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Screening of *Bacillus thuringiensis* Isolates Recovered from Diverse Habitats in India for the Presence of Insect and Nematode-active *cry* Genes

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Abstract

Novel *cry* genes with different structure and mode of action are potential candidates for resistant management strategies. Isolation of novel genes needs thorough screening of large number of native *Bacillus thuringiensis* (Bt) isolates collected from diverse habitats and ecotypes. In the present study, 63 Bt isolates recovered from different habitats in India have been investigated for their insecticidal and nematicidal *cry* gene content. Bt isolates were screened through Polymerase Chain Reaction (PCR) using degenerate primers for the presence of *cry*1, *cry*1A and nematode-active *cry* genes. Among the collection of 63 Bt isolates 21 were found positive for the presence of *cry*1-type genes, 49 for *cry*1A-type genes and 20 for nematode-active *cry* genes. Nine Bt isolates were found positive for all three types of *cry* genes. The study also show that PCR based screening method is rapid and highly useful for characterization of Bt isolates to reveal their insecticidal spectrum.

Highlights

- Sixty three native Bt isolates were screened for the presence of insect and nematode-active *cry* Genes.
- Twenty one were found positive for the presence of *cry*1-type genes, 49 for *cry*1A-type genes and 20 for nematode-active *cry* genes.

Keywords: *Bacillus thuringiensis*, PCR screening, *cry*1-type genes, *cry*1A-type genes, nematicidal *cry* genes

Introduction

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that produces crystal inclusions during the sporulation phase. The crystals comprise one or more Cry proteins (δ-endotoxins) that are specifically toxic to insect orders such as *Lepidoptera*, *Diptera*, and *Coleoptera* and also to some nematodes, mites, and protozoa (Schnepf *et al.*, 1998; Bravo *et al.*, 2011). Cry proteins act by binding to receptors and subsequent insertion into the brush border membrane in the midgut of susceptible insects, leading to disruption of osmotic balance, cell lysis and eventually death of the insect (Kaur, 2000; Bravo *et al.*, 2011). Bt has been used as a successful biological insecticide for more than 100 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests (Nester *et al.*, 2002; Romeis *et al.*, 2006). The efficacy and host range of the strain is dependent on the *cry* genes the strain carries (Schnepf *et al.*, 1998; Aly, 2011).

Usually the Bt strains carry a combination of *cry* genes and therefore become effective against different insect pests. Therefore, identifying the *cry* genes carried by a strain provides a clue regarding the utility of the strain against different groups of insects.

However, there is a threat of eventual development of resistance in insects upon large-scale cultivation of transgenic crops (Shelton *et al.*, 2002; Kaur and Gujar, 2004). Development of second-generation Bt transgenic crops requires new insecticidal genes for stacking or pyramiding wherein more than one insecticidal genes are used in combination as a resistance management strategy (Zhao *et al.*, 2003; Kaur, 2006). Therefore, the isolation of novel Bt strains and characterizing them for their insecticidal gene content may lead to the discovery of novel genes with higher toxicity which can provide an alternative to cope up with emergence of insect resistant population against currently deployed limited number of *cry*-type gene (s).

Since the first *cry* gene was cloned from *Bt* ssp. *Kurstaki* HD-1 in 1981 (Schnepf and Whiteley, 1981) the search for new *cry* genes is an ongoing effort worldwide and so far more than 500 different *cry* gene sequences have been classified into 67 groups (Cry1–Cry67) (Crickmore *et al.*, 2010). To identify novel *cry* genes Bt strains have been isolated worldwide from diverse habitats, including soil (Kaur and Singh, 2000a), stored grains (Meadows *et al.*, 1992), phyllospheres (Kaur and Singh, 2000b; Jara *et al.*, 2006) and other miscellaneous habitats (Martinez and Caballero, 2002; Uribe *et al.*, 2003; Apaydin *et al.*, 2005). Among the techniques used in profiling the *cry* genes carried by a strain, PCR based approach is proved to be useful for its sensitivity, reproducibility and rapidity. Therefore, different PCR based methods have been deployed to identify the *cry* genes in *Bt* strains (Porcar and Juarez-Perez, 2003; Beron *et al.*, 2005; Kaur, 2006; Thammasittirong and Attathom, 2008).

The present study was conducted to screen our collection of native Bt isolates for the presence of insect and nematode-active *cry* genes. For that lepidopteron-active genes of *cry*1 family and various nematicidal *cry* genes have been amplified through PCR using degenerate primer sets.

Materials and Methods

Bacterial isolates and strains

Sixty three native Bt isolates recovered from diverse agricultural and non-agricultural locations in India in the corresponding author Dr. S. Kaur's laboratory were used in this study (Table 3). Bt strains used as reference in this study were kindly provided by Dr. D.R. Ziegler, Director, Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA to Dr. S. Kaur (Table 1).

Growth media for bacterial Strains

Luria Bertani Agar (LA) and Luria Bertani Broth (LB) were used for the growth of Bt isolates and strains.

Oligonucleotide PCR primers

A set of general primers (gral-cry1) designed as per Bravo *et al.*, (1998) was used in PCR amplification for the detection of partial *cry*1-type genes and a set of specific primers designed as per Rolle *et al.* (2005) was used for detection of partial *cry*1A-type genes. In addition to insecticidal *cry* genes, Bt isolates were also screened for nematode-active *cry* genes using a set of general primers (gral-nem) designed as per Bravo *et al.*, (1998) (Table 2).

Table 1: List of Bt reference strains

Si. No.	Bt strains	BGSC code	Original code
1.	*B. thuringiensis* subsp. *taumanoffi*	4N1	HD 201(B-30-2)
2.	*B. thuringiensis* subsp. *thuringiensis*	4A6	1715
3.	*B. thuringiensis* subsp. *aizawai*	4J2	HD137 (HDB-24)
4.	*B. thuringiensis* subsp. *israelensis*	4Q5	4Q2-72
5.	*B. thuringiensis* subsp. *darmstadiensis*	4M2	HD199(102)
6.	*B. thuringiensis* subsp. *aizawai*	4J4	HD11
7.	*B. thuringiensis* subsp. *kurstaki*	4D1	HD1
8.	*B. thuringiensis* subsp. *Sotto*	4E3	*sotto*
9.	*B. thuringiensis* subsp. *Kenyae*	4F1	HD136
10.	*B. thuringiensis* subsp. *Alesti*	4C3	HD4 (*B. alesti* 143)

Table 2: Characteristics of primer sets used in PCR amplification

Primer pair	Gene(s) recognized	Product size (bp)	Sequence	References
gral-cry1	*cry*1Aa, *cry*1Ad, *cry*1Ab, *cry*1Ae, *cry*1Ac, *cry*1Af, *cry*1Ba, *cry*1Bb, *cry*1Bc, *cry*1Ca, *cry*1Cb, *cry*1Da, *cry*1Db, *cry*1Ea, *cry*1Fa, *cry*1Eb and *cry*1Fb	543-594	Forward; 5'CTGGATTTAC AGGTGGGGATAT3' Reverse; 5'TGAGTCGCTTCGCATA TTTGACT3'	Bravo *et al.* (1998)
U/19-merL/18-mer	*cry*1A	450	Forward; 5'-CAAGATGGGCACGCA AGAC-3' Reverse; 5'-ACGACCCGGACAGAC ACG-3'	Rolle *et al.* (2005)
gral-nem	*cry*5Aa, *cry*5Ab, *cry*5Ac, *cry*5B, *cry* 12A, *cry*14A and *cry*21Aa	474-489	Forward; 5'TTACGTAAATTGGTC AATCAAGCAAA3' Reverse; 5'AAGACCAAATT CAATACCAGGGTT 3'	Bravo *et al.* (1998)

Genomic DNA extraction

Genomic DNA was extracted from Bt isolates by the method modified by Kalman *et al.* (1993). A single colony from a freshly streaked plate was incubated into 5 ml LB medium containing penicillin (10 µg ml^{-1}) and incubated at 28°C overnight with shaking at 150 rpm. This starter culture was added to 50 ml LB medium and incubated at 28^0C with shaking at 150 rpm to an optical density of 0.8 at 600 nm. The cells were harvested by centrifuging at 7000 rpm for 10 min (Beckman JA 20 rotor) at 4°C. The cell pellet was washed in 5 ml TES buffer (10 mM tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) and suspended in 5 ml of resuspension solution (25% sucrose, 25 mM Tris-HCl, 25 mM EDTA, pH 8.0) containing 1 mg ml^1 lysozyme. The cell suspension was incubated at 37°C for 1 hour. 10% SDS was added to the suspension to a final concentration of 2% and the suspension was incubated at 50^0C for 15 min and then at 4°C overnight. The suspension was centrifuged at 10,000 rpm for 15 min and the supernatant was carefully taken out. DNA in the supernatant was precipitated with 2 volumes of ethanol. DNA was resuspended in 10 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 M NaCl, 10 µg ml^{-1} RNase and 0.6 mg ml^{-1} Proteinase K and incubated at 30°C for 30 min. The mixture was extracted with phenol-chloroform (1:1) and DNA was precipitated with ethanol. DNA pellet was washed once with 70% ethanol, air-dried and dissolved in 300 ìl of TE buffer.

Plasmid DNA extraction

Plasmid DNA was isolated from Bt isolates and reference strains by using Qiagen Plasmid Midi kit (Qiagen, Germany). A single colony from a freshly streaked plate was inoculated into 5 ml LB medium containing penicillin (10 µg ml^{-1}) and incubated at 28°C with shaking at 150 rpm. Overnight grown cultures were pelleted by centrifugation at 7000 rpm for 10 min at 4°C. Method as described in the supplier's manual was followed, with the modification of preheating of elution buffer to 50°C for isolation of large plasmids. DNA pellet was dissolved in 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Plasmid DNA was size fractionated on 0.8% agarose gel along with 1 kb DNA ladder (MBI Fermentas, Germany). DNA bands were observed under UV in the gel documentation system (Alpha gel imager).

PCR analysis

PCR reaction was carried out for amplification of *cry*1-type, *cry*1A-type and nematode-active *cry* genes using general set of primers. PCR was carried out in a reaction mixture of 25 ìL containing DNA template 50 ng; PCR buffer with $(NH_4)_2SO_4$ and $MgCl_2$ (10X) 2.5 µl; deoxy ribonucleotide triphosphate (dNTPs) (2mM) 2.5 µl; primers (1 µM) 1 µl each; Taq DNA polymerase 1.0 U and sterile distilled water in thermal cycler (BioRad). Amplification of lepidopteron-active *cry*1-type genes and nematode active *cry* genes were carried out using general primer sets gral-cry1 and gral-nem, respectively, for 30 cycles of denaturation at 94°C for 1 min, primer annealing at 43°C for 1 min and extension at 72°C for 1.5 min. Amplification of *cry*1A-type genes with a set of specific primers (U/19-

mer and L/18-mer) was carried out for 30 cycles with parameters similar to earlier except primer annealing which is at 54°C. In each PCR, first cycle of denaturation was performed for 2 min and the last cycle of extension was performed for 10 min. PCR products were visualized on 1.5% agarose gel using 1 kb DNA ladder marker.

Results and Discussion

Detection of partial *cry*1-type genes

Sixty three native Bt isolates and 10 reference Bt strains were subjected to PCR amplification using the gral-cry1 primer set to detect various genes of *cry*1 gene family. The PCR amplicon of expected size of about 550 bp with varying intensity corresponding to the highly conserved region of cry1 gene family was generated in 21 native Bt isolates and 6 reference Bt strains (Table 3). Care was taken to avoid non-specific amplification and for that *B. thuringiensis* subsp. *israelensis* (4Q5) was used as a negative control and as expected amplicon of 550 bp was not found. One representative gel picture is shown here for 24 Bt isolates and strains (Fig. 1).

Detection of cry1A-type genes

Same set of Bt isolates along with Bt reference strains were also screened through PCR for detection of various genes of *cry*1A gene family using specific primer set (U/19-mer and L/18-mer). The prominent PCR amplicon of expected size 450 bp corresponding to the highly conserved region of *cry*1A gene family was observed in 49 native Bt isolates and 6 reference Bt strains (Table 3). *B. thuringiensis* subsp. *israelensis* (4Q5) was used as a negative control and amplification of 450 bp band was not found. One representative gel picture is shown here for 24 Bt isolates and strains (Fig. 2).

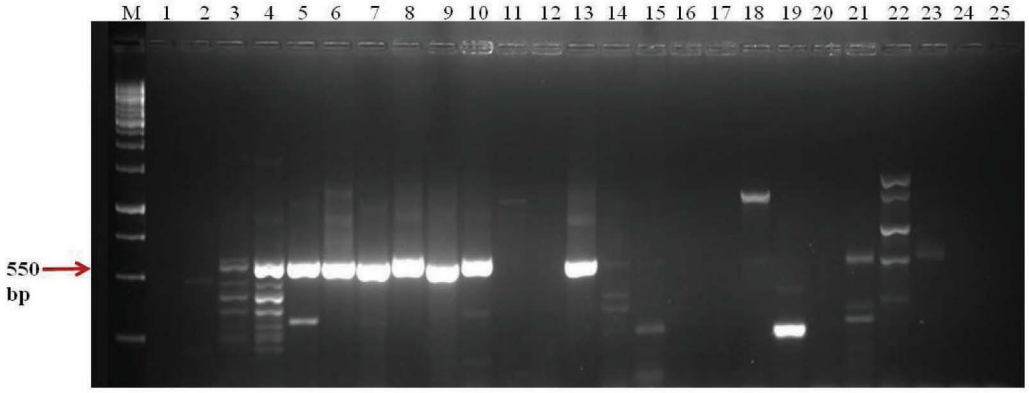

Fig. 1: Representative picture of PCR amplification for *cry*1-type genes in Bt isolates and strains. M: 1kb DNA Ladder; 1: SK-217; 2: SK-957; 3: SK-980; 4: SK-935; 5: SK-223; 6: 4N1; 7: 4A6; 8: 4J2; 9: 4J4; 10: 4C3; 11: 4M2; 12: 4Q5; 13: SK-711; 14: SK-921; 15: SK-219; 16: SK-1; 17: SK-4; 18: SK-9; 19: SK-13; 20: SK-20; 21: SK-28; 22: SK-82; 23: SK-84; 24: SK-88 and 25: SK-222

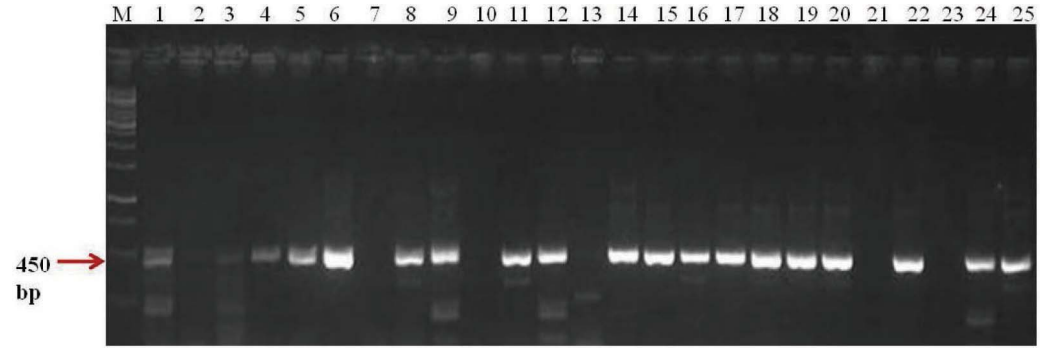

Fig. 2: Representative picture of PCR amplification for *cry*1A type genes in Bt isolates and strains. M: 1kb DNA Ladder; 1: 4A6; 2: 4Q5; 3: SK-84; 4: SK-217; 5: SK-944; 6: SK-223; 7: SK-405; 8: SK-942; 9: SK-301; 10: SK-721; 11: SK-921; 12: SK-973; 13: SK-930; 14: SK-629; 15: SK-722; 16: SK-922; 17 SK-851; 18: SK-958; 19: SK-977; 20: SK-980; 21: SK-995; 22: SK-13; 23: SK-794; 24: SK-222 and 25: SK-935.

Table 3: Prevalence of insect and nematode-active *cry* gene(s) in native Bt isolates

Si. No.	Bt Isolates	Source	Set 1*	Set 2*	Set 3*
1.	SK-1	Chickpea field, Rohtak, HR	-	+	-
2.	SK-4	Chickpea field, Rohtak, HR	-	-	-
3.	SK-9	Cotton field, Agreoha, UP	-	+	-
4.	SK-13	Cotton field, Malout, PB	-	+	-
5.	SK-20	Cotton field, Malout, PB	-	-	-
6.	SK-28	Soil near spring, HP	+	-	-
7.	SK-82	Grain dust,Bhareri, HP	+	+	+
8.	SK-84	Soil, Baramulla, J&K	+	-	-
9.	SK-88	Chickpea and mustard field, Dungarpur, RJ	-	-	-
10.	SK-110	Chickpea field, HP	-	+	+
11.	SK-217	Chickpea phyllosphere, IARI, ND	-	+	-
12.	SK-219	Chickpea phyllosphere, IARI, ND	-	+	-
13.	SK-222	Chickpea phyllosphere, IARI, ND	-	+	-
14.	SK-223	Pea phyllosphere, IARI, ND	+	+	+
15.	SK-232	Pea phyllosphere, IARI, ND	-	+	+
16.	SK-301	Field of nematode infestion, IARI, ND	-	+	-
17.	SK-304	Field of nematode infestion, IARI, ND	-	+	-
18.	SK-305	Field of nematode infestion, IARI, ND	-	+	-
19.	SK-405	Chilli field black soil, Vemboor, TN	-	-	-
20.	SK-449	Chilli field black soil, Vemboor, TN	-	+	-
21.	SK-463	Chilli field black soil, Vemboor, TN	-	+	+
22.	SK-617	Cattle shed, Vemboor, TN	-	-	+
23.	SK-629	Cattle shed, Vemboor, TN	-	+	-
24.	SK-677	Kitchen garden, Alahbad	+	+	-
25.	SK-678	Kitchen garden, Alahbad	+	+	+
26.	SK-711	Red gram field, Lam, Guntur, AP	+	+	+
27.	SK-721	Soil from cotton field, Lam, Guntur, AP	-	-	-
28.	SK-722	Soil from cotton field, Lam, Guntur, AP	-	+	+
29.	SK-741	Cotton seeds Var: LK-389, Guntur, AP	-	+	-
30.	SK-753	Chickpea seeds, Lam, Guntur, AP	-	-	-
31.	SK-754	Chickpea seeds, Lam, Guntur, AP	+	+	+
32.	SK-792	Chilly seeds Warehouse, Guntur, AP	-	+	-
33.	SK-794	Chilly seeds Warehouse, Guntur, AP	+	-	
34.	SK-851	Wheat field, Burdwan, WB	-	+	-
35.	SK-921	Desert soil, Sriganganagar, RJ	-	+	+
36.	SK-922	Desert soil, Sriganganagar, RJ	-	+	+
37.	SK-930	Desert soil, Sriganganagar, RJ	+	-	-
38.	SK-935	Desert soil, Sriganganagar, RJ	+	+	+
39.	SK-942	Desert soil, Sriganganagar, RJ	-	+	-
40.	SK-944	Desert soil, Sriganganagar, RJ	-	+	-
41.	SK-952	Cotton field, Sriganganagar, RJ	+	+	-
42.	SK-953	Cotton field, Sriganganagar, RJ	+	+	+
43.	SK-956	Cotton field, Sriganganagar, RJ	-	+	+
44.	SK-957	Cotton field, Sriganganagar, RJ	-	+	+
45.	SK-958	Cotton field, Sriganganagar, RJ	+	+	-
46.	SK-959	Desert soil, Sriganganagar, RJ	-	+	-
47.	SK-960	Desert soil, Sriganganagar, RJ	+	+	-
48.	SK-962	Desert soil, Sriganganagar, RJ	-	+	-
49.	SK-973	Cotton field, Sriganganagar, RJ	-	+	+
50.	SK-977	Cotton field, Sriganganagar, RJ	+	+	-
51.	SK-980	Cotton field, Sriganganagar, RJ	+	+	+

Contd.

Si. No.	Bt Isolates	Source	Set 1*	Set 2*	Set 3*
52.	SK-995	Cotton field, Sriganganagar, RJ	-	-	-
53.	SK-996	Grain dust, Sriganganagar, RJ	+	+	-
54.	SK-1007	Insect infested wheat grain, FCI godown, RJ	-	+	+
55.	SK-1008	Insect infested wheat grain, FCI godown, RJ	+	+	-
56.	SK-1009	Insect infested wheat grain, FCI godown, RJ	-	-	-
57.	SK-1025	Barren land, RJ	+	+	-
58.	SK-1026	Barren land, RJ	-	+	-
59.	SK-1027	Barren land, RJ	-	+	-
60.	SK-1028	Barren land, RJ	+	+	+
61.	SK-1034	Soil, Beminar, J&K	-	+	-
62.	SK-1035	Soil, Beminar, J&K	-	-	-
63.	SK-1036	Soil, Beminar, J&K	-	+	-

AP: Andra Pradesh, RJ: Rajasthan, WB: West Bengal, ND: New Delhi, J&K: Jammu and Kashmir, HP: Himachal Pradesh, TN: Tamil Nadu, PB: Punjab, HR: Haryana
Set 1*: PCR amplification for *cry*1-type genes using primer set 'gral-cry1'
Set 2*: PCR amplification for *cry*1A-type genes using primer set 'U/19-mer_L/18-mer'
Set 3*: PCR amplification for nematode-active *cry* genes using primer set 'gral-nem'
(+) : Represents the presence of the expected amplicon
(-) : Represents the absence of the expected amplicon

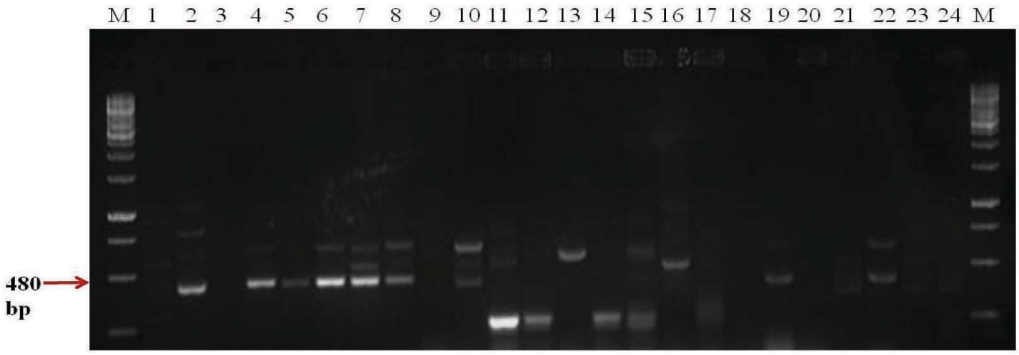

Fig. 3: Representative picture of PCR amplification for nematode-active *cry* genes in Bt isolates and strains. M: 1kb DNA Ladder; 1: SK-9; 2: SK-223; 3: SK-219; 4: SK-957; 5: SK-921; 6: SK-935; 9: SK-4J4; 10: SK-232; 11: SK-1; 12: SK-4; 13: SK-13; 14: SK-20; 18: SK-88; 19: SK-110; 20: SK-217; 21: SK-222; 22: SK-232; 23 and SK-301.

Detection of nematode-active *cry* genes

To characterise collection of native Bt isolates for the presence of the nematicidal *cry*-type genes like *cry*5Aa, *cry*5Ab, *cry*5Ac, *cry*5B, *cry*12A, *cry*14A and *cry*21Aa, another set of general primers (gral-nem) was used in PCR amplification. The expected band of 480 bp with varying intensity was observed in 20 native Bt isolates and 4 Bt strains (Table 3). One representative gel picture is shown here for 24 Bt isolates and strains (Fig. 3).

Isolation of native Bt isolates from diverse habitats and ecotypes is of utmost importance to identify novel insecticidal *cry* genes. It is emphasized that discovery of novel *cry* genes with new or broad activity spectra or higher toxicity is important for the development of new products and the management of insect resistance (Xue *et al*., 2008; Darsi *et al*., 2010). Among the various molecular techniques, PCR screening with degenerate primers has proven to be a very useful and rapid method for detection of the *cry* genes (Porcar and Juarez-Perez, 2003; Kaur, 2006).

In the present study, a collection of native Bt isolates were screened for the presence of insecticidal and nematicidal *cry*-type genes through PCR using degenerate primer sets. Twenty one Bt isolates were found positive with amplicon of expected size when amplified with gral-cry1 primers while 49 Bt isolates were found positive when amplified with primer set (U/19-mer and L/18-mer). For amplification

of nematode-active *cry* genes a set of general primers (gral-nem) was used and 20 isolates were found positive. In the PCR amplification for *cry*1-type genes and nematode-active *cry* genes comparatively less number of isolate were found positive and many dense bands of unexpected size were also observed. This may be due to higher degeneracy and broad specificity of gral-cry1 and gral-nem primers and dense amplicons of unexpected size might be novel *cry*-type genes which need to be further characterized. Large numbers of positive isolates with specific amplicon were found with the primers specific to *cry*1A-type genes due to less degeneracy and higher specificity in the primer sequences.

This may be very useful preliminary study of strain characterization for *cry* gene content in their genome before isolation of specific full length genes. The isolates found positive in this study may be further used for isolation of corresponding full length gene by using gene specific primers or TAIL-PCR. On the basis of this study one full length gene has been amplified in the Bt isolate SK 711 by PCR using primers specific to *cry*1Aa,b,c (Meena *et al.*, 2012). The gene was cloned, sequenced and classified as *cry*1Ac33 by the *Bacillus thuringiensis* Nomenclature Committee (http://www.biols.susx.ac.uk/Home / Neil_Crickmore/Bt/). Further studies of screening of native Bt isolates and cloning of novel full length genes are going on in our laboratory which may be highly useful to tackle the problem of emergence of insect resistance towards limitedly utilized *cry* genes in transgenic plants.

Conclusion

A collection of 63 native Bt isolates were characterized to reveal their insecticidal and nematicidal *cry* gene content. Different set of degenerate primers specific to various genes of different gene families were used for PCR amplification. In the study, 21 Bt isolates were found positive for *cry*1, 49 for *cry*1A and 20 for nematode-active *cry*-type gene.

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