The experiment was carried out in three replications with 12 larvae per replication

cumulative mean percent mortality of 97 % after 5 days of feeding on the crystal protein smeared diet while the Cry1Ab from standard strain (HD1) showed 86 % mortality after the same period. Larvae on control diet or the diet smeared with the protein isolated from the *E. coli* cells carrying only basal pET29a(+) vector showed no mortality (Table 1). Therefore, we infer that *cry1Ab26* gene could be a potential member of *cry* genes for developing transgenic plants with imparted resistance against lepidopteran pests.

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