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Distribution of *CRY1* and *CRY2* Genes in *Bacillus thuringiensis* Isolates from India

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A B S T R A C T

Bacillus thuringiensis (*Bt*) based biopesticides are a viable alternative to chemical pesticides. A total of 150 isolates with a Bt index of 0.17 having crystalline inclusions were identified as *Bacillus thuringiensis*. Here, we report the distribution of lepidopteran-specific *cry1* and *cry2* genes in native isolates of *Bacillus thuringiensis*. Twenty-seven isolates showed amplification with *cry1* and *cry2* universal primers, whereas 123 isolates did not show the presence of any of the *cry1* and *cry2* genes. The restriction fragment length polymorphism analysis of the PCR amplicons revealed that all 27 isolates may carry only one variant of the *cry1* and *cry2* family genes, while multiple fragments were observed in the *cry1* type gene of *Bacillus thuringiensis* var. *kurstaki* HD-1. The partial and full-length DNA sequence analysis confirmed that the putative *cry* genes of twenty-seven Bt isolates and the reference strain matched the known insecticidal crystal protein-encoding genes. Three out of twenty-seven isolates presented nucleotide substitutions, deletions, and insertions in the receptor binding and pore-forming domains of *cry1* and *cry2* genes. The natural variations found in the receptor-binding and pore-forming domains of the genes isolated in this study may lead to increased insecticidal activity of the *cry* proteins. The method used in this study to identify and characterize new insecticidal *cry* genes may prove useful in discovering novel *cry* genes to overcome insect resistance.

***Key words*:** *Bacillus thuringiensis, Cry* genes PCR, RFLP, DNA sequence

Providing food security for an exponentially growing population is a primary challenge. It is estimated that there will be 9.2 billion people by 2050, which would require a 70% increase in food production [21]. The competition from insect pests substantially hampers this process. Annually, one-fifth of the world's crop production is lost, resulting in heavy economic losses. Most devastating insects belong to the order Lepidoptera [47]. *Helicoverpa armigera* is one of the lepidopteran pests that can attack more than 180 plant species [57] across Asia, Europe, Africa, and Australia. These insect pests cause damage worth two billion dollars annually.

The most common method used for controlling insect pests is the application of chemical insecticides. Unfortunately, chemical insecticides invariably show long-term residual action and toxicity to a wide range of organisms leading to public and environmental concerns. The use of Bt spore preparations as an alternative to chemical pesticides has the potential to be highly effective and environment-friendly. Bt is a soil microbe that produces *cry* and *cyt* insecticidal crystal proteins (ICPs) [54] and vegetative insecticidal proteins (Vip) [19] that are toxic to the specific orders of insect pests [22, 31, 25, 41, 50]. Bt strains have been isolated from several heterogeneous environments, including soil [7, 32, 66], stored grains [39], phyllosphere [33], and other miscellaneous habitats [3], [38], [58], [65]. Bt produces ICPs during sporulation. Several ICPs have been identified that are highly toxic to coleopteran, lepidopteran, and dipteran insect species [28]. It has been reported that more than 200 ICP-coding genes have been cloned, sequenced, and classified based on their amino acid homology into 74 *cry* and 2 *cyt* types [15]. The *cry* genes consist of three domains. The first domain is responsible for the formation of pores in the insect's midgut; the second domain binds in a specific way to epithelial cells of the insect's midgut; the third domain stabilizes the bond between toxin and receptor, resulting in osmotic discrepancy, and finally death of the insect [40]. The *cry1* and *cry2* proteins exhibit the most robust activity against Lepidopteran insects; *cry3*, *cry7*, and *cry8* are toxic to Coleoptera; while *cry4* and *cry11* are highly active against Diptera. Some of the *cry* gene encoded ICPs also show toxicity to more than one insect order, e.g., *cry1I*, which is toxic to both Lepidoptera and Coleoptera [62]. In addition, Bt strains active against nematodes, mites, and protozoa have also been isolated [15], [16], [54], [67]. However, many insect pests show no

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susceptibility to *cry* ICPs or are poorly controlled by the *cry* proteins.

The evolution of resistance to Bt-crops in the field has been documented for at least five insect species [5], [26], [59], [60], [64]. *H. armigera* is a lepidopteran insect spread throughout the world and is a pest of numerous crops, primarily cotton (*Gossypium spp*.). Due to its recidivist nature in evolving resistance to conventional insecticides, it poses a threat to transgenic cotton in Australia, India, and China [23], [68]. There has been evidence in Australia [1], India [34], and China [20], [37] that this species has the potential to develop resistance to Bt toxin (c*ry1Ac*). Resistance to *cry1Ac* has also been reported in Indian populations of *Pectinophora gossypiella* [18] and *Helicoverpa zea* [11], [2], [60]. *H. armigera* had become resistant to the Bt toxin *cry2Ab* and was found to have happened due to evolutionary mutations in a protein of the ABC transporter subfamily A of the insect [62]. In another study, it was reported that PBW larval incidence on *Bt* II cotton was high at 28.85–72.49% during 2014–2017 [69].

There are currently more than 50,000 strains of Bt isolated from diverse environments worldwide. These strains exhibit a variable level of toxicity against different insect pests. Recent outbreaks due to insect resistance development have made it even more critical to find novel insecticidal genes/strains despite the availability of many Bt strains and their insecticidal genes [42], [44], [51]. In general, insect pests develop resistance to Bt due to alterations in any of the steps involved in the mechanism of action of Bt *cry* proteins, such as solubilization, proteolytic processing, passage through the peritrophic membrane, receptor binding, membrane insertion, pore formation, and osmotic lysis of midgut cells [27], [65]. Saleem and Shakoori [52] found that changes in a few amino acids can alter the toxicity spectrum of *cry* proteins. A change in the loop I of the receptor-binding domain of *cry3A* N-endotoxin enhanced its toxicity in coleopteran larvae [55]. Rajamohan *et al*. [4] documented that amino acid substitutions in loop II increased toxicity against gypsy moth larvae 8-fold. It also increased binding affinity to brush border membrane vesicles (BBMV) in the midgut of gypsy moths fourfold. Lee [35] isolated the strains harbouring natural variations with the increased toxic activity of the ICPs. Protein solubility in insect guts may be improved by the amino acid changes caused by missense mutations. As a result of the modifications in the solvent-exposed region of the protein, *cry3Aa*'s pK decreased, allowing a greater solubility in *D. virgifera* gut fluid and enhanced activity [55]. Accordingly, variations in the toxicity of *cry* proteins are genetically determined and can be controlled by inducing mutations in *cry* genes or isolating *cry* genes with natural variations. Thus, this study is aimed to isolate lepidopteran-specific novel *cry1* and *cry2* ICP-encoding genes that are specifically toxic to *H. armigera.*

MATERIALS AND METHODS

Bacillus thuringiensis isolates

In this study, 150 isolates of Bt that we previously identified were used to screen *cry* genes. The *Bacillus thuringiensis* serovar *kurstaki* var. HD-1 (henceforth mentioned as Btk HD-1) shown to be toxic against lepidopteran insects like *Spodoptera* sp. and *Helicoverpa* sp. [8], was used as a reference strain in this study.

Total DNA isolation

DNA was extracted using a procedure similar to that described by Delecluse *et al*. [17] with some modifications. The Bt strains were grown in 1 mL of Luria Bertani (LB) (Himedia # M1245) medium and, vegetative cells were harvested at the exponential phase (A650, OD 1.5). The cells were suspended in 1/10 volume of Lysis buffer [Tris-HCL (0.1 M) (pH 8.0), EDTA (0.1 M), NaCl (0.15 M), and 1/10 volume lysozyme (10 mg/mL)] and incubated at 37 °C for 30 min. Following lysis, samples were treated with 10 µL RNase (10 mg/mL) and incubated at 70 °C for 20 min. Finally, 10 µL of Proteinase K (10 mg/mL) was added to the suspension and incubated at 45 °C for 2 h. The supernatant collected after centrifugation at 12000 rpm at 4 °C for 10 min was mixed with equal volumes of ice-cold isopropanol. The suspension was centrifuged at 14000 rpm at 4 °C for 15 min. The pellet was washed once with 70% ethyl alcohol and air-dried. The air-dried pellet was suspended in 10 mM TE buffer (pH 8.0) containing 0.1 M Tris-HCl and 1 mM EDTA.

Screening the Bt isolates for cry1 and cry2 genes

The *cry1 and cry2* genes were amplified by polymerase chain reaction (PCR) using universal primers (Table 1). Amplification was carried out in a 25 µL reaction mixture containing 2.5 µL of *Pfu* DNA Polymerase Buffer (10 X) with $MgSO_4$ (20 mM), 50 pmol each primer, 0.2 µL dNTPs (25 mM each), and 1 µL DNA template (100 ng/µL), 0.5 µL (1.0 U) of *Pfu* DNA Polymerase (Promega, USA), and nuclease-free water to a final volume of 25 µL. The PCR reactions were performed in a thermal cycler (Eppendorf, Germany). The amplification protocol included a denaturation step of 4 min at 94 °C, thirty-five cycles of denaturation (94 °C for 40 sec), annealing (45 °C for 50 sec for *cry1* and 59 °C for 1 min for *cry2*), and extension (1–2 min) at 72 °C, followed by a final extension step at 72 °C for 7 min. The amplified product was analyzed on 1% agarose gel made in TBE buffer. Electrophoresis was carried out at 80 V in 1X TAE buffer for 60 minutes. An appropriate DNA marker was run alongside in a lane. To visualize DNA bands, gels were viewed under ultraviolet light (λ = 254 nm), and images were captured using a Gel Imaging System (Bio-Rad, USA.).

Table 1 Sequences of universal primers used for amplification of the cry1 and cry2 genes

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Product size	Reference
Cry1	MDATYTCTAKRTCTTGACTA TRACRHTDDBDGTATTAGAT	45	1.5–1.6 kb	Juarez-Perez *et al*. [30]
Cry2	GTTATTCTTAATGCAGATGAATGGG CGGATAAAATAATCTGGGAAATAGT	59	698–701 bp	Ben-Dov *et al*. [6]

Restriction fragment length polymorphism analysis of the PCR amplicons

Restriction fragment length polymorphism (RFLP) of PCR amplicons was performed using the method of Patel and Ingle [46] with some minor modifications. RFLP was performed to determine if the amplicons contained different *cry* family gene fragments. Based on an in-silico analysis of the *cry* genes (data not shown) available in the gene bank database, two restriction enzymes, HindIII and AflII, were selected. The PCR amplified products were added directly into the reaction mix

containing 1 µL fast digest buffer and 10 U of the fast digest restriction enzyme per reaction. The entire reaction mixture was diluted with nuclease-free water to a final volume of 20 µL and incubated at 37 °C for 30 min. The digested products were analysed on a 2% agarose gel containing 0.5 µg/mL ethidium bromide. The electrophoresis was carried out at 100 V in 1X TAE running buffer for 60 minutes. An appropriate DNA marker was run alongside in a lane. Gels were viewed under UV light (λ = 254 nm), and images were captured using the Gel Imaging System (Bio-Rad, USA).

Cloning and sequence determination

"A" tailing of blunt-end PCR Amplicons

The amplicons generated using high fidelity proofreading polymerase (*pfu*) lack *"*A*"* overhangs which are required to clone the fragments into a TA cloning vector. So, to add adenine residues to the PCR fragments, a reaction was set up by adding 2 µL of blunt-ended DNA fragment (from PCR), 2 µL of *Taq* Polymerase Buffer (10 X) with 15 mM $MgCl_2$, 2 µL dATP (1 mM), 1 µL *Taq* DNA polymerase (3 U/µL) (Genie, Bengaluru), and nuclease-free water to a final volume of 10 µL to a 0.2 mL PCR tube and incubated at 70 °C for 15-20 min in the thermal cycler (Eppendorf, Germany). The PCR fragments were then purified using the QIAquick PCR Purification Kit (Qiagen # 28104) and further used for cloning into the TOPO vector.

Cloning of PCR fragments into TOPO vector

The cloning reaction was set up by adding 2 µL of fresh PCR product tailed with adenine overhangs, 1 µL salt solution, 2 µL water, and finally 1 µL TOPO vector to a 1.5 mL polypropylene tube (Tarsons, India). The contents in the tube were gently mixed and incubated for 5 min at room temperature (22–23 °C). The reaction mixture was then transferred to ice and mobilized into chemically competent *E. coli* cells (one-shot TOP10 competent cells, Invitrogen, USA). The transformed *E. coli* cells were spread over LB agar containing kanamycin (50 µg/mL), IPTG (100 mM), and X-Gal (20 mg/mL) and incubated overnight at 37 °C. The white/light blue colonies were analysed by colony PCR using M13 primers. The PCR amplicons produced by M13 primers were sequenced using the Sanger method at Eurofins Genomics India Ltd., Bangalore. Amplicons were sequenced in both forward and reverse directions. The nucleotide sequences were then subjected to homology search using the BLAST program of NCBI (National Centre for Biotechnology Information).

PCR amplification of full-length cry type genes

Oligonucleotide primers designing

The nucleotide sequences with the best match to the partial *cry* type sequences were used as templates for designing primers for amplification of full-length *cry* genes (Table 2). The full-length *cry* genes were isolated only from the isolates whose *cry* genes showed variation at the nucleotide level. Btk HD-1 was used as a reference strain.

PCR amplification

Amplification of *cry* genes was carried out in a 20 µL reaction mixture containing 2.5 µL of *Pfu* DNA Polymerase Buffer (10X) with $MgSO_4$ (20 mM), 50 pmol of each primer, 0.2 µL of dNTPs (25 mM each), and 1 µL DNA template (100 ng/µL), 0.5 µL (1.0 U) of *Pfu* DNA Polymerase (Promega, USA), and nuclease-free water to a final volume of 20 µL. All PCR reactions were performed in a thermal cycler (Eppendorf AG, Germany) and the PCR parameters were: initial denaturation of 94 °C for 5 min followed by 30 thermal cycles of denaturation at 94 °C for 1 min, annealing for 50 sec (refer Table 2 for annealing temperature), and extension at 72 °C for 2 min, with a final extension step of 15 min at 72 °C. PCR products were analyzed in a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide. The electrophoresis was carried out at 80 V along with a 1 kb size DNA ladder. The gels were viewed under ultraviolet light (λ = 254 nm) to visualize the bands, and images were captured using a Gel Imaging System (Bio-Rad, USA.).

Table 2 Primer sequences for the amplification of full-length cry genes in Bt isolates and the reference strain

Isolate	Primer code	Gene	Direction	NCBI hit	Primer sequence (5'-3')	Annealing temp
BRI-86	86C1	*Cry1*	Forward Reverse	KF938681	ATGGATAACAATCCGAACATC CTATTCCTCCATAAGGAG	53.5
BRI-118	118C1	*Cry1*	Forward Reverse	KF938681	ATGGATAACAATCCGAACATC CTATTCCTCCATAAGGAG	55
BRI-148	148C1	*Cry1*	Forward Reverse	MN729491	ATGAAACTAAAGAATCAA CTACATGTTACGCTCAAT	50
BRI-86	86C2	*Cry2*	Forward Reverse	MK184479	ATGAATAGTGTATTGAATAG TTAATAAAGTGGTGAAATATTAG	50
BRI-118	118C2	*Cry2*	Forward Reverse	EU623976	ATGAATAGTGTATTGAATAG TTAATAAAGTGGTGAAATATTAG	50
BRI-148	148C2	*Cry2*	Forward Reverse	MK184479	ATGAATAGTGTATTGAATAG TTAATAAAGTGGTGAAATATTAG	50
Btk HD-1	HD-1C1	*Cry1*	Forward Reverse	CP009999	ATGGATAACAATCCGAAC CTATTCCTCCATAAGGAG	53.5
Btk HD-1	HD-1C2	*Cry2*	Forward Reverse	CP009999	ATGAATAGTGTATTGAATAGCGG TTAATAAAGTGGTGAAATATTAG	53.6

Cloning and sequence determination of full-length cry genes

Cloning DNA fragments into TA cloning vector

The adenine residues were added to the PCR blunt end fragments following the method explained earlier. The purified DNA fragments were cloned into the Pcr 2.1-TOPO TA (Invitrogen, USA) cloning vector following the kit manufacturer's instructions. The clones were then mobilized into chemically competent *E. coli* cells (One-shot TOP10 competent cells, Invitrogen, USA). The transformed *E. coli* cells were spread over LB agar containing kanamycin (50 µg/Ml), IPTG (100 Mm), and X-Gal (20 mg/Ml) and incubated overnight at 37°C. The white/light blue colonies were analyzed by colony PCR using M13 primers (Table 3).

Nucleotide sequence determination

The M13 primers amplified PCR amplicons were sequenced by the Sanger sequencing method. Nucleotide sequence homology search and analysis were done using the BLAST program of NCBI (National Centre for Biotechnology Information). The sequences were analysed to compare with all previously reported *cry*1 and *cry2* gene sequences.

Table 3 M13 universal primers

Primer	Direction	Primer sequence (5'-3')	Annealing temperature (°C)
M13	Forward	GTAAAACGACGGCCAG	52
M13	Reverse	CAGGAAACAGCTATGAC	

RESULTS AND DISCUSSION

Screening of Bt isolates for cry1 and cry2 genes

The universal primers amplified prominent partial DNA fragments of ~1500 and ~700 bp in the Bt isolates carrying *cry1* and *cry2* type genes (Fig 1-2). Twenty-seven Bt isolates and the reference strain were found to carry both the *cry* genes. The *cry1* and *cry2* related sequences were detected in five isolates, each from Andhra Pradesh and Uttar Pradesh, three each from Haryana, Himachal Pradesh, Maharashtra, and Rajasthan, and four from Tamil Nadu. While only one isolate from Gujarat showed amplification for both genes (Table 4).

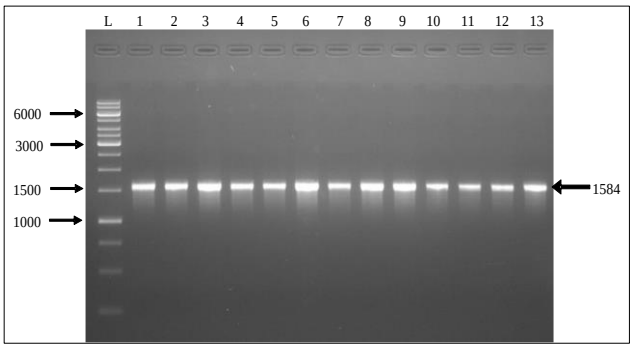

Fig 1 Representative image of the *cry1* gene amplified in Bt isolates and Btk HD-1. Lanes: L: DNA molecular weight marker (1 kb), 1: Btk HD-1, 2: BRI-5, 3: BRI-11, 4: BRI-46, 5: BRI-52, 6: BRI-69, 7: BRI-74, 8: BRI-86, 9: BRI-111, 10: BRI-118, 11: BRI-124, 12: BRI-136, and 13: BRI-148

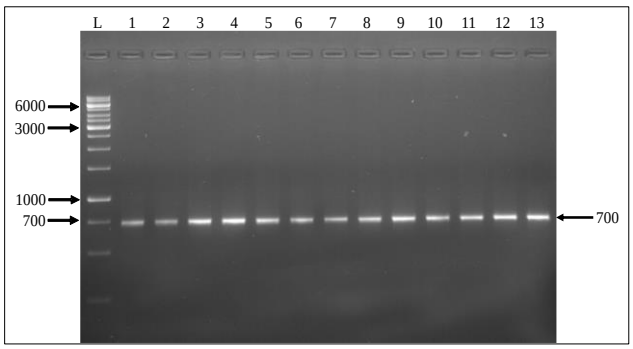

Fig 2 Representative image of the *cry2* gene amplified in Bt isolates and Btk HD-1. Lanes: L: DNA molecular weight marker (1 kb), 1: Btk HD-1, 2: BRI-5, 3: BRI-11, 4: BRI-46, 5: BRI-52, 6: BRI-69, 7: BRI-74, 8: BRI-86, 9: BRI-111, 10: BRI-118, 11: BRI-124, 12: BRI-136, and 13: BRI-148

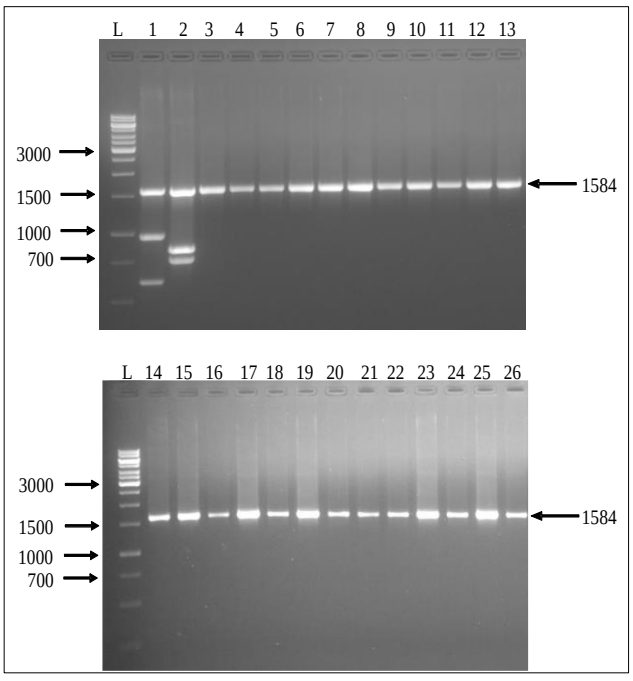

Fig 3 Representative image showing RFLP analysis of 1.6 kb *cry1* type gene PCR amplicons. L: DNA molecular weight marker (1 kb), Lanes: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 with the PCR amplicons of: Btk HD-1, 2: BRI-5, 3: BRI-11, 4: BRI-46, 5: BRI-52, 6: BRI- 69, 7: BRI-74, 8: BRI-86, 9: BRI-111, 10: BRI-118, 11: BRI-124, 12: BRI-136, and 13: BRI-148 respectively digested with HindIII. Lanes: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 with the PCR amplicons of 1: Btk HD-1, 2: BRI-5, 3: BRI-11, 4: BRI-46, 5: BRI-52, 6: BRI- 69, 7: BRI-74, 8: BRI-86, 9: BRI-111, 10: BRI-118, 11: BRI-124, 12: BRI-136, and 13: BRI-148 respectively digested with AflII

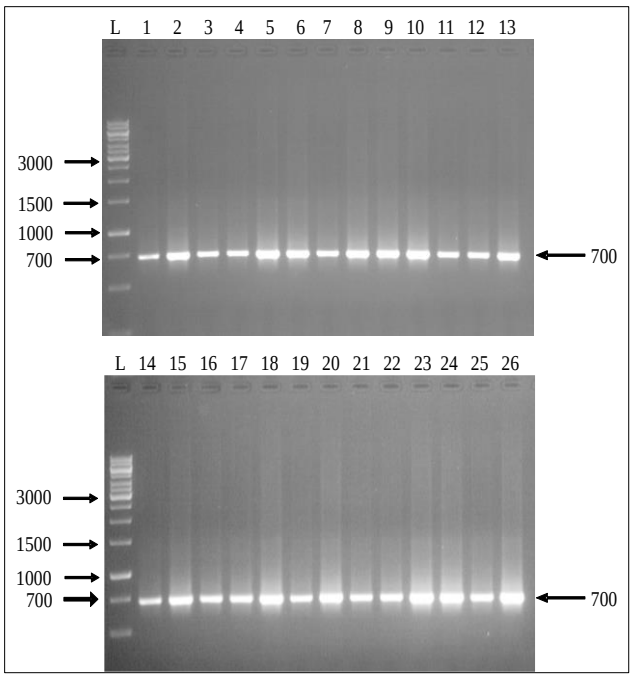

Fig 4 Representative image showing RFLP analysis of 700 bp *cry2* type gene PCR amplicons. L: DNA molecular weight marker (1 kb), Lanes: 1,3,5 ,7 ,9, 11, 13, 15, 17, 19, 21, 23, 25 with the PCR amplicons of: Btk HD-1, 2: BRI-5, 3: BRI-11, 4: BRI-46, 5: BRI-52, 6: BRI- 69, 7: BRI-74, 8: BRI-86, 9: BRI-111, 10: BRI-118, 11: BRI-124, 12: BRI-136, and 13: BRI-148 respectively digested with HindIII. Lanes: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 with the PCR amplicons of 1: Btk HD-1, 2: BRI-5, 3: BRI-11, 4: BRI-46, 5: BRI-52, 6: BRI- 69, 7: BRI-74, 8: BRI-86, 9: BRI-111, 10: BRI-118, 11: BRI-124, 12: BRI-136, and 13: BRI-148 respectively digested with AflII

RFLP analysis of cry type gene amplicons

The RFLP analysis of the PCR products using HindIII and AflII restriction enzymes demonstrated two different kinds of PCR-RFLP profiles in the *cry1* type gene of Btk HD-1, indicating that there could be multiple *cry1*-like genes. Restriction digestion was not seen in any of the *Bt isolates' cry1* and *cry2* type gene fragments (Fig 3-4). This indicates that only one *cry1* and *cry2* type gene might be present in the Bt isolates.

Cloning and sequence determination of partial cry genes

Polymerase chain reaction (PCR) amplification of the recombinant pCR 2.1-TOPO plasmids carrying partial *cry1 and cry2* type genes using M13 primers (Table 3) produced 1800 and 900 bp fragments, respectively. Each M13 PCR amplicon has an additional 200 bp which is part of the M13 regions of the pCR 2.1-TOPO plasmid. The blast analysis of the DNA sequences revealed that they belong to *cry* gene family members and are 97-100% similar to previously reported *cry1* and *cry2* genes. The c*ry1* partial sequences of BRI-86, BRI-118, and BRI-148 were 99.49, 98.93, and 98.02% identical to those of the known *cry1* genes, respectively. The *cry1*-like gene of the isolate BRI-86 and BRI-118 matched to *cry1A* type gene, while that of the isolate BRI-148 matched to *cry1I* type gene. The *cry2* partial sequences of isolates BRI-86, BRI-118, and BRI-148 were 99.43, 99.43, and 98.86% identical to known *cry2* gene sequences. All other isolates and the reference strain had *cry1* and *cry2* gene sequences that matched 100% with known *cry* gene sequences (Table 4).

Table 4 Blast analysis data of partial cry type genes isolated from Bt isolates and Btk HD-1

Isolate	Location	ICP Mol. Wt. (KDa)	*cry1* gene hit (Accession no.)	Percent similarity	*cry2* gene hit (Accession No.)	Percent similarity
BRI-5	Andhra Pradesh	130 & 60	GU322939	100	KJ710647	100
BRI-6	Andhra Pradesh	130 & 60	KF938681	100	MK184479	100
BRI-11	Andhra Pradesh	130 & 60	KP122960	100	KJ710647	100
BRI-15	Andhra Pradesh	130 & 60	GU322939	100	MK184479	100
BRI-16	Andhra Pradesh	130 & 60	EU282379	100	FJ032364	100
BRI-31	Gujarat	130 & 60	GU322939	100	FJ032364	100
BRI-34	Haryana	130 & 60	EU282379	100	MK184479	100
BRI-40	Haryana	130 & 60	GU322939	100	EU623976	100
BRI-42	Haryana	130 & 60	KF938681	100	MK184479	100
BRI-46	Himachal Pradesh	130 & 60	KF938681	100	FJ032364	100
BRI-51	Himachal Pradesh	130 & 60	EU282379	100	FJ032364	100
BRI-52	Himachal Pradesh	130 & 60	GU322939	100	KJ634087	100
BRI-74	Maharashtra	130 & 60	GU322939	100	FJ032364	100
BRI-75	Maharashtra	130 & 60	KF938681	100	EU623976	100
BRI-69	Maharashtra	130 & 60	EU282379	100	KJ710647	100
BRI-86	Rajasthan	130 & 60	KF938681	99.49	MK184479	99.43
BRI-94	Rajasthan	130 & 60	KF938681	100	EU623976	100
BRI-111	Rajasthan	130 & 60	GU322939	100	KJ710647	100
BRI-118	Tamil Nadu	130 & 60	KF938681	98.93	EU623976	99.43
BRI-121	Tamil Nadu	130 & 60	KJ619662	100	KJ710647	100
BRI-123	Tamil Nadu	130 & 60	EU282379	100	MK184479	100
BRI-124	Tamil Nadu	130 & 60	GU322939	100	EU909454	100
BRI-133	Uttar Pradesh	130 & 60	EU282379	100	EU623976	100
BRI-136	Uttar Pradesh	130 & 60	GU322939	100	KJ710647	100
BRI-137	Uttar Pradesh	130 & 60	KJ619662	100	MK184479	100
BRI-147	Uttar Pradesh	130 & 60	KJ619662	100	KJ710647	100
BRI-148	Uttar Pradesh	130 & 60	MN729491	98.02	MK184479	98.86
Btk HD-1	Reference strain	130 & 60	CP009999	100	CP009999	100

PCR amplification of full-length cry genes

Primers designed for isolation of full-length *cry1* gene (Table 2; primer code - 86C1 and 118C1) have amplified 3.5 kb fragment in isolates BRI-86, BRI-118, and Btk HD-1, while the oligonucleotide primers (Table 2; primer code - 148C1) designed to isolate *cry1* gene in BRI-148, amplified 2.1 kb fragment (Fig 5). The *cry2* primers (Table 2; primer code – 86C2, 118C2, 148C2, HD-1C1, and HD1C2) amplified a 1.9 kb fragment in all the Bt isolates and Btk HD-1 (Fig 6).

Cloning and sequence determination of full-length cry type genes

Cloning polymerase chain reaction (PCR) amplicons into TA cloning vector

The amplicons were tailed with adenine residues following the method described earlier. The purified full-length *cry* gene fragments were cloned into the TA cloning vector following the kit manufacturer's instructions. The clones were then mobilized into chemically competent *E. coli* (one-shot TOP10 competent cells, Invitrogen, USA). The positive transformants were selected by screening the white/light blue colour colonies using M13 primers.

Nucleotide sequence determination of full-length cry genes

Plasmid DNA was isolated from the clones carrying full-length *cry* genes. The PCR amplification of the recombinant pCR 2.1-TOPO plasmids carrying inserts using M13 primers produced fragments of 3.7 kb in BRI-86, BRI-118 isolates and

Btk HD-1, while the fragment size in BRI-148 was only 2.3 kb. The M13 primers produced a fragment of 2.1 kb in the pCR 2.1-TOPO_*cry2* plasmids of all the isolates, including Btk HD-1. Since the *cry1*-like gene fragments of Btk HD-1 had multiple fragments (as observed in RFLP analysis), all the white/light blue colonies of the *E. coli* cells transformed with pCR 2.1-TOPO_*cry1* were screened using M13 primers and confirmed with HindIII and AflII restriction digestions. Eight of the 22 colonies screened carried a plasmid with the *cry* gene having both HindIII and AflII sites, ten colonies neither had the restriction site for HindIII nor AflII, and four colonies did not have the insert. With this, it is clear that Btk HD-1 contains two *cry1*-like genes. The blast analysis of the *cry-type* sequences revealed that the *cry1* and *cry2* sequences matched the known *cry* gene sequences. The *cry1* type sequences of the isolates BRI-86 and BRI-118 had 99.29% and 98.70% similarities with the *cry1Ac* gene, respectively (GenBank Accession No. KF938681). Even though the *cry1* type sequences from both isolates BRI-86 and BRI-118 showed high homology with the *cry1Ac* gene (GenBank Accession No. KF938681), the nucleotide variations in both genes did not match with each other. The *cry* type gene sequence of BRI-148 showed 97.41% similarity with *cry1I* (GenBank Accession No. KF560475).

The *cry2* type sequences of all three isolates showed 98.74%, 98.53%, and 99.42% similarity to the *cry2Ab* gene (GenBank Accession No. MK184479). While the *cry2* type sequences from three isolates showed similarity with the same gene in the NCBI database, each gene sequence had nucleotide variations at different positions. Among two *cry1*-like genes in Btk HD-1, one matched to *cry1Ac* and the other to *cry1Aa* with a 100% similarity score. The *cry2* type sequence of Btk HD1 matched to *cry2Ab* sequence in the NCBI database with 100% similarity (Table 5).

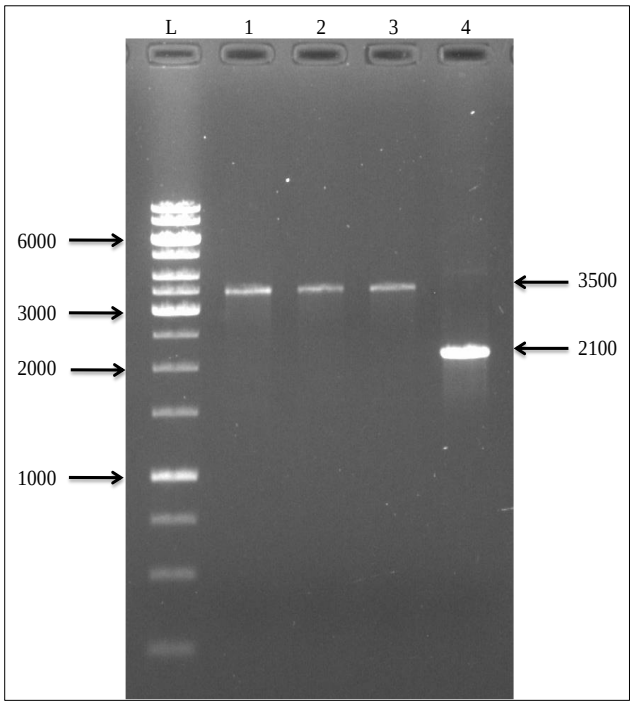

Fig 5 PCR amplification of full-length *cry1* gene. Lanes L: DNA molecular weight marker (1 kb); 1- Btk HD-1, 2- BRI-86, 3- BRI-118, and 4- BRI-148

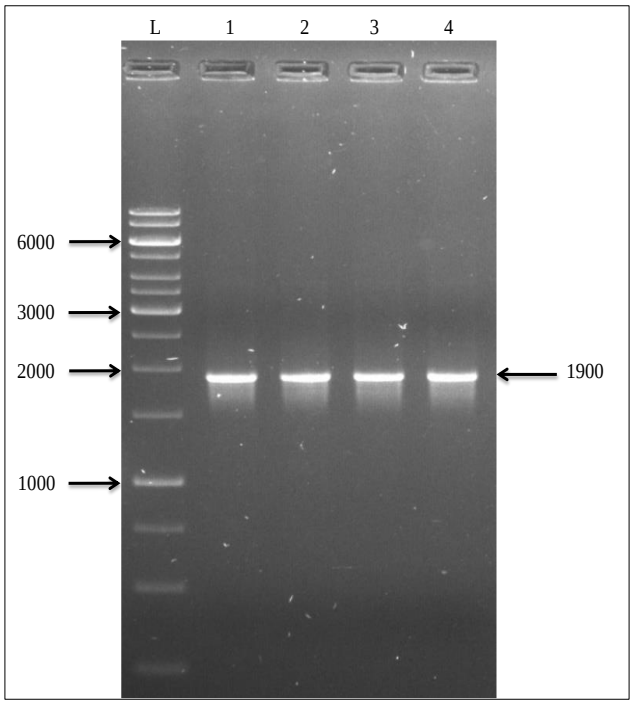

Fig 6 PCR amplification of full-length *cry2* gene. Lanes L: DNA molecular weight marker (1 kb); 1- Btk HD-1, 2- BRI-86, 3-BRI-118, and 4- BRI-148

Table 5 Blast analysis data of full-length cry type genes isolated from Bt isolates and Btk HD-1

Isolate	Gene	Blast hit accession number	Coverage (%)	Identity (%)
BRI-86	Cry1	KF938681	100	99.29
BRI-118	Cry1	KF938681	100	98.70
BRI-148	Cry1	KF560475	100	97.41
BRI-86	Cry2	MK184479	100	98.74
BRI-118	Cry2	MK184479	100	98.53
BRI-148	Cry2	MK184479	100	99.42
Btk HD-1	Cry1	CP009999	100	100.00
Btk HD-1	Cry2	CP009999	100	100.00

The annotation of the nucleotide substitutions and indels revealed that a few variations resulted in missense mutations, while others have led to silent mutations (Table 6). There were no frameshift mutations in any of the genes. The NCBI CDD (NCBI Conserved Domain Database) analysis of full-length genes showed that the *cry* genes in all three Bt isolates had missense mutations in the insect receptor binding and pore-forming domains. BRI-86_*cry1* gene had 5, 3, and 2 missense mutations in domains I, II, and III, respectively, while the BRI-118_*cry1* gene had 8, 7, and 6 missense mutations in the domains I, II, and III, respectively. BRI-148_*cry1I* had 6, 5, and 19 missense mutations in the domains I, II, and III, respectively. Similarly, *cry2* genes also had missense mutations in the receptor-binding and pore-forming domains. BRI-86_*cry2* gene had 2, 11, and 10 missense mutations in domains I, II, and III, respectively, while BRI-118_*cry2* gene had 1 and 3 missense mutations in the domains I and II, respectively. BRI-148_*cry2* had 2, 8, and 1 missense mutations in the domains I, II, and III, respectively (Table 6).

Table 6 Annotated data of the nucleotide variations identified in cry genes isolated from Bt isolates

Isolate	Gene	Position	Nucleotide		Amino acid change	Mutation
			Ref	Alt		
BRI-86	*Cry1*	9	C	T	Nil	Silent
BRI-86	*Cry1*	30	C	G	W10C	Missense
BRI-86	*Cry1*	108	C	T	Nil	Silent
BRI-86	*Cry1*	132	A	G	Nil	Silent
BRI-86	*Cry1*	166	T	A	I56F	Missense
BRI-86	*Cry1*	336	A	T	D112E	Missense
BRI-86	*Cry1*	533	T	C	S178F	Missense
BRI-86	*Cry1*	603	C	T	Nil	Silent
BRI-86	*Cry1*	675	T	A	E225D	Missense
BRI-86	*Cry1*	726	T	A	E242D	Missense
BRI-86	*Cry1*	1025	G	T	V342G	Missense
BRI-86	*Cry1*	1026	A	G		
BRI-86	*Cry1*	1231	GAT/GAC	DEL		
BRI-86	*Cry1*	1657	G	A	I553V	Missense
BRI-86	*Cry1*	1659	A	T		
BRI-86	*Cry1*	2362	C	A	K788Q	Missense
BRI-86	*Cry1*	2493	GAT	DEL	Q993H	Missense
BRI-86	*Cry1*	2979	T	A		
BRI-86	*Cry1*	3197	A	G	C1066Y	Missense
BRI-86	*Cry1*	3473	A	T	V1158E	Missense
BRI-86	*Cry1*	3474	A	T		
BRI-118	*Cry1*	26	A	G	G9E	Missense
BRI-118	*Cry1*	27	A	T		
BRI-118	*Cry1*	60	GTA	DEL		
BRI-118	*Cry1*	90	T	C	Nil	Silent
BRI-118	*Cry1*	189	TGG	DEL		
BRI-118	*Cry1*	230	A	C	P77Q	Missense
BRI-118	*Cry1*	231	A	T		
BRI-118	*Cry1*	336	T	G	L112F	Missense
BRI-118	*Cry1*	518	T	G	G173V	Missense
BRI-118	*Cry1*	534	A	G	Nil	Silent
BRI-118	*Cry1*	576	GAT	DEL		
BRI-118	*Cry1*	593	G	C	A198G	Missense
BRI-118	*Cry1*	594	C	G		
BRI-118	*Cry1*	776	A	G	R259Q	Missense
BRI-118	*Cry1*	777	A	T		
BRI-118	*Cry1*	926	G	C	A309G	Missense
BRI-118	*Cry1*	1064	T	C	A355V	Missense
BRI-118	*Cry1*	1065	G	T		
BRI-118	*Cry1*	1176	T	A	K392N	Missense
BRI-118	*Cry1*	1357	CAT	DEL		
BRI-118	*Cry1*	1373	A	G	S458N	Missense
BRI-118	*Cry1*	1439	G	C	A480G	Missense
BRI-118	*Cry1*	1580	G	A	Q527R	Missense
BRI-118	*Cry1*	1581	G	A		
BRI-118	*Cry1*	1605	C	A	Q535H	Missense
BRI-118	*Cry1*	1785	GAC	DEL		
BRI-118	*Cry1*	2418	GAT	DEL		
BRI-118	*Cry1*	2647	A	G		
BRI-118	*Cry1*	2648	A	G	G883K	Missense
BRI-118	*Cry1*	2649	A	T		
BRI-118	*Cry1*	2994	A	T	H998Q	Missense
BRI-118	*Cry1*	3483	GAC	DEL		
BRI-148	*Cry1I*	42	T	A	K14N	Missense
BRI-148	*Cry1I*	68	A	C	A23D	Missense
BRI-148	*Cry1I*	69	T	C		
BRI-148	*Cry1I*	101	A	G	R34Q	Missense
BRI-148	*Cry1I*	102	A	T		
BRI-148	*Cry1I*	140	A	G	C47Y	Missense
BRI-148	*Cry1I*	1623	T	A	L541F	Missense
BRI-148	*Cry1I*	1625	G	A	D542G	Missense
BRI-148	*Cry1I*	1626	G	C		
BRI-148	*Cry1I*	1627	G	A	T543D	Missense
BRI-148	*Cry1I*	1628	A	C		
BRI-148	*Cry1I*	1631	T	A	K544I	Missense
BRI-148	*Cry1I*	1732	G	A	T578A	Missense
BRI-148	*Cry1I*	1737	T	G	M579I	Missense
BRI-148	*Cry1I*	1739	A	G	S580N	Missense
BRI-148	*Cry1I*	1743	A	T	H581Q	Missense
BRI-148	*Cry1I*	1744	G	A	N582G	Missense
BRI-148	*Cry1I*	1745	G	A		
BRI-148	*Cry1I*	1747	A	C	L583N	Missense
BRI-148	*Cry1I*	1748	A	T		
BRI-148	*Cry1I*	1749	T	A		
BRI-148	*Cry1I*	1946	A	C	A649D	Missense
BRI-148	*Cry1I*	1947	T	C		
BRI-148	*Cry1I*	2078	A	T	F693Y	Missense
BRI-148	*Cry1I*	2145	C	A	K715N	Missense
BRI-86	*Cry2*	612	A	G		Silent
BRI-86	*Cry2*	778	T	C	P260S	Missense
BRI-86	*Cry2*	1208	G	A	E403G	Missense
BRI-86	*Cry2*	1222	G	C	P408A	Missense
BRI-86	*Cry2*	1314	A	C	F438L	Missense
BRI-86	*Cry2*	1319	G	T	I440R	Missense
BRI-86	*Cry2*	1321	C	G	A441P	Missense
BRI-86	*Cry2*	1324	T	G	V442L	Missense
BRI-86	*Cry2*	1356	T	A	R452S	Missense
BRI-86	*Cry2*	1400	T	A	E467V	Missense
BRI-86	*Cry2*	1410	A	T	S470R	Missense
BRI-86	*Cry2*	1436	A	C	A479E	Missense
BRI-86	*Cry2*	1454	A	T	L485H	Missense
BRI-86	*Cry2*	1583	A	C	T528N	Missense
BRI-86	*Cry2*	1666	A	C	P556T	Missense
BRI-86	*Cry2*	1675	G	C	L559V	Missense
BRI-86	*Cry2*	1696	T	C	H566Y	Missense
BRI-86	*Cry2*	1756	C	A	T586R	Missense
BRI-86	*Cry2*	1757	G	C		
BRI-86	*Cry2*	1758	T	C		
BRI-86	*Cry2*	1776	C	G	M592I	Missense
BRI-86	*Cry2*	1777	G	A	R593G	Missense
BRI-86	*Cry2*	1779	T	A		
BRI-86	*Cry2*	1875	A	G	Nil	Silent
BRI-118	*Cry2*	51	T	A	K17N	Missense
BRI-118	*Cry2*	68	C	A	Q23P	Missense
BRI-118	*Cry2*	95	A	C	A32D	Missense
BRI-118	*Cry2*	125	A	T	I42K	Missense
BRI-118	*Cry2*	127	A	C	Q43K	Missense
BRI-118	*Cry2*	612	A	C	Nil	Silent
BRI-118	*Cry2*	613	T	G	V205L	Missense
BRI-118	*Cry2*	852	G	T	H284Q	Missense
BRI-118	*Cry2*	1174	A	G	D392N	Missense
BRI-118	*Cry2*	1187	A	G	G396E	Missense
BRI-118	*Cry2*	1435	G	C	Q479E	Missense
BRI-118	*Cry2*	1444	T	G	A482S	Missense
BRI-118	*Cry2*	1564	T	G	A522S	Missense
BRI-118	*Cry2*	1652	C	T	F551S	Missense
BRI-118	*Cry2*	1653	A	T		
BRI-118	*Cry2*	1657	G	C	R553G	Missense

BRI-148	*Cry1I*	155	C	G	R52P	Missense
BRI-148	*Cry1I*	156	G	T		
BRI-148	*Cry1I*	160	C	G	L54V	Missense
BRI-148	*Cry1I*	162	T	A		
BRI-148	*Cry1I*	166	G	C	L56A	Missense
BRI-148	*Cry1I*	167	C	T		
BRI-148	*Cry1I*	168	A	T		
BRI-148	*Cry1I*	173	C	A	K58T	Missense
BRI-148	*Cry1I*	338	A	C	P113Q	Missense
BRI-148	*Cry1I*	339	A	T		
BRI-148	*Cry1I*	414	GAT	DEL	S174F	Missense
BRI-148	*Cry1I*	521	T	C	D205G	Missense
BRI-148	*Cry1I*	614	G	A		
BRI-148	*Cry1I*	615	A	T	P288Q	Missense
BRI-148	*Cry1I*	863	A	C		
BRI-148	*Cry1I*	864	A	T	A413V	Missense
BRI-148	*Cry1I*	1238	T	C	R461Q	Missense
BRI-148	*Cry1I*	1382	A	G		
BRI-148	*Cry1I*	1383	G	T		
BRI-148	*Cry1I*	1539	T	C	Nil	Silent
BRI-148	*Cry1I*	1577	GGT	DEL		
BRI-148	*Cry1I*	1589	G	C	A530G	Missense
BRI-148	*Cry1I*	1619	C	T	I540T	Missense
BRI-148	*Cry1I*	1620	A	C		
BRI-118	*Cry2*	1664	C	G	C555S	Missense
BRI-118	*Cry2*	1685	A	G	S562N	Missense
BRI-118	*Cry2*	1703	C	G	G568A	Missense
BRI-118	*Cry2*	1750	G	A	R584G	Missense
BRI-118	*Cry2*	1760	T	C	S587F	Missense
BRI-118	*Cry2*	1764	A	T	Nil	Silent
BRI-118	*Cry2*	1766	A	C	A589D	Missense
BRI-118	*Cry2*	1780	A	C	H594N	Missense
BRI-118	*Cry2*	1800	T	G	K600N	Missense
BRI-118	*Cry2*	1802	C	G	C601S	Missense
BRI-118	*Cry2*	1805	A	T	V602D	Missense
BRI-118	*Cry2*	1875	A	G	Nil	Silent
BRI-148	*Cry2*	510	A	T	H170Q	Missense
BRI-148	*Cry2*	561	T	A	L187F	Missense
BRI-148	*Cry2*	857	A	G	R286Q	Missense
BRI-148	*Cry2*	1109	T	C	S370L	Missense
BRI-148	*Cry2*	1115	C	G	R372P	Missense
BRI-148	*Cry2*	1119	T	A	L373F	Missense
BRI-148	*Cry2*	1181	A	G	R394Q	Missense
BRI-148	*Cry2*	1211	T	C	S404L	Missense
BRI-148	*Cry2*	1222	G	C	P408A	Missense
BRI-148	*Cry2*	1238	G	T	V413G	Missense
BRI-148	*Cry2*	1875	A	G	Nil	Silent

This study aimed to characterize and analyse the distribution of lepidopteran-specific *cry1* and *cry2* genes in the native Bt isolates identified from the soil samples collected from diverse locations in India. The genes belonging to *cry1* and *cry2* groups encode ICPs specific to lepidopteran larvae [10]. The distribution of Bt and characterization of *cry* genes have already been studied in a few regions of India [53], [56], but there is very little information available about its distribution in both the north and southern parts of the country. Therefore, studying the distribution of Bt in all four zones of India is useful for planning future Bt deployment strategies. There are several molecular techniques available for detecting *cry* genes; Polymerase Chain Reaction (PCR) can potentially be a valuable and rapid method for characterization and selection of Bt strains [6], [30].

Universal primers were used to screen 150 Bt isolates for the presence of *cry* genes belonging to *cry1* and *cry2* families. Out of 150 isolates, 123 isolates (82%) did not show any amplification with the universal primers. There is a possibility that the primer recognition region of *cry* genes might get truncated due to frequent recombinations in the genome. Due to this *reason, cry* genes may not be amplified in some of the Bt isolates [36]. Twenty-seven isolates (18%) contained both *cry1* and *cry2* type genes. A minimum of two isolates from each state, except Gujarat, had *cry1* and *cry2* type genes. This shows the widespread presence of Bt isolates harbouring *cry1* and *cry2* type genes, which is in agreement with observations of Ben-Dov *et al.* [6]; Bravo *et al.* [9]; Uribe *et al.* [63]; Stobdan *et al.* [58]*;* Armengol *et al.* [4]; Ramalakshmi and Udayasuriyan [49]. Depending on the geographical source, there was a difference in the frequency of isolates carrying *cry1* and *cry2* type genes. This was evident from the number of isolates identified from samples collected from Gujarat and Rajasthan. Only one isolate was identified from 12 samples from Gujarat, while three isolates were from 30 samples from Rajasthan. Uribe *et al.* [63] have reported an ecologically favoured distribution of *cry1* and *cry2* genes in croplands. Also, numerous studies have shown that *cry* genes are geographically distributed [9], [12-13]. Therefore, this study confirms that *cry1* and *cry2* type genes are distributed differentially across various regions in India. RFLP analysis of *cry1* and *cry2* type PCR amplicons revealed that only one type of *cry1 and cry2* gene is present in the Bt isolates, which is in agreement with the findings of Crickmore *et al.* [15]; Hofte and Whiteley [28] detected a single *cry1Ac* gene in the strain HD-73. However, the c*ry1* type PCR amplicon of the reference strain showed multiple fragments after digestion with HindIII and AflII, indicating that the strain has more than one type of *cry1* gene. Crickmore *et al.* [15]; Hofte and Whiteley [28], also identified cry*1Aa* and *cry1Ac* genes in Btk HD-1. The sequencing of PCR amplified partial, and full-length amplicons further confirmed that there are two *cry1* genes (*cry1Ac* and *cry1Aa*) and one *cry2* (*cry2Ab*) in the reference strain. In the present study, the sequence of *cry1* type genes of BRI-86 and BRI-118 matched with *cry1Ac,* and that of BRI-148 matched with *cry1Ia.* The *cry2* type sequences of all three isolates matched with *cry2Ab* genes in the NCBI database. The nucleotide blast and the conserved domain search analysis of the putative *cry* genes revealed that all the sequences had nucleotide variations in the receptor-binding and pore-forming domains. These findings indicate that the *cry* genes in three BRI Bt isolates may be unique and encode novel insecticidal proteins.

Researchers attempted to generate and screen *cry* mutant libraries for enhanced binding and toxicity via phage display [14], [24], [29], [43]. A few amino acid changes can alter the toxicity spectrum of *cry* proteins [52]. It was reported that a change in loop I of the receptor-binding domain of *cry3A* endotoxin increased its toxicity to coleopteran larvae [55], also amino acid substitutions in loop 2 of Domain II increased the toxicity and binding affinity for brush border membrane vesicles (BBMV) by 8 and 4-fold, respectively in gypsy moth [48]. The amino acid changes caused by missense mutations may improve protein solubility in insect guts. *Cry3Aa*'s pK decreased due to mutations in the protein's solvent-exposed region, allowing for greater solubility in *D. virgifera* gut fluid and enhanced activity [55]. The Bt *cry1B*, protein which was altered by using saturation mutagenesis approach, resulted in a 30-fold increase in the activity over the original protein and was found to protect corn from *Hemophilus zea* [55]. Unlike other

genes, the *cry* genes identified in this study have natural variations in the domains that govern receptor binding and pore formation. Lee [35] isolated the strains harbouring natural variations with the increased toxic activity of the ICPs. Adaptative mechanisms may have led to these natural variations as a result of evolution and may have happened selectively in particular regions of the genes. Thus, these *cry-type* genes may be encoding proteins with unique insecticidal properties against *H. armigera*. Hence, these genes can be used as a potential gene bank to replace existing genes for which *H. armigera* has gained resistance.

CONCLUSION

Historically, toxins derived from the Bt bacteria have been used to control insect pest populations, usually through foliar sprays, but transgenic crops have been used more recently. It is crucial to understand their mode of action to maximize their potential. Some insect pests are not susceptible to *cry* toxins, while others acquire resistance due to high selection pressure. In these cases, the absence of toxicity is likely due to interference with the crucial steps in toxin action. The regular search for novel *cry* genes with genetic variations may help counter limitations of toxicity with regards to the mechanism of action of *cry* toxin against the targeted pests. Including such novel toxins into current management strategies will maximize the use of Bt as a tool for crop protection and disease management. In this study, we confirm that the *cry1* and *cry2* genes identified in three Bt isolates have nucleotide substitutions and indels predominantly in the receptor binding and pore-forming domains. Researchers have found that most *cry* toxins are nontoxic, mainly due to their low affinity for binding to receptors. Evolutionary mutations have taken place within the genes encoding for the receptors, which has led to the development of an escape mechanism. Therefore, searching for novel *cry* genes that encode toxins with a high affinity for changing insect mid-gut receptors is imperative. The missense variations in the receptor-binding domains of the *cry* genes identified in this study may promote irreversible binding of the toxin to the receptors and induce osmolysis. It may also help overcome insect resistance to *cry1* and *cry2* toxins. However, to ascertain whether the new toxins are novel, it is necessary to test them against *Helicoverpa armigera*.

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