Pakistan J. Zool., pp 1-9, 2023. DOI: https://dx.doi.org/10.17582/journal.pjz/20230516110558

Cry2Aa Delta-Endotoxin Confers Strong Resistance Against Brinjal Fruit and Shoot Borer in Transgenic Brinjal (*Solanum melongena* L.) Plants

Shruti Yadav[1,2]*, Kamana Singh[2], Pratap Adinath Divekar[3] and Suhas Gorakh Karkute[1,3]*

[1]*ICAR-National Institute for Plant Biotechnology, New Delhi, India*
[2]*Deshbandhu College, University of Delhi, New Delhi, India*
[3]*ICAR-Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh, India*

Shruti Yadav and Kamana Singh have made equal contributions in this article.

Article Information
Received 16 May 2023
Revised 05 June 2023
Accepted 23 June 2023
Available online 04 September 2023 (early access)

Authors' Contribution
SY and SGK conceptualized the work. SY and KS conducted all the experiments. PAD analysed the data. SY and SGK wrote the manuscript. All the authors read and approved the final manuscript.

Key words
Brinjal, Shoot and fruit borer, *cry2Aa*, *Leucinodes orbonalis*, Resistance, Transgenic

ABSTRACT

Eggplant or brinjal (*Solanum melongena* L.) is an important fruit vegetable of Solanaceae family that has originated from India and is widely cultivated in tropical and temperate regions across the world. The major constraint for brinjal production is severe damage caused by a Lepidopteran insect pest brinjal shoot and fruit borer (BSFB; *Leucinodes orbonalis*) leading to almost 60% losses. In the present study, a codon-modified synthetic *Cry2Aa* gene was introduced into brinjal through *Agrobacterium* mediated transformation. Presence of *Cry2Aa* gene was confirmed by PCR and Southern blot analysis showed single copy insertions in plants of six independent transgenic events. *Cry2Aa* gene was highly expressed in transgenic plants and its protein level was as high as 30.94 µg/g in fresh leaves and 20.57µg/g in fruits. Insect bioassay showed the BSFB larval mortality between 90% to 100%. Altogether it was observed that expression of the Cry2Aa protein in the shoots and fruit of transgenic brinjal lead to high BSFB larval mortality. Thus, *Cry2Aa* is a potential alternate gene to presently used *Cry1Ac* gene which can also be used for managing the resistance breakdown.

Online First Article

INTRODUCTION

Brinjal or eggplant (*Solanum melongena* L.) is one of the major solanaceous crops of tropics and sub-tropics acclimatized to different agro-climatic zones grown in South East Asia. It is rich in minerals, vitamins and is a great source of total water-soluble sugars, amide proteins and free reducing sugars among other nutrients (Alam *et al.*, 2003). India is the second largest producer of brinjal worldwide, after China. Brinjal is the fourth largest crop after potato, onion and tomato in terms of consumption in Indian scenario. In India, it is cultivated on 747 thousand hectares with an annual production of 12.98 million tonnes second advance estimates 2021-2022 https://pib.gov.in/). More than 70 species of insect attack brinjal (Subbarathnam and Butani, 1982), shoot and fruit borer (BSFB) being the most destructive insect pest which is not only responsible for a substantial yield loss (85-90%) (Patnaik, 2000), but it also decreases the value of the product, making the product less lucrative.

Therefore, it is crucial for sustainable brinjal production that this pest be controlled. The first choice of farmers is synthetic pesticides, despite the availability of eco-friendly and sustainable pest management options like host plant resistance (HPR) (Divekar *et al.*, 2019), plant secondary metabolites (Divekar *et al.*, 2022), bio-control agents (Dukare *et al.*, 2020), and defence proteins (Divekar *et al.*, 2022). Control by application of insecticides is often ineffective since the larvae remain concealed within fruits and shoots, and therefore, escape contact with the insecticide. The uncontrolled use of insecticides by farmers has also resulted in the development of insect resistance, higher production costs, and potential risks to

* Corresponding author: suhaskarkute@gmail.com, kbshrutiyadav@gmail.com
0030-9923/2023/0001-0001 $ 9.00/0

Copyright 2023 by the authors. Licensee Zoological Society of Pakistan.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

the environment and human health (Gaur and Chaudhary, 2009). The development of BSFB-resistant cultivars has been significantly hampered by the absence of any natural sources of resistance to BSFB in any of the cultivated and conventionally cross-compatible species of *Solanum*.

The delta-endotoxin encoded by insecticidal crystal protein (*Cry*) genes of the gram-negative bacteria *Bacillus thuringiensis* is known as the most effective proteinaceous insecticide used in agriculture (Kumar and Kumar, 2010). BSFB resistant transgenic brinjal lines have been made possible by plant genetic engineering employing *Cry* genes from *B. thuringiensis* (Kumar *et al*., 1998). In India, several public and private sector research institutions have developed transgenic brinjal resistant to BSFB, by transferring different version of *Cry* genes (*Cry1Ab* in cv. Pusa Purple Long, Kumar *et al*. (1998); *Cry1Ac* in cv. Kashi Taru, Pal *et al*. (2009); *Cry1Aa3* in cv. Kashi Taru, Rai *et al*. (2013) and five brinjal hybrids are derived from transgenic BSFB resistant lines (carrying *Cry1Ac* gene) by Maharastra Hybrid Seeds Company (Mahyco, Jalna, India). Considering the fact that very limited study has been made on the efficacy of *Cry2Aa* gene particularly in brinjal, we have made an effort to develop BSFB resistant transgenic brinjal lines in the cultivar Pusa Hybrid 6 (male), which could be further used for stacking multiple *Cry* genes into one variety. Cry2Aa protein has very high toxic activity against larvae of Lepidoptera as well as Diptera (Morse *et al*., 2001) providing broad spectrum insect resistance to transgenic plants.

MATERIALS AND METHODS

Construction of the plant transformation binary vector

Synthetic plant codon-optimized *Cry2Aa* gene was excised from pBluescript KS (+) vector using *Kpn*I *and Xba*I restriction sites and cloned into the respective sites present in the pBinAR binary vector and confirmed by restriction digestion analysis. *Cry2Aa* gene was driven under the control of the potato ubiquitin promoter and OCSA terminator. *Neomycin phosphotransferase* (*nptII*) marker gene present in the binary vector, regulated by NOS promoter and NOS terminator was used as a plant selection marker. The pBinAR-*Ubi-Cry2Aa* binary vector constructs were mobilized into *Agrobacterium tumefaciens* EHA105 by freeze-thaw method. *Agrobacterium* harboring vector was inoculated in YEM media with 50 mg/l kanamycin (HI Media) and 10 mg/l rifampicin (HI Media) and grown overnight at 28 ºC on a rotary shaker (220 rpm). The *Agrobacterium* cells were harvested by centrifugation at 5000 g for 10 min at room temperature and further the cells were resuspended in 20 ml Murashige and Skoog (MS) basal salts as inoculation medium (IM) (Fig. 1).

Culture conditions and Agrobacterium *mediated transformation*

Seeds of brinjal cv. Pusa Hybrid 6 (Male parent) were surface sterilized by rinsing for 1 min in 70% ethanol, followed by three washes with autoclaved double distilled water. Further, the seeds were rinsed using 0.1% commercial Clorox (5.25% sodium hypochlorite) with 0.01% Tween 20 for about 10 min, followed by three washes with autoclaved distilled water (5 min per rinse). Seeds were then sowed and germinated on half strength MS medium with pH 5.8. The cultures in all the experiments were incubated at 25 ºC with a 16 h photoperiod. Cotyledonary leaves of 1 mm size in diameter were excised from 12 day old seedlings and kept on pre-culture medium (MS salts, 3% sucrose, 0.1 mg/l α-naphthalene acetic acid and 2 mg/l 6-benzylaminopurine) with pH 5.8 for two days. Later, the explants were submerged in *Agrobacterium* suspension for 10 min and incubated in the dark at 28 ºC. After inoculation, the excess of bacterial suspension was removed, and the explants were shifted to the same pre-culture medium for next two days for co-cultivation. The explants were selected for transformants on selection medium (MS salts, B5 vitamins, 3% sucrose, 2 mg/l BAP, 0.1 mg/l IAA, 500 mg/l cefotaxime and 100 mg/l kanamycin), and the surviving proliferating explants were transferred to regeneration medium (MS salts, B5 vitamin, 3% sucrose, 1.0 mg/l Zeatin, 100 mg/l kanamycin and 500mg/l cefotaxime). The regenerated shoot buds were then shifted to shoot induction medium (MS salts, B5 vitamin, 3% sucrose, 0.1 mg/l α-naphthalene acetic acid, 1 mg/l 6-benzylaminopurine, 100 mg/l kanamycin and 500 mg/l cefotaxime). The regenerated shoots were further transferred to rooting medium (MS salts, B5 Vitamin, 3% sucrose, kanamycin 50 mg/l and cefotaxime 300 mg/l) devoid of growth regulators. Further, these plantlets were hardened and transferred to controlled glasshouse conditions and allowed to set seeds.

Molecular characterization of transgenic plants

The putative transgenic plants were confirmed by amplification of *Cry2Aa* and *nptII* genes by PCR and by Southern hybridization. The gene specific primers used for *Cry2Aa* were F 5' TCAGGGACGTGATCCTCAACGC-3' and R 5' TCGCCCTGGTTGCCGAACTT-3' and for *nptII* were F 5' GCTTGGGTGGAGAGGCTATT 3' and R 5' AGAACTCGTCAAGAAGGCGA 3' for amplification of 1kb and 728bp fragments, respectively.

For Southern hybridization, 10 µg of total genomic DNA from the transgenic and wild-type brinjal plants was digested with *Hind*III restriction enzyme and subjected to electrophoresis on 0.8% agarose gel. Further, the DNA was transferred to a nylon membrane (Hybond-N+; Amersham

Online First Article

Co. Ltd., NJ, USA) by capillary blotting. *Cry2Aa* gene probe of 1 kb was PCR amplified and purified using QI PCR purification kit (Qiagen, USA). The probe was radiolabelled with α [^{32}P]-dCTP by random priming. All Southern blot analysis and hybridizations were performed using Rapid-Hyb buffer according to the manufacturer's instructions (Amersham Co. Ltd., NJ, USA). The hybridization was performed at 60ºC in Rapid-Hyb buffer plus the denatured probe. Later, the membrane was exposed to X-ray film and was placed in an intensifying cassette under dark conditions and stored at -80ºC for 48-72 h and the exposed X-ray film (Fujifilm, Kodak) was developed to visualize the results.

Segregation analysis

Segregation of the *Cry2Aa* transgene in T_1 progeny seedlings was analyzed for each of the four independent transformation events. T_1 generation seeds from self-pollinated T_0 fruits were germinated in pots filled with soilrite. The genomic DNA were extracted from leaf tissues of 30-days old seedlings and PCR was performed using the *Cry2Aa* gene-specific primers as described earlier.

qRT- PCR analysis

Total RNA from the leaves of transgenic *Cry2Aa* and wild type plants were isolated using Spectrum Total Plant RNA Isolation Kit (Sigma-Aldrich) following the manufacturer's instructions. Quality and quantity of RNA was analyzed by gel electrophoresis and Nanodrop spectrophotometer (Thermo Scientific, USA) respectively. cDNA was synthesized with 1µg of total RNA using SuperScript-III cDNA Synthesis System (Invitrogen, Carlsbad, USA). Quantitative real-time PCR was performed in Stratagene Mx3005P detection unit, using VeriQuest SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, USA). For each sample, PCR assays were carried out in triplicates. Melting curve analysis was used to confirm the specificity of the method. The PCR cycles used were as follows: 95° C for 3 min, then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Brinjal *18S* gene was used as an internal control with primers 5'-CCGCGGAAGTTTGAGGCAATAAC-3' and 5'-CGGCAAGGCTATAAGCTCGTTGAA-3'. and for specific amplification of *Cry2Aa*, 5'-AAGAACAACATCTACGCCGCCAAC-3, and 5'-TGAAGGTACGCGTCTGGTTGTTCA-3' were used as forward and reverse primers respectively. For each sample, PCR assays were performed in triplicates. The relative expression of *Cry2Aa* was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

ELISA

A double antibody sandwich enzyme linked immunosorbent assay (ELISA) was used to detect the presence of *Cry2Aa* protein expressed in the leaves of transgenic plants. The experiment was performed with double sandwich quantitative Cry2Aa ELISA plate (Envirologix, Portland, USA). Total protein from the leaf samples of transgenic and wild type brinjal plants was extracted using the protein extraction buffer (Envirologix). The leaf extract was diluted to fit in the linear range of the provided *Cry2Aa* standards, and steps were performed essentially according to manufacturer's instructions. Halo MPR-96 microplate reader (Dynamica, 23 Ottawa, Canada), was used to read the plate at 450 nm. The *Cry2Aa* standard supplied in the kit was used for quantification.

Western blotting

Young leaves of transgenic *Cry2Aa* brinjal and wild-type plants were used for the experiment. The sample was extracted using a protein extraction buffer (50 mM Tris buffer, 150 mM NaCl, 0.001 M PMSF, β-mercaptoethanol, pH 8). The proteins were quantified using Bradford reagent (Bio-Rad, Hercules, USA) and protein concentrations were determined against a standard of bovine serum albumin. About 100 ng crude protein samples were then run on 10% SDS-PAGE gel with a Dual Mini Slab Chamber (Bio-Rad). This protein gel was transferred to Immobilon-P membrane (Millipore, Billerica, USA) using a Mini Trans-Blot electrophoretic cell (Bio-Rad) by applying 40 V for 2 hours in a cold room and further transferred to a blocking solution having skimmed milk powder (5%) in 1X Tris-buffered saline (TBS). It was incubated with primary antibody (mouse anti-cry2Aa antigen, Envirologix) at 1:4000 dilution, and secondary antibody (goat anti-mouse IgG alkaline phosphatase conjugate, GeNei, Bengaluru, India) at 1:4000 dilutions for one hour each at room temperature followed by washes. The signal was detected using BCIP/NBT substrate (Sigma-Aldrich, St Louis, USA) for 5-10 min.

Insect bioassay

Insect bioassay was performed to assess the efficacy of the Cry2Aa protein in the transgenic plants. Detached leaves and fruits from T_2 generation transgenic events (T) and wild type of Pusa Hybrid 6 (C) were used. A shoot bioassay was performed with 4 cm long detached shoots of 30-day old seedlings, grown under glasshouse conditions (28± 2ºC; 70±5% RH); three plants from each transgenic event were used for the experiment. Three mm thick aseptic fruit slices were made from the detached fruits. Each fruit slice/shoot piece was placed in a petri dish (90 X 15 mm) containing water-soaked Whatman paper. Further, laboratory-reared pre-weighed third-instar Leucinodes larva were released into the petri dishes. Later,

Online First Article

larval mortality or the weight of surviving larvae was measured on the fruit slices and shoot pieces of both the transgenic events (T) and the wild type (C). The weight gain was recorded after 4 days, and larval mortality was noted on day six.

RESULTS

Transgenic brinjal overexpressing Cry2Aa *gene*

About 300 cotyledonary leaf explants of Pusa Hybrid-6 brinjal variety were co-cultivated with *Agrobacterium EHA105* strain harboring pBinAR-*cry2Aa*. It was observed that, after three weeks of incubation on the selection medium, around 55% explants formed green calli along the cut surface of the infected leaves whereas wildtype explants (non-infected) completely failed to either proliferate (in to shoots) or show any regeneration response. Wild type explants completely died after three weeks of incubation on the selection medium. The proliferating shoots recovered on selection medium were further transferred to regeneration medium, followed by shoot induction medium and then rooting medium containing 100 mg/l kanamycin and 250 mg/l cefotaxime, to obtain completely regenerated plantlets, which later acclimatized successfully in glass house conditions.

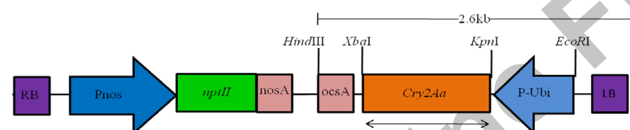

Fig. 1. The T-DNA map of binary vector pBinAR-*cry2Aa*. LB: left border; RB: right border; P-potato: Potato ubiquitin promoter; OCS Ter: terminator sequence of octopine synthase; NOS: nopaline synthase promoter; *nptII:* neomycin phosphotransferase; and Nos Ter: terminator sequence of nopaline synthase.

Molecular characterization of transgenic plants

Putative *cry2Aa* brinjal transformants were subjected to PCR analysis. The results of PCR amplifications showed expected amplicon of 1 kb and 750 bp for the *cry2Aa* and *nptII* genes respectively in transformed plants whereas; as expected no amplification was detected in the wildtype plants (Fig. 2A, B). A total of 16 plants out of 22 examined were positive for the presence of *cry2Aa* and *nptII* gene. To determine the copy number and integration pattern of the transgenes, southern hybridization analysis was performed (Fig. 2C). The analysis revealed the presence of eight independent transgenic events (E1, E2, E3, E4, E5, E6, E7 and E8) of which six lines (E1, E3, E4, E5, E6, E7) showed a single copy of T-DNA insertion while, lines E2 and E8 showed a triple and double copy of T-DNA insertion respectively. As expected, for the left border junction fragment, the hybridization signals were different in sizes and more than 2.6 kb size. Single copy transgene harboring fertile lines, E1, E3, E4 and E7 were further used for molecular and biochemical analysis in this study.

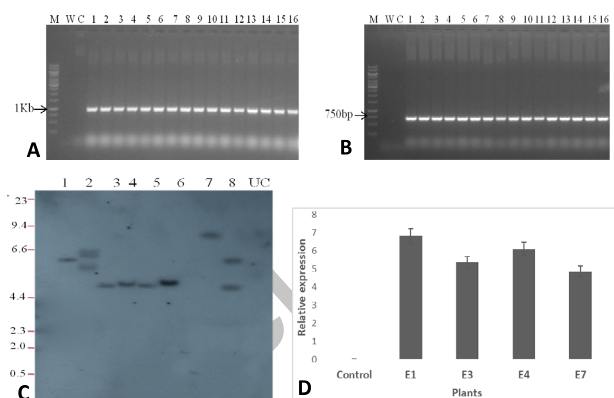

Fig. 2. Molecular confirmation and expression analysis of *cry2Aa* in transgenic brinjal plants. PCR amplification of the *cry2Aa* (A) and *nptII* gene (B) from genomic DNA of putative transgenic plants (T_0). Lanes: M1, 1-kb DNA ladder; W, water control; C, genomic DNA from wildtype plant; 1-16, putative transgenic plants. (C) Southern blot analysis of transgenic plants. Lane 1, 3, 4, 5, 6 and 7 shows single copy integration, Lane 2 shows triple copy integration while lane 8 shows double copy integration M: λ DNA/*Hind*III ladder (Thermo Scientific). The left border (LB) junction fragment of the T-DNA is greater than 2.6 kb in pBinAR-UBI:Cry2Aa binary vector (the distance between *HindIII* and LB). (D) The qRT-PCR analysis shows relative fold change in gene expression of cry2Aa transcript in T2 generation of transgenic brinjal plants. The expression of cry2Aa was determined by using 18S rRNA as internal control. E1, E3, E4 and E7 represent T2 generation transgenic lines.

Segregation analysis

The T_0 transgenic plants were grown in glass house under controlled atmospheric condition like wild type plants. The T_0 plants were selfed and after maturity seeds were harvested. The fertile T_1 progenies were grown, and genomic DNA from these plants was isolated and PCR were performed with *cry2Aa* primers. Segregation analysis of four tested independent events (E1, E3, E4, and E7) showed three out of four lines segregated nearly in 3:1 Mendelian segregation ratio indicating one transgene locus (Fig. 3) (Table I) however, one line (E7) showed some distortion from Mendelian segregation ratios (Table I).

Online First Article

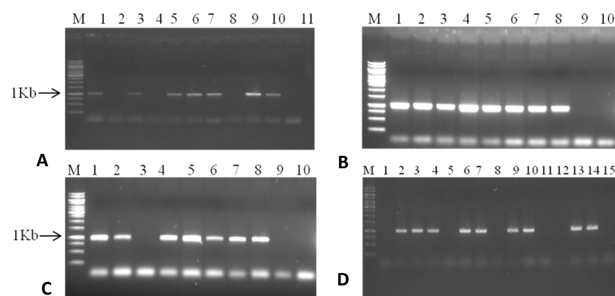

Fig. 3. Segregation analysis of T_1 Cry2Aa brinjal plants. Randomly collected leaf samples from different plants of line E1, E3, E4, and E7 have been used to test the segregation by PCR. (A) E1 *cry2Aa* gene-specific PCR; (B) E3 *cry2Aa* gene-specific PCR; (C) E4 *cry2Aa* gene-specific PCR; (D) E7 *cry2Aa*. M, 1 kb ladder.

Table I. Segregation of *cry2Aa* gene among T_1 progenies of four independent transformation events of eggplant Pusa Hybrid-6.

Transgenic event	No. of seedling		χ^2 value (3:1)
	PCR positive	PCR Negative	
Event E1	7	4	0.382
Event E3	8	2	3.88
Event E4	7	3	2.5
Event E7	9	6	0.355

X^2 0.05 df1=3.841. Table shows the segregation analysis of different transgenic events E1, E3, E4 and E7 using the *Cry2Aa* gene primers.

Expression analysis of transgenic brinjal plants

The relative expression levels of *cry2Aa* transcript were analyzed by qRT-PCR in the four events (Fig. 2D). The four selected transgenic events showed different *Cry2Aa* expression levels; the event E1 showed the highest expression level followed by the event E4, E3 and E7. Protein expression levels of brinjal Cry2Aa plants were analyzed with Cry2Aa ELISA kit (Fig. 4A). The average expression levels in terms of percentage of TSP (total soluble protein) of Cry2Aa was minimum in transgenic line E7 and maximum levels of protein expression found in the line E1. Western blot analysis was performed to detect the presence of cry2Aa protein and to confirm the obtained ELISA results (Fig. 4B). A band corresponding to 66 kDa reacted with the polyclonal antibodies raised against Cry2Aa protein.

Insect bioassay

Non-significant differences were noted in transformed and non-transformed brinjal line in terms of pre-weight BSFB, *L. orbonalis* larvae in shoots (F = 3.241, p= 0.06) and fruits (F = 2.745, p= 0.089). Significant differences were noted in terms of larval mortality of BSFB on shoot as well as fruit tissues (shoot mortality: (F = 380.443, p<0.001), fruit mortality: (F = 252.477, p<0.001) when fed on transformed and non-transformed control brinjal (Table II). The mean mortality of BSFB fed on shoot of transgenic brinjal line was significantly higher (83.33 to 91.11%) in comparison to control (6.67). Similarly, in case of fruit tissues, the transformed brinjal plants exhibited 100% mean mortality, whereas, it was only 3.33 % for non-transformed plants.

Table II. Larval mortality on shoot and fruit of non-transgenic and transgenic brinjal plants after six days of feeding.

Treatment	Larval mortality (%)	
	Shoot	Fruit
E1	86.67±1.92bc	100.00±0.00b
E3	91.11±2.22c	100.00±0.00b
E4	88.89±1.11bc	100.00±0.00b
E7	83.33±1.92b	100.00±0.00b
Control	6.67±1.92a	3.33±1.92a
F	380.443	252.477
P	<0.001	<0.001

Mean values in the same column followed by a different letter (a-c) are significantly different on the basis of Tukey's test (P ≤0.05).

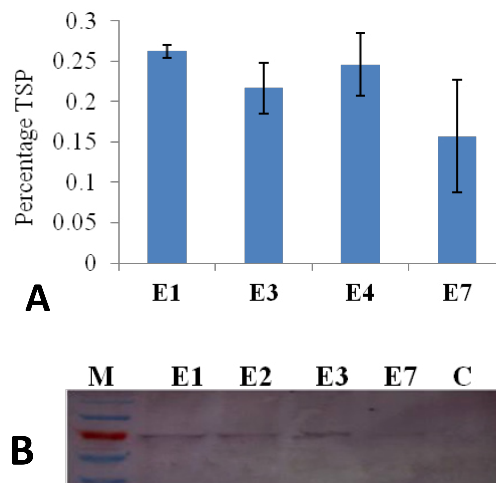

Fig. 4. Protein expression analysis of transgenic brinjal plants. Protein expression profiling of different transgenic plants: E1, E2, E3 and E7 represent different lines of *Cry2Aa* transgenic plants. (A) Quantitative ELISA (B) Western analysis, E1 transgenic lines showes higher concentration of Cry2Aa protein accumulation whereas E7 accumulate less Cry2Aa protein.

Online First Article

Table III. Growth of BSFB third-instar larvae reared on shoot and fruit of transformed brinjal.

Treat-ment	Shoot bioassay				Fruit bioassay			
	Pre-weight (mg)	Weight 4 DAI (mg)	Weight gain (mg)	Weight gain (%)	Pre-weight (mg)	Weight 4 DAI (mg)	Weight gain (mg)	Weight gain (%)
E1	15.00±0.42[a]	23.97±0.84[a]	9.12±0.92[a]	59.87±4.95	14.40±0.76[a]	25.47±0.66[a]	11.60±0.29[a]	77.37±5.16
E3	15.80±1.01[ab]	28.60±0.69[b]	12.63±1.27[a]	82.83±14.47	17.47±0.64[b]	27.90±1.12[a]	10.53±0.52[a]	59.70±1.56
E4	16.60±1.10[ab]	25.60±1.36[ab]	8.47±0.13[a]	54.50±2.29	16.10±1.27[ab]	25.80±1.04[a]	9.40±0.80[a]	61.40±8.71
E7	14.07±0.81[a]	26.80±0.81[ab]	12.47±1.22[a]	92.49±17.10	14.10±0.93[a]	25.40±0.70[a]	11.43±1.00[a]	81.62±12.42
Control	18.10±0.78[b]	48.77±2.25[c]	30.33±0.98[b]	169.36±1.26	16.93±0.81[ab]	59.33±2.97[b]	41.90±2.97[b]	251.68±22.14
F	3.241	59.722	51.286	-	2.745	91.872	93.3	-
P	0.060	<0.001	<0.001	-	0.089	<0.001	<0.001	-

Mean values in the same column followed by a different letter (a-c) are significantly different on the basis of Tukey's test ($P \leq 0.05$).

The mean weight of BSFB larvae at 4 Days after feeding (DAF) on fruit slices from the transformed brinjal plants was found in the range of 25.40-27.90 mg, which was significantly lower than larval weight on fruit slices from non-transformed control brinjal (48.77 mg). Similarly, the mean weight of larvae at 4 DAF on shoots from the transformed brinjal plants was ranged in between 23.97-28.60 mg, which was significantly lower than larval weights measured on shoots from non-transformed control samples (59.33 mg) (Table III). The *L. orbonalis* larvae reared on transgenic shoots (9.12-12.63 mg) or fruit slices (9.40-11.60 mg) showed significantly less weight gain which resulted into high larval mortality.

DISCUSSION

Brinjal is one of the widely grown vegetable crops in Asia and is susceptible to many diseases and pests including the fruit and shoot borer resulting in huge economic losses. *Bacillus thuringiensis* is being efficiently used as a source of Cry toxins for insect pest management in crops. The Bt or Cry formulations are safe for other natural enemies of insect pests (Anil and Sharma, 2010) but it has certain limitations as not being cost effective nor providing long term defense. With the advent of transgenic technology for the genetic manipulation of plant species, an enormous progress is observed in basic plant research. Transgenic crop plants expressing different Cry proteins were able to achieve insect resistance and displayed improved yield and reduced use of synthetic pesticides (Perlak *et al*., 2001; James, 2011). *Bt* transgenic crops are more reliable against target pests as the Cry toxin has a prolonged expression.

The insecticidal gene from *Bacillus thuringiensis* was incorporated in brinjal with *CryIIIb* and *Cry1Ab* to make it resistant to Colorado beetle and brinjal shoot and fruit borer respectively. These transgenic plants showed significantly higher yields in the field trails (Arpaia *et al*., 1998; Kumar *et al*., 1998). Sanyal *et al*. (2005) has successfully transformed chickpea with *Cry2Aa* and *Cry1Ac* with commendable expressions which can further form the basis for combination of these genes to form more robust transgenic lines. Transgenic brinjal with *Cry1Ac* gene has been commercially released in Bangladesh and by 2018-19 it was cultivated ~~by~~ on the area of 1,213.3 ha (Shelton *et al*., 2020).

The perusal of pertinent literature shows that not enough evidences of any definite risk is associated with the genetically modified crops if used with proper implementation of regulatory mechanism. Insecticides are not very effective in control of *L. orbonolis* as it is concealed within the fruit and excessive use of pesticides leads to development of resistance among the target and non-target pests. However, there will always be a certain concern regarding the resistance development in the target pest and secondly lower use of insecticides may not be able to curtail the outbreak of the secondary pest such as mealybugs, aphids and other sucking pest species. (Nagrare *et al*., 2009) for which gene stacking can be an effective method to counter it. This emphasizes the need of alternative and strong Cry genes that can be used in transgenic programmes for managing the resistance development in insects.

Cry2Aa protein acquires broad insect specificity by displaying high level of definite activity against two insect orders; Lepidopteron and Diptera (Yamamoto and McLaughlin, 1981). The *Cry2Aa* nucleotide sequence comprises an open reading frame of 1902 bp that encodes a polypeptide of 633 amino acids. Morse *et al*. (2001) reported that since Cry2Aa protein is way smaller (66kDa) as compared to the Cry1 class proteins (approximately 137kDa) they exhibit higher expression hence higher level of toxicity. Cry2Aa toxins are more lethal compared to the other Cry toxins for the agriculturally important Lepidoptera and display a low level of cross-resistance

Online First Article

in Cry1a- resistant insects (Kota *et al*., 1999). Moreover, to increase the transgene expression in plants for the bacterial gene, plant preferred codon optimization is a major barrier and was succeeding in tobacco, tomato and potato (Perlak *et al*., 1991, 1993). Hence to improve expression of the bacterial *Cry2Aa* gene in plants, various limiting factors like the AT content, codon composition and polyadenylation signals were optimized, and care was taken so as not to disturb the amino acid output of the *Cry* gene (Perlak *et al*., 2001). The related studies show that the growth rates, chlorophyll content, flowering or seed formation is not affected by the high expression rates of the Cry2Aa in the transgenic plants (Fearing *et al*., 1997).

Agrobacterium-mediated genetic transformation in brinjal cv. Pusa Hybrid 6 (male parent) was carried out using cotyledonary leaves as explants and *Agrobacterium* strain harboring pBinAR binary vector containing plant codon optimized *Cry2Aa* gene. Transformed explants were screened and regenerated efficiently with kanamycin (100mg/l) as reported earlier for leaf explants (Purushothaman *et al*., 2013). After transformation only 79% survived from the cotyledonary leaf explants and after two subcultures only 62% showed shoot initiation. Elongated shoots were selected on rooting medium and showed 93% rooting efficiency. Rooted plants were eventually hardened and grown on vermi culite for 15 days and were successfully established in the green house. Southern hybridization analysis, with *Cry2Aa* probe revealed four individual lines with single copy integration of the transgene. These lines with single copy transgene were further selected for molecular and phenotypic studies, to avoid the possibility of transgene silencing in multiple copy transgenics (Hobbs *et al*., 1993). ELISA analysis of the transgenic events indicated the expression of Cry2Aa. Quantitative estimation of Cry2Aa protein was carried out with an absorbance of 450 nm. The transgenic line, E4 showed the highest protein accumulation, and the protein concentrations varied between 23 to 35-ng/ml in the transformed plants. The results were concurrent with the qRT-PCR results, and collaboratively explained the high mortality rate observed for line E4.

Insect bioassay tests revealed that the larvae weight gain after 4 days feed was significantly higher on wild type as compared to the transgenic lines, further proceeding to the mortality data after 6 days, larvae fed on transformed plants exhibited highest mean mortality (100% for lines E1 and E4) as compared to the wild type plants, concluding that the larvae reared on transgenic shoot and fruit pieces did not increase in weight or starved and displayed a high mortality rate. Similar results in terms of bioassay and expression levels have been reported in case of *Cry1Ac* gene for BSFB resistant transgenic brinjal (Pal *et al*., 2009), transgenic brinjal expressing *cry1Aa3* (Rai *et al*., 2013), *CryIIIB* overexpression in brinjal (Chen *et al*., 1995) and *Cry1A(b)* gene for diamondback moth (*Plutella xylostella* L.) resistance in *Brassica oleracea* L. var. capitata (Bhattacharya *et al*., 2002). This suggests that the *Cry2Aa* gene can be effectively used in development of transgenic brinjals having strong and durable resistance against brinjal shoot and fruit borer.

CONCLUSION

Present study shows that *Cry2Aa* is the potential gene with better and durable resistance against BSFB. Significant changes in larval periodic weight, weight gain, and mortality confirmed the results obtained in qRT-PCR and ELISA. Overall, these results demonstrated that *Cry2Aa*-mediated BSFB resistance was present in all the tested transgenic lines. Considering the extensive use of insecticides to control BSFB, host-plant resistance via *Cry2Aa*- transformed plants may provide a first line of defence in an integrated pest management programme. Moreover, the developed Pusa Hybrid 6 lines can be used for pyramiding other toxins, so as to delay resistance development as well to enhance toxicity to BSFB.

ACKNOWLEDGEMENT

The authors are thankful to the Director, ICAR-NIPB for providing the necessary facilities to carry out the work.

Funding

This work was supported by ICAR-National Institute for Plant Biotechnology, New Delhi, India.

Ethical approval

The research work does not contain any studies with human participants and animals performed by any of the authors.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCE

Alam, S.N., Rashid, M.A., Rouf, F.M.A., Jhala, R.C., Patel, J.R., Satpathy, S., Shivalingaswamy, T.M., Rai, S., Wahundeniya, I., Cork, A., Ammaranan,

Online First Article

C. and Talekar, N.S., 2003. Development of an integrated pest management strategy for eggplant fruit and shoot borer in South Asia. *Tech. Bull*., **28**: AVRDC publication No. 03-548. pp. 56.

Anil and Sharma, C.P., 2010. Bioefficacy of insecticides against *Leucinodes orbonalis* on brinjal. *J. environ. Biol.,* **31**: 399-402.

Arpaia, S., Chiriatti, K. and Giorio, G., 1998. Predicting the adaptation of Colorado potato beetle (Coleoptera: Chrysomelidae) to transgenic eggplants expressing CryIII toxin: The role of gene dominance, migration, and fitness costs. *J. econ. Ent.,* **91**: 21-29. https://doi.org/10.1093/jee/91.1.21

Bhattacharya, R.C., Viswakarma, N., Bhatt, S.R., Kirti, P.B. and Chopra, V.L., 2002. Development of insect-resistant transgenic cabbage plants expressing a synthetic cryIA(b) gene from *Bacillus thuringiensis*. *Curr. Sci*., **83**: 146-150.

Chen, Q., Jelenkovic, G., Chin, C.K., Billings, S., Eherhardt, J., Goffreda, J.C. and Day, P., 1995. Transfer and transcriptional expression of coleopteran CryIIIB endotoxin gene of *Bacillus thuringiensis* in eggplant. *J. Soc. Hortic. Sci.,* **120**: 921-927. https://doi.org/10.21273/JASHS.120.6.921

Divekar, P., Kumar, P. and Suby, S.B., 2019. Screening of maize germplasm through antibiosis mechanism of resistance against *Chilo partellus* (Swinhoe). *J. Ent. Zool. Stud.,* **7**: 1115-1119.

Divekar, P.A., Narayana, S., Divekar, B.A., Kumar, R., Gadratagi, B.G., Ray, A., Singh, A.K., Rani, V., Singh, V., Singh, A.K., Kumar, A., Singh, R.P., Meena, R.S. and Behera, T.K., 2022. Plant secondary metabolites as defense tools against herbivores for sustainable crop protection. *Int. J. mol. Sci.,* **23**: 2690. https://doi.org/10.3390/ijms23052690

Divekar, P.A., Rani, V., Majumder, S., Karkute, S.G., Molla, K.A., Pandey, K.K., Behera, T.K. and Govindharaj, G.P.P., 2022. Protease inhibitors: An induced plant defense mechanism against herbivores. *J. Pl. Growth Regul*., **2022**: 1-17. https://doi.org/10.1007/s00344-022-10767-2

Dukare, A., Paul, S., Mhatre, P.H. and Divekar, P.A., 2020. Biological disease control agents in organic crop production system. In: *Pesticide contamination in freshwater and soil environs*: *Impacts, threats, and sustainable remediation* (Hard ISBN: 9781771889537). Apple, https://doi.org/10.1201/9781003104957-10

Fearing, P.L., Brown, D., Vlachos, D., Meghji, M. and Privalle, L., 1997. Quantitative analysis of *CryIA* (b) expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. *Mol. Breed*., **3**: 169-176. https://doi.org/10.1023/A:1009611613475

Gaur, K. and Chaudhary, B., 2009. *The development and regulation of Bt brinjal in India (Eggplant/Aubergine)*. ISAAA Brief No. 38.

Hobbs, S.L., Warkentin, T.D. and DeLong, C.M., 1993. Transgene copy number can be positively or negatively associated with transgene expression. *Pl. mol. Biol*., **21**: 17-26. https://doi.org/10.1007/BF00039614

IHD, Indian Horticulture Database. 2018. National Horticulture Board, Ministry of Agriculture. Government of India, Gurgaon, India. pp. 148.

James, C., 2011. Global status of commercialized Biotech/GM crops - ISAAA Briefs No **43**, Ithaca, NY, USA.

Kota, M., Daniell, H., Varma, S., Garczynski, S.F., Gould, F. and Moar, W.J., 1999. Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proc. natl. Acad. Sci. U.S.A*., **96**: 1840–1845. https://doi.org/10.1073/pnas.96.5.1840

Kumar, B.K. and Kumar, P.A., 2010. *Pests and pathogens: Management strategies*. BS Publication, Hyderabad.

Kumar, P.A., Mandaokar, A., Srinivasu, K., Chakraborty, S.K., Bisaria, S., Sharma, S.K., Kaur, S. and Sharma, R.P., 1998. Insect resistant transgenic brinjal plants. *Mol. Breed.,* **4**: 33e37. https://doi.org/10.1023/A:1009694016179

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, **25**: 402-408. https://doi.org/10.1006/meth.2001.1262

Morse, R.J., Yamamoto, T. and Stroud, R.M., 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure*, **9**: 409-417. https://doi.org/10.1016/S0969-2126(01)00601-3

Nagrare, V.S., Kranthi, S., Biradar, V.K., Zade, N.N., Sangode, V., Kakde, Shukla, G.R.M., Shivare, D., Khadi, B.M., and Kranthi, K.R., 2009. Widespread infestation of the exotic mealybug species, *Phenacoccus solenopsis* (Tinsley) (Hemiptera: Pseudococcidae), on cotton in India. *Bull. entomol. Res*., **99**: 537-541. https://doi.org/10.1017/S0007485308006573

Pal, J.K., Singh, M., Rai, M., Satpathy, S., Singh, D.V. and Kumar, S., 2009. Development and bioassay of

Online First Article

Cry1Ac-transgenic eggplant (*Solanam melongena* L.) resistant to shoot and fruit borer. *J. Hortic. Sci. Biotechnol*., **84**: 434–438. https://doi.org/10.1080/14620316.2009.11512545

Patnaik, H.P., 2000. Flower and fruit infestation by brinjal shoot and fruit borer, *Leucinodes orbonalis* Guen damage potential vs. weather. *Veg. Sci*., **27**: 82-83.

Perlak, F.J., Oppenhuizen, M., Gustafson, K., Voth, R., Sivasupramaniam, S., Heering, D., Carey, B., Ihrig, R.A. and Roberts, J.K., 2001. Development and commercial use of Bollgard® cotton in the USA–early promises versus today's reality. *Pl. J*., **27**: 489-501. https://doi.org/10.1046/j.1365-313X.2001.01120.x

Perlak, F.J., Roy, L.F., Duff, A.D., Sylria, L., McPherson and Fischoff, D.A., 1991. Modification of the coding sequences enhances plant expression of insect control protein genes. *Proc. natl. Acad. Sci. U.S.A.,* **88**: 3324-3328. https://doi.org/10.1073/pnas.88.8.3324

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Peterson, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Blever, D. and Fischoff, D.A., 1993. Genetically improved potatoes: Protection from damage by Colorado potato beetles. *Pl. mol. Biol*., **22**: 313-321. https://doi.org/10.1007/BF00014938

Purushothaman, G., Sudhkar, D. and Jothieswari, M., 2013. Engineering insect resistance in brinjal against fruit and shoot borer *Leucinodes orbonalis*. *Int. J. Adv. Res. Technol*., **6**: 206-217.

Rai, N.P., Rai, G.K., Kumar, S., Kumari, N. and Singh, M., 2013. Shoot and fruit borer resistant transgenic eggplant (*Solanum melongena* L.) expressing cry1Aa3 gene: Development and bioassay. *Crop Prot.*, **53**: 37-45. https://doi.org/10.1016/j.cropro.2013.06.005

Sanyal, I., Singh, A.K., Kaushik, M. and Amla, D.V., 2005. Agrobacterium-mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis* cry1Ac gene for resistance against pod borer insect *Helicoverpa armigera*. *Pl. Sci.*, **168**: 1135-1146. https://doi.org/10.1016/j.plantsci.2004.12.015

Shelton, A.M., Sarwer, S.H., Hossain, M.J., Brookes, G. and Paranjape, V., 2020. Impact of Bt brinjal cultivation in the market value chain in five districts of Bangladesh. *Front. Bioengin. Biotechnol.*, **498**. https://doi.org/10.3389/fbioe.2020.00498

Subbarathnam, G.V. and Butani, D.K.,1982. Chemical control of insect pest complex of brinjal. *Entomology*, **7**: 97-100.

Yamamoto, T. and McLaughlin, R.E., 1981. Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. *Biochem. biophys. Res. Commun*., **103**: 414-421. https://doi.org/10.1016/0006-291X(81)90468-X

Online First Article