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ORIGINAL ARTICLE

Transgenic pigeonpea events expressing Cry1Ac and Cry2Aa exhibit resistance to *Helicoverpa armigera*

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Abstract
Key message **Independent transgenic pigeonpea events were developed using two *cry* genes. Transgenic Cry2Aa-pigeonpea was established for the first time. Selected transgenic events demonstrated 100% mortality of *Helicoverpa armigera* in successive generations.**
Abstract Lepidopteran insect *Helicoverpa armigera* is the major yield constraint of food legume pigeonpea. The present study was aimed to develop *H. armigera*-resistant transgenic pigeonpea, selected on the basis of transgene expression and phenotyping. *Agrobacterium tumefaciens*-mediated transformation of embryonic axis explants of pigeonpea cv UPAS 120 was performed using two separate binary vectors carrying synthetic *Bacillus thuringiensis* insecticidal crystal protein genes, *cry1Ac* and *cry2Aa*. T_0 transformants were selected on the basis of PCR and protein expression profile. T_1 events were exclusively selected on the basis of expression and monogenic character for *cry*, validated through Western and Southern blot analyses, respectively. Independently transformed 12 Cry1Ac and 11 Cry2Aa single-copy events were developed. The level of Cry-protein expression in T_1 transgenic events was 0.140–0.175% of total soluble protein. Expressed Cry1Ac and Cry2Aa proteins in transgenic pigeonpea exhibited significant weight loss of second–fourth instar larvae of *H. armigera* and ultimately 80–100% mortality in detached leaf bioassay. Selected Cry-transgenic pigeonpea events, established at T_2 generation, inherited insect-resistant phenotype. Immunohistofluorescence localization in T_3 plants demonstrated constitutive accumulation of Cry1Ac and Cry2Aa in leaf tissues of respective transgenic events. This study is the first report of transgenic pigeonpea development, where stable integration, effective expression and biological activity of two Cry proteins were demonstrated in subsequent three generations (T_0, T_1, and T_2). These studies will contribute to biotechnological breeding programmes of pigeonpea for its genetic improvement.

Keywords Gene pyramiding · In vitro shoot grafting · Insect bioassay · Multiple shooting · Pigeonpea transformation

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Introduction

Pigeonpea (*Cajanus cajan* (L). Millsp.) is cultivated in semi-arid as well as rainfed regions, spanning mostly the developing nations. It is the sixth most important grain legume in the world and India contributes for 90% of the global production. This legume crop is an important source of protein for impoverished population of the world. Although production of pigeonpea has increased worldwide to 4.85 million tonnes, the yield per hectare has declined over the last decade (FAO 2014). India alone has to import

200,000 tonnes of grains annually to cope with the ever growing market demand of dry and split pigeonpea seeds (dahl) (Shiferaw et al. 2008). The major reason behind this slump in production is the susceptibility of this crop to the devastating Lepidopteran pest *Helicoverpa armigera* or the pod borer. This polyphagous pest is responsible for extensive economic loss to the tune of US$ 300 million, annually (Shanower et al. 1999).

Low level of genetic diversity in pigeonpea reflected by the absence of resistant germplasm to insect infestations was the main reason for failure in the conventional breeding strategies for its improvement. This narrow genetic base makes pigeonpea more prone to a variety of insect attacks, especially pod borer or *H. armigera*. Farmers resorted to excessive use of pesticides in the face of surging demands (Rondon et al. 2007). This practice has been a serious cause of public concern related to food safety and environmental pollution. Furthermore, Lepidopteran pests have a tendency to develop resistance against these compounds. In this context, genetic engineering was found to be an alternative strategy for crop improvement and new variety development.

Bacillus thuringiensis endotoxin (Bt) Cry1Ac has been preferred by researchers for developing resistance against Lepidopteran pests (Sanahuja et al. 2011). With the commercialization of various Bt transgenic crops, there were also reports of their decreasing efficacy towards pests due to field-evolved resistance (Tabashnik et al. 2013). Transgenic plant expressing more than one toxin, each with unique mode of action is of utmost importance to deal this obstacle. Cry2Aa toxin has been previously used in the development of genetically modified rice and chickpea (Bashir et al. 2004; Chen et al. 2005; Acharjee et al. 2010). Mode of action of Cry2Aa was found to be different to Cry1Ac owing to the limited sequence homology and different receptor binding epitopes (Morse et al. 2001). They interacted with different receptor binding sites in the insect gut epithelium, where there were no chance of cross-reactivity between these two proteins (English et al. 1994; Morse et al. 2001; Hernandez-Rodriguez et al. 2008). Moreover, Cry1Ac-resistant larvae did not survive on Bt-cotton plants producing Cry2Ab protein (Tabashnik et al. 2002). Thus, stacking of *cry1Ac* and *cry2Aa* in one crop is supposed to be effective for establishment of durable resistance management strategy against the devastating *H. armigera*.

Despite numerous efforts in last two decades, few reports of transgenic Bt-pigeonpea have been published (Krishna et al. 2010; Ghosh et al. 2014a). The *cry* genes that have been utilized until date are synthetic *cry1E-C* (replacement of 58 amino acids from Cry1Ea with 70 homologous amino acid of Cry1Ca), *cry1Ab, cry1Ac*, and chimeric *cry1AcF* (fusion of N-terminal and domain II from *cry1Ac* and the C-terminal domain from c*ry1F*) (Surekha et al. 2005; Sharma et al. 2006; Krishna et al. 2011; Ramu et al. 2012). There has been inadequate evidences for validation of reported Cry1Ac transgenic pigeonpea events in terms of stability of protein expression and rate of insect mortality (Ghosh et al. 2014a). The present study delineates a reproducible strategy of *Agrobacterium tumefaciens*-mediated transformation of pigeonpea with two important cry genes, *cry1Ac* and *cry2Aa*. Established independent events of *cry1Ac* and *cry2Aa* transgenic pigeonpea expressed cry proteins at optimum level over three generations, corroborated by their remarkable effect on *H. armigera* larval mortality and weight loss.

Materials and methods

Seeds of the pigeonpea cultivar UPAS 120 obtained from the Indian Institute of Pulses Research (IIPR), Kanpur, India, were used for explant preparation. *A. tumefaciens* AGL-1 strain was used for plant transformation. Eggs of *H. armigera* were obtained from the National Bureau of Agricultural Insect Resources (NBAIR), Bangalore, India and used for larval mortality bioassay experiments.

Plant transformation

The plasmids pBINAR-*cry1Ac* and pBINAR-*cry2Aa*, obtained as generous gift from Dr. P. Ananda Kumar, NRCPB, New Delhi, were used for *A. tumefaciens*-mediated transformation. The plasmids contained the gene of interest, *cry1Ac* or *cry2Aa*, under the influence of *CaMV35S* promoter and *nopaline synthase* (*nos*) terminator. *Neomycin phosphotransferase II* (*nptII*) was used as selectable marker gene, flanked by *nos* promoter and terminator (Fig. 1). *A. tumefaciens*-mediated pigeonpea transformation was performed using embryonic axis explants as described by Ghosh et al. (2014b). Transformed explants were cultured on shoot regeneration medium [modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1 mgl^{-1} 6-benzylaminopurine (BAP) and 0.2 $mgl^{-1}\alpha$-naphthaleneacetic acid] containing 100 mgl^{-1} kanamycin. After 6 weeks of incubation in regeneration medium, they were transferred to elongation medium [modified MS medium supplemented with 0.5 mgl^{-1} BAP and 0.5 mgl^{-1} gibberellic acid] containing 100 mgl^{-1} kanamycin and incubated for another 5 weeks. Properly elongated shoots were then grafted on non-transgenic rootstock and subsequently established in greenhouse.

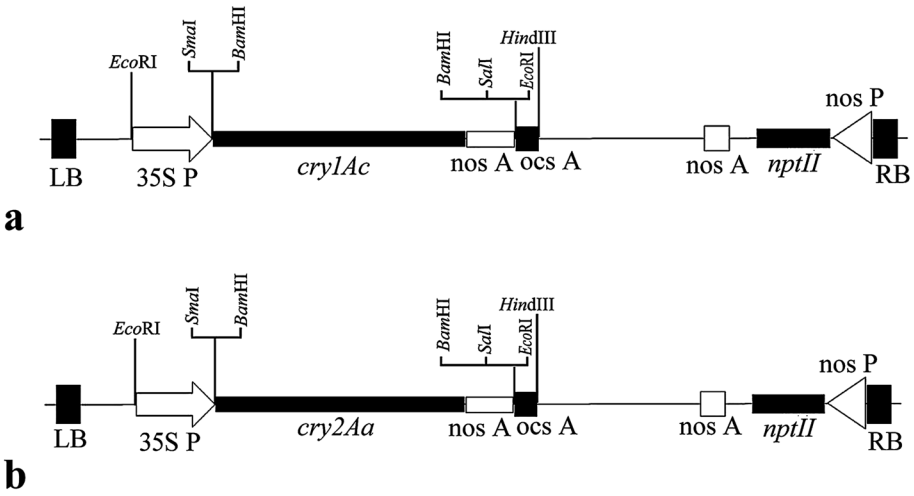

Fig. 1 Schematic representation of T-DNA region of binary vectors used for transformation. **a** pBINAR-*cry1Ac* and **b** pBINAR-*cry2Aa. LB*, left border of T-DNA; *35S P*, cauliflower mosaic virus *35S* promoter; *nos A, nopaline synthase* polyA terminator; *ocs A, octopine synthase* terminator; *nptII,neomycin phosphotransferase II*; *nos P*, *nopaline synthase* promoter; *RB*, right border of T-DNA

Table 1 Details of primers used for PCR analysis

Gene	Sequence	Amplicon size (bp)
nptII	Forward-5′GAGGCTATTCGGCTATGACTG 3′	700
	Reverse-5′ATCGGGAGCGGCGATACCGTA 3′	
cry1Ac	Forward-5′TTGACCACAGCTATCCCATT 3′	662
	Reverse-5′CAACGATACGTTGTTGTGGA 3′	
cry2Aa	Forward-5′CGAACAGTTCCTCAACCAGAGG 3′	600
	Reverse-5′CACAGAACTGGCCGTTCCTCTA 3′	

PCR analysis

Genomic DNA was extracted from leaves of transgenic pigeonpea plants of T_0, T_1, T_2, and T_3 generations following a modified CTAB protocol reported earlier by Chakraborti et al. (2006). Genomic DNA was also isolated from tissue culture regenerated, untransformed, control plants. The integration of *cry1Ac* or *cry2Aa* genes in pigeonpea genome, along with *nptII* in putative transformants, was examined by PCR using genomic DNA as template. Specific forward and reverse primers were designed to amplify the transgenes, *cry1Ac*/*cry2Aa* and selection marker, *nptII* (Table 1). The PCR conditions were, 95 °C for 5 min, followed by 35 cycles at 95 °C for 45 s, 57 °C for 45 s, and 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR amplified products were analysed in 0.8% agarose gel, stained with ethidium bromide, and visualised on a UV transilluminator (UVP, LMS-20).

Qualitative detection of Cry protein using lateral flow strip

Expression of Cry proteins in T_0 transformants was confirmed preliminarily using QuickStix™ strips (Envirologix, USA) meant for detecting Cry1Ac and Cry2A. Leaves of Bollgard II cotton plants were used as positive transgenic control, whereas untransformed pigeonpea plants were used as negative control. Control and transgenic leaf samples were crushed in buffer solution provided by manufacturer and lateral flow strips were dipped in slurry. It was kept for about 10 min, and observed for development of control and test bands as mentioned in manufacturer's manual.

Western blot analysis

About 50 mg of fresh leaf tissue from plants of T_0, T_1, and T_2 generations and untransformed control plants were ground in liquid nitrogen, to which 1 ml of extraction buffer (50 mM Tris Cl, 10 mM ethylenediaminetetraacetic acid, 0.05% Triton-X, 200 µM phenylmethylsulphonylfluoride, 10 µM Leupeptin, and 1 µM Pepstatin) was added. After centrifugation, supernatant containing total soluble protein (TSP) was collected and protein concentration was determined (Bradford 1976). Five hundred nanogram of *Escherichia coli* expressed and purified Cry protein (Cry1Ac/Cry2Aa) was used as positive control. Forty microgram of crude protein from each sample was separated by SDS-PAGE and blotted onto positively charged Hybond C Membrane (Amersham Sciences, UK). After blocking, the membrane was probed with anti-Cry1Ac/ anti-Cry2Aa polyclonal primary antibody (Amar Immunodiagnostics, India) at 1:10,000 dilution and anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (Sigma-Aldrich, USA) as secondary antibody at 1:10,000 dilution. Bound secondary antibodies were detected by enhanced chemiluminescence

(ECL kit, Amersham Biosciences, UK) reagents and developed on Kodak film.

Segregation analysis of T_1 progenies

T_1 seeds were collected from individual T_0 plants. They were imbibed in water overnight, and seed coats were removed and submerged in 100 mgl^{-1} kanamycin for 5 h. Similarly, untransformed seeds were also treated under the same conditions. After 5 h of treatment, seeds were soaked in sterile tissue paper and sown on sterile soilrite. Three-week-old healthy seedlings were scored for kanamycin resistance (Kan^R), while the non-germinated or deformed seedlings were scored for kanamycin sensitivity (Kan^S). Resistant plants were allowed to grow and compared with untransformed plants treated with (negative control) and without (positive control) kanamycin. They were compared on the basis of their morphological differences, viz., plant height, number of leaves and leaflets, and formation of primary and secondary roots. Segregation patterns in the progeny plants were calculated and validated through χ^2 test using the Statistica Software v. 10.0 (StatSoft 2010).

Southern blot analysis

Southern blot analysis was performed according to Sambrook et al. (1989). Genomic DNA was isolated from young, green leaves of T_1 and T_2 plants. Tissue culture regenerated untransformed plant was used as control. Approximately 20 µg genomic DNA was digested with *Hin*dIII and separated on 0.8% (w/v) agarose gel and blotted onto positively charged nylon membrane (Hybond N+) (Amersham Biosciences, UK), following depurination, alkali denaturation, and neutralization. An amplicon of 662 bp obtained from pBINAR-*cry1Ac* vector was used as probe for hybridization with digested genomic DNA from Cry1Ac plants. Similarly, an amplicon of 600 bp obtained from pBINAR-*cry2Aa* vector was used as probe for hybridization with digested genomic DNA from Cry2Aa plants. Membrane was hybridized overnight with [α-^{32}P]-dCTP-labelled *cry1Ac*/ *cry2Aa* specific probes at 68 °C. After hybridization, the membrane was washed with 2X SSC buffer (3 M sodium chloride and 0.3 M tri sodium citrate, dihydrate) and 0.1% sodium dodecyl sulphate (SDS) at room temperature for 1 h and at 68 °C for another hour using 0.1X SSC, 0.1% SDS, and then, the membrane was exposed to Kodak X-ray film, stored at −80 °C for 5 days, and developed subsequently.

In addition, membrane containing blotted DNA of T_2 plants was probed with Digoxigenin (DIG) labelled above-mentioned DNA probes (Roche, DIG DNA Labelling and Detection Kit, version 19, 2004). Membrane was hybridized according to manufacturer's instructions and incubated in dark, overnight. Reaction was stopped when desired band intensities were achieved on membrane.

Quantification of Cry proteins from total soluble protein extracts

Protein extracted from leaves of single-copy T_1 and T_2 lines was used for quantification analysis by indirect ELISA. Leaves from untransformed plants were used as control. Twenty microgram of crude protein extract of transgenic leaves and purified Cry proteins (serially diluted from 50 to 5 ng) were added to ELISA coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide; pH 9.5). They were coated onto 96-well microtiter plate and incubated overnight at 4 °C. After incubation, wells were washed thrice with 1X PBST (1X phosphate buffered saline and 0.1% Tween-20) and blocked with 5% (w/v) bovine serum albumin (BSA) in PBS for 2 h at 37 °C. The wells were again washed thrice with PBST, and incubated with anti-Cry1Ac/anti-Cry2Aa primary antibody in PBS for 1 h at 37 °C, followed by incubation in HRP-conjugated anti-rabbit antibody in PBS for 1 h at 37 °C after washing four times with PBST. Then, O-phenylenediamine hydrochloride tablets (OPD, Sigma-Aldrich, USA) dissolved in citrate buffer were added to each well. After the development of colour, the absorbance was read with a microplate reader (BioRad, CA, USA) at 415 nm.

Detached leaf bioassay of *H. armigera*

Eggs of *H. armigera* were hatched and reared on artificial diet (Armes et al. 1992) for 2–5 days. Second instar larvae were used for no-choice detached leaf feeding bioassay. Leaflets of 30-day-old individual T_1 and T_2 transgenic events and untransformed plants were placed in 3% water-agar in insect breeding boxes in five replicates. In each box, two healthy second instar larvae were placed. Each experiment was conducted for three times and percentage of larval mortality was recorded over a period of 7 days. Larvae fed on untransformed pigeonpea leaves served as control. The percentage of living insects was calculated for each bioassay box at 24 h intervals. Mean and standard error of survival percentage of larvae were calculated. Data were combined from the three independent repeat experiments and compared using analysis of variance (ANOVA, $p = 0.05$).

Furthermore, to determine the effect of Cry expression on the growth of *H. armigera*, detached leaf bioassay was conducted to observe the weight loss of third–fourth instar larvae over a period of 13 days. Each insect breeding box contained single larva, and five such boxes were used per experiment. Each experiment was conducted for three times. Weight of larvae was measured at every 24 h,

and mortality was recorded. Larvae fed on untransformed leaves served as control.

Immunohistofluorescence analysis

Transverse leaflet sections of T_3 transgenic events and untransformed plants were incubated in 10% (v/v) trichloroacetic acid at 4 °C for 2 h, followed by washing with 3:1 ethanol: acetic acid for three-to-four times or until complete removal of chlorophyll. The sections were then incubated successively through a series of graded ethanol to water 90, 70, 50, and 30% (v/v), respectively, each of 15 min duration and kept in blocking solution [3% (w/v) BSA in 1X PBS] at room temperature for 2 h. The tissue samples were then subjected to anti-Cry1Ac/anti-Cry2Aa antibody (1:10,000) dissolved in BSA for overnight at 4 °C. After washing with 1x PBS, the sections were treated with anti-rabbit IgG-FITC conjugated (1:20,000) (Sigma-Aldrich, USA) secondary antibody for 1 h at room temperature. Finally, leaf sections were examined under a fluorescence microscope (Axio Scope, Carl Zeiss, Germany) using excitation filter of 450–490 nm for FITC.

Results

Pigeonpea transformation and PCR analysis of T_0 transgenic plants

Regenerated transformed shoots were grafted on non-transgenic rootstock after selection and established in greenhouse. Putative T_0 transgenic plants were identified by PCR analysis. Fifty-two Cry1Ac and 55 Cry2Aa T_0 plants successfully amplified 700 bp fragment of the *nptII* gene when specific primers were used to detect the selectable marker gene. All those T_0 plants responded positively when *cry1Ac* or *cry2Aa* specific primers were used to amplify 662 bp or 600 bp internal fragment, respectively (Fig. 2).

Selection of primary transformants using Cry expression profile

T_0 putative transformants positive for PCR analysis were further screened on the basis of their protein expressing capabilities. Lateral flow devices in the form of Quick-Stix strips were inducted to rapidly detect the expression of Cry proteins at preliminary stage of analysis. Twenty-one Cry1Ac and 32 Cry2Aa lines exhibited prominent control and test bands on the strips (Fig. 3 a, b). These batches of plants were primarily selected for the next round of screening by Western blot. Transformants that did not exhibit test bands were discontinued for further analyses. Western blot analysis of strip test-positive T_0 transformants revealed 14 Cry1Ac and 18 Cry2Aa lines exhibiting the presence of ~66 and ~68 kDa bands, respectively (Fig. 3 c, d). No such corresponding bands were observed in case of the untransformed plant sample taken as negative control. T_0 lines positive for PCR, strip assay, and Western blot were selected for further investigations. They were allowed to self-pollinate under restricted conditions in biocontainment facilities, and T_1 seeds were harvested.

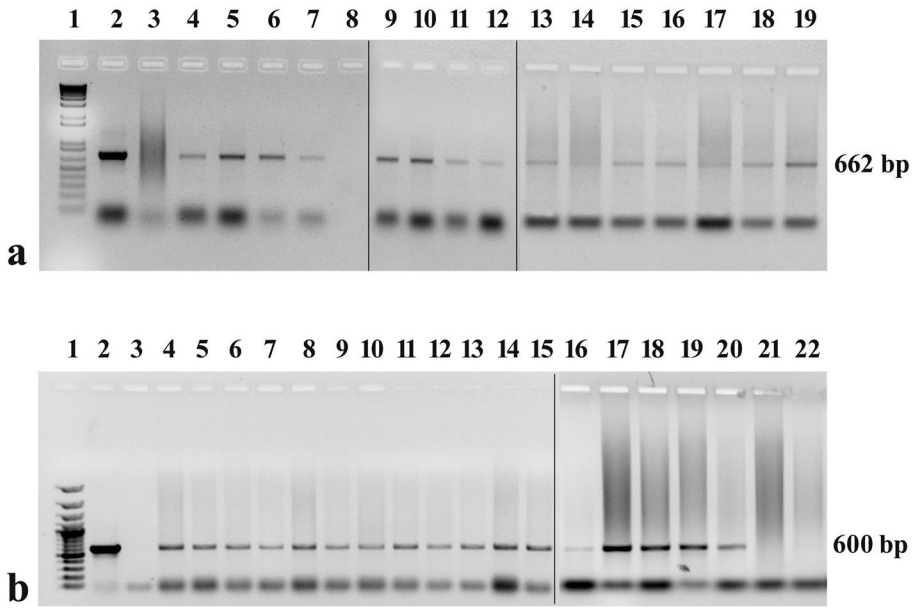

Fig. 2 Representative PCR profile of putative T_0 transformants. **a** Lanes *4–19*, amplification of *cry1Ac* specific 662 bp fragment. **b** Lanes *4–22*, amplification of *cry2Aa* specific 600 bp fragment. Lanes *1, 2*, and *3*, DNA ladder, positive and negative control, respectively

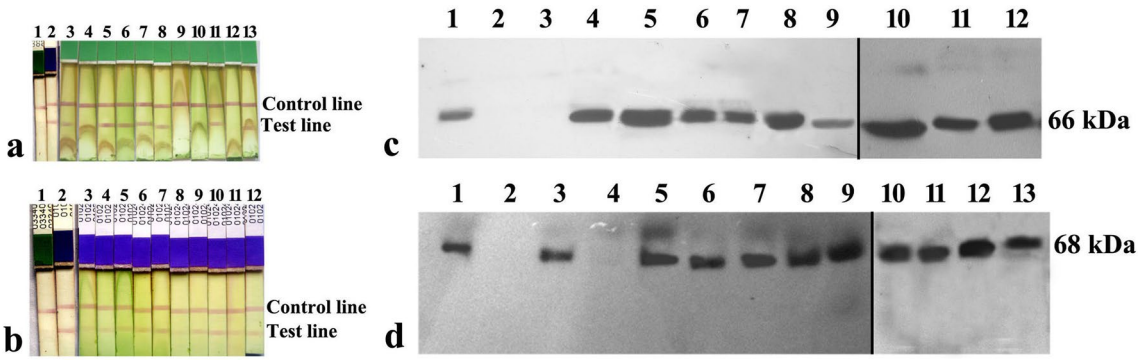

Fig. 3 Lateral flow strip test (**a, b**) and Western blot analysis (**c, d**) performed on total soluble protein of T_0 putative transformants. **a** Lanes *3–13*, Cry1Ac transformants. **b** Lanes *3–12*, Cry2Aa transformants. Lanes *1* and *2* (**a, b**), untransformed plant as negative control and Bollgard II cotton as positive control, respectively. **c** Lane *1*, purified Cry1Ac (~66 kDa) as positive control; lanes *3–17*, Cry1Ac transformants. **d** Lane 1, purified Cry2Aa (~68 kDa) as positive control; lanes *3–17*, Cry2Aa transformants. Lane *2* (**c, d**), untransformed plant as negative control

Segregation analysis of T_1 progenies and inheritance of transgene

Segregation pattern of T_1 seeds derived from self-fertilization of the T_0 plants (14 Cry1Ac and 18 Cry2Aa lines) was studied by germinating them in the presence of 100 mgl^{-1} kanamycin. Later, those were sown on soilrite. Positive control seeds (not treated with kanamycin) germinated normally and attained optimum height (~14 cm) within 3 weeks. Transgenic seedlings were comparable with positive control plants with distinct cotyledonary leaves. These seedlings later matured into green plants with expected height, leaf shape, and number without deformities. Long primary tap roots with numerous secondary roots were observed in these plants (Supplementary Fig. 1). Seeds of null segregant lines failed to set appropriate cotyledonary leaves and were distinctly dwarfed and stunted in appearance with deformed rudimentary roots. They were unable to attain the desired height within 3 weeks. Leaflets were shrivelled up and the apical buds started to disintegrate, ultimately leading to death. Negative control seeds (treated with kanamycin) responded similarly to the null segregants. They failed to germinate showing deformed cotyledonary leaves and absence of primary roots.

After 3 weeks of germination, 12 Cry1Ac and 15 Cry2Aa T_1 events showed segregation in 3:1 ratio (Kan^R:Kan^S) according to Chi-square test ($p \geq 0.05$) (Table 2). These T_1 transformants were chosen for PCR analysis with *nptII* specific primers, which yielded 700 bp amplicon. Similarly, PCR with *cry1Ac* or *cry2Aa* specific primers resulted in the amplification of 662 or 600 bp fragment, respectively. The individual progenies of these T_1 events were identified for their molecular attributes and grown to maturity in greenhouse to obtain T_2 and T_3 seeds.

Molecular analyses of *cry*-pigeonpea in T_1 generation

Western blot analysis of leaf extracts of matured T_1 progeny lines demonstrated that Cry1Ac/Cry2Aa protein was expressed efficiently in the transgenic pigeonpea lines. TSP obtained from T_1 progenies indicated the presence of ~66 and ~68 kDa bands of expressed Cry1Ac and Cry2Aa proteins, respectively, in transgenic events (Fig. 4). No such band was observed in case of untransformed plant samples when probed with anti-cry1Ac/cry2Aa antibody. T_1 progenies obtained from 12 Cry1Ac and 11 Cry2Aa T_0 parental events were able to express Cry1Ac and Cry2Aa proteins, respectively.

Genomic DNA of 12 Cry1Ac and 11 Cry2Aa Western-positive T_1 events was digested with *Hin*dIII, due to the presence of unique *Hin*dIII site located at the 3′ end of the *ocs* terminator. After hybridization with radiolabelled gene probes, all Cry1Ac and Cry2Aa events showed single-copy transgene integration (Fig. 5). The hybridization signal of T_1 transgenic plants unveiled single-copy insertion of *cry1Ac* ranging between 4 and 9 kb. Integration of single-copy *cry2Aa* ranged between 3 and 9 kb. Untransformed plant did not show any hybridization signal in both occasions. Purified PCR product of *cry* genes (662 bp and 600 bp for *cry1Ac* and *cry2Aa*, respectively) as well as *Hin*dIII digested binary vectors (~14 kb) used as positive controls in this experiment showed positive signals.

The kanamycin-resistant and Western blot positive single-copy transgenic events were analysed by indirect ELISA. Twelve events expressed Cry1Ac in the range of 0.150–0.172% of TSP and another 11 events exhibited Cry2Aa in the range of 0.138–0.170% of TSP (Fig. 6).

Table 2 Segregation analysis of 14 Cry1Ac and 18 Cry2Aa T_1 transgenic lines of pigeonpea

Cry1Ac T_0 plants	T_1 seeds	Ratio (Kan^R:Kan^S)	χ^2 value	p*	Cry2Aa T_0 plants	T_1 seeds	Ratio (Kan^R:Kan^S)	χ^2 value	p*
142-6	25	2.12:1	0.653	0.419	129-1	25	1.5:1	3.000	0.083
142-7	25	2.12:1	0.653	0.419	129-2	16	0.33:1	21.333	0.000
142-7(1)	22	1.44:1	2.970	0.085	137-2	19	1.37:1	2.965	0.085
142-10	10	1.5:1	3.333	0.273	137-4	23	1.87:1	1.174	0.279
142-10(1)	16	1.3:1	3.000	0.083	137-5	25	1.5:1	3.000	0.083
142-11	25	1.7:1	1.613	0.204	137-7	25	1.5:1	3.000	0.083
142-12	20	0.42:1	0.267	0.000	137-8	19	1.37:1	2.965	0.085
143-1S	25	2.6:1	0.120	0.729	137-10	10	1.5:1	1.200	0.273
143-2S	27	2:1	1.000	0.317	137-11	20	1.85:1	1.067	0.302
143-8S	25	2.58:1	2.400	0.729	137-12	18	1.25:1	3.630	0.057
143-11S	15	1.5:1	1.800	0.180	137-13	38	0.58:1	29.500	0.000
143-43M	10	1.5:1	1.200	0.273	137-15	18	1.25:1	3.630	0.057
143-43M(1)	25	2.58:1	0.120	0.729	137-20	24	1.4:1	3.556	0.059
143-44M	25	0.66:1	3.000	0.000	140-3	25	1.5:1	3.000	0.083
					140-10	25	0.5:1	20.28	0.000
					140-14	25	2.57:1	0.12	0.729
					140-17	25	2.12:1	0.653	0.419
					70-2	17	2.4:1	0.176	0.674

*12 Cry1Ac and 15 Cry2Aa T_1 lines exhibited 3:1 segregation according to Chi-square test, ($p \geq 0.05$)

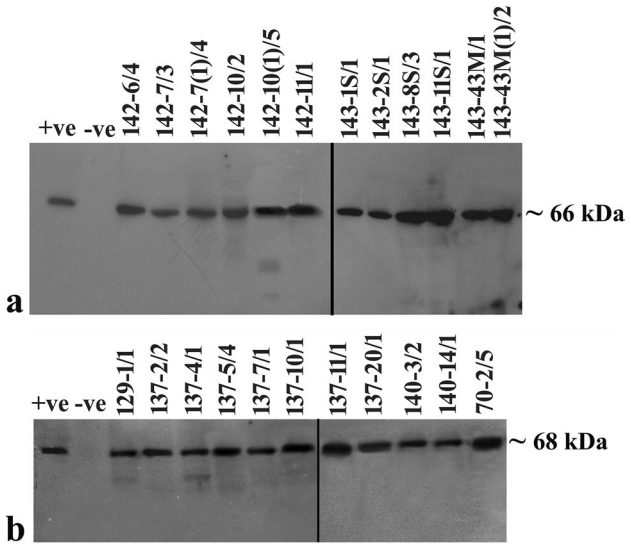

Fig. 4 Western blot analysis of T_1 transgenic pigeonpea events. **a** Total soluble protein (TSP) of 12 Cry1Ac events. Lane '+ve', purified Cry1Ac (~66 kDa) as positive control; lane –ve, untransformed plant as negative control. **b** TSP of 11 Cry2Aa events. Lane '+ve', purified Cry2Aa (~68 kDa) as positive control; lane −ve, TSP from untransformed plant as negative control

Transgene integration and expression analyses in T_2 generation

*Hin*dIII digested genomic DNA of T_2 progenies was probed with non-radioactive DIG DNA probes. All T_2 progenies exhibited single-copy integration of transgene. Three progenies each from T_1 Cry1Ac [143-2S/1 and 143-43M(1)/2] and Cry2Aa events [137-5/4 and 70-2/5] exhibited single-copy band identical to the parental line (Fig. 7a–d). Untransformed plant did not show any hybridization signal.

Western blot analysis of leaf extracts of mentioned T_2 progenies demonstrated that Cry1Ac/Cry2Aa protein was expressed efficiently in these pigeonpea lines similar to their T_1 parental events (Fig. 7e, f). ELISA revealed T_2 progenies of mentioned events contained Cry1Ac in the range of 0.160–0.171% of TSP, and Cry2Aa in the range of 0.156–0.172% of TSP.

Efficacy of the transformed plants against *Helicoverpa armigera*

Biological efficacy of 12 Cry1Ac and 11 Cry2Aa T_1 events carrying single-copy transgene was analysed. The survivability of second instar larvae inside the bioassay setup was monitored at an interval of 24 h (Fig. 8). Untransformed plants were used as negative control. The difference in survival percentage of the insects incubated on T_1 leaves was statistically significant after 72 h in comparison with control leaves according to ANOVA analysis ($p \geq 0.05$). Insect survival on untransformed pigeonpea was between 98–100%. Three independent events of Cry1Ac and two events of Cry2Aa were found to have 100% mortality on

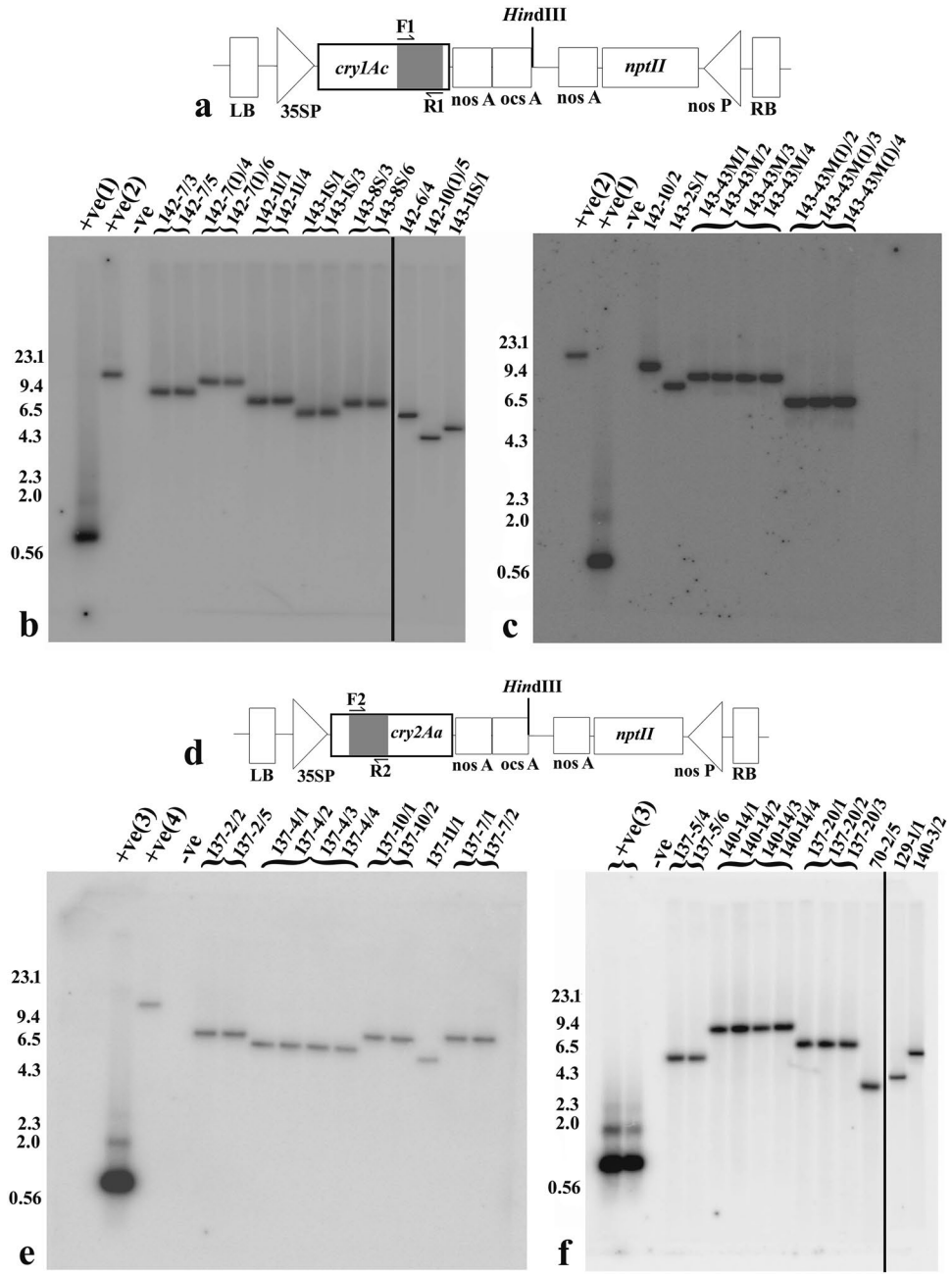

Fig. 5 Southern blot analysis of T_1 transgenic pigeonpea events. **a** Schematic representation of *Hin*dIII recognition site and 662 bp probe sequence (marked in *grey*) obtained from binary vector pBINAR-*cry1Ac* using probe specific forward (F1) and reverse (R1) primers. **b, c** *Hin*dIII digested genomic DNA of T_1 progenies of 12 Cry1Ac transgenic events. Lanes +ve(*1*) and +ve(*2*), PCR purified 662 bp *cry1Ac* specific amplicon, and *Hin*dIII digested 14.3 bp pBINAR-*cry1Ac* plasmid, respectively, as positive controls. **d** Schematic representation of *Hin*dIII recognition site and 600 bp probe sequence (marked in *grey*) obtained from binary vector pBINAR-*cry2Aa* using probe specific forward (F2) and reverse (R2) primers. **e, f** *Hin*dIII digested genomic DNA of T_1 progenies of 11 Cry2Aa transgenic events. Lanes +ve(*3*) and +ve(*4*), PCR purified 600 bp *cry2Aa* specific amplicon and *Hin*dIII digested 14.4 bp pBINAR-*cry2Aa* plasmid as positive controls, respectively. Lane −ve, *Hin*dIII digested genomic DNA of untransformed plant as negative control

the second instar larvae at the end of the bioassay period (Table 3). Five Cry1Ac and four Cry2Aa events exhibited mortality between 80–90% (Table 3). In rest of the events, i.e., four Cry1Ac and five Cry2Aa, mortality was below 80%. T_1 events exhibiting more than 80% mortality were selected for further analysis and their T_2 progenies were taken for bioassay studies.

Three Cry1Ac T_2 progenies each from two T_1 events [143-2S/1 and 143-43M(1)/2] were observed to have 90–100% mortality. Similarly, three Cry2Aa T_2 progenies each from two T_1 events [137-5/4 and 70-2/5] exhibited 100% mortality (Fig. 9a, c). The difference in survival percentage of the insects incubated on T_2 leaves was found to be statistically significant after 72 h in comparison with control leaves according to ANOVA analysis ($p \geq 0.05$). Development of surviving larvae that fed on transgenic plants was critically impaired when correlated with the control larvae. Drastic weight loss followed by untimely death of third–fourth instar larvae was observed within 13 days. (Fig. 9b, d; Fig. 10). Loss of weight was recorded to be statistically significant after 9th day of incubation in comparison with control plant, according to ANOVA analysis ($p \geq 0.05$). Weight of larvae fed on

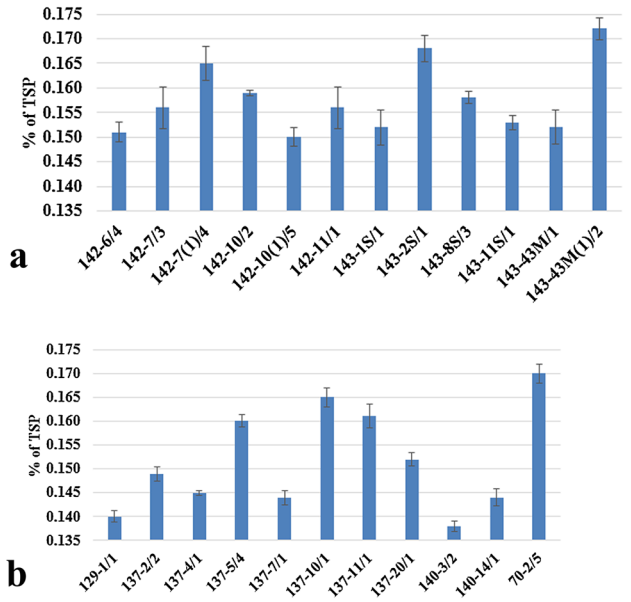

Fig. 6 Quantitative estimation of accumulated transgenic protein through ELISA in T_1 events, expressed as percentage of total soluble protein (TSP). **a** Expression of Cry1Ac between 0.150–0.172% of TSP. **b** Expression of Cry2Aa between 0.138–0.170% of TSP

control plants increased gradually. They matured normally on their host, and were able to pupate.

Immunohistofluorescence study of T_3 plants

The spatial expression patterns of *cry1Ac* and *cry2Aa* driven by *CaMV35S* promoter in PCR positive (data not shown), T_3 transgenic events [143-43M(1)/2/2/1 and 70-2/5/2/1] were determined by in situ immunohistofluorescence localization of Cry proteins in TCA-fixed leaf tissue sections. Immunohistofluorescence analysis of the leaf tissue sections showed the expected constitutive expression pattern with green fluorescence spreading in all tissue types (Fig. 11). Leaf sections revealed the presence of Cry1Ac in trichomes, epidermis, cortex, and vascular bundles. Similar expression pattern was found in case of Cry2Aa leaf sections. No Cry accumulation was detected in the tissues of untransformed control plant.

Discussion

Non-availability of insect-resistant traits in the germplasm of pigeonpea had restricted its genetic improvement. The described study delineates a reproducible strategy to develop transgenic pigeonpea with two important *cry* genes, *cry1Ac* and *cry2Aa*. The transgenic events were screened on the basis of the following criteria: (1) ability of transgenic plants to express Cry protein; (2) Mendelian segregation pattern in T_1 progenies; (3) single-copy insertion status; and (4) high rate of insect mortality. The putative transformants of pigeonpea were selected on the basis of Cry expression in T_0 generation. It involved the usage of QuickStix™ strips for rapid detection of Cry1Ac and Cry2Aa proteins in primary transformants those amplified the transgenes through PCR. These lateral flow devices were designed to give yes/no result, whether the sample contained the desired protein at or above a standard threshold level (Lipton et al. 2000). Usage of strips was found to be reliable during quick detection of Bt proteins in transgenic corn, Bollgard® cotton and Roundup-Ready® soybean (Bernardi et al. 2015; Stave 2002; Lipp and Anklam 2000). This helped to narrow down the search for the lines having detectable transgene expression, instead of carrying many primary transformants to the next generation.

It is an essential requirement of any transgenic research to prove faithful transmission of integrated transgene in the successive generations of the transgenic plants. The study was undertaken to determine the inheritance and segregation of the *cry* insecticidal gene in transgenic pigeonpea events. The kanamycin-resistant phenotype in Cry1Ac—and Cry2Aa—pigeonpea segregated as simple dominant Mendelian trait. Twelve Cry1Ac and 15 Cry2Aa T_1 lines exhibited 3:1 segregation pattern. Stable expression of *cry* gene in putative T_1 single-copy lines was confirmed by Western blot analysis before proceeding to Southern blot. Twelve Cry1Ac and 11 Cry2Aa lines that showed 3:1 segregation pattern in kanamycin selection demonstrated Cry1Ac and Cry2Aa expression, respectively. Transformation is considered to be a random event and each transgenic plant is the result from a separate insertion event (Kohel et al. 2000). The number and sites of gene insertions into the plant genome were found to influence gene expression in transgenic plants (Benedict et al. 1996). In the present study, Southern blot hybridization with *Hin*dIII digested genomic DNA in selected T_1 progenies indicated copy number of T-DNA integration events. Twelve Cry1Ac [142-6/4, 142-7/3, 142-7(1)/4, 142-10/2, 142-10(1)/5, 142-11/1, 143-1S/1, 143-2S/1, 143-8S/3, 143-11S/1, 143-43M/1 and 143-43M(1)/2] and 11 Cry2Aa [129-1/1, 137-2/2, 137-4/1, 137-5/4, 137-7/1, 137-10/1, 137-11/1, 137-20/1, 140-3/2, 140-14/1, and 70-2/5] events carried single-copy T-DNA integrations, concurring the findings in segregation analysis. Furthermore, Southern blot hybridization in selected T_2 events indicated stable foreign gene integration. The size of each insert was unique in individual event confirming independent origin of the transgenic lines. Earlier, in the field of transgenic pigeonpea development, Sharma et al. (2006) performed Southern blot of four single-copy T_1 Cry1Ab events; Surekha et al. (2005) reportedly identified 18 Southern positive, single-copy T_1 Cry1E-C plants and Krishna et al. (2011) demonstrated four T_0 Cry1Ac

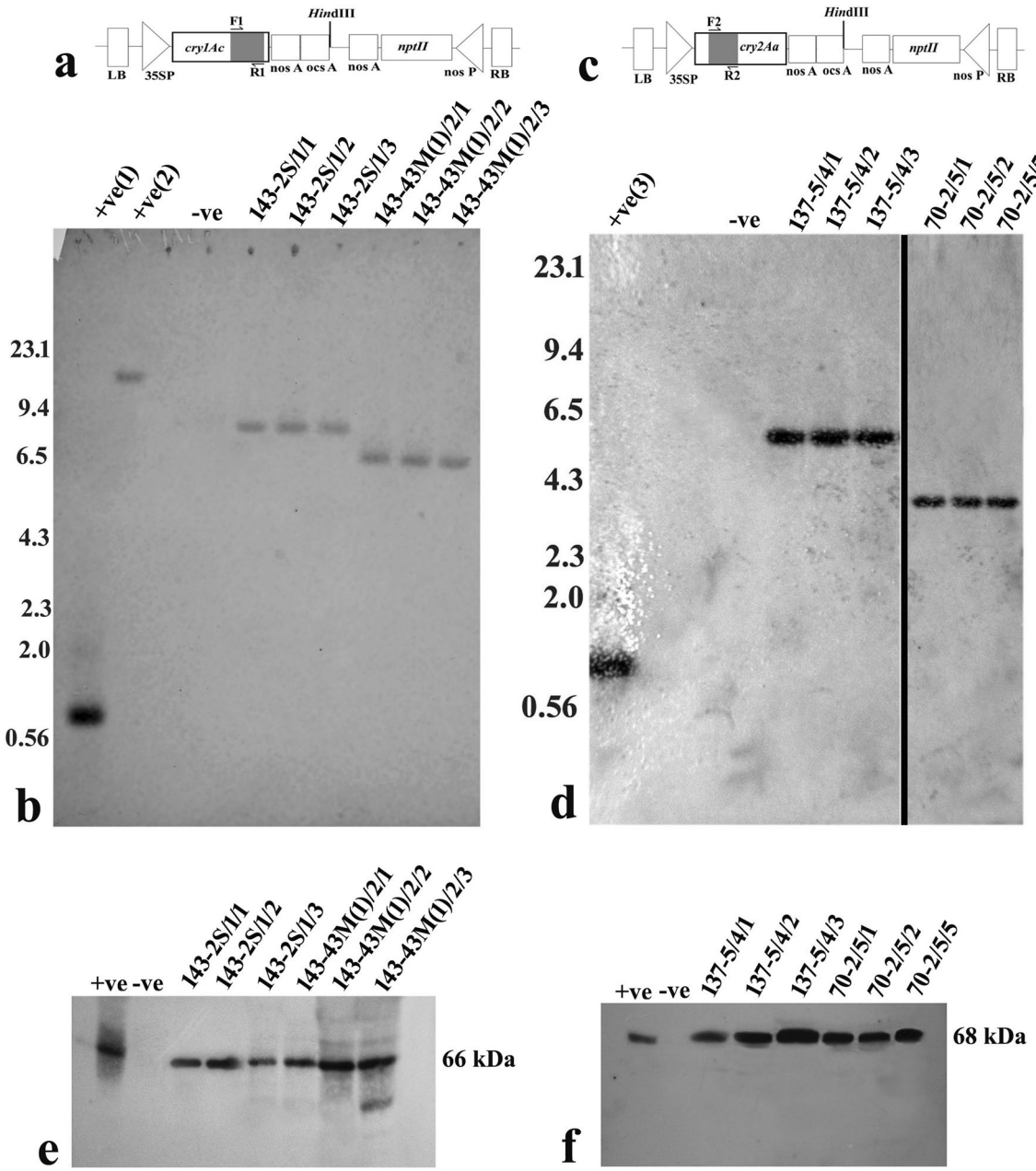

Fig. 7 Southern (**a**–**d**) and Western blot (**e, f**) analyses of T_2 transgenic pigeonpea events. **a** Schematic representation of *Hin*dIII recognition site and 662 bp probe sequence (marked in *grey*) obtained from binary vector pBINAR-*cry1Ac* using probe specific forward (F1) and reverse (R1) primers. **b** *Hin*dIII digested genomic DNA of T_2 progenies of two Cry1Ac transgenic events. Lanes +ve(*1*) and +ve(*2*) PCR purified 662 bp *cry1Ac* specific amplicon and *Hin*dIII digested 14.3 bp pBINAR-*cry1Ac* plasmid, respectively, as positive controls. **c** Schematic representation of *Hin*dIII recognition site and 600 bp probe sequence (marked in *grey*) obtained from binary vector pBINAR-*cry2Aa* using probe specific forward (F2) and reverse (R2) primers. **d** *Hin*dIII digested genomic DNA of T_2 progenies of two Cry2Aa transgenic events. Lanes +ve(*3*) PCR purified 600 bp *cry2Aa* specific amplicon. Lane –ve (**a, b**), *Hin*dIII digested genomic DNA of untransformed plant as negative control. **e** Total soluble protein (TSP) of T_2 progenies of two Cry1Ac events. Lane +ve, purified Cry1Ac (~66 kDa) as positive control. **f** TSP of T_2 progenies of two Cry2Aa events. Lane '+ve', purified Cry2Aa (~68 kDa) as positive control. Lane –ve (**c, d**), TSP from untransformed plant as negative control

events with single-copy integration. Previously, Ramu et al. (2012) performed extended analysis during the production of transgenic pigeonpea containing the fused *cry1Ac-F* gene. In T_3 generation, they designated four transgenic lines to be promising on the basis of larval mortality bioassay, but Southern analysis was reportedly done on three selected plants at T_3 stage, while data revealed two events, with different integration patterns.

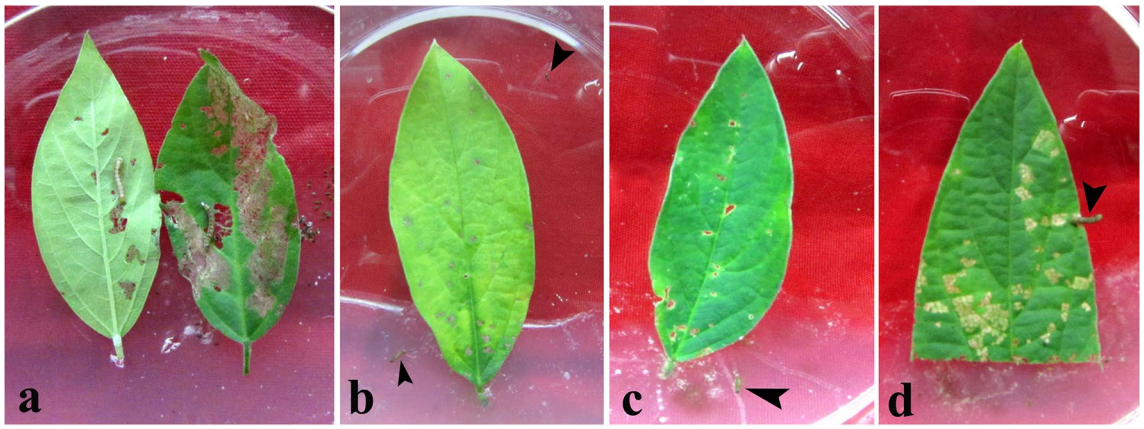

Fig. 8 Representative images illustrating detached leaf bioassay performed on T_1 transgenic pigeonpea events to determine rate of mortality of *Helicoverpa armigera* second instar larvae. **a** Insects feeding on untransformed plant as control. **b–d** Events 143-2S/1 (Cry1Ac), 143-43M(1)/2 (Cry1Ac), and 70-2/5 (Cry2Aa), respectively, exhibiting mortality of larvae. *Arrowheads* indicate dead insects

Western blot analysis, followed by ELISA, of T_2 events confirmed the stable expression of *cry* genes. Expression study of transgenic events revealed that Cry expression remained constitutive, stable, and consistent through three generations. Among all T_2 progenies, the highest level of Cry1Ac expression was detected in, 143-43M(1)/2/2 (0.171% of TSP), whereas maximum Cry2Aa expression was recorded in 70-2/5/2 (0.172% of TSP). Deployment of expression based selection ensured the identification of high expressing events in the present study. Furthermore, immunohistofluorescence study confirmed the constitutive localization of Cry1Ac and Cry2Aa proteins in the leaf tissues of transgenic pigeonpea. Previously, researchers demonstrated the use of *CaMV35S* promoter to drive the constitutive expression of Cry1Ab (Sharma et al. 2006) and haemagglutinin (Satyavathi et al. 2003) in transgenic events of pigeonpea at considerably higher level (0.1–0.49% of TSP). Amidst very few reports of recovery of transgenic pigeonpea with trait genes, Surekha et al. (2005), Sharma et al. (2006), and Ramu et al. (2012) reported the expression and inheritance of Cry1 E-C, Cry1Ab, and Cry1AcF, respectively, beyond the T_0 generation. Although, expression of Cry1Ac in pigeonpea had been reported by Krishna et al. (2011), the study was limited to T_0 generation only. Among other legumes, Stewart et al. (1996) reported the expression of Cry1Ac at 0.0046% in transgenic soybean and Kar et al. (1997) reported the constitutive expression of Cry1Ac up to 0.0045% in chickpea. Chen et al. (2005) reported the presence of Cry2Aa at 12.11 μg g^{-1} of leaf fresh weight in transgenic rice. In the present study, we report for the first time the stable expression of Cry1Ac in transgenic pigeonpea beyond the T_0 generation. This is also the first ever report of T_1 transgenic pigeonpea events expressing Cry2Aa.

Table 3 Percentage mortality of T_1 events against *H. armigera* in detached leaf bioassay

Cry1Ac events	Mortality (%)	Cry2Aa events	Mortality (%)
142-10/2	100	70-2/5	100
143-2S/1	100	137-5/4	100
143-43M(1)/2	100	137-10/1	90
142-7/3	90	137-11/1	90
142-7(1)/4	90	137-7/1	80
143-8S/3	90	137-20/1	80
142-11/1	80	137-4/1	70
143-1S/1	80	129-1/1	60
143-43M/1	60	137-2/2	50
142-10(1)/5	50	140-3/2	40
143-11S/1	50	140-14/1	40
142-6/4	30		

The biology and ecology of *H. armigera* had been extensively reviewed, and general features did not differ when pigeonpea was used as a host (Fitt 1989). Females were found to oviposit on leaves at night and fecundity was high, with up to 3000 eggs reported from a single female (Sison and Shanower 1994). This made leaves of pigeonpea the primary target of growing pod borer larvae. Larvae of first–third instar generally feed on leaves and gradually advance to other parts of the plant including flowers and pods. In this context, the use of constitutive *CaMV35S* promoter to express insecticidal proteins throughout the plant body to confer maximum resistance against pod borer was found to be essential.

In the described study, eight Cry1Ac events [142-10/2, 143-2S/1,143-43M(1)/2, 142-7/3, 142-7(1)/4, 143-8S/3, 142-11/1, and 143-1S/1] showed 80–100% mortality against the second instar *H. armigera* larvae. In addition, six Cry2Aa events [70-2/5, 137-5/4, 137-10/1, 137-11/1,

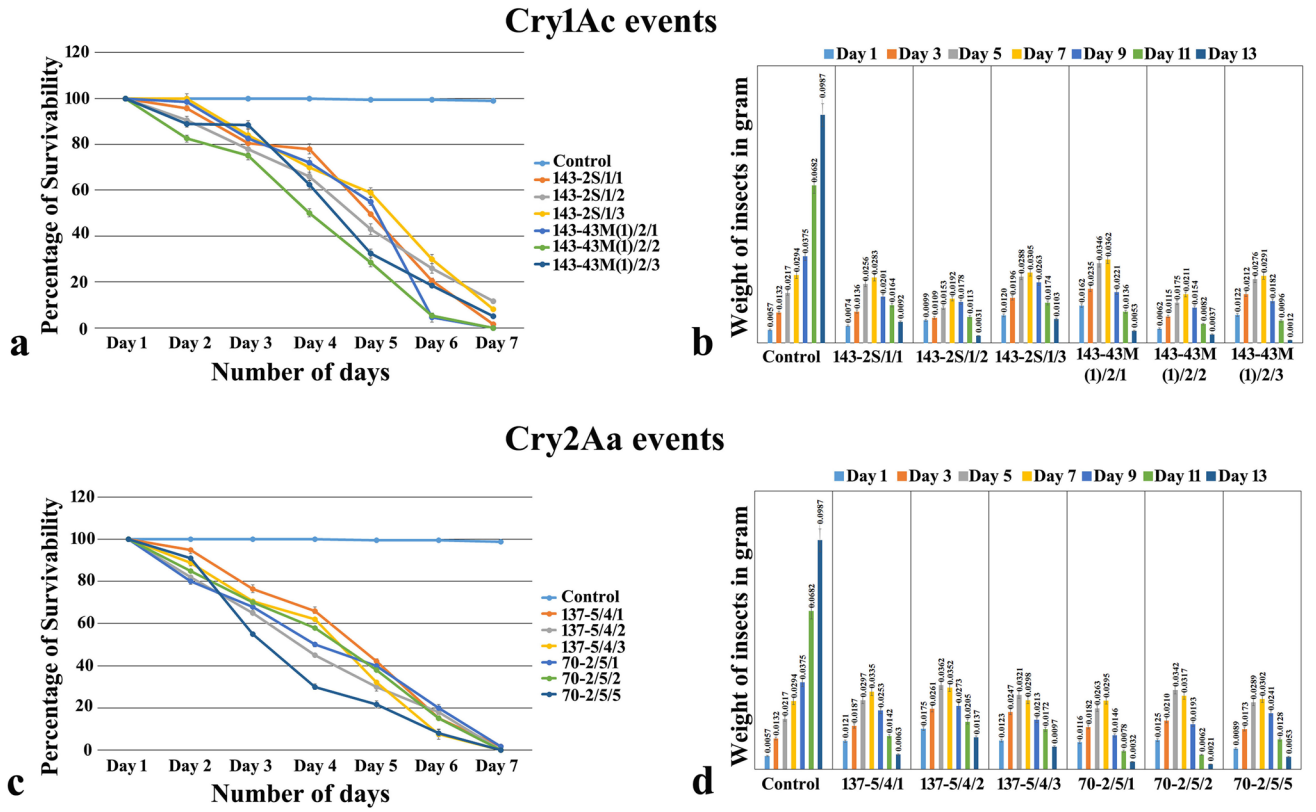

Fig. 9 Bioassay of T_2 events against *H. armigera*. **a, b** T_2 progenies of two Cry1Ac transgenic events manifesting 90–100% larval mortality and weight loss, respectively, in comparison with untransformed control. **c, d** T_2 progenies of two Cry2Aa transgenic events manifesting 99–100% larval mortality and weight loss, respectively, in comparison with untransformed control

137-7/1, and 137-20/1] demonstrated 80–100% mortality of the second instar larvae. Some of the mentioned events exhibited drastic weight loss of the fourth instar larvae leading to death. T_1 events selected based on 100% larval mortality were transferred to T_2 generation to check the inheritance of phenotype. The evidences of bioassay experiment on transgenic pigeonpea by previous workers exhibited mortality of *Spodoptera litura* to be 80% on Cry1E-C events (Surekha et al. 2005) while Cry1Ac T_0 plants demonstrated 55% mortality of *H. armigera* (Krishna et al. 2011). Ramu et al. (2012) reported bioassay of T_2 and T_3 pigeonpea expressing Cry1AcF, where mortality of *H. armigera* ranged between 80–100%. Recently, Chakraborty et al. (2016) reported about 90% mortality of *H. armigera* larvae fed on chickpea events expressing Cry1Ac under the control of RuBisCo small subunit promoter. Earlier, 100% larval mortality of *H. armigera* in chickpea (Acharjee et al. 2010) and yellow stem borer in rice (Chen et al. 2005) were demonstrated using constitutive expression of Cry2Aa.

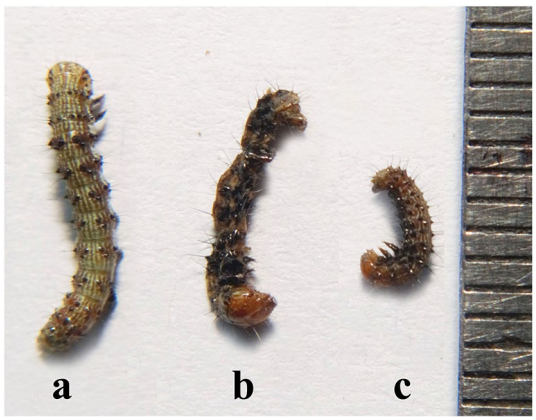

Fig. 10 Morphology of *H. armigera* larvae at the end of detached leaf bioassay period, performed for demonstration of weight loss. **a** Larvae fed on untransformed plant as control. **b, c** Larvae fed on events 143-43M(1)/2/2 and 70-2/5/2, respectively

This is a novel report of transgenic pigeonpea development, successfully expressing Cry2Aa protein at levels lethal to *H. armigera*. The approach of screening T_1 transgenic pigeonpea events on the basis of antibiotic selection and protein expression phenotypes helped to narrow down the search for promising events. The events were later on confirmed by Southern blot to ensure their monogenic character, followed by extensive bioassay

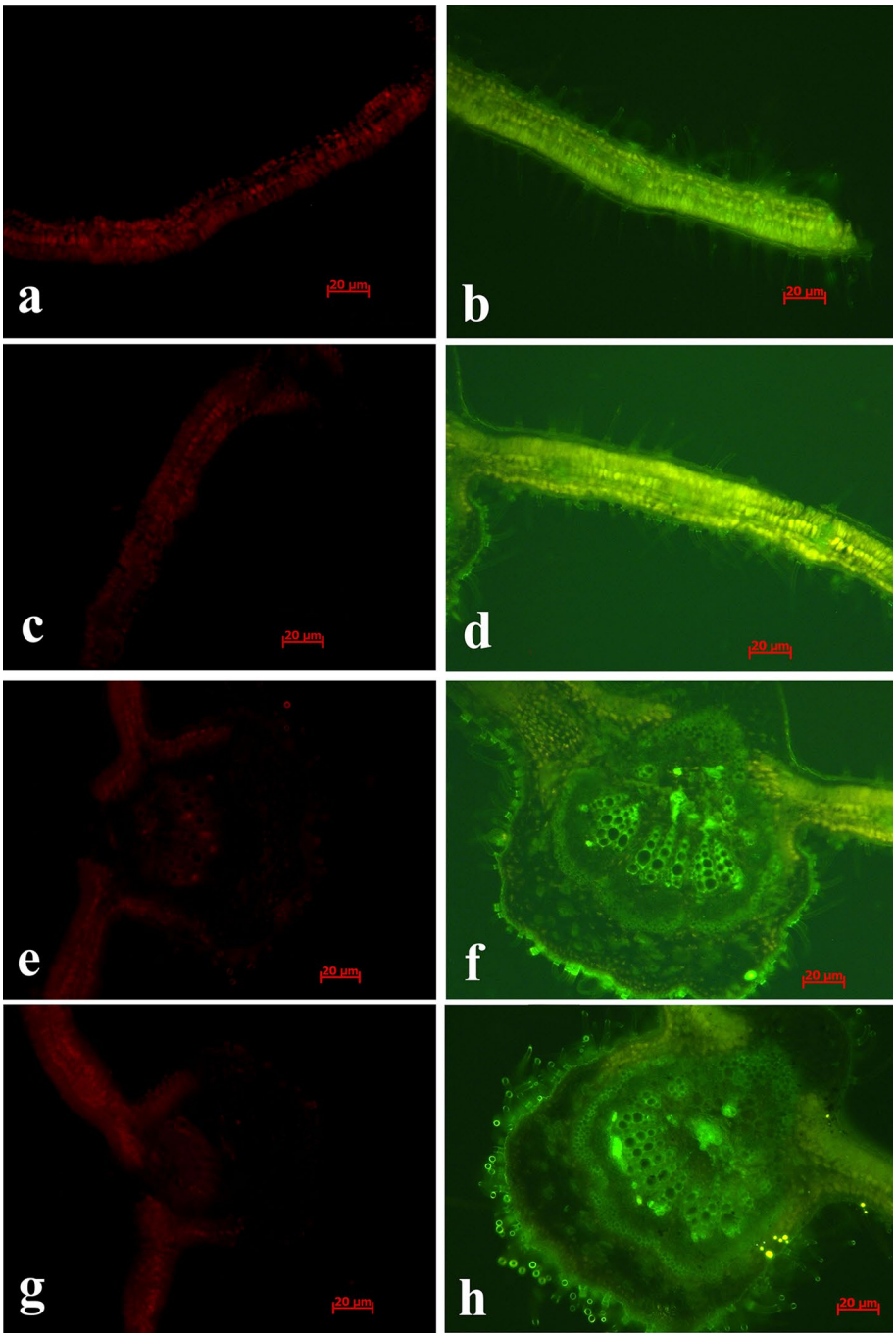

Fig. 11 Immunohistofluorescence localization of Cry proteins in transverse section of leaf of T_3 events, 143-43M(1)/2/2/1 (Cry1Ac) and 70-2/5/2/1 (Cry2Aa). The presence of Cry protein is indicated by green fluorescence. Lamina of untransformed control plant (**a, c**), Cry1Ac (**b**) and Cry2Aa (**d**) events. Vascular bundle of leaf of untransformed control plant (**e, g**), Cry1Ac (**f**), and Cry2Aa (**h**) events

experiments confirming efficiency of the expressed proteins. The expression of Cry proteins in three successive generations of transgenic pigeonpea conferred substantial resistance against *H. armigera*, not only in terms of increased mortality, but also weight loss of larvae. Furthermore, the *cry1Ac* and *cry2Aa* genes were successfully inherited to T_3 generations. These Cry-transgenic events can serve as resources for the development of pyramided pigeonpea in the near future. A multi-toxin deployment system is supposed to enhance the efficacy of transgenic pigeonpea and eventually slow down the rate of resistance evolution in pod borers.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Author contribution statement GG, SG, AP, and DC conceived and designed the experiments. GG and SG conducted all the experiments. GG and DC drafted the manuscript. SD, RKC, and DC were responsible for data analysis, manuscript editing, and supervision of the work. All authors read and approved the final manuscript.

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