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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Clean gene technology to develop selectable marker-free pod borer-resistant transgenic pigeon pea events involving the constitutive expression of Cry1Ac

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Abstract

The most crucial yield constraint of pigeon pea is susceptibility to the pod borer *Helicoverpa armigera*, which causes extensive damage and severe economic losses every year. The *Agrobacterium*-mediated plumular meristem transformation technique was applied for the development of *cry1Ac* transgenic pigeon pea. Bioactivity of the *cry1Ac* gene was compared based on integration and expression driven by two promoters, the constitutive *CaMV35S* promoter and the green-tissue-specific *ats1A* promoter, in those transgenic events. The transgenic events also contained the selectable marker gene *nptII* flanked by *loxP* sites. Independent transgenic events expressing the Cre recombinase gene along with a linked *bar* selection marker were also developed. Integration and expression patterns of both *cry1Ac* and *cre* were confirmed through Southern and western blot analysis of T_1 events. The constitutive expression of the Cry1Ac protein was found to be more effective for conferring resistant activity against *H. armigera* larvae in comparison to green-tissue-specific expression. Constitutively expressing Cry1Ac T_1 events were crossed with Cre recombinase expressing T_1 events. The crossing-based Cre/*lox*-mediated marker gene elimination strategy was demonstrated to generate *nptII*-free Cry1Ac-expressing T_2 events. These events were subsequently analyzed in the T_3 generation for the segregation of *cre* and *bar* genes. Five Cry1Ac-expressing T_3 transgenic pigeon pea events were devoid of the *nptII* marker as well as *cre-bar* genes. *H. armigera* larval mortality in those marker-free T_3 events was found to be 80–100%. The development of such *nptII* selectable marker-free Cry1Ac-expressing pigeon pea transgenics for the first time would greatly support the sustainable biotechnological breeding program for pod borer resistance in pigeon pea.

Key points

- *Constitutiveexpression of Cry1Ac conferred complete resistance against Helicoverpa armigera*
- *Green-tissue-specific expression of Cry1Ac conferred partial pest resistance*
- *Cre/lox-mediated nptII elimination was successful in constitutively expressing Cry1Ac transgenic pigeon pea events.*

Keywords *ats1A* promoter · *Bar* · Constitutive promoter · Cre protein · Cre/*lox* recombination · Insect bioassay · Marker elimination · *nptII* · Plumular meristem transformation

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Introduction

Pigeon pea (*Cajanus cajan* (L.) Millspaugh), popularly known as red gram (arhar) is an important pulse crop grown predominantly in developing countries of semiarid tropical and subtropical regions of the world. According to FAO (2019), pigeon pea is accepted as the sixth most important edible grain legume with worldwide production of 5.61 million tonnes per year. This has a broad influence on the overall crop yield, which is 7.9 thousand kg per hectare area, much less than the potential yield. Over the last few decades,

pigeon pea production is facing global demand to meet food and nutritional security and also proving its usefulness for various purposes such as fuel, fodder, building material, etc. (Ghosh et al. 2014). Based on worldwide data, India holds the topmost position as a pigeon peaproducing country. Around 3.31 million tonnes of pigeon pea are produced annually in India, where it is the second most important pulse. As a whole, India contributes about 75% of global production (FAO 2019). Although pigeon peaharvesting areas have considerably increased, the yield has remained stagnant for the last 6 decades mainly due to the high susceptibility of the cultivars to various biotic stress (Varshney et al. 2012). Additionally, in comparison to other crops, limited efforts have been directed to generate precise knowledge on the genetic inheritance of stress-resistant traits in pigeon pea. This also affected crop yield and further limited the efficacy of crop improvement programmes (Varshney et al. 2010).

The major yield constraint of pigeon pea is the lepidopteran pest *Helicoverpa armigera*, which severely damages the plant growth and productivity and results in significant economic losses of over $400 million per year (Sreekanth et al. 2014). Despite several control strategies, the use of chemical pesticides has been found to be very effective for managing pests over large areas. However, extensive usage of hazardous chemicals has detrimental effects on human health and other organisms and finally resulted in environmental degradation (Mishra et al. 2018). Increased public concerns regarding the probability of destructive environmental consequences associated with these harmful pesticides prompted the search for improved methods of insect pest resistance. This situation has drawn the attention of the scientific community to adopt eco-friendly and environmentally safe technologies to improve crop protection for sustainable agriculture (Mishra et al. 2018). However, limited genetic variability in the primary gene pool coupled with poor exploitation of genetic resources is considered a major obstacle in the genetic improvement of pigeon pea (Varshney et al. 2012). Due to cross incompatibility with wild relatives and lack of genetic resistance in the gene pool, the primary objective of pigeon pea breeding to develop insect-resistant varieties has not been succeeded (Sharma et al. 2009). In recent years, modern biotechnological approaches have provided a new avenue to develop improved cultivars by overcoming severe bottlenecks associated with conventional breeding (Venkata et al. 2019). Transgenic technology has proven to be beneficial for this crop to incorporate agronomically convenient traits, which have a positive impact on yield, productivity, and nutritional security of the worldwide human population (Saxena et al. 2015). Based on recombinant DNA technology, the successful integration of several foreign genes has rendered new opportunities for the development of resistant pigeon pea cultivars with inbuilt resilience to withstand the biotic stress factors (Ghosh et al. 2014). Among them, the introduction of *Bacillus thuringiensis*-encoded ∂-endotoxins or Cry proteins into different crop species remained the most advantageous choice for researchers to provide good and long-term potentiality against the infestation of various lepidopteran insects (Ghosh et al. 2017). Highly specific ∂-endotoxins to a particular insect group have been justified by previous workers to forego the testing on nontarget organisms. This also facilitated its effective utilization as a biologically secured and most reliable molecular device for crop improvement (Kumar et al. 2008). Over the last few decades, limited attempts have been made in pigeon pea for successful integration of the *cry* gene to drive the constitutive expression of a protein that could exhibit a maximum range of insect mortality (Krishna et al. 2011; Ramu et al. 2012; Das et al. 2016; Kaur et al. 2016; Ghosh et al. 2017; Singh et al. 2018; Sarkar et al. 2021). Among them, Cry1Ac has been preferred by researchers due to its high entomotoxicity toward the lepidopteran group of insects, followed by Cry1Aa, Cry1Ab, and Cry2Aa (Perlak et al. 1990). Additionally, due to different receptor-binding potentials, using more than one Cry protein in transgenic development is the need of the future to obtain durable resistance to the lepidopteran pest (Ghosh et al. 2017). The toxicity level is directly correlated with the activity and occurrence of the gene in host plant tissue. However, the expression of such a foreign protein could be controlled by a specific promoter to manage the metabolic load in plants.

In such circumstances, the potentiality of a suitable promoter should be assessed for successful genetic transformation. The correct choice of the promoter was proven to be a crucial component in transgenic plant development through regulating the transcription in the host cell and also influencing the transgene expression level in a spatiotemporal manner (Bakhsh et al. 2011). The great majority of studies conducted in pigeon pea thus far have largely relied on the Cauliflower mosaic virus 35S (*CaMV35S*) constitutive promoter (Ghosh et al. 2014). This promoter is widely used in transformation technology for driving the expression of genes in almost all tissues, and also the promoter activity is independent of any environmental and developmental conditions. Alternatively, constitutive expression of transgenes, under certain conditions may interfere with normal processes in a plant and can exhibit undesirable phenotypes during plant growth and development (Potenza et al. 2004). Advancement in *Agrobacterium tumefaciens*-mediated foreign gene delivery system has enabled the researchers to use suitable tissue-specific promoters to adjust the transgene expression pattern. In pigeon pea, a few reports are available on the usage of tissue-specific promoters like a flower- and leaf-specific double-enhanced *CaMV35S* promoter (*CaMV35SDE*), seed-specific phaseolin, and *Arabidopsis thaliana* 2S2 albumin promoters to regulate the transgene expression pattern (Sharma et al. 2006; Thu et al. 2007). The use of

ribulose 1,5-bisphosphate carboxylase small subunit promoters along with their chloroplast-directed transit peptides were found to be attractive candidates for obtaining high gene expression levels specifically in green tissue by targeting foreign protein into the chloroplast. If the green-tissue-specific promoter is used for the expression of Cry1Ac in pigeon pea, the accumulation of the protein in seed grains can be avoided. Additionally, the expression of Cry1Ac protein was proven to be increased around 10 to 20-fold in tobacco, under the control of *A. thaliana* ribulose 1,5-bisphosphate carboxylase (*rubisco*) small subunit (*ats1A*) promoter along with the transit peptide (Wong et al. 1992). The use of the *ats1A* promoter was proven to delineate a high level of green-tissue-specific expression of the *bar* gene in the cotton plants (Kumar and Timko 2004). Further, this promoter was found to be advantageous in transgenic soybean and chickpea plants for conferring complete resistance to target pests (Miklos et al. 2007; Acharjee et al. 2010). In the present report, the *ats1A* promoter was utilized in pigeon pea to delineate a confined expression of Cry1Ac protein mostly in the green-tissue-specific regions preferred by the lepidopteran insects.

Despite the scientific advances made in crop improvement, the presence of a selectable marker gene (SMG) has been considered a major limiting factor for the public acceptance of genetically modified (GM) crops and also hindered their commercialization (Miki and McHugh 2004). During the last few decades, several ecological and food-safety-related issues have been raised by environmentalists and consumers over the spreading of resistance markers into related wild species, bacterial strains, and non-transgenic crops (Upadhaya et al. 2010). Considering the worldwide data, it can be mentioned that transgenic products were subjected to strict regulatory measures before their commercial release. Several biosafety assessments have been done to evaluate the impact of GM crops on the environment and human nutrition. Different types of assessment methods such as allergenicity, acute oral toxicity, in vitro degradation studies, bioinformatics studies, animal dietary exposure, nutritional assessment, and also environmental risk assessment are performed following the rules of food safety authorities (Mondal et al. 2011). In addition to the gene of interest, the selectable marker gene in transgenic crops should also be evaluated by the rigid framework of biosafety assessments before commercialization (Ramessar et al. 2007). It also increases the cost of transgenic production. Therefore, the removal of *nptII* was found to be convenient to avoid biosafety and environmental risk assessment studies associated with the gene and marker-free transgenic plants are more acceptable for commercialization.

Additionally, after the accomplishment of each transformation, the elimination of the marker gene not only increases the public acceptability of GM crops, but it would also facilitate the pyramiding of multiple desirable genes into the same plant (Jaiwal et al. 2002; Yau et al. 2013). All these factors motivated the researchers to apply clean gene technologies for marker-free transgenic development and its subsequent application in the commercial sector (Upadhyaya et al. 2010). Several strategies were implemented for the elimination of selectable marker genes, among them the Cre/*lox*-mediated site-specific recombination strategy has been widely adopted in the crop system (Upadhaya et al. 2010; Bala et al. 2013). Cre recombinase recognizes the specific DNA sequences to be eliminated, which is flanked by two directly repeated asymmetric 34 bp *loxP* sites and induces precise recombination for successful DNA excision. In the present report, the tissue-specific and constitutive expression of Cry1Ac was compared in transgenic pigeon pea. The Cre/*lox* recombination strategy was utilized for the removal of the *nptII* marker gene from transgenic events. Further elimination of the *cre* gene and the *bar* selectable marker was successfully accomplished by genetic segregation in pod borer-resistant, T_3 Cry1Ac transgenic events.

Materials and methods

Biological resources

Seeds of ICPL 87119 (Asha), a cultivar of pigeon pea, were collected from the Indian Institute of Pulses Research, Kanpur, India. The AGL1 strain (Lazo et al. 1991) of *A. tumefaciens* harboring binary plasmids was used for plant transformation. Bioassay experiments were performed with *H. armigera* larvae. Eggs of *H. armigera* were obtained from the National Bureau of Agricultural Insect Resources (NBAIR), Bangalore, India, in a sterile insect box.

Pigeon pea transformation

Three binary plasmid vectors, 35S-cry, ats-cry, and 35S-cre were used for the described study. These plasmids were used in *Agrobacterium*-mediated plumular meristem transformation to develop the Cry1Ac and Cre transgenic pigeon pea events. The synthetic *cry1Ac* gene (NCBI Accession No: KF630361.1) was cloned separately into the binary vector pBK11 (Chakraborti et al. 2008) to construct two new vectors 35S-cry and ats-cry. pBK11 contained the *CaMV35S* promoter-controlled neomycin phosphotransferase II (*nptII*) gene flanked by *loxP* sites. An *Eco*RI- and *Hin*dIII-digested 2.8 kb *cry1Ac* gene cassette driven by the *CaMV35S* constitutive promoter and the *nos* terminator was cloned into pBK11. The construct was designated as 35S-cry (~ 8.3 kb, Fig. 1a). For the development of the second vector ats-cry, *Bam*HI- and *Eco*RI-digested *cry1Ac* gene was first blunted and then ligated into the *Bam*HI-digested, blunted, and

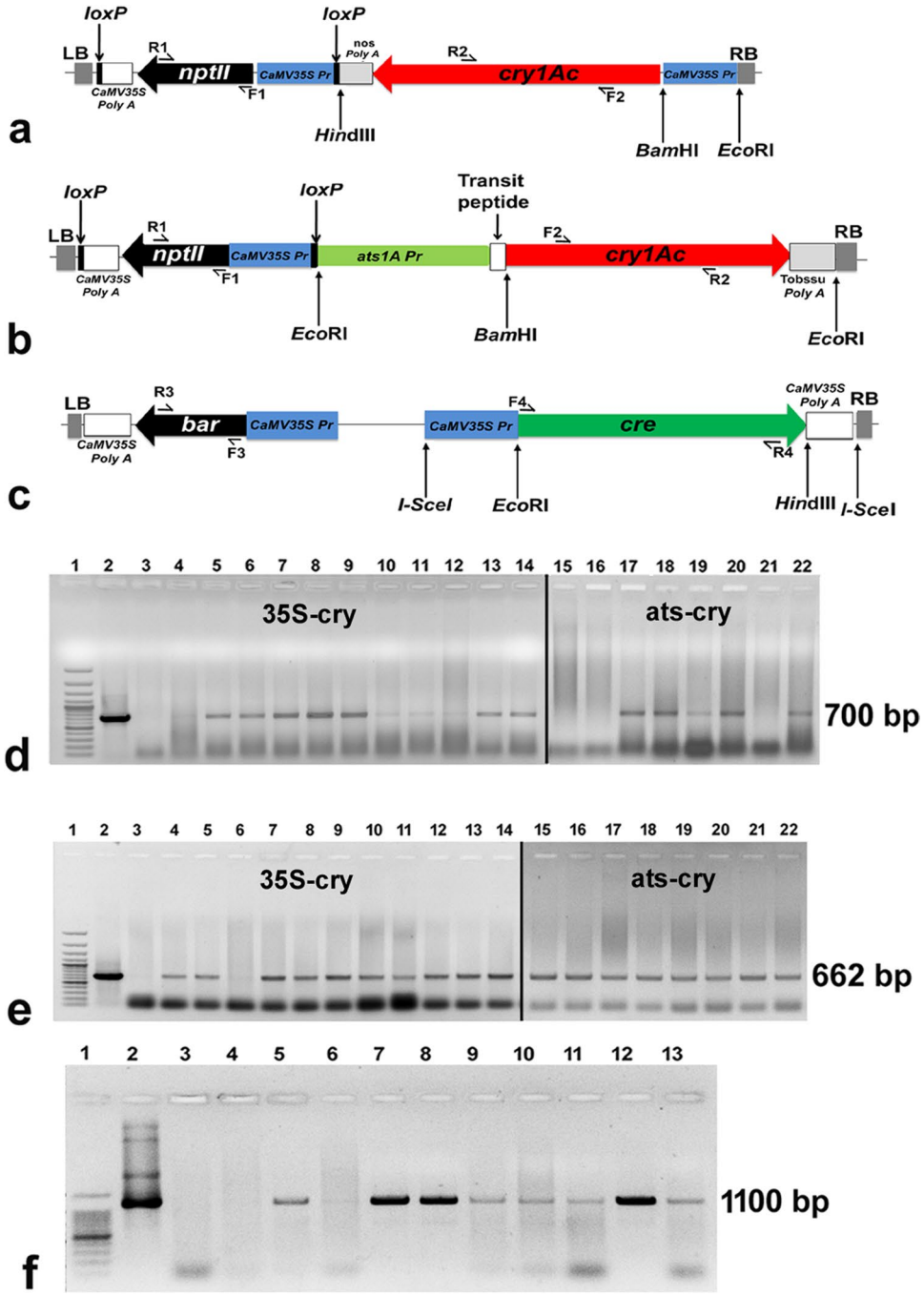

Fig. 1 Binary vectors and analysis of T_0 plants. Schematic representation of the T-DNA region of binary vectors; **a** constitutive promoter-driven *cry1Ac*, designated as 35S-cry, and **b** green- tissue-specific promoter-driven *cry1Ac*, designated as ats-cry. F1/R1 and F2/ R2 are *nptII* and *cry1Ac* specific primers, respectively, **c** schematic representation of the T-DNA region of the 35S-cre binary vector showing *bar* and *cre* specific primers F3/R3 and F4/R4, respectively. LB, left border of T-DNA; RB, right border of T-DNA; *CaMV35S Pr*, Cauliflower mosaic virus 35S promoter; *ats1A Pr*, *Arabidopsis thaliana* rubisco small subunit promoter; *CaMV35S* Poly A, Cauliflower mosaic virus 35S terminator; *nos* Poly A, *nopaline synthase* terminator; and *Tobssu* Poly A, *Nicotiana tabacum* small subunit terminator. **d**, **e** Representative PCR amplification of *nptII*-specific 700 bp and *cry1Ac*-specific 662 bp sequences in established Cry1Ac T_0 transformants. **f** PCR amplification of T_0 35S-cre events showing the presence of 1100 bp *cre*-specific amplicon. Lanes 1–3, DNA ladder, respective binary plasmid as positive and untransformed pigeon pea DNA as negative controls, respectively

dephosphorylated pWM5 binary vector (Tabe et al. 1995). The resulted 5′-*ats1A*-transit peptide-*cry1Ac-Tobssu* Poly A-3′ (~4.0 kb) gene cassette was taken out from the pWM5 vector through *Eco*RI digestion and then ligated into *Eco*RI-digested pBK11 to develop the vector ats-cry of ~9.5 kb (Fig. 1b). The third binary vector 35S-cre was constructed with an *I-SceI*-digested *cre* gene cassette from pBK15 (Chakraborti et al. 2008), which was cloned into pCAMBIA3300I (Chakraborti et al. 2008) to develop a 35S-cre vector of ~10.6 kb size. The designated vector containing the Cre recombinase gene was under influence of the *CaMV35S* promoter, and the herbicide-resistant *bar* gene was present as a selectable marker (Fig. 1c). All the vectors were mobilized into the AGL1 strain of *A. tumefaciens*. Decapitated embryonic axis-based explants were utilized for *Agrobacterium*-mediated plumular meristem transformation as described by Ganguly et al. (2018). *Agrobacterium* infection followed by 250 mg l^{-1} cefotaxime wash of explants and subsequent transfer to the artificial soil. After 4–5 weeks, T_0 plant lines were established in the soil and allowed for self-fertilization

in the greenhouse to obtain T_1 seeds. The untransformed control pigeon pea events were also developed under the same condition without *Agrobacterium* infection.

Polymerase chain reaction (PCR) of pigeon pea DNA samples

Fresh young leaves of established pigeon pea transgenic events of T_0, T_1, T_2, and T_3 generations were taken for genomic DNA isolation following the cetyl trimethyl ammonium bromide (CTAB) method as described by Chakraborti et al. (2006). PCR was performed with the extracted DNA samples to determine the integration of the *cry1Ac* gene along with *nptII* selectable markers in both 35S-cry and ats-cry transgenic events. Similarly, the presence of the *cre* gene along with *bar* selectable marker was analyzed. Primers were developed to amplify *nptII*, *cry1Ac*, *cre*, and *bar* gene-specific sequences (Supplementary Table S1). 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 with a final extension at 72 °C for 10 min. In all PCR analyses, untransformed pigeon pea genomic DNA and plasmid DNA of respective binary vectors were taken as negative and positive controls, respectively.

Selection and establishment of T_1 transgenic events

T_1 seeds were obtained from 35S-cry, ats-cry, and 35S-cre T_0 pigeon pea events. Kanamycin-mediated stringent selection of T_1 seeds from 35S-cry and ats-cry was performed (Ganguly et al. 2017). After peeling the seed coat, overnight imbibed seeds were allowed for 100 mg l^{-1} kanamycin treatment for 5 h and subsequently sown onto artificial soil. Antibiotic treated and non-treated seeds of untransformed pigeon pea events were served as controls. Simultaneously, for 35S-cre events established T_1 plants were screened for herbicide tolerance through a glufosinate painting assay with 2.0 mg l^{-1} solution of glufosinate ammonium on two out of three leaflets of each leaf.

Western blot analysis

Total soluble protein (TSP) was extracted from 1-month-old T_1 and T_3 Cry1Ac events as well as 35S-cre T_1 events according to the method described by Ghosh et al. (2017). Protein concentration was measured (Bradford 1976). Purified Cry1Ac, obtained from the bacterial overexpression system, was used as a positive control (Ghosh et al. 2017). Around 40 µg TSP of each plant sample was separated on 12% SDS-PAGE and subsequently transferred on to HybondC membrane (Amersham Sciences, Amersham, Buckinghamshire, UK) by electro-blotting. Then, the membrane was kept in a blocking solution overnight at 4 °C. On the next day, the membrane was allowed to probe with anti-Cry1Ac or Cre polyclonal primary antibody at 1:10,000 dilution for 1 h. Then, the membrane was incubated with anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (Sigma-Aldrich, USA) as a secondary antibody at 1:10,000 dilution. Chemiluminescence (ECL kit, Amersham Biosciences, Amersham, Buckinghamshire, UK) reagents were used to detect the bound secondary antibody, developed on Kodak film.

Enzyme-linked immunosorbent assay (ELISA) of soluble protein extracts

Western positive pigeon pea events of T_1 and T_3 generations were selected for quantification of transgenic protein through indirect ELISA from the extracted crude protein of Cry1Ac events. ELISA experiments were undertaken following the method described by Ghosh et al. (2017). Microtitre wells were coated with 20 µg of isolated soluble protein of transgenic events along with serially diluted purified Cry1Ac (5–50 ng) in ELISA coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide; pH 9.5) for 18 h at 4 °C. After washing with 1X PBST [1X phosphate-buffered saline (PBS) and 0.1% Tween-20], microtitre plates were kept for 2 h in blocking solution [PBS with 5% (w/v) bovine serum albumin (BSA)] at 37 °C. Coated protein samples were allowed to bind with anti-Cry1Ac primary antibody and HRP-conjugated anti-rabbit secondary antibody. Finally, the wells were kept in citrate buffer containing *Oo*-Phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, USA) for color development. Data were recorded with a BioRad (Hercules, CA, USA) microplate reader at 415 nm. Three biological replications were used per experiment, and each experiment was performed thrice. Untransformed plant protein was used as a negative control in all experiments. All the experimental data were combined and compared through ANOVA ($p = 0.05, 0.01, 0.001, 0.0001$).

Southern blot hybridization

Southern hybridization experiments were carried out with transgenic events of T_1, T_2, and, T_3 generations using both radioactive and nonradioactive methods (Ghosh et al. 2017). Forty micrograms of isolated genomic DNA was digested with restriction endonucleases and run in 0.8% (w/v) agarose gel electrophoresis and subsequently blotted onto positively charged nylon membrane following depurination, alkali denaturation, and neutralization. PCR-amplified probe sequence was labeled with [α^{32}P] dCTP in radioactive method, whereas digoxigenin (DIG, Roche Applied Science, Indianapolis, USA) labeled probe was used for nonradioactive Southern blotting. Gel-extracted 662 bp amplicon of the *cry1Ac* gene obtained from 35S-cry and ats-cry binary

vectors along with the 1100 bp amplicon of the *cre* gene from the 35S-cre plasmids served as a probe as well as one of the positive controls in the hybridization method. Digested binary plasmids and untransformed pigeon pea plant DNA were used as second positive and negative controls, respectively.

Larval mortality assay of *H. armigera*

Detached leaf-feeding bioassay was performed with freshly hatched 2nd instar larvae of *H. armigera* fed on an artificial diet (Armes et al. 1992). Leaflets of 2 to 3-month-old 35S-cry and ats-cry pigeon pea transgenic plants of T_1 and T_3 generations were placed in insect-breeding boxes containing 3% water agar. In every experiment, one healthy 2nd instar larva was kept in each box, and ten such boxes were taken. The survival of 2nd instar larvae was monitored at an interval of 24 h till the 7th day. Untransformed plant leaves were kept as a negative control. Each experiment was repeated three times, and the data was recorded to calculate the mean ± standard error for the percentage of larval survivability. Alternatively, weight loss of 3rd–4th instar larvae was recorded to confirm the Cry1Ac toxicity on larval growth over 13 days. A single larva was kept in one box, and five such boxes were taken per experiment. Data were recorded every 24 h by measuring the larval weight in grams. Each experiment was performed three times. All experimental data were combined and compared through ANOVA ($p = 0.05, 0.01, 0.001, 0.0001$).

Immunohistofluorescence localization of transgenic protein

Transverse leaflet sections of T_1 Cry1Ac and Cre transgenic pigeon pea events along with untransformed negative control plants were allowed for 2 h incubation at 4 °C with 10% (v/v) trichloroacetic acid. This was followed by 3–4 times washing with 3:1 ethanol: acetic acid to remove the chlorophyll. A series of ethanol grades (90, 70, 50, and 30% (v/v) to water) were allowed to treat the leaf sections, 15 min each, and then incubated for 2 h in blocking solution [3% (w/v) BSA in 1X PBS] at room temperature. Then, leaf sections were kept in a BSA solution containing an anti-Cry1Ac/-Cre antibody (1:10,000) for 18 h at 4 °C. Then samples were washed with 1X PBS followed by 1 h incubation with anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugated (1:20,000) (Sigma-Aldrich, St. Louis, USA) secondary antibody at room temperature. The treated samples were visualized under a fluorescence microscope (Axio Scope, Carl Zeiss, Jena, Germany) using an excitation filter of 450–490 nm for FITC. All the experiments were performed in triplicates using 4 single-copy transgenic events each from 35S-cry, ats-cry, and 35S-cre categories.

Histological study of leaf tissues

T_1 transgenic and untransformed control leaves of pigeon pea (cv. ICPL-87119) were taken for histological study using aniline blue strain to find out anatomical differences in comparison to *Nicotiana tabacum* (cv. Petit Havana SR1) and *A. thaliana* (Col-0) leaf, according to the protocol mentioned by Schenk and Schikora (2015). All the leaf samples were treated with 1:3 acetic acid and ethanol solution for 1 h until the chlorophyll was completely removed, and the material became transparent. Then, the samples were washed with 150 mM K_2HPO_4 (pH 9.5) for 30 min followed by incubation for 2 h with 150 mM K_2HPO_4 and 0.01% aniline blue solution in a dark condition. After the treatment, the leaf samples were embedded in 50% glycerol and visualized under a fluorescence microscope using the excitation filter of 360–420 nm. All the experiments were repeated twice using 3 samples from each plant system.

Crossing of T_1 pigeon pea events

Single-copy T_1 35S-cry events showing 80–100% larval mortality were selected for the crossing. Ten independent 35S-cry transgenic lines were reciprocally crossed with four individual 35S-cre plants, and T_2 hybrid seeds were obtained. Established hybrid pigeon pea T_2 events were further evaluated to confirm the elimination of marker genes using PCR and Southern blot hybridization and subsequently analyzed for detection of protein activity through western blot and insect bioassay.

Establishment of *nptII*-eliminated T_2 hybrids

Genomic DNA of the T_2 hybrid events was extracted, and PCR analysis was carried out to determine the presence or absence of the *nptII* gene. The *nptII* negative samples were allowed for another round of polymerase chain reaction with *cre*, *cry1Ac*, and *bar* gene-specific primers. Finally, all *nptII*-eliminated transgenic events were confirmed by Southern blot analysis. Parental lines of those hybrid events were also kept as control along with the other positive and untransformed negative controls. Established Southern positive single-copy T_2 transgenic plants were allowed for selfing, and T_3 seeds were obtained.

Molecular analysis of T_3 events to establish marker-free pigeon pea transgenics

The harvested seeds from *nptII*-eliminated Cry1Ac-expressing T_2 hybrid events were germinated to obtain T_3 plants. These plants were allowed for PCR analysis to determine the removal of the *cre* and *bar* genes, which was subsequently confirmed by Southern blot hybridization.

Western blot analysis and larval mortality bioassay were performed in those marker-free transgenic events.

Results

Plumular meristem transformation and characterization of T_0 transformants

Agrobacterium-mediated plumular meristem transformation was carried out in pigeon pea using 3 binary vectors, designated as 35S-cry, ats-cry, and 35S-cre (Fig. 1a, b, c). After establishment in the artificial soil, 270 Cry1Ac (35S-cry and ats-cry) and 60 35S-cre T_0 events were found to be PCR positive detected through the successful amplification of transgenes (Supplementary Table S1, Fig. 1d, e, f). Binary plasmid, used for transformation, was kept as a positive control, whereas a negative control was served by untransformed plant genomic DNA in respective PCR analyses. The T_1 seeds obtained from these T_0 lines were taken for further analysis.

Selection of T_1 progenies

Germinated T_1 seeds from each Cry1Ac T_0 event were screened by treating them with 100 mg l^{-1} kanamycin (Fig. 2a). After 3 weeks, antibiotic-resistant T_1 plants showed the desired height and were found to be morphologically comparable with non-selected untransformed control plants, whereas antibiotic-treated non-transgenic T_1 lines were distinctly shorter with an undeveloped root system. Fifty kanamycin-resistant Cry1Ac T_1 progenies were established, which were found to be PCR positive through the successful amplification of transgenes, *nptII*, and *cry1Ac* (Fig. 2b, c).

Glufosinate ammonium leaf painting assay was performed on T_1 progenies of each 35S-cre event along with untransformed pigeon pea plants as control (Fig. 2d). Painted leaflets in the control plants exhibited necrosis after 3 days, whereas no detrimental effect of the herbicide was observed in leaflets of T_1 plants. T_1 progenies from 20 T_0 lines were allowed for glufosinateammonium painting assay. Twenty-five T_1 progenies were established after the assay. Twenty-one plants were found to be positive for the presence of a *bar* and *cre* gene-specific fragments identified through PCR (Fig. 2e, f).

Expression analyses of T_1 transgenics

Western blot analysis was performed using TSP extracted from PCR-positive T_1 plants of Cry1Ac (35S-cry and ats-cry) and 35S-cre events. All the PCR-positive T_1 Cry1Ac events were found to express ~ 66 kDa Cry1Ac protein (Fig. 3a). Among these plants, 40 T_1 events were 35S-cry, whereas 10 events were ats-cry. Purified Cry1Ac obtained from bacterial overexpression was used as a positive control. Simultaneously, ~ 33 kDa Cre protein was found to be expressed in all PCR positive 35S-cre T_1 events (Fig. 3b). No such band was exhibited by untransformed negative controls.

A quantitative assessment of Cry1Ac protein accumulation in transgenic pigeon pea leaves was performed through indirect ELISA analysis. Purified Cry1Ac, obtained from bacterial overexpression, was used for serial dilution, which was required for standard curve preparation. Out of 40 selected 35S-cry transgenic events, a significantly higher ($p < 0.001$) level of Cry1Ac expression was observed in 14 T_1 lines (22–62 µg g^{-1} FW (fresh weight)), and alternatively a lower amount of protein accumulation (6–28 µg g^{-1} FW) was noticed in all ats-cry T_1 transgenic events (Fig. 3c, d). Untransformed plant protein was kept as a negative control.

Immunohistofluorescence study of T_1 progenies

The spatial expression patterns of transgenic proteins in T_1 transgenic events were detected through in situ immunohistofluorescence localization of leaflet tissue sections. The distinguishable accumulation of Cry1Ac protein was observed in 35S-cry and ats-cry T_1 events under the control of *CaMV35S* and *ats1A* promoters, respectively (Fig. 3e). Leaflet sections of constitutive promoter-driven T_1 events revealed the presence of Cry1Ac protein in the form of green fluorescence in all tissues. Alternatively, the expression of the Cry protein was found to be confined to trichomes and the outer epidermal area of the leaflet in *ats1A* promoter-driven Cry1Ac events. No fluorescence signal was detected in untransformed control plant tissues. Simultaneously, single-copy T_1 plants of 35S-cre events demonstrated the constitutive expression pattern of Cre protein throughout the leaflet tissues (Fig. 3f). Untransformed negative control pigeon pea leaves did not show Cre protein expression.

Southern blot analysis of T_1 progenies

PCR and western positive T_1 transgenic events were allowed for Southern blot analysis to confirm the integration and copy number of transgenes. Genomic DNA of 35S-cry and 35S-cre transgenic lines were digested with *Hin*dIII, whereas ats-cry transgenic DNA samples were digested with *Bam*HI restriction endonucleases. PCR-purified *cry1Ac* (for 35S-cry and ats-cry transgenics) and *cre* (for 35S-cre transgenics) sequences along with digested binary plasmids served as positive controls in Southern hybridization experiments. The hybridization signal of T_1 Cry1Ac events showed the single-copy

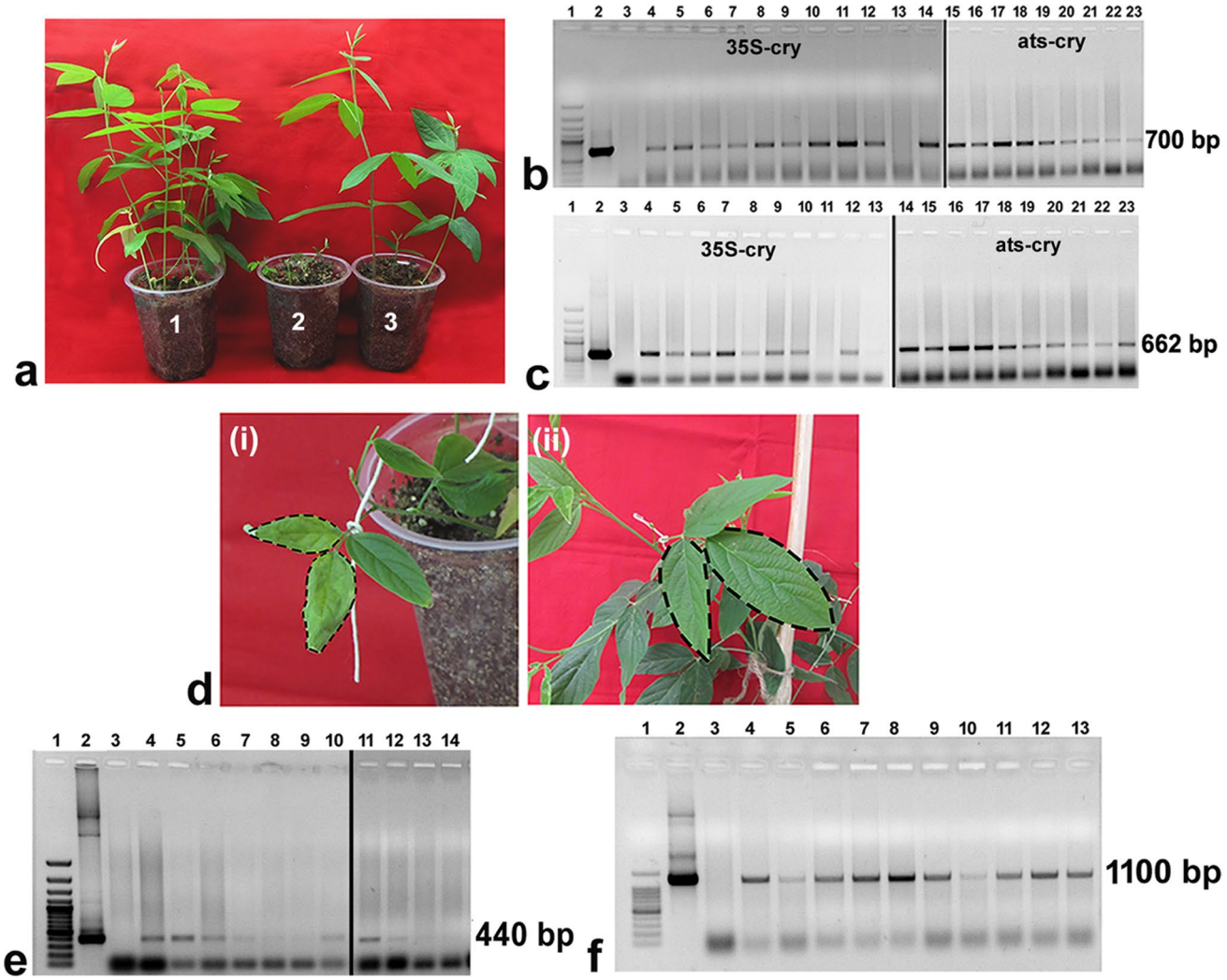

Fig. 2 Selection and establishment of T_1 transgenics. **a** Germination of T_1 Cry1Ac seeds treated with 100 mg l^{-1} kanamycin. Pot 1, non-treated untransformed events, pot 2, treated untransformed plants, and pot 3, established T_1 transgenic events. **b**, **c** Representative PCR analysis of *nptII*-specific 700 bp and *cry1Ac*-specific 662 bp sequences in *cry1Ac* T_1 plants. **d** Glufosinate painting assay on *cre* T_1 plants, (i) leaflets of untransformed plant, and (ii) T_1 events painted with 2.0 mg l^{-1} glufosinate ammonium (marked with dotted lines). **e**, **f** PCR amplification of 35S-cre events showing the presence of 440 bp bar and 1100 bp *cre*-specific fragments. Lanes 1–3, DNA ladder, binary plasmid as positive and untransformed pigeon pea DNA as negative controls, respectively

integration in the range of 4–6 kb (Fig. 4a, b, and c), and T_1 pigeon pea plants of 35S-cre events exhibited single-copy insertion of the *cre* gene between 6 and 9 kb (Fig. 4d). Twenty-one 35S-cry (Fig. 4a, b) and 4 ats-cry T_1 (Fig. 4c) events were found with single-copy integration of the *cry1Ac* gene, whereas 15 35S-cre events were confirmed as single-copy transgenic lines with the integration of the *cre* gene (Fig. 4d). Eight T_1 plants of Cry1Ac events (35S-cry and ats-cry) and 3 plants of 35S-cre events exhibited double-copy insertion of the transgene. Genomic DNA of untransformed negative control plants exhibited no hybridization signal.

Insecticidal activity of Cry1Ac protein in T_1 transgenic pigeon pea

Cry1Ac-expressing 35S-cry and ats-cry pigeon pea T_1 events of both single- and double-copy integrations were used for detached leaf bioassay to study the insecticidal activity against *H. armigera.* The survival of 2nd instar larvae inside the breeding boxes was monitored at 24 h intervals till the 7th day. Data were compared with untransformed negative control pigeon pea events. Three 35S-cry single-copy events (222–10/1, 222–10/2, and 237–1/5) exhibited 100% mortality of the *H. armigera* larvae, and also more than 80% mortality was recorded in nine 35S-cry single-copy events (Fig. 5a). Alternatively, mortality in green

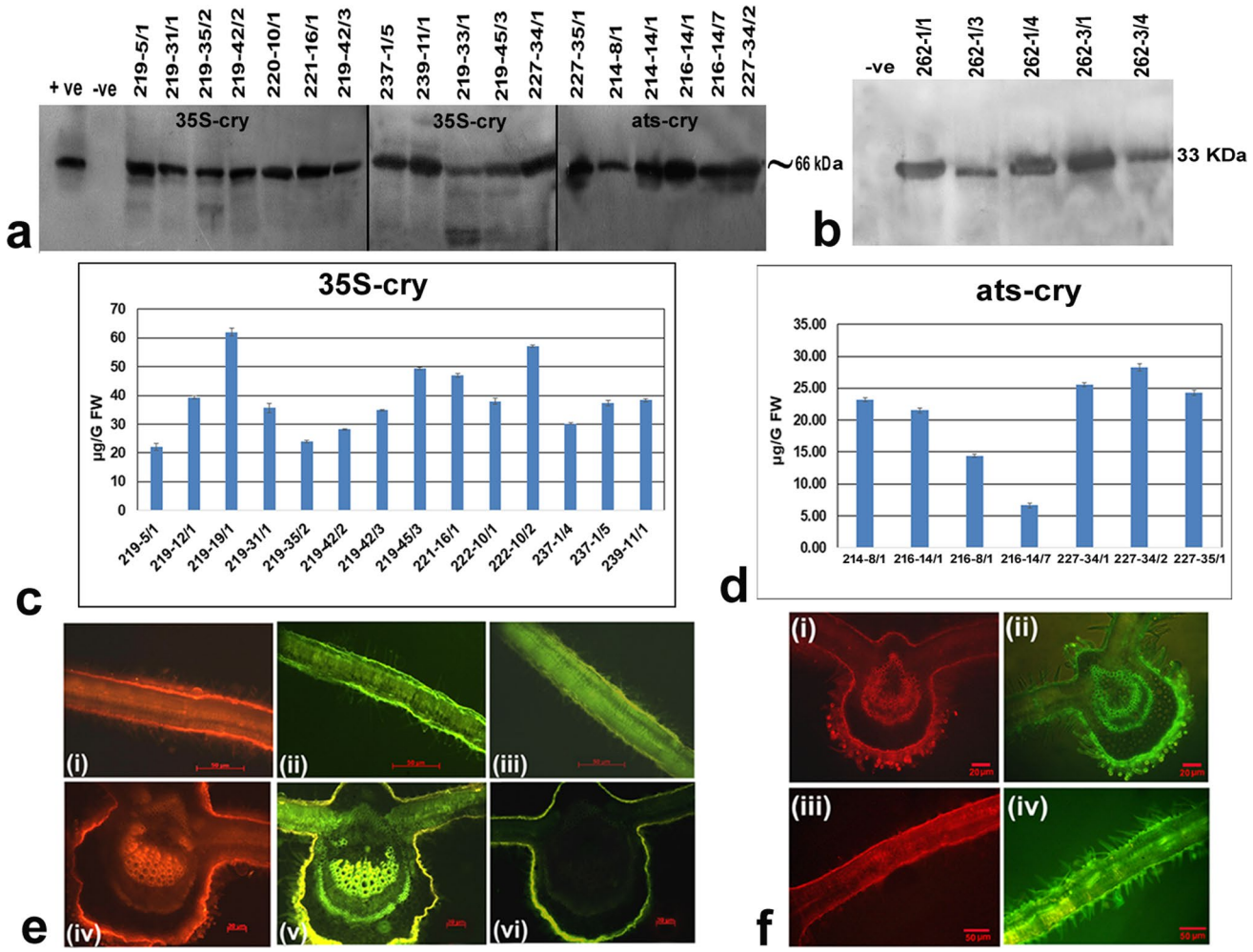

Fig. 3 Expression analysis of T_1 transgenic events. **a** Representative western blot analysis of total protein extract from 35S-cry and ats-cry events; lane + ve purified Cry1Ac as the positive control; lane − ve, untransformed pigeon pea plant as the negative control. **b** Representative western blot analysis of 35S-cre T_1 events with the untransformed plant as the negative control. **c** Expression level of transgenic Cry1Ac protein in 35S-cry events. **d** Expression level of Cry1Ac protein in ats-cry transgenic events. **e** Immunohistofluorescence localization of Cry1Ac in the leaflets of T_1 events indicated by green fluorescence; lamina of (i) untransformed control, (ii) 35S-cry, and (iii) ats-cry leaflets; vascular bundle of (iv) untransformed control, (v) 35S-cry, and (vi) ats-cry leaflets. **f** Immunohistofluorescence localization of Cre recombinase protein in the transverse section of leaflets from 35S-cre events; vascular bundle of (i) untransformed control and (ii) 35S-cre leaflets; lamina of (iii) untransformed control and (iv) 35S-cre leaflets

tissue promoter-driven 8 ats-cry transgenic T_1 events ranged between 40 and 60% (Fig. 5b). After 3 days of incubation, the percentage of insect mortality was found to be statistically significant in 35S-cry T_1 events compared to control plants according to the analysis of variance (ANOVA, $p < 0.0001$). Another bioassay was conducted with 3rd to 4th instar larvae to determine the toxicity of Cry1Ac protein on larval growth. Third to fourth instar larvae fed on leaflets of 35S-cry T_1 events demonstrated gradual weight loss at each 24 h interval, till the 13th day, compared to untransformed leaflets, where the larvae exhibited normal growth till maturity and developed into a pupa (Fig. 5c). Statistically, significant weight loss was noted on the 7th day ($p < 0.0001$). Green tissue promoter-driven Cry1Ac events did not show weight loss of the 3rd–4th instar larvae, rather a small amount of weight gain was recorded (Fig. 5d).

Histological analysis of pigeon pea, tobacco, and *Arabidopsis* leaf

The anatomy of transgenic and untransformed control pigeon pea (cv. ICPL 87119) leaf was studied in comparison to *N. tabacum* (cv. Petit Havana SR1) and *A. thaliana* (Col-0). Sections of both transformed and untransformed pigeon pea leaflets revealed that a large number of lignified cells were distributed in broad vascular bundles of midribs and veinlets. These lignified tissues showed green fluorescence of aniline blue (Fig. 5e). Alternatively, the fluorescence

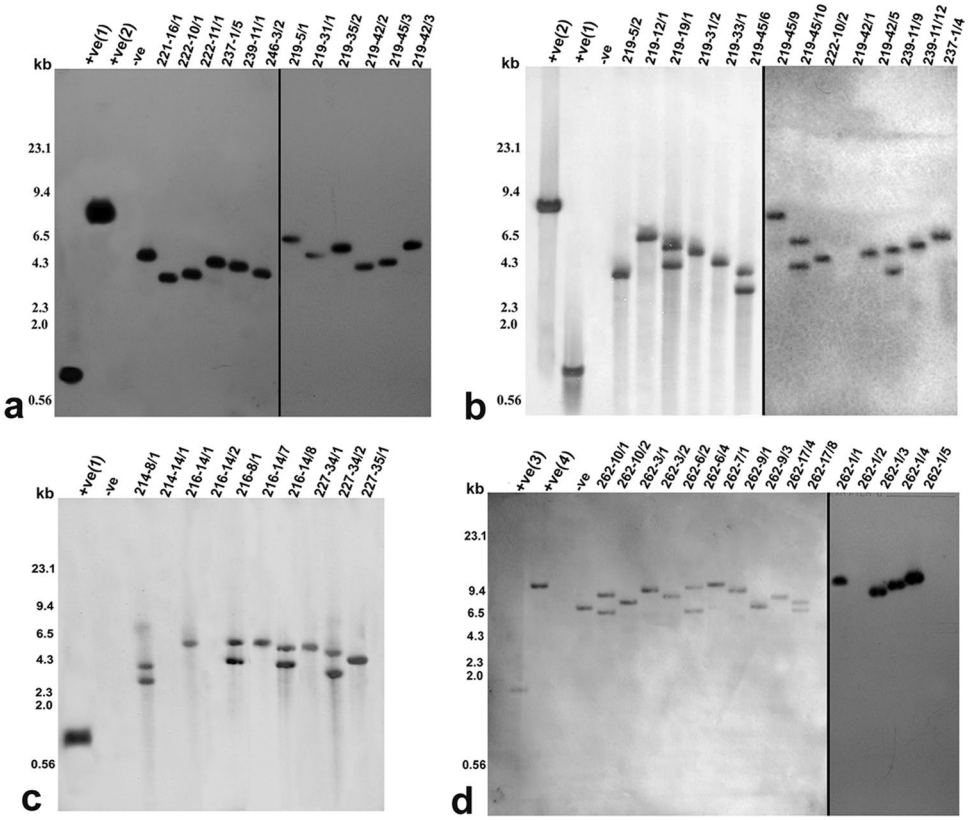

Fig. 4 Southern blot analysis of established Cre/*lox* T_1 transgenic events. **a** Radioactive Southern blot hybridization of *Hin*dIII-digested genomic DNA of 12 T_1 35S-cry transgenic pigeon pea events. **b** Nonradioactive Southern blot analysis of *Hin*dIII digested genomic DNA of 14 T_1 35S-cry transgenic pigeon pea events. **c** *Bam*HI-digested genomic DNA of 10 ats-cry T_1 transgenic events probed using the nonradioactive method. **d** *Hin*dIII-digested genomic DNA of 16 35S-cre T_1 transgenic events probed using the nonradioactive method. Lanes + ve (1), + ve (2), + ve (3), and + ve (4) are PCR-purified 662 bp *cry1Ac* probe specific sequence, *Hin*dIII-digested 8.6 kb 35S-cry plasmid DNA, PCR-purified 1100 bp *cre* gene sequence and *Hin*dIII-digested 10.6 kb 35S-cre plasmid DNA as positive controls, respectively; lane – ve genomic DNA of the untransformed plant as the negative control

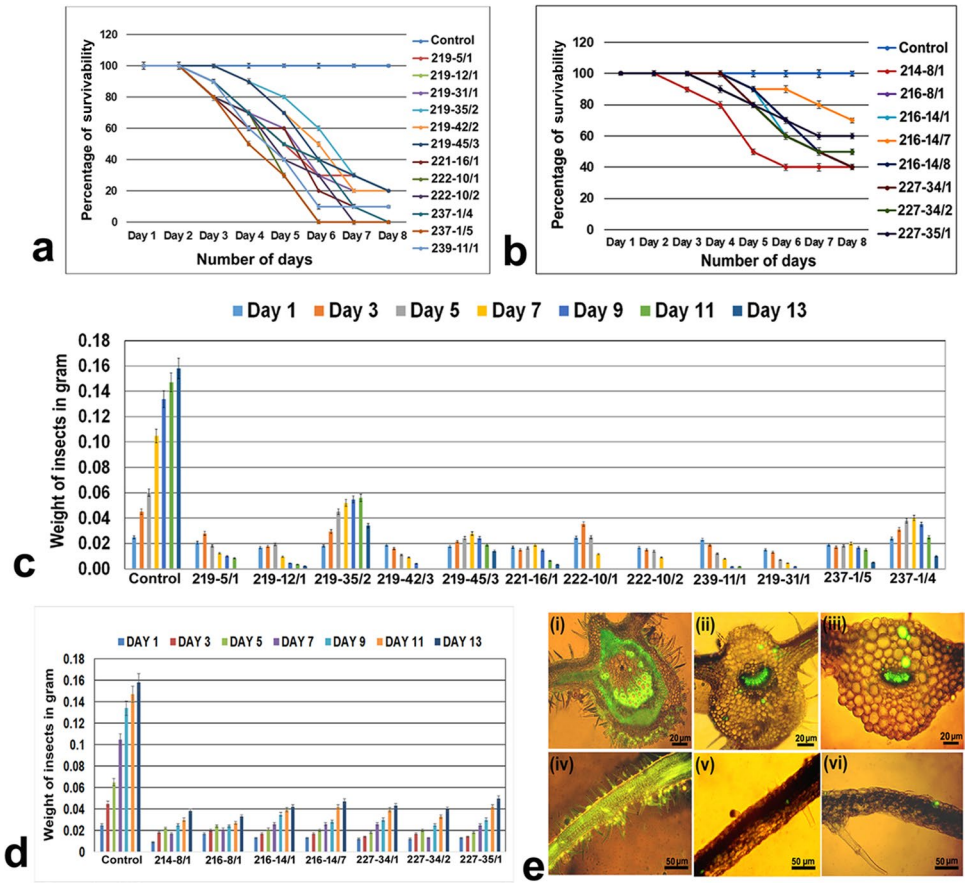

Fig. 5 Insect bioassay and histological study leaf transverse sections. **a**, **b** Established T_1 plants of 35S-cry and ats-cry transgenic events demonstrating 80–100% and 50–60% larval mortality, respectively. **c** Weight loss bioassay of 12 T_1 35S-cry events in comparison to untransformed control. **d** Weight loss bioassay of 8 T_1 ats-cry events. **e** Fluorescence study of leaf sections in three plant species; vascular bundles and lamina of pigeon pea (i and iv), *Nicotiana tabacum* (ii and v), and *Arabidopsis thaliana* (iii and vi), respectively

signal was found to be confined to relatively smaller areas in vascular bundles with a limited number of lignified cells in *N. tabacum* and *A. thaliana* leaf sections. No fluorescence signal was detected in non-lignified areas of all leaf sections. The anatomical difference between these three plant systems revealed that leaflets of pigeon pea had more lignified cells.

Molecular analysis of *nptII* elimination in T_2 hybrids

Ten independent single-copy 35S-cry T_1 events exhibiting 80–100% larval mortality were reciprocally crossed with five independent 35S-cre T_1 events selected based on the western positive expression profile. T_2 seeds of each hybrid event were collected separately, and independent PCR reactions were carried out using *nptII*-, *cry1Ac*-, and *cre*-specific primers to screen the hybrid events. Among 30 T_2 plants, five events (222–10/1 × 262–1/4, 221–16/1 × 262–1/4, 239–11/1 × 262–1/4, 219–12/1 × 262–1/4, 219–45/3 × 262–1/4) were found to be negative for the *nptII* marker gene, whereas they successfully amplified *cry1Ac*, *cre*, and *bar* genes (Supplementary Fig. S1, S2a). The absence of *nptII* (Fig. 6a), as well as the presence of *cry1Ac* (Fig. 6b) genes in those T_2 hybrid events, were confirmed through Southern blot analysis. 35S-cry parental lines of those hybrid events showed the presence of both *nptII* and *cry1Ac* genes. PCR-purified fragments of respective genes and *Hin*dIII-digested 8.6-bp 35S-cry binary plasmid were used as positive controls. One 35S-cry hybrid line with a non-eliminated *nptII* gene (219–5/1 × 262–10/1) was also taken as positive control and the untransformed plant as a negative control in those Southern blot experiments. Western blot analysis revealed the expression of Cry1Ac in those five *nptII*-eliminated T_2 hybrid plants (data not shown).

Analyses of Cry1Ac-expressing marker-free T_3 transgenic plants

T_3 seeds were obtained from the self-pollinated *nptII*-eliminated T_2 hybrid events. The established T_3 progenies were allowed for another round of molecular analysis to determine the absence of *cre-bar* gene cassette to fulfill the goal of marker-free transgenic establishment in pigeon pea. Fifteen progenies of each *nptII*-eliminated T_2 hybrid plant were screened by PCR analysis, and among them, 6 independent T_3 events (222–10/1 × 262–1/4/2/3, 221–16/1 × 262–1/4/1/2, 221–16/1 × 262–1/4/1/9, 239–11/1 × 262–1/4/2/8, 219–12/1 × 262–1/4/2/4, 219–45/3 × 262–1/4/2/5) were found to be negative for *cre* and *bar* genes, followed by successful amplification of *cry1Ac* gene (Table 1, Supplementary Fig. S2b, S3). These PCR positive T_3 Cry1Ac events were further confirmed by Southern blot analysis for *cre*, *nptII*, and *cry1Ac* genes (Fig. 6c, d, and e). Single-copy integration of the *cry1Ac* gene was found in the transgenic events along with the absence of *nptII* and *cre* genes. Western blot analysis exhibited the Cry1Ac expression in the abovementioned 6 marker-free events (Fig. 7a), and protein accumulation was found to be around 31–43 μg g^{-1} FW (Fig. 7b). These events were further allowed for insect mortality and weight loss bioassay on *H. armigera* larvae. The data on mortality of the 2nd instar larvae and the weight loss of the 3rd-4th instar larvae was found to be statistically identical ($p > 0.05$) in the marker-free T_3 transgenic plants to their respective T_1 parental events (Fig. 7c, d). More than 80% mortality was found in these 6 marker-free T_3 plants, among them 222–10/1 × 262–1/4/2/3 exhibited 100% mortality of *H. armigera* (Fig. 7e, i), whereas 90% mortality was found in 221–16/1 × 262–1/4/1/9 and 239–11/1 × 262–1/4/1/8 plants (Fig. 7f, g). All marker-free transgenic events exhibited gradual weight loss of the 3rd–4th instar larvae, at each 24 h interval till the 13th day, and statistically significant weight loss was observed at the 7th day ($p < 0.0001$) (Fig. 7d). Untransformed pigeon pea events were kept as a negative control in all bioassay experiments (Fig. 7h, j).

Discussion

Recent productivity data indicates that pigeon pea suffers from massive yield loss due to *H. armigera* infestations (Choudhary et al. 2013). Over the last few decades, several Cry toxins have been introduced in pigeon pea to incorporate pod borer resistance (Ghosh et al. 2017). However, in the context of safety assessment, the removal of selectable marker genes is necessary to avoid various issues regarding the commercialization of transgenic crops (Upadhyaya et al. 2010). The ever-increasing requirement for the production of improved crop varieties has expanded interest in researchers to develop marker-free transgenics, which has the potential to improve the acceptability of genetically modified products at the market level. The here described Cre/*loxP* recombination strategy executed through the crossing technique was found suitable concerning the plumular meristem transformation system used in this work, where T_0 antibiotic selection was avoided to get a maximum number of primary transformants. The marker elimination through co-transformation using a super binary vector, as demonstrated in another legume chickpea, cannot be utilized with the present transgenic development method of pigeon pea as the co-transformation system is based on the selection of primary transformants. Although several reports are available on Cre/*loxP*-mediated marker-free transgenic development in model and crop plants (Dale and Ow 1991; Hoa et al. 2002; Srivastava and Ow 2003; Zhang et al. 2003; Kopertekh et al. 2004; Sreekala et al. 2005; Chakraborti et al. 2008; Sengupta et al. 2010; Bala et al. 2013; Sarkar

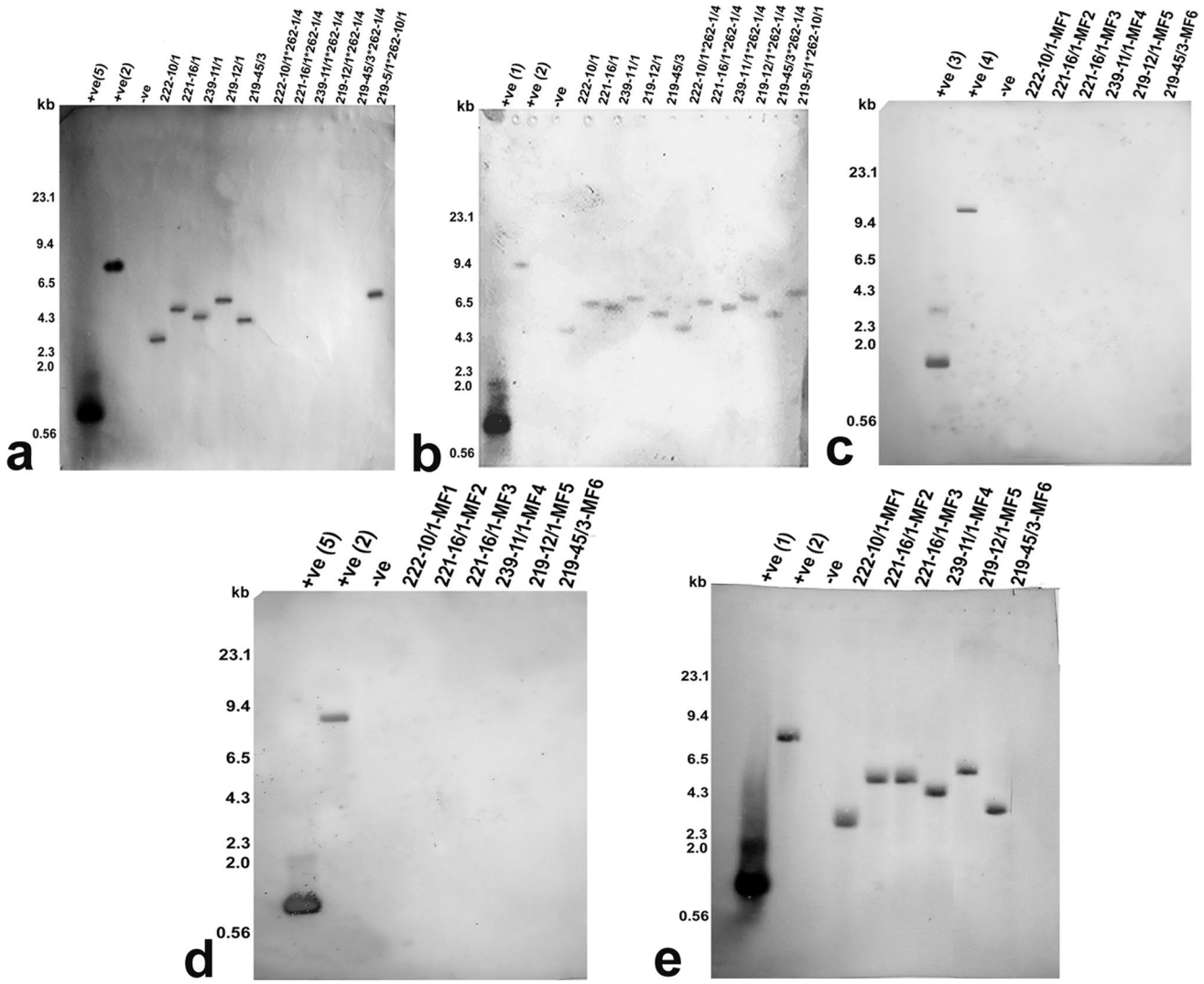

Fig. 6 Southern blot analysis of marker-eliminated transgenic events. **a**, **b** *Hin*dIII-digested genomic DNA of T_2 events probed with *nptII*- and *cry1Ac*-specific sequences, respectively. **c**, **d**, and **e** *Hin*-dIII digestion of *nptII*-eliminated T_3 progeny lines probed with *cre*, *nptII*, and *cry1Ac*-specific sequences, respectively. Lane + ve (1) and + ve (2), + ve (3), + ve (4), and + ve (5) represent PCR purified probe sequences of the *cry1Ac* gene, *Hin*dIII-digested binary plasmid 35S-cry, PCR-purified probe sequence of *cre* gene, *Hin*dIII-digested binary plasmid 35S-cre, PCR-purified probe sequence of *nptII* gene, respectively, as positive controls and lane − ve represents genomic DNA of the untransformed plant as a negative control

Table 1 Details of the marker-free transgenic pigeon pea plants

T_0 35S-cry event	T_1 35S-cry event	T_1 35S-cre event	T_2 hybrid	Marker-free 35S-cry T_3 plant	Marker-free 35S-cry T_3 plant name
219–12	219–12/1	262–1/4	219–12/1 × 262–1/4	219–12/1 × 262–1/4–4	219–12-MF1
219–45	219–45/3	262–1/4	219–45/3 × 262–1/4	219–45/3 × 262–1/4–5	219–45-MF2
221–16	221–16/1	262–1/4	221–16/1 × 262–1/4	221–16/1 × 262–1/4/-2	221–16-MF3
				221–16/1 × 262–1/4–9	221–16-MF4
222–10	222–10/1	262–1/4	222–10/1 × 262–1/4	222–10/1 × 262–1/4–3	222–10-MF5
239–11	239–11/1	262–1/4	239–11/1 × 262–1/4	239–11/1 × 262–1/4–8	239–11-MF6

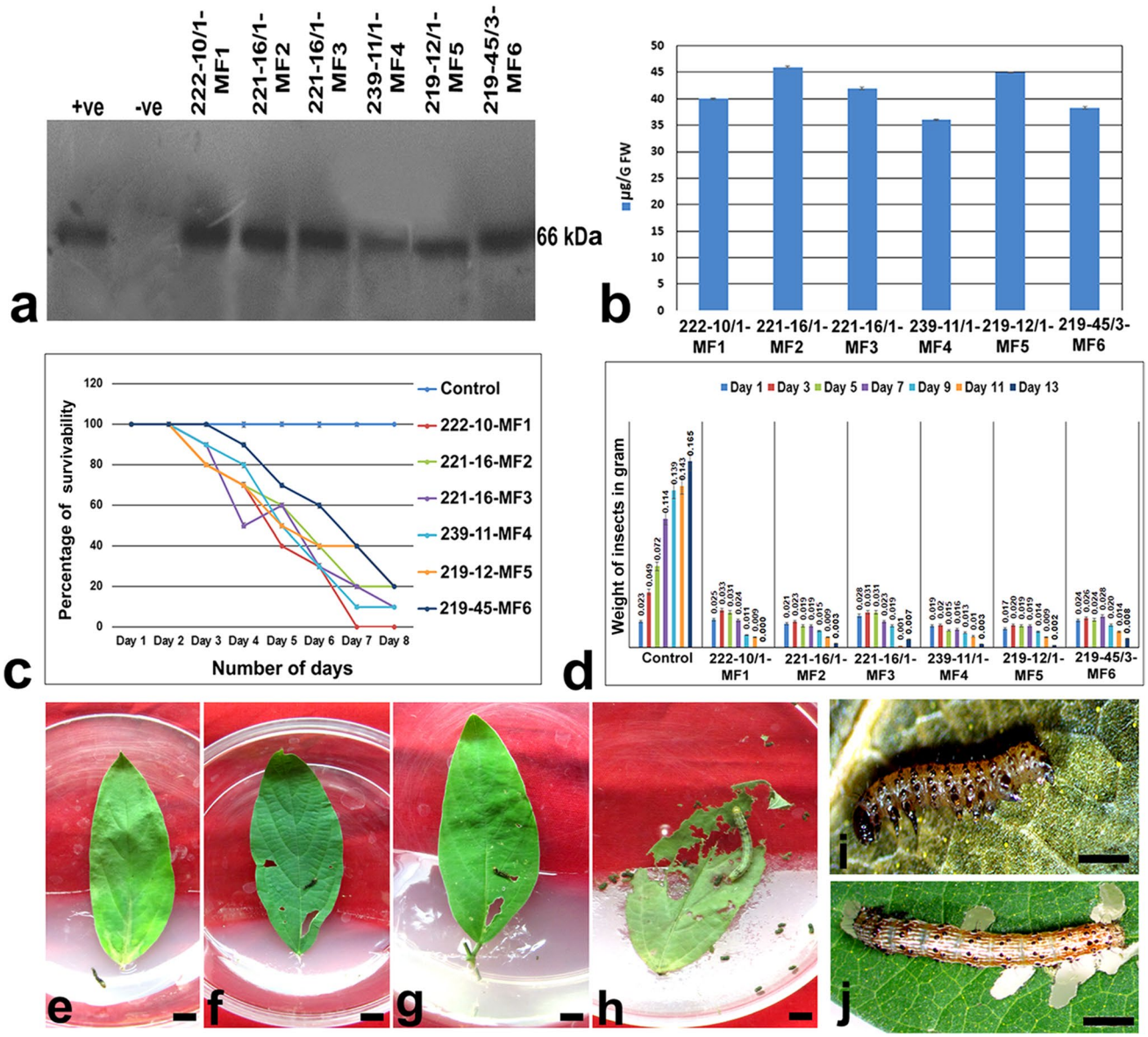

Fig. 7 Expression analysis and bioassay of Cry1Ac protein in T_3 transgenic events. **a** Western blot of marker-eliminated T_3 events with purified Cry1Ac protein of-66 kDa and untransformed plant protein as positive (+ve) and negative (−ve) controls, respectively. **b** Quantification of Cry1Ac protein in T_3 plants to record the expression level at 32–48 µg g^{-1} FW. **c** and **d** Marker-free T_3 events demonstrating 80 − 100% larval mortality and significant weight loss, in comparison to untransformed control, respectively. **e** and **i** Death of 2nd and 4th instar larvae, respectively, fed on the event with 100% larval mortality. **f** and **g** Death of 2nd instar larvae fed on the events with 90% larval mortality. **h** and **j** Feeding habit of 2nd and 4th instar larvae, respectively, on untransformed control leaves. The bar represents 5 mm

et al. 2021), there is no published report on *nptII* selectable marker elimination and simultaneous Cry1Ac gene expression using the Cre/*loxP* system in legume crops till date.

In the majority of plant systems, transformation vectors have been developed where the foreign gene is cloned under the control of the *CaMV35S* constitutive promoter due to its strong expression potentiality. Alternatively, the green-tissue-specific *ats1A* promoter was proven to delineate a high level of tissue-specific expression of a transgene in the cotton plant compared to the expression driven by *CaMV35S* (Kumar et al. 2004). The present study was undertaken to verify the expression of Cry1Ac under the influence of *CaMV35S* and *ats1A* promoters independently, along with the incorporation of *lox*P-flanked *nptII* to develop marker-free transgenic pigeon pea events. The promoter activities were compared based on protein accumulation in T_1 transgenic events and differential insecticidal activity against *H. armigera* larvae.

Although PCR positive T_0 transformants obtained from *A. tumefaciens*-mediated plumular meristem transformation produced a sufficient amount of seeds, some T_1 progenies were supposed to be non-transgenic due to the chimeric pattern of their parental events. Therefore, antibiotic-mediated stringent selection of T_1 transgenic events was performed. As a result, segregation analysis could not be performed in the T_1 generation, and hence no χ^2 test is presented for T_1 segregation. Constitutive promoter-driven Cry1Ac accumulation in T_1 transgenic plants was found to be higher than green tissue promoter-controlled expression, and this constitutive expression pattern of Cry1Ac was successfully inherited in T_2 and T_3 generations. Among several tissue culture-based pigeon pea transformation studies, few reports are available where the expression and inheritance of Cry proteins (Cry1Ac, Cry1Ab, Cry1-EC, and Cry2Aa) were analyzed beyond the T_1 generations (Surekha et al. 2005; Sharma et al. 2006; Ghosh et al. 2017). Krishna et al. (2011) reported the expression study of Cry1Ac protein through ELISA analysis from the different parts of established transgenic plants in T_0 generation. Alternatively, a tissue culture-independent transformation study of pigeon pea demonstrated 3–15 µg g^{-1} FW of Cry1AcF protein accumulation till the T_2 generation (Ramu et al. 2012). In all these studies, the Cry protein expression was driven by the *CaMV35S* promoter. Through a study on transgenic tobacco, the *ats1A* promoter together with a chloroplast transit peptide was found to exhibit a high expression of the Cry1Ac protein. The expression was reported to be around 1% of the total leaf protein (Wong et al. 1992). Another report demonstrated the development of transgenic cotton plants where the chloroplast-specific expression of Cry1Ac and Cry2Aa proteins using transit peptides was found to be 0.673 µg g^{-1} and 0.568 µg g^{-1} tissue, respectively (Muzaffar et al. 2015). In a recent report, the *ats1A* promoter-driven expression of truncated Cry1Ac protein was found to be relatively higher (30–79 µg g^{-1} FW) in transgenic *Arabidopsis* plants (Hazarika et al. 2019). In the present study, a higher accumulation of Cry protein (22–62 μg^{-1} FW) was observed in all leaf tissues due to the constitutive activity of the *CaMV35S* promoter, whereas protein accumulation was found to be restricted to the outermost epidermal regions in green tissue promoter containing ats-cry pigeon pea events. The histological analysis of pigeon pea leaflets demonstrated the presence of more lignified tissue in comparison to the leaves of *N. tabacum* and *A. thaliana*. This further justified the observation of lesser Cry1Ac accumulation under the influence of *ats1A* in pigeon pea. This is the first report where green-tissue-specific expression of Cry1Ac protein was analyzed in pigeon pea using the *ats1A* promoter conjugated with chloroplast transit peptide.

Nine constitutively expressing T_1 Cry1Ac events exhibited 80–100% mortality of 2nd instar *H. armigera* larvae along with drastic weight loss of 3rd–4th instar larvae. This mortality data was found to be comparable to the previous studies based on *cry* genes. According to a previous report, the constitutive expression of Cry1Ac protein conferred insect resistance, and only 55% mortality was recorded in T_0 transgenic lines (Krishna et al. 2011). Ramu et al. (2012) performed the insect bioassay in T_1 and successive two generations of pigeon pea where constitutive expression of Cry1AcF protein showed 80–100% mortality of *H. armigera* (Ramu et al. 2012). Through tissue culture-based transgenic development of pigeon pea, the current group reported a high level of insect-resistant activity of Cry1Ac and Cry2Aa proteins through mortality and weight loss bioassay of *H. armigera* larvae in successive T_1 and T_2 generations, although there was no provision of SMG elimination from those transgenic events (Ghosh et al. 2017). On contrary, the insecticidal activity of Cry1Ac protein under the control of the green-tissue-specific promoter was found to be lower, and thus these events were not continued for marker elimination studies. These results clearly illustrated that use of constitutive promoter will be more suitable for cry-transgenic development in pigeon pea.

The use of the Cre/*lox* system was found to be effective on a wide range of plants (Kerbech et al. 2005). Cre recombinase events were independently established to generate marker-free transgenic pigeon pea plants, following the crossing between these cre- and *lox*-transgenic plants. The crossing-mediated marker elimination strategy was reported to be more convenient than the cre-mediated auto-excision method (Konig 2003). This is the first report in a legume system where detailed analyses of cre-transgenics were performed at both integration and expression levels. According to a previous study, the highly expressive Cre-recombinase protein is required for precise and complete excision of the *lox*-flanked marker gene (Bayley et al. 1992).

Reciprocal crossing between single-copy 35S-cry and 35S-cre events was performed, and T_2 hybrid events were found to be *nptII* marker negative. The T_1 transgenic plants were mixtures of homozygous and hemizygous progenies, which were utilized for crossing. Therefore, the segregation pattern could not be predicted in the T_2 generation and hence no χ^2 test is presented for T_2 segregation. All the marker-free events were allowed to self-pollinate for the segregation of *cre*, and linked *bar* genes in the T_3 generation, and molecular analyses confirmed the absence of *cre* and linked *bar* genes in all those events. Previously, in a study by Sarkar et al. (2021), the elimination of the *bar* selectable marker was accomplished in transgenic pigeon pea, which was expressing Cry1Ab under the green-tissue-specific rubisco small subunit promoter. However, the expression level of Cry1Ab protein was not monitored, and the study demonstrated marker elimination of a sole transgenic event with partial resistance in larval bioassay. This finding could be explained

by the limited activity of the green-tissue-specific promoter to accumulate the inadequate amount of Cry toxin in pigeon pea. This was similar to our observation that the rubisco small subunit promoter-mediated Cry toxin expression was unable to provide desired resistance against *H. armigera* in pigeon pea.

This is the first report in pigeon pea where constitutive and tissue-specific expressions of the *cry1Ac* gene and their insecticidal activities were compared. Bioassay and protein expression study indicated that constitutive expression of Cry1Ac in transgenic pigeon pea plants conferred complete resistance against *H. armigera*, whereas *ats1A* promoter-driven Cry1Ac demonstrated partial resistance. The reason for this partial resistance could be attributed to the lesser accumulation of this Cry toxin as a whole in lignified, leathery leaves of ats-cry pigeon pea. This information could influence the choice of promoters in the future for transgenic pigeon pea development. The key feature of this research work was the establishment of transgenic events by a tissue culture-independent transformation protocol to overcome the recalcitrant nature of this particular legume along with a kanamycin-based T_1 selection procedure to avoid chimeric interference. The application of appropriate Cre*/lox* binary vectors was proven to be successful for the precise elimination of the marker gene. The manifestation of the insect-resistant ability of integrated the *cry1Ac* gene product was effectively maintained up to the T_3 generation in 5 marker-free events. The implementation of such a marker-free transgenic development could be extended to a multi-toxin approach using both *cry1Ac* and *cry2Aa* for durable resistance. This strategy has the potential to increase commercial acceptance of genetically modified crops addressing marker gene-related environmental and food safety issues.

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

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Data availability All data generated or analyzed during this study are included in this published article [and its Supplementary Information files].

Declarations

Ethics approval This article does not contain any studies with human participants. The authors did not perform any animal-based experiments for this work.

Consent for publication Review work is presented in this manuscript with the consent of all authors.

Conflict of interest The authors declare no competing interests.

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