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Prevalence of cry2-type genes in *Bacillus thuringiensis* isolates recovered from diverse habitats in India and isolation of a novel cry2Af2 gene toxic to *Helicoverpa armigera* (cotton boll worm)

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1 **Prevalence of *cry2*-type genes in *Bacillus thuringiensis* isolates recovered from diverse**
2 **habitats in India and isolation of a novel *cry2Af2* gene toxic to *Helicoverpa armigera***
3 **(cotton boll worm)**

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8 **Running Title:**

9 Novel *cry2Af2* gene from Bt

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Abstract

Insecticidal *cry* and *vip* genes from *Bacillus thuringiensis* (Bt) have been used for control of lepidopteran insects in transgenic crops. However, novel genes are required for gene pyramiding to delay evolution of resistance to the currently deployed genes. Two PCR-based techniques were employed for screening of *cry2*-type genes in 129 Bt isolates from diverse habitats in India and 27 known Bt strains. The *cry2Ab*-type genes were more prevalent than *cry2Aa*- and *cry2Ac*-type genes. Correlation between source of isolates and abundance of *cry2*-type genes was not observed. Full length *cry2A*-type genes were amplified by PCR from nine Bt isolates and four Bt strains. The genes from Bt isolates SK-758 from Sorghum grain dust and SK-793 from Chilli seeds warehouse, Andhra Pradesh, were cloned and sequenced. The gene from SK-758 (NCBI GenBank accession GQ866915) was novel, while that from SK-793 (NCBI GenBank accession GQ866914) was identical to *cry2Ab1* gene. *Bacillus thuringiensis* Nomenclature Committee (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/BT/) named these genes as *cry2Af2* and *cry2Ab16* respectively. The *cry2Af2* gene was expressed in *Escherichia coli*, and found to be toxic towards *Helicoverpa armigera*. The *cry2Af2* gene will be useful for pyramiding in transgenic crops.

Key words: *Bacillus thuringiensis*, *cry2* genes, *cry2Af2*, *Helicoverpa armigera*, insecticidal genes

46 Introduction

47 *Bacillus thuringiensis* (Bt) is an aerobic, Gram-positive, spore-forming, facultative bacterial
48 pathogen that produces parasporal crystals containing one or more insecticidal crystal
49 proteins (Cry), which are selectively toxic to insects (Schnepf et al. 1998; Frankenhuyzen
50 2013; Adang et al. 2014). Bt has been used as a biopesticide in agriculture, forestry and
51 mosquito control (Kaur 2000). Synthetic *cry* genes, modified for plant-preferred codon usage,
52 have been transferred into crop plants for insect resistance (Koziel et al. 1993). Bt transgenic
53 corn, cotton, tomato, potato and soybean have been developed (James 2013). Search for
54 novel *cry* and *vip* (vegetative insecticidal proteins) genes from native Bt isolates is an on-
55 going effort worldwide (Kaur 2006; Crickmore et al. 2014,
56 http://www.biols.susx.ac.uk/Home/Neil_Crickmore/BT/).

57 *Helicoverpa armigera* is a polyphagous pest of several crops such as cotton, pigeon
58 pea, chickpea, sorghum and tomato worldwide, which has developed resistance to chemical
59 pesticides (Subramanian and Mohankumar 2006). Of the different types of *cry* genes, *cry1Ac*
60 has been found to be most toxic towards *H. armigera* (Liao et al. 2002). However,
61 development of resistance in *H. armigera*, exposed to *cry1Ac* gene upon large scale
62 cultivation of first generation transgenic crops, is a disconcerting possibility (Kaur 2012;
63 Pardo-López et al. 2013; Fabrick et al. 2014). Gene pyramiding, deploying more than one
64 type of insecticidal genes, having different receptors in the insect midgut, is the best strategy
65 to delay the onset of resistance (Tabashnik et al. 2013; Carriere et al. 2015). The *cry2*-type
66 genes are promising candidates for gene pyramiding due to difference in structure and mode
67 of action. In heliothine type insects such as *H. armigera*, the Cry2-type proteins have been
68 shown to have binding sites different from those of other Cry proteins deployed in transgenic
69 crops such as Cry1Ac, Cry1F and Vip3A proteins (Gouffon et al. 2011). The *cry1Ac*-
70 resistant *H. armigera* did not show cross resistance to *cry2Aa* (Akhurst et al. 2003).

71 Furthermore, synergistic effect of combination of Cry1Ac and Cry2Ab proteins has been
72 observed towards *H. armigera* (Ibargutxi et al. 2008).

73 Novel *cry2*-type genes have been identified by PCR and cloned in many laboratories in
74 India and elsewhere (Misra et al. 2002; Kumar and Udayasuriyan 2004; Sauka et al. 2005,
75 Beard et al. 2008; Lin et al. 2008; Shu et al 2013; Somwatcharajit et al. 2014). PCR-based
76 identification of *cry* genes was first developed by Carozzi et al. (1991) and has remained the
77 method of choice due to its sensitivity, rapidity and ease of performance (Porcar and Juarez-
78 Perez 2003). In this study, 129 native Bt native isolates, recovered from different locations in
79 India (Table S1), and 27 known Bt strains (Table S2) have been screened for presence of
80 *cry2*-type genes, using two different sets of primers designed from conserved regions of *cry2*-
81 type genes. Full length amplification of *cry2*-type genes has been carried out using open
82 reading frame (ORF) primers, and a novel *cry2Af2* gene has been isolated.

83 **Materials and Methods**

84 **Bacterial strains and plasmids**

85 One hundred and twenty nine Bt isolates recovered from diverse habitats in India
86 (Kaur and Singh 2000) were used (Table S1, Fig S1). Twenty seven known Bt strains and
87 *E. coli* strain harbouring cloned *cry2Aa1* gene (ECE 126) obtained from *Bacillus* Genetic
88 Stock Centre (BGSC), Department of Biochemistry, Ohio State University, Columbus, Ohio
89 43210, USA were used as reference strains (Table S2). The vector pGEM-T (Promega) was
90 used for cloning of *cry2*-type genes and the vector pET29a (+) (Novagen) was used for
91 protein expression. *E. coli* strains DH5 α and BL21 (DE3) were used for transformation with
92 cloning and expression vectors respectively.

93 **Isolation of plasmid DNA from Bt**

94 Plasmid DNA was isolated from native Bt isolates and known Bt strains using
95 alkaline lysis method (Sambrook et al. 1989).

96 **Oligonucleotide primers for screening and full length amplification of *cry2*-type genes**

97 Bt isolates and reference strains were screened for the presence of *cry2* class (family)
98 and subfamily (specific type) genes using two different sets of primers designed as per Ben-
99 Dov et al. (1997) and Masson et al. (1998, Table S3). For full length amplification of *cry2*-
100 type genes, ORF primers were designed from known *cry2* genes in NCBI
101 (www.ncbi.nlm.nih.gov) database. For cloning into pET29a(+) vector, an *EcoRI* site at 5' end
102 and a *HindIII* site at 3' end of amplified PCR product were designed (Table S4). PCR was
103 performed as: initial denaturation of 3 min at 94°C, followed by 30 cycles each of
104 denaturation at 94°C for 1 min, annealing at 42°C (or according to T_m of primers) for 1
105 min, and extension at 72°C for 1-2 min (according to size of amplified product, 1 min per 1
106 kb). Amplification was completed with a final extension step at 72°C for 10 min.

107 PCR products were visualized by agarose gel electrophoresis and documented with a
108 Gel Doc 2008 gel system (Alpha Innotech Gel Doc System).

109 **Cloning and sequence analysis of *cry2*-type genes**

110 Full length *cry2A*-type gene PCR products, amplified from the native isolates SK-758
111 and SK-793, were purified by gel elution, examined by agarose gel electrophoresis, cloned
112 into pGEM-T vector (Promega) and transformed into *E. coli* DH5α by heat shock method
113 (Sambrook et al. 1989). The positive clones were confirmed by restriction analysis. Both
114 genes were sequenced by primer walking three times on ABI-sequencer from three confirmed
115 clones of each gene. Sequence analysis was performed with BLAST (National Centre for
116 Biotechnology Information NCBI) and MultAlin programme.

117

118 **Expression of *cry2Af2* gene in *E. coli***

119 Protein extraction was carried out by a method modified from that of Lee et al.
120 (1992). Recombinant *E. coli* clone carrying *cry2Af2* gene was inoculated in five mL LB with
121 50 µg mL⁻¹ kanamycin and grown overnight at 37°C. One mL culture was re-inoculated in
122 100 mL LB, at 37°C with shaking at 180 rpm in shaker incubator (Kuhner, Germany) till
123 OD 600 nm reached 0.6 (4 h), and was induced with 1 mM IPTG at time intervals of 4 h, 6 h
124 and 12 h.

125 **Rearing of *H. armigera***

126 The neonates of *H. armigera* were collected from chickpea fields from IARI. The larvae were
127 allowed to grow until pupation on artificial diet. Pupae were collected and kept in cage for
128 adult emergence. The adults were paired, kept in a cage and given 5 per cent honey solution
129 for egg laying, on markin cloth. The eggs thus collected, were separated day-wise and
130 allowed to hatch. The neonates were transferred to chickpea based artificial diet. The insects
131 were reared at 25±2°C and 60-70 per cent RH. The pupae were separated and kept in a jar at
132 25±2°C until adult emergence. The adults were allowed to mate and lay eggs. Insects used in
133 bioassays were reared for about 6 months in laboratory on artificial diet.

134 **Bioassay**

135 Toxicity of Cry2Af2 protein towards neonate larvae of *H. armigera* reared in
136 laboratory was evaluated. Total protein of recombinant clones was prepared by Lactose-
137 Acetone co-precipitation method (Dulmage et al. 1970). Protein content was estimated with
138 Quant-iT[™] Protein Assay Kit (Invitrogen). Bioassays were performed with neonate larvae
139 under rearing conditions of 16 h light, 8 h dark photoperiod at 25±2°C, by diet incorporation
140 method, at 1 ppm and 5 ppm concentration. Three replications with ten neonates each, were

141 used for each treatment. The mortality was observed each day for 7 days. Corrected mortality
142 was calculated as per Abbott's formula (Abbott 1925). For reference, MVPII formulation,
143 containing 19.7% Cry1Ac toxin, received from Monsanto India Ltd, and ECE 126, *E. coli*
144 recombinant clone carrying *cry2Aa1* gene, were used as a standards for comparison.
145 Statistical analysis was done with ANOVA.

146 **Results**

147 **Determination of presence of *cry2* genes**

148 ***i) Determination of presence of *cry2* gene family and type genes using triplex PCR strategy***

149 Native Bt isolates and Bt strains used as reference were screened for presence of *cry2*
150 genes using triplex PCR strategy of Masson et al. (1998) which employs a set of family
151 primers designed from conserved regions of *cry2* gene family and amplifying a 1.5 kb
152 product (set 1) and a set (set 2) of gene type specific primers (Table S3). In screening with
153 primer set 1, out of 129 native Bt isolates, 29 (22.4%) were found to be positive and out of
154 27 reference Bt strains, 8 (29.6%) were positive (Fig. 1a, 1b, Table S1, S2). The positive
155 isolates and strains were screened further in a triplex PCR strategy using family primer set 1
156 and either of set 2 primers as additional reverse primer for amplification of expected product
157 of 694 bp, indicating presence of *cry2Aa* or *cry2Ab*-type genes. The *cry2Ab*-type genes were
158 found to be more prevalent than *cry2Aa*-type genes, as out of 29 *cry2*-positive Bt isolates, 20
159 Bt isolates (68.9%) were positive for *cry2Aa*-type genes, while 27 (93.1%) Bt isolates were
160 positive for *cry2Ab*-type genes (Table S5, Fig. 1c). Out of 8 Bt strains, four (50%) were
161 positive for *cry2Aa*-type and five (62.5%) were positive for *cry2Ab*-type genes (Table S6).

162 ***ii) Determination of presence of *cry2* gene family and type genes using universal and type-specific primers***

PCR strategy of Ben-Dov et al. (1997) for the detection of *cry2* genes employs a universal primer set comprising of a common forward primer and a specific reverse primer for the amplification of *cry2* family (primer set 3) or *cry2Aa*, *cry2Ab* or *cry2Ac*-type genes (primer sets 4, 5 and 6) based on C-terminal variable regions with product size 700 bp, 498 bp, 546 bp or 725 bp respectively (Table S3). These primer sets were seen to detect a greater number of positive isolates as compared with the triplex PCR strategy (Table S1). Out of 129 native isolates, 56 (43.4%) were found positive and out of 27 reference strains, 12 (44.4%) were positive in the *cry2* family-specific screening with the primer set 3 (Fig. 1d, 1e, Table S1, S2).

In the *cry2*-type gene-specific screening with primer sets 4, 5 and 6, out of 56 positive Bt isolates, 22 (39%) each, were positive for *cry2Aa* and *cry2Ac*-type genes, and 37 (66%) were positive for *cry2Ab*-type genes. In Bt reference strains also, *cry2Ab*-type genes were seen to be more prevalent with all 12 (100%) Bt strains positive for *cry2Ab*, while nine (75%) were positive for *cry2Aa* and six (50%) positive for *cry2Ac*-type genes (Fig. 1f, 1g, 1h, Table S7, S8).

Frequency distribution of *cry2A*-type genes in Bt

Native Bt isolates recovered from diverse habitats such as cultivated soil, desert soils, grains dust, insect/ nematode infected soils, phyllosphere, cattle sheds and barren land were examined for abundance of *cry2* type genes. Out of 56 isolates from cultivated soils, 34 (60.7%) isolates were positive for *cry2* gene family using either primer set 1 (Masson et al 1998) or primer set 3 (Ben-Dov et al 1997) in this study (Table S1). Out of 22 isolates collected from desert soil, 17 (77.2%) were positive, while out of 20 native isolates collected from grains/ grain dust samples, 8 (40%) isolates were positive and out of 9 isolates collected from phyllosphere, 4 (44.4%) isolates were positive for *cry2* gene family (Table

189 S1, S2). There were only four isolates (SK-617, SK-621, SK-629 and SK-1014) from cattle
190 shed out of which, one (25%) isolate (SK-621) was positive. Out of seven isolates collected
191 from barren land, none showed the presence of *cry2* gene family.

192 Further, isolates which were positive for *cry2* gene family using both PCR techniques were
193 tested for *cry2A* group type-specific screening, and *cry2Ab* was found to be abundant over
194 *cry2Aa*- and *cry2Ac*-type genes (Fig. 2).

195 The source-wise frequency distribution of Bt isolates was determined. Out of 85 Bt
196 isolates from soil, 44 (51.76%) isolates were positive for *cry2* gene family. Out of these 44
197 positive isolates from soil, 18 (40.9%) isolates showed presence of *cry2Aa*- and 28 (63.6%)
198 showed presence of *cry2Ab*-type genes (Table S6, S8). Only one isolate showed presence of
199 *cry2Ac*-type genes.

200 Among 20 isolates from grain dust samples, eight (40%) isolates showed presence of
201 *cry2* gene family, out of which, four (50%) isolates showed presence of *cry2Aa*, five (62.5%)
202 isolates showed presence of *cry2Ab*-type genes and none of isolates showed presence of
203 *cry2Ac*-type genes.

204 The third source of native isolates was from phyllosphere, with 11 isolates, out of
205 which, four (36.3%) isolates showed presence of *cry2* gene family and four isolates (SK-217,
206 SK-220, SK-222 and SK-229, Table S1) showed presence of both *cry2Aa*- and *cry2Ab*-type
207 genes in the triplex PCR (Table S6). However, these isolates showed no amplification with
208 universal primer sets 4, 5 and 6 (Table S8). Out of 11 Bt isolates from insects/ nematodes
209 infected soil, four (50%) isolates were positive for *cry2* gene family (Table S1) amongst
210 which, two (25%) isolates (SK-304, SK-305) were positive for both *cry2Aa*- and *cry2Ab*-type
211 genes (Table S6). Out of four isolates from cowshed, one (25%) isolate showed presence of
212 *cry2Aa*- and *cry2Ab*-type genes.

213

Isolation of full length *cry2*-type genes using ORF Primers

Bt isolates found positive with *cry2*-type-specific screening were tested for amplification with ORF primers designed for amplification of full length *cry2Aa*, *cry2Ab* and *cry2Ac*-type genes (Table S4). In the *cry2Aa*-type amplification, out of total 38 Bt isolates and reference strains positive for *cry2Aa*-type genes, full length PCR product was observed in four Bt isolates (SK-767, SK-793, SK-915 and SK-953) and two Bt strains- Bt subsp. *aizawai/pacifics* (4J2) and Bt subsp. *tolworthi* (4L3) (Fig. S2). In the *cry2Ab*-type amplification, out of 58 isolates, five Bt isolates (SK-758, SK-781, SK-793, SK-943 and SK-793) and two Bt strains- Bt subsp. *kenyae* (4F1) and Bt subsp. *galleriae* (4G6) were found positive for full length *cry2Ab*-type genes (Fig. S3). However, none of the isolates showed amplification for full length *cry2Ac*-type genes, even though 27 isolates had been found positive for *cry2Ac*-type screening.

Cloning of full length *cry2*-type genes and sequence analysis

Full length genes were amplified from Bt isolates SK-758 and SK-793 using *cry2Ab* ORF primers, cloned into pGEM-T cloning vector (Promega), confirmed by restriction analysis and sequenced (Fig. 3a, 3b, 3c). Sequence analysis revealed that the gene cloned from SK-758 (NCBI GenBank accession GQ866915) was novel while the gene isolated from SK-793 (NCBI GenBank accession GQ866914) was identical to *cry2Ab1* gene. Nomenclature for these genes was obtained from *Bacillus thuringiensis* Nomenclature Committee (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/BT/) as *cry2Af2* and *cry2Ab16* respectively.

Comparison of deduced amino acids sequence of *cry2Af2* gene with that of *cry2Af1* gene isolated by Beard et al. (2008), showed a difference of two amino acids, namely Aspartic acid (D) instead of Asparagine (N) at positions 86 and 140 of Cry2Af1 protein (Fig.

4). The *cry2Ab*-type gene cloned from Bt isolate SK-793 was 1.92 Kb as was expected for *cry2Ab*-type genes. Sequence alignment was done with all known *cry2Ab*-type genes and was found to be identical to *cry2Ab1* gene cloned by Widner and Whiteley (1989).

Protein expression in *E. coli*

Since *cry2Ab16* was identical to *cry2Ab1* gene, the novel 1881 bp *cry2Af2* gene isolated from Bt isolate SK-758 from Sorgham grain dust, Guntur, Andhra Pradesh, was expressed in *E. coli* to evaluate its toxicity against *H. armigera*. The *cry2Af2* gene insert in pGEM-T cloning vector was excised using *EcoRI*/ *HindIII* restriction, gel purified and cloned into pET29a(+) expression vector. The positive clone was confirmed using restriction analysis (Fig. 3d). The pET-29a(+)-*cry2Af2* recombinant clone induced with IPTG for 6 h showed good expression as compared with that induced for 4 h and 12 h. The expressed protein was analysed on SDS-PAGE and expected size 70.5 kDa protein band was observed (Fig. 5).

Determination of efficacy of Cry2Af2 protein against *H. armigera*

Mortality of neonate *H. armigera* larvae upon treatment with 1 ppm concentration of acetone powder preparation of recombinant *E. coli* BL21 (DE3) cells expressing *cry2Af2* gene was observed to be 16.6% after four days and 30% after seven days. At 5 ppm concentration, mortality was observed to be 30% after four days and 40% after seven days (Table 1). The difference between mortality at both concentrations, as compared with control, was statistically significant. With ECE126, *E. coli* strain expressing *cry2Aa1* gene, 20% and 23.3 % mortality at 1 ppm after four and seven days, respectively, was observed, while mortality was 33.3% and 36.3% at 5 ppm after four and seven days, respectively (Table 1). Toxicity observed in the Cry2Af2 protein treatment was comparable to that of Cry2Aa1 treatment. MVPII formulation was used as a standard for comparison and found to have 100%

264 mortality at both 1 ppm and 5 ppm concentration after 4 days, whereas no mortality was
265 observed in control (Table 1).

266 Discussion

267 Several PCR based methods have been developed for identification of *cry* genes from
268 Bt isolates. In this study, the efficacy of two PCR methods, an Exclusive triplex PCR strategy
269 (Masson et al. 1998) and a universal primer PCR strategy (Ben-Dov et al. 1997) has been
270 compared. Bt isolates recovered from diverse habitats in India had genotypic diversity
271 (Katara et al. 2012). Screening for the presence of *cry2*-type genes was performed using six
272 sets of primers, designed from the conserved region of these genes, as per Ben-Dov et al.
273 (1997) and Masson et al. (1998). Bt isolates observed to be positive in the *cry2*-type family
274 specific screening, were further examined for type-specific screening using different sets of
275 primers.

276 The primer sets designed by Ben-Dov et al. (1997) correspond to the highly conserved region
277 located towards the C terminal of *cry2*-type genes and thus, detected a higher number of
278 positive isolates, as compared to the primer sets of Masson et al. (1998). Though the
279 frequency distribution of *cry2*-type genes in native Bt isolates indicated abundance of
280 *cry2Ab*-type genes, it did not show any clear correlation between the source of isolate and
281 abundance of *cry2*-type genes. Sauka et al. (2005) have also found that *cry2Aa/2Ab* profile
282 was most abundant (94.9%) irrespective of source in a Bt isolate collection from Argentina.

283 In *cry2Ab*-type screening, 49 Bt isolates and strains were found to be positive with
284 universal primers, out of which, five Bt isolates and two strains were positive for full length
285 gene amplification. In *cry2Aa*-type screening, 31 Bt isolates and Bt strains were found to be
286 positive with universal primers, out of which, four isolates and two Bt strains were positive
287 with ORF primers. Amplification of full length *cry2*-type genes was, thus, achieved in four
288 isolates and two Bt strains with *cry2Aa*-type ORF primers and five isolates and two Bt strains

289 with *cry2Ab*-type ORF primers. None of isolates showed amplification of full length *cry2Ac*-
290 type genes even though 29 Bt isolates and strains were found to be positive in screening with
291 *cry2Ac*-type-specific primers. The sequences of cloned genes were submitted to NCBI and
292 *Bacillus thuringiensis* Nomenclature Committee ([www.lifesci.sussex.ac.uk/home/nei-](http://www.lifesci.sussex.ac.uk/home/nei-crickmore)
293 [crickmore](http://www.lifesci.sussex.ac.uk/home/nei-crickmore)). The *Bacillus thuringiensis* Nomenclature Committee has provided names
294 *cry2Ab16* (NCBI GenBank accession GQ866914) and *cry2Af2* (NCBI GenBank accession
295 GQ866915) for the genes isolated from Bt isolates SK-793, and SK-758 respectively.

296 Sequence differences may impact full length amplification of gene, even though the
297 ORF primers were designed from the relatively conserved flanking regions of *cry2*-type
298 genes. It is likely that the genes, which had different flanking sequences, were not amplified.
299 In most of the isolates, either PCR products of smaller than expected size or no amplification
300 was observed. This could be due to presence of truncated genes or non-specific amplification,
301 even though care was taken to keep the annealing temperature high. Since the 3' sequence of
302 primers is critical for successful amplification, variation in the sequence of *cry2A*-type genes
303 corresponding to 3' region of primers may impact amplification. A change of one or two
304 bases at this region, resulting in a mismatch between primer and template, may lead to
305 closely related genes not being detected (Porcar and Juarez-Perez 2003). Variation may also
306 occur due to insertion of mobile genetic elements (Kronstad and Whiteley 1984; Lereclus et
307 al. 1986). Insertion sequences encoding putative transposases have been found downstream
308 of a cryptic *cry2Ab* gene (Hodgman et al. 1993). It may also be the case that full length *cry2*-
309 type genes may be absent in isolates showing presence of partial *cry2A*-type PCR products.

310 Amplification of *cry2*-type genes was not observed with genomic DNA of Bt isolates
311 and strains used as template, corroborating the reports that *cry* genes are generally plasmid
312 borne (Gonzalez et al. 1982).

New types of *cry2A*-type genes have been isolated from Bt isolates in other laboratories as well (Lima et al. 2008; Sevim et al. 2012; Shu et al. 2013). The toxicity level of *cry2A*-type genes differs, depending on differences in deduced amino acid sequence. There is 87% sequence homology between *cry2Aa* and *cry2Ab* genes with the latter being more toxic to *H. zea* (Dankocsik et al. 1990). Cry2Aa protein is toxic to both lepidopteran and dipteran insects while Cry2Ab, Cry2Ac and Cry2Ad have been reported to be toxic to only lepidopteran insects. Variation of a single amino acid can result in significant difference in level of toxicity of Cry proteins (Rajamohan et al. 1996). A *cry2Ab7* gene was isolated from a Bt isolate and shown to have toxicity towards *H. armigera* (Jain et al. 2006) while the *cry2Aa4* gene from Bt subsp. *kenyae* (HD 549) was found to have very low toxicity towards *H. armigera* (Misra et al. 2002). The *cry2Ab27* gene from Bt subsp. *aizawai* was also found to be toxic to *H. armigera* (Somwatcharajit et al. 2014).

The *cry2A*-type genes having toxicity towards target insects are good candidates for gene pyramiding in transgenic crops (Ibargutxi et al. 2008; Lin et al. 2008; Li and Bouwer 2014). Lab selected resistant *H. armigera* strain, developed resistance to Cry1Ac after 16 generations, and was also resistant to Cry1Ab but not to Cry2Aa or Cry2Ab (Liao et al. 2002; Akhurst et al. 2003). Beard et al. (2008) observed that Cry2Afl protein had toxicity toward lab developed Cry1Ac-resistant strain of *H. armigera*, although it was much less toxic than either Cry2Aa or Cry2Ab proteins. Sensitivity of Cry1Ac-resistant *H. armigera* to Cry2A toxins reiterates the usefulness of *cry2A*-type genes. Although Cry2Aa, Cry2Ab and Cry2Ae proteins shared a common binding site in *H. armigera*, it was not shared by Cry1Ac, (Hernandez-Rodriguez et al. 2008). Thus, Cry2A-type proteins are potential candidates for gene pyramiding for control of *H. armigera*. Nevertheless, it has also been proposed that additional mechanisms of development of resistance may account for the observed cross

337 resistance to Cry2Aa protein in lab-selected *Heliothis zea* resistant to Cry1Ac (Jurat-Fuentes
338 et al. 2003).

339 Comparison of deduced amino acid sequence of *cry2Af2* gene isolated in this study,
340 with that of *cry2Af1* gene showed change at 2 positions, with Aspartic acid (D) instead of
341 Asparagine (N) at positions 86 and 140 (Fig.4). Both the changes are in domain I of deduced
342 three dimensional structure of Cry2Af2 protein and may have a role in its toxicity. Detailed
343 structure function analysis of this protein based on molecular modelling is underway. Since
344 the *cry2Af2* gene is toxic towards against *H. armigera*, with toxicity comparable to that of
345 ECE 126 strain carrying *cry2Aa1* gene, it may be useful in gene pyramiding to overcome the
346 development of resistance in insects. There are differences in amino acids in all three
347 domains compared to that of the holotype Cry2Aa1 protein. Transgenic cotton with *cry1Ac*
348 and *cry2Ab2* genes has been developed (Sivasupramainiam et al. 2008). The *cry2Af2* gene
349 isolated in this study will be useful in control of *H. armigera*, deployed in combination with
350 the *cry1Ac18* gene previously isolated in our lab (Kaur and Allam 2006).

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512

513 **Table 1.** Toxicity of *cry2Af2* gene against *H. armigera* neonate larvae.

Treatment	Dose (ppm)	% mortality at 4 th d	% mortality at 7 th d
Cry2Af2	1	16.67	30
	5	30	40
MVP-II	1	100	100
	5	100	100
ECE126*	1	20	23.33
	5	33.33	36.66
Control†		0.00	0.00

514 **cry2Aa1* gene expressed in *E. coli*. †sterile double distilled water. Thirty larvae, as ten larvae
 515 in three replicates each, were used per treatment.

516

517

518 **Figure legends:**

519 **Figure 1.** Agarose gel electrophoresis of PCR products of native Bt isolates and strains.
 520 Isolates and strains shown are a representative sub-set of results obtained. **a, b)** Screened
 521 using family specific primers for *cry2* genes designed as per Masson et al. (1998). The
 522 expected band size is 1.5 Kb for the presence of *cry2* gene family. Lane 4D1-4H3: reference
 523 Bt strains, lane13-977: native Bt isolates, lane M: 1Kb DNA ladder. **c)** Screened with triplex
 524 PCR for presence of *cry2* family and *cry2Ab*-type specific genes. Lane 4A6-4K1: reference
 525 Bt strains, lane 1-944: native Bt isolates (for brevity SK prefix not written with the numbers),
 526 lane M: 1Kb DNA ladder marker. **d, e)** Screened for *cry2* genes using family specific primers
 527 designed as per Ben-Dov et al. (1997). The expected band size is 700 bp for the presence of

528 *cry2* gene family. Lane 4N1-4K1: reference Bt strains, lane 110-996: native Bt isolates, lane
529 M: DNA Ladder Mix marker. **f, g, h**) Screened for *cry2*-type genes using type-specific
530 primers designed as per Ben-Dov et al. (1997) for *cry2Aa*, *cry2Ab* and *cry2Ac* respectively.
531 Lane 4L3-4W1: reference Bt strains, lane 28-978: native Bt isolates, lane M: 1Kb DNA
532 ladder marker (1f) and 100 bp plus DNA ladder marker (1g, h).

533 **Figure 2.** Frequency distribution of *cry2*-type genes in Bt isolates using type-specific
534 primers. Out of 68 isolates, 31 showed presence of *cry2Aa* genes, 49 and 29 isolates showed
535 presence of *cry2Ab* and *cry2Ac* genes respectively.

536 **Figure 3.** Cloning of two genes amplified using *cry2Ab*-type ORF primers from native Bt
537 isolates SK-793 and SK-758 in pGEM-T vector. (a) agarose gel electrophoresis of gel eluted
538 PCR products of full length genes. Lane 1: SK-793, lane 2: SK-758. (b) Restriction analysis
539 of recombinant pGEM-T-SK-793 clone. Lane 1: Undigested pGEM-T-SK-758, lane 2:
540 pGEM-T-SK-793 digested with *EcoRI*, lane 3: pGEM-T-SK-793 digested with *EcoRI* and
541 *HindIII*. (c) Restriction analysis of recombinant pGEM-T-SK-758 clone. Lane 1: Undigested
542 pGEM-T-SK-758, 2; pGEM-T-SK-758 digested with *EcoRI*, 3; pGEM-T-SK-758 digested
543 with *EcoRI* and *HindIII*. Lane M: 1 Kb DNA ladder. **(d)** Restriction analysis of recombinant
544 expression vector pET-29a(+)-*cry2Af2*. Lane 1: pET-29a-*cry2Af2* digested by *EcoRI*, Lane 2:
545 pET-29a-*cry2Af2* digested with *EcoRI* and *HindIII* (5.3 kb vector and 1881 bp insert) lane M:
546 1 Kb DNA ladder.

547

548 **Figure 4.** Comparison of deduced amino acids sequences of *cry2Af2* gene isolated from SK-
549 758 native Bt isolate with that of *cry2Ab16* gene from SK-793 native Bt isolate and *cry2Af1*
550 gene.

551 **Figure 5.** SDS-PAGE analysis of *cry2Af2* gene expressed in *E. coli* (BL21). Lane 1: BL21
552 cells without IPTG induction, lane 2: BL21 cells with IPTG induction, lane 3: pET29a-BL21

(DE3) cells without IPTG induction, lane 4: pET29a-BL21 cells with IPTG induction, lane 5: pET29-*cry2Af2*-BL21 (DE3) cells without IPTG induction, lane 6, 7 and 8: pET29-*cry2Af2*-BL21 (DE3) cells with IPTG induction at 4 h, 6 h and 12 h respectively. Lane M: protein standard marker.

Legends for supporting information:

Table S1. Screening of *cry2* family genes in native Bt isolates recovered from different sources from diverse locations in India with primers designed as per Masson *et al.* (1998) and Ben-Dov *et al.* (1997).

Table S2. Screening of *cry2* family genes in Bt reference strains with primers designed as per Masson *et al.* (1998) and Ben-Dov *et al.* (1997)

Table S3. Primers used for PCR screening for the presence of *cry2* genes

Table S4. ORF primers for amplification of full length *cry2* type genes

Table S5. Screening of reference Bt strains for the presence of *cry2Aa* and *cry2Ab*-type genes using Triplex PCR with primers designed as per Masson *et al.* (1998)

Table S6. Screening of native Bt isolates for the presence of *cry2Aa* and *cry2Ab*-type genes using Triplex PCR with primers designed as per Masson *et al.* (1998)

Table S7. Screening of reference Bt strains for the presence of *cry2*-type genes by using type-specific primers designed as per Ben-Dov *et al.* (1997)

Table S8. Screening of native Bt isolates for the presence of *cry2*-type genes by using type-specific primers designed as per Ben-Dov *et al.* (1997)

Figure S1. Distribution of native Bt isolates recovered from different sources. Total numbers of isolates from each type of source are shown.

Figure S2. Agarose gel electrophoresis of PCR products of full length *cry2Aa*-type genes amplified using ORF primers. Lanes 4A6-4F1: reference Bt strains, lanes 217-951: native Bt isolates. Lane M: 1 Kb DNA ladder marker.

Figure S3. Agarose gel electrophoresis of PCR products of full length *cry2Ab*-type genes amplified using ORF primers. Lanes 4B2-4G6: reference Bt strains, lanes 20-758: native Bt isolates. Lane M: 1 Kb DNA ladder marker.

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Figure 1

Figure 2

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Figure 3

Figure 4

Figure 5

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Prevalence of *cry2*-type genes in *Bacillus thuringiensis* isolates recovered from diverse habitats in India and isolation of a novel *cry2Af2* gene toxic to *Helicoverpa armigera* (cotton boll worm)

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Figure S3. Agarose gel electrophoresis of PCR products of full length *cry2Ab*-type genes amplified using ORF primers. Lanes 4B2-4G6: reference Bt strains, lanes 20-758: native Bt isolates.. Lane M: 1 Kb DNA ladder marker.

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Table S1 Screening of *cry2* family genes in native Bt isolates recovered from different sources from diverse locations in India with primers designed as per Masson *et al.* (1998) and BenDov *et al.* (1997).

S. No.	Isolate	Location*	Screening with primer set 1 (Masson <i>et al.</i> 1998)	Screening with primer set 3 (BenDov <i>et al.</i> 1997)
1	SK-1	Chickpea field soil, Rohtak, HN	-	-
2	SK-3	Chickpea field soil, Rohtak, HN	-	-
3	SK-4	Chickpea field soil, Rohtak, HN	-	-
4	SK-5	Cotton field soil, Agroha, dist. Hissar, HN	-	-
5	SK-9	IARI, chickpea field soil, DL	-	-
6	SK-13	Cotton field soil, Malout, PB	-	-
7	SK-20	Cotton field soil, Malout, PB	-	+
8	SK-28	Cotton field soil, Malout, PB	+	+
9	SK-32	Cotton field soil, Malout, PB	+	-
10	SK-51	Orchard soil, Shimla, HP	-	+
11	SK-63	Orchard soil, Shimla, HP	-	-
12	SK-82	Grain dust, Bhareri, Shimla, HP	+	-
13	SK-84	Grain dust, Bhareri, Shimla, HP	-	-
14	SK-88	Grain dust, Bhareri, Shimla, HP	-	-
15	SK-94	Grain dust, Bhareri, Shimla, HP	-	-
16	SK-105	Chickpea field soil, IARI, DL	-	+
17	SK-110	Chickpea field soil, IARI, DL	-	+
18	SK-113	Chickpea field soil, IARI, DL	-	+
19	SK-213	Phyllosphere, Chickpea field, IARI, DL	-	-
20	SK-214	Phyllosphere, Chickpea field, IARI, DL	-	-
21	SK-217	Phyllosphere, Chickpea field, IARI, DL	+	-
22	SK-219	Phyllosphere, Chickpea field, IARI, DL	-	-
23	SK-220	Phyllosphere, Chickpea field, IARI, DL	+	-
24	SK-222	Phyllosphere, Pigeon pea field, IARI, DL	+	-
25	SK-223	Phyllosphere, Pigeon pea field, IARI, DL	-	-
26	SK-229	Phyllosphere, Pigeon pea field, IARI, DL	+	-
27	SK-232	Phyllosphere, Mung bean field, IARI, DL	-	-
28	SK-301	Nematodes infected field soil, IARI, DL	-	-
29	SK-302	Nematodes infected field soil, IARI, DL	-	-
30	SK-303	Nematodes infected field soil, IARI, DL	-	-
31	SK-304	Nematodes infected field soil, IARI, DL	+	-
32	SK-305	Nematodes infected field soil, IARI, DL	+	-
33	SK-307	Nematodes infected field soil, IARI, DL	-	-
34	SK-617	Cattle Shed, Vemboor, TN	-	-
35	SK-621	Cattle Shed, Vemboor, TN	-	+
36	SK-629	Cattle Shed, Vemboor, TN	-	-
37	SK-669	Kitchen garden soil, Allahabad, UP	-	+
38	SK-671	Kitchen garden soil, Allahabad, UP	-	-
39	SK-677	Kitchen garden soil, Allahabad, UP	-	+
40	SK-678	Kitchen garden soil, Allahabad, UP	-	+

41	SK-680	Soil , Sangam river, Allahabad, UP	-	+
42	SK-684	Soil, Sangam river Allahabad, UP	-	+
43	SK-700	Cotton field soil, Lam, Guntur, AP	+	+
44	SK-701	Chickpea field soil, Lam, Guntur, AP	+	-
45	SK-721	Cotton field soil, Lam, Guntur, AP	-	+
46	SK-722	Cotton field soil, Lam, Guntur, AP	-	+
47	SK-729	Cotton Seed <i>var.</i> LK861, Lam, Guntur, AP	-	-
48	SK-739	Cotton Seed <i>var.</i> LK689, Lam, Guntur, AP	-	-
49	SK-741	Cotton Seed <i>var.</i> LK689, Lam, Guntur, AP	-	-
50	SK-750	Chickpea seeds, Lam, Guntur, AP	-	-
51	SK-751	Chickpea seeds, Lam, Guntur, AP	-	-
52	SK-754	Chickpea seeds, Lam, Guntur, AP	-	+
53	SK-758	Sorghum grain dust, Guntur, AP	+	-
54	SK-764	Sorghum grain dust, Guntur, AP	-	-
55	SK-767	Sorghum grain dust, Guntur, AP	-	+
56	SK-768	Sorghum grain dust, Guntur, AP	-	+
57	SK-781	Sorghum grain dust, Guntur, AP	-	+
58	SK-783	Sorghum grain dust, Guntur, AP	-	-
59	SK-786	Sorghum grain dust, Guntur, AP	-	-
60	SK-791	Chilli seeds Warehouse, Nallapadu, Guntur, AP	-	-
61	SK-792	Chilli seeds Warehouse, Nallapadu, Guntur, AP	+	+
62	SK-793	Chilli seeds Warehouse, Nallapadu, Guntur, AP	+	+
63	SK-794	Chilli seeds Warehouse, Nallapadu, Guntur, AP	-	-
64	SK-851	Wheat field soil, Burdwan, WB	+	+
65	SK-853	Wheat field soil, Burdwan, WB	-	-
66	T-27	Soil, Laddakh ,JK	-	-
67	T-94	Soil, Laddakh, JK	-	-
68	SK-911	Desert soil, Location 4, Khyaliwala, Sriganganagar, RJ	-	-
69	SK-915	Desert soil, Location 4, Khyaliwala, Sriganganagar, RJ	+	+
70	SK-916	Desert soil, Location 4, Khyaliwala, Sriganganagar, RJ	-	+
71	SK-918	Desert soil, Location 4, Khyaliwala, Sriganganagar, RJ	-	-
72	SK-920	Desert soil, Location 4, Khyaliwala, Sriganganagar, RJ	-	+
73	SK-921	Desert soil, Location 4, Khyaliwala, Sriganganagar, RJ	+	+
74	SK-922	Desert soil, Location 2, Khyaliwala,	-	+

		Sriganganagar, RJ		
75	SK-934	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	
76	SK-935	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	+
77	SK-936	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	-
78	SK-937	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	-
79	SK-938	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	+	-
80	SK-941	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	+	-
81	SK-942	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	+
82	SK-943	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	+
83	SK-944	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	+	+
84	SK-945	Desert soil, Location 3, Khyaliwala, Sriganganagar, RJ	-	+
85	SK-947	Cotton field 1, Khyaliwala, Sriganganagar, RJ	-	+
86	SK-948	Cotton field 1, Khyaliwala, Sriganganagar, RJ	+	+
87	SK-949	Cotton field 1, Khyaliwala, Sriganganagar, RJ	-	+
88	SK-950	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	-	+
89	SK-951	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	-	+
90	SK-952	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	+	+
91	SK-953	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	+	+
92	SK-954	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	-	+
93	SK-955	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	+	+
94	SK-956	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	+	+
95	SK-957	Cotton field soil 1, Khyaliwala, Sriganganagar, RJ	-	+
96	SK-958	Desert soil, Location 1, Khyaliwala, Sriganganagar, RJ	+	-
97	SK-959	Desert soil, Location 1, Khyaliwala, Sriganganagar, RJ	-	+
98	SK-960	Desert soil, Location 1, Khyaliwala, Sriganganagar, RJ	-	+

99	SK-961	Desert soil, Location 1, Khyaliwala, Sriganganagar, RJ	-	+
100	SK-962	Desert soil, Location 1, Khyaliwala, Sriganganagar, RJ	+	+
101	SK-963	Desert soil, Location 1, Khyaliwala, Sriganganagar, RJ	-	+
102	SK-973	Cotton field soil 2, Khyaliwala, Sriganganagar, RJ	+	+
103	SK-977	Cotton field soil 2, Khyaliwala, Sriganganagar, RJ	-	-
104	SK-978	Cotton field soil 3, Khyaliwala, Sriganganagar, RJ	-	+
105	SK-980	Cotton field soil 3, Khyaliwala, Sriganganagar, RJ	-	+
106	SK-986	Cotton field soil 3, Khyaliwala, Sriganganagar, RJ	-	-
107	SK-995	Cotton field soil 4, Khyaliwala, Sriganganagar, RJ	-	+
108	SK-996	Cotton field soil 4, Khyaliwala, Sriganganagar, RJ	+	+
109	SK-1007	Insect infected wheat field, Sriganganagar, RJ	-	+
110	SK-1008	Insect infected wheat field, Sriganganagar, RJ	-	-
111	SK-1009	Insect infected wheat field, Sriganganagar, RJ	-	-
112	SK-1013	Insect infected wheat field, Sriganganagar, RJ	-	+
113	SK-1014	Cow shed sample, Sriganganagar, RJ	-	-
114	SK-1026	Barren land, Sriganganagar, RJ	-	-
115	SK-1027	Barren land, Sriganganagar, RJ	-	-
116	SK-1028	Barren land, Sriganganagar, RJ	-	-
117	SK-1034	Barren land, Sriganganagar, RJ	-	-
118	SK-1035	Soil sample, Baramulla, JK	-	-
119	SK-1036	Soil sample, Baramulla, JK	-	-
120	SK-1050	Maize field soil, Motali, Udaipur, RJ	-	-
121	SK-1051	Maize field soil, Motali, Udaipur, RJ	-	-
122	SK-1052	Maize field soil, Motali, Udaipur, RJ	-	-
123	SK-	Maize field, Motali, Udaipur, RJ	-	-

	1055			
124	SK-1056	Maize field soil, Motali, Udaipur, RJ	-	+
125	SK-1064	Maize field, Bankoda, Dungarpur, RJ	-	-
126	SK-1060	Barren land. Lolakpur, Dungarpur, RJ	-	-
127	SK-1065	Maize field soil, Bankoda, Dungarpur, RJ	-	-
128	SK-1066	Maize field soil, Bankoda, Dungarpur, RJ	-	-
129	SK-1067	Maize field soil, Bankoda, Dungarpur, RJ	-	-
		Total positive for <i>cry2</i> family genes	29	56

*Native isolates recovered from different states in India, HN: Haryana, PB: Punjab, HP: Himachal Pradesh, DL: New Delhi, TN: Tamil Nadu, UP: Uttar Pradesh, AP: Andhra Pradesh, JK: Jammu and Kashmir, RJ: Rajasthan, WB: West Bengal. +: positive for *cry2* gene family, -: no amplification (negative) of *cry2* gene family.

Table S2 Screening of *cry2* family genes in Bt reference strains obtained from *Bacillus* Genetic Stock Centre (BGSC), Department of Biochemistry, Ohio State University, Columbus, Ohio 43210, USA with primers designed as per Masson *et al.* (1998) and BenDov *et al.* (1997)

S. No.	Strain	Location	Screening with primer set 1 (Masson <i>et al.</i> 1998)	Screening with primer set 3 (BenDov <i>et al.</i> (1997)
1	4M2	<i>darmstadiensis</i> , isolated in England	+	-
2	4W1	<i>kumamotoensis</i> , isolated in Japan from <i>B. mori</i> colony; antisera standard	-	+
3	4L3	<i>tolworthi</i> , wild type isolate	+	+
4	4M1	<i>darmstadiensis</i> isolated in England	-	-
5	4A6	<i>Thuringiensis</i> , Wild type isolate	+	-
6	4C3	<i>alest,i</i> , isolated in France from <i>Bombyx mori</i> ; antisera st	-	-
7	4N1	<i>Toumanoffi</i> , isolated in England from <i>Galleria mellonella</i>	-	+
8	4S2	<i>indiana</i> ,,, wild type isolate	-	-
9	4F1	<i>Kenya</i> , isolated in England from <i>Corcyra cephalonica</i>	+	+
10	4D1	<i>kurstaki</i> , isolated in US	+	+
11	4Q5	<i>israelensis</i> Plasmid cured mutant; bears only 72 mDal plasmid	-	+
12	4J2	<i>aizawai/pacificus</i> isolated in England from <i>Plodia interpunctella</i>	-	+
13	4O1	<i>dakota</i> wild type isolate	-	+
14	4Y1	<i>tochigiensis</i> isolated in Japan from soil; antisera standard	-	
15	4B1	<i>finitimus</i> wild type isolate	+	+
16	4B2	<i>finitimus</i> isolated in the US from <i>Malacosoma distria</i>	+	+
17	4J3	<i>aizawai/pacificus</i> isolated in England from <i>Plodia interpunctella</i>	-	-
18	4T1	<i>wuhanensis</i> no flagellar antigen	-	-
19	4E3	<i>sotto/dendrolimus</i> isolated in Canada	-	-

S. No.	Strain	Location	Screening with primer set 1 (Masson <i>et al.</i> 1998)	Screening with primer set 3 (BenDov <i>et al.</i> (1997))
20	4M3	<i>darmstadiensis</i> isolated in England	-	-
21	4U1	<i>kyushuensis</i> isolated in Japan from <i>B. mori</i> colony; antisera	-	-
22	4D22	<i>kurstaki</i> HD-73 Crystal minus derivative of <i>Bacillus thuringiensis</i>	-	-
23	4H2	<i>canadensis</i> isolated in Canada; antisera standard	-	-
24	4G6	<i>galleriae</i> isolated in USSR from <i>Galleria mellonella</i> ;	+	+
25	4J4	<i>aizawai/pacificus</i> isolated in Japan from <i>Heliothis assulta</i>	-	-
26	4L1	<i>tolworthi</i> isolated in England from <i>Cadra cautella</i>	-	-
27	4K1	<i>tohokuensis</i> isolated in the US; antisera standard	-	+
		Total positive for <i>cry2</i> family genes	8	12

+ = positive for *cry2* gene family, - = no amplification (negative) of *cry2* gene family.

Table S3 Primers used for PCR screening for the presence of *cry2* genes *

Primer set	Sequence	Specificity	Expected product size
1	F 5'-TAAAGA AAGTGG GGA GTCTT-3' R 5'-AACTCCATCGTTATT TGT AG-3'	<i>cry2</i> gene family	1.5 Kb
2 [†]	R 5'-TCTCATAGGGGCGACTAATC-3' R 5'- TGATATAGGTGCATCTCCGT-3'	<i>cry2Aa</i> -type <i>cry2Ab</i> -type	694 bp 694bp
3	F5'-GTTATTCTTAATGCAGATGAATGGG-3' R 5'-CGGATA AAATAATCTGGGAAA TAGT-3'	<i>cry2</i> gene family	700 bp
4	F 5'-GTTATTCTTAATGCAGATGAATGGG-3' R 5'-GAG ATT AGT CGC CCC TAT GAG-3'	<i>cry2Aa</i> -type	498bp
5	F 5'-GTTATTCTTAATGCAGATGAATGGG-3' R 5'-TGGCGTTAACAATGGGGGGAGAAAT-3'	<i>cry2Ab</i> -type	546 bp
6	F 5'-GTTATTCTTAATGCAGATGAATGGG-3' R 5'-GCGTTGCTAATAGTCCCAACAACA-3'	<i>cry2Ac</i> -type	725 bp

*Primer sets 1 and 2 were designed as per Masson *et al.* (1998) and primers sets 3-6 as per BenDov *et al.* (1997). [†]Each primer was used with primer set 1 as a triplex PCR reaction in screening for *cry2Aa*- and *cry2Ab*-type genes.

Table S4 ORF primers for amplification of full length *cry2*-type genes

S.No.	Primer name	Sequence (5'-3')*	Specificity
1	SK_0BF SK_0BR	CGGAATTCATGAATAGTGTATTGAATAGCGGAAG CCAAGCTTATAAAGTGGTGAAATATTAGTTGGTAC	<i>cry2Ab</i>
2	SK_0AF SK_0AR	CGGAATTCATGAATAATGTATTGAATAGTGGAAGAAC CCAAGCTTATAAAGTGGTGGAAGATTAGTTG	<i>cry2Aa</i>
3	SK_0CF SK_0CR	CGGAATTCATGAATACTGTATTGAATAACGGAAG CCAAGCTTATAAAGTGGTGGAAGATTAGTTGGAAC	<i>cry2Ac</i>

*The underline depicts *Eco*RI site in the forward primer and *Hind*III site in the reverse primer to facilitate cloning in pET29a(+) expression vector.

Table S5 Screening of reference Bt strains for the presence of *cry2Aa*-and *cry2Ab*-type genes using triplex PCR with primers designed as per Masson *et al.* (1998)

S. No.	Reference strains	<i>cry2Aa</i> -type	<i>cry2Ab</i> -type
1	4A6	+	+
2	4F1	-	+
3	4M2	+	+
4	4L3	-	-
5	4B1	+	-
6	4B2	-	+
7	4G6	+	+
8	4D1	-	-
Total positive	8	4	5

+ Presence of expected amplicon, - absence of expected amplicon

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Table S6 Screening of native Bt isolates for the presence of *cry2Aa* and *cry2Ab*-type genes using Triplex PCR with primers designed as per Masson *et al.* (1998)

S. No.	Native isolates	<i>cry2Aa</i> -type	<i>cry2Ab</i> -type
1	SK-28	+	+
2	SK-32	+	+
3	SK-82	+	+
4	SK-217	+	+
5	SK-220	+	+
6	SK-222	+	+
7	SK-229	+	+
8	SK-304	+	+
9	SK-305	+	+
10	SK-700	-	+
11	SK-701	-	+
12	SK-792	+	+
13	SK-793	-	-
14	SK-758	-	-
15	SK-851	-	+
16	SK-915	+	+
17	SK-921	-	+
18	SK-938	+	+
19	SK-941	-	+
20	SK-944	-	+
21	SK-948	+	+
22	SK-952	+	+
23	SK-953	+	+
24	SK-955	+	+
25	SK-956	+	+
26	SK-958	+	+
27	SK-962	+	+
28	SK-973	+	+
29	SK-996	-	+
Total positive		20	27

+ Presence of expected amplicon, - absence of expected amplicon

Table S7 Screening of reference Bt strains for the presence of *cry2*-type genes by using type-specific primers designed as per BenDov *et al* (1997)

S. No	Reference strains	<i>cry2Aa</i>	<i>cry2Ab</i>	<i>cry2Ac</i>
1	4N1	+	+	+
2	4J2	+	+	-
3	4Q5	-	+	-
4	4K1	-	+	-
5	4L3	+	+	-
6	4F1	+	+	+
7	4B1	+	+	+
8	4B2	-	+	-
9	4G6	+	+	+
10	4D1	+	+	-
11	4O1	+	+	+
12	4W1	+	+	+
Total positive	12	9	12	6

+ Presence of expected amplicon, - absence of expected amplicon

Table S8 Screening of native *Bt* isolates for the presence of *cry2*-type genes by using type-specific primers designed as per BenDov *et al* (1997)

S. No	Native isolates	<i>cry2Aa</i>	<i>cry2Ab</i>	<i>cry2Ac</i>
1	SK-20	+	+	+
2	SK-28	+	-	-
3	SK-51	+	+	-
4	SK-105	+	-	-
5	SK-110	-	-	-
6	SK-113	-	-	-
7	SK-621	-	+	+
8	SK-669	-	-	+
9	SK-677	-	-	+
10	SK-678	-	+	+
11	SK-680	+	+	+
12	SK-684	-	+	-
13	SK-700	-	+	-
14	SK-721	+	+	+
15	SK-722	-	+	-
16	SK-758	-	+	*
17	SK-767	+	-	-
18	SK-768	-	+	-
19	SK-781	+	+	-
20	SK-792	-	-	-
21	SK-793	-	+	-
22	SK-853	-	-	-
23	SK-915	+	+	-
24	SK-916	+	+	-
25	SK-920	+	+	-
26	SK-921	+	+	-
27	SK-922	+	-	-
28	SK-935	+	+	-
29	SK-942	-	+	+
30	SK-943	+	+	-

31	SK-944	-	+	+
32	SK-945	+	+	-
33	SK-947	-	-	-
34	SK-948	-	+	+
35	SK-949	-	+	+
36	SK-950	-	-	*
37	SK-951	+	-	-
38	SK-952	-	+	+
39	SK-953	-	+	+
40	SK-954	-	-	+
41	SK-955	-	+	+
42	SK-956	+	+	+
43	SK-957	-	-	+
44	SK-959	-	-	-
45	SK-960	-	+	-
46	SK-961	-	+	+
47	SK-962	-	+	+
48	SK-963	-	+	-
49	SK-973	+	+	+
50	SK-978	+	+	+
51	SK-980	+	+	-
52	SK-995	+	+	+
53	SK-996	-	+	+
54	SK-1007	-	-	-
55	SK-1013	-	-	-
56	SK-1056	-	-	-
Total positive		22	37	22

* Variant amplicon than the expected PCR product size, + presence of expected band, - absence of expected band

Supplementary figure legend:

Figure S1 Distribution of **native** Bt isolates recovered from different sources. Total numbers of isolates from each type of source are shown.

Figure S1

Figure S2 Agarose gel electrophoresis of PCR products of full length (a) *cry2Aa*-type genes amplified using ORF primers. Lanes 4A6-4F1: reference Bt strains, lanes 217-951: native Bt isolates. Lane M: 1 Kb DNA ladder marker.

Figure S2

Figure S3. Agarose gel electrophoresis of PCR products of full length *cry2Ab* genes amplified using ORF primers. Lanes 4B2-4G6: reference Bt strains, lanes 20-758: native Bt isolates. Lane M: 1 Kb DNA ladder marker.

Figure S3