CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Inheritance and expression analyses of *cry1Ab* gene in transgenic pigeonpea tolerant to *Maruca* pod borer

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Abstract: *Development of transgenic crops with stable gene inheritance and expression over generations is important for effective deployment at field level. In present study, T_1 plants of pigeonpea cultivars AL15 and AL201 were evaluated for the presence and expression of cry1Ab gene and protection against Maruca pod borer. Cry1Ab protein in transgene carrying T_1 plants ranged from 0.72 to 0.87 µg g-1 flower tissue. In vitro insect bioassay demonstrated up to 49.17 and 53.80% loss in larval body weight after four days of infesting T_1 transgenic flowers and pods, respectively. Further, no adults emerged from the pupae of larvae fed on transgenic plants 15-537 and 201-344. All T_2 progeny plants of 15-537 exhibited cry1Ab presence; likewise, all T_3 progeny plants derived from homozygous T_2 plant (15-537-5) displayed presence and expression of transgene, thus establishing stable transgene integration in T_1 plants, followed by its stable inheritance and expression in T_2 and T_3 generations.*

Keywords: *Cajanus cajan, ELISA, insect bioassay, loss in larval weight, transgene expression*

INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp.) is an important legume crop grown in semi-arid tropics with an overall production of 5.47 m tons (FAO 2021). India tops in the area (3.6 m ha) under pigeonpea cultivation. The crop is cultivated for human consumption as it bears protein-rich (21%) seeds as well as for animal feed, green manure and soil conservation (Krishna et al. 2010). Various conventional breeding approaches and molecular techniques have enabled tailoring of genetic architecture of pigeonpea suitable for present-day challenges. Despite this, the crop productivity is hampered due to various abiotic and biotic stresses (Rana et al. 2016). Among biotic stresses, damage caused by insect pests and disease-causing pathogens pose major constraint in stagnating pigeonpea yield. The maximum devastation in yield losses worldwide is caused by *Maruca vitrata* or spotted pod borer (Gopali et al. 2010, Chatterjee et al. 2019) as the insect feeds inside the webbed masses of flowers and pods at an early stage of plant growth (Taggar 2014). The bottlenecks in developing resistance against *M. vitrata* in pigeonpea are limited resistant genetic resources and cross incompatibility with wild species, which hinder the success of conventional breeding approaches (Choudhary et al. 2013).

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In this context, genetic transformation is an effective approach for development of pigeonpea cultivars having integral insect resistance. Among various transgenes, the most widely studied, *cry* genes from *Bacillus thuringiensis*, are known to possess insecticidal activities, specifically against lepidopteran insects (Eapen et al. 2008). In a study, Cry1Ab protein was found to be more effective against second instar larvae of *Maruca* pod borer when the larvae were fed with artificial diet containing the toxic proteins Cry1Aa, Cry1Ab, Cry2Aa, Cry1Ac and Cry1Ca (Srinivasan et al. 2008). Transgenic pigeonpea plants carrying different *cry* genes, such as *cry1AcF*, *cry1Ac* and *cry2Aa*, have been developed by Ramu et al. (2011), Kaur et al. (2016) and Singh et al. (2018). Likewise, *cry1Ab* carrying T_0 pigeonpea transformants that were raised to next generations and characterized in present study were developed in our laboratory (Singh et al. 2021). T_1 plants were analyzed for transgene presence through PCR, transgene expression through semi-quantitative reverse transcription PCR (RT-PCR), Cry1Ab protein content by enzyme linked immunosorbent assay (ELISA) and transgene efficacy through *in vitro* insect bioassay. Further, T_2 and T_3 plants (raised from seeds of selfed T_1 and T_2 plants) were analyzed for transgene segregation and inheritance through PCR, and expression by qualitative ELISA. The highlighting features of the study were: i) development of morphologically normal T_1 transgenic pigeonpea plants that retarded the growth of *M. vitrata* larvae with no adult emergence from the pupae, ii) homozygosity of T_1 and T_2 plants for *cry1Ab* gene, and iii) stable inheritance and expression of the transgene in T_3 generation.

MATERIAL AND METHODS

Polymerase chain reaction analysis on T_1 plants

Total genomic DNA was extracted from young leaves (100 mg weight) of screenhouse-grown one-month-old putative T_1 and non-transgenic (NT) pigeonpea plants using CTAB method described by Murray and Thompson (1980). Genomic DNA was analyzed for the presence of *cry1Ab* under the control of maize ubiquitin promoter through PCR using transgene specific internal primers 5'-CTATCCCATTGTTCGCAGTCCA-3' (forward) and 5'-GTGTCCAGACCAGTAATACTCTCC-3' (reverse). The analysis was performed in 20 µL reaction mixture containing 75 ng genomic DNA template (3 µL), 1 unit *Taq* DNA polymerase (4 µL), 0.5 mM of dNTPs (1 µL), 25 mM $MgCl_2$ (1.2 µL), 1´ reaction buffer (4 µL), 0.3 µM of forward and reverse primer (1.2 µL each) and nuclease free water (4.4 µL) in a thermal cycler (Eppendorf Master cycler, Germany). The reaction profile comprised of 35 cycles with strand separation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, extension at 72 °C for 1 min along with a final extension at 72 °C for 7 min. PCR products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light in a gel documentation system (Analytik Jena, Germany).

Semi-quantitative reverse transcription-PCR analysis on PCR positive T_1 plants

Total RNA from tender apical leaves (100 mg weight) of 3-month-old T_1 plants showing the presence of *cry1Ab* and NT plant was isolated using Spectrum plant total RNA kit (Sigma-Aldrich, USA) according to manufacturer's instructions. RNA was analyzed on 1.2% denaturing agarose gel prepared in 1´ MOPS buffer (200 mM MOPS, 80 mM sodium acetate and 10 mM EDTA); 1 µg of RNA was used for cDNA synthesis by Verso kit (Thermo Scientific, USA).

RT-PCR analysis for cDNA confirmation was carried out by amplifying *26S rRNA* (GenBank accession no. AY283368) using forward 5'-CACAATGATAGGAGGAGCCGAC-3' and reverse 5'-CAAGGGAACGGGCTTGGCAGAATC-3' primers. The analysis was performed in 10 µL reaction mixture containing 30 ng cDNA (2 µL), 25 µM $MgCl_2$ (0.5 µL), 5 µM each of forward and reverse primer (0.75 µL), master mix (3.5 µL) and sterile water (2.5 µL) as per Kaur et al. (2016). The reaction was carried out in a programmable thermocycler according to conditions described for PCR. Thereafter, RT-PCR to check transgene transcription was performed with *cry1Ab* specific primers, and the amplicons were electrophoresed and visualized.

Quantitative ELISA on RT-PCR positive T_1 plants

Cry1Ab content in flower tissues of 3½-month-old T_1 plants showing transcript accumulation and NT (AL 15 and AL 201) plants was estimated through sandwich ELISA using QuantiPlate kit™ (EnviroLogix, USA). The estimation was carried out as per description in our previous study (Singh et al. 2021). Briefly, the protocol involved homogenization and dilution of T_1 flower tissues (20 mg each) in extraction buffer. Thereafter, the samples were loaded in microtiter

plate wells and incubated with enzyme conjugate. The plate was washed thrice with wash buffer, substrate was added, allowed to react and the plate was then read at 450 and 600 nm in an ELISA plate reader (Tecan Infinite® 200 Pro, Switzerland). The protein content in µg/g (ppm) was calculated using the formula: [protein content in ppb × dilution factor (11) × weight of flower tissue (20 mg)]/1000. The data were analyzed for mean ± standard deviation in Microsoft Excel 2010 software at default settings.

In vitro insect bioassay on *cry1Ab* expressing T_1 transgenic plants

The efficacy of *cry1Ab* expressing T_1 plants for protection against *M. vitrata* was tested through *in vitro* feeding assay performed on flowers and pods using second instar insect larvae. The webbed flowers/buds of unsprayed field-grown pigeonpea plants infested with larvae were plucked, brought to Pulses Entomology Laboratory, Department of Plant Breeding & Genetics, and used for collection of second instar larvae using a Camel-hair brush. These were starved for 4 h and weighed using an electronic balance, thereafter one larva was released on ten freshly collected flowers from each T_1 plant. The flowers were placed on a clean Whatman filter paper inside a plastic cup (height = 6 cm, diameter = 11 cm), covered with a plastic lid having holes and incubated in a B.O.D. incubator (Remi, India) at 28 °C and 70% relative humidity. Simultaneously, a single larva was released on four freshly collected T_1 pods in a similar fashion. As a control, the flowers and pods from NT plants with single larva were also incubated separately for comparison.

The data were recorded four days after infestation (DAI) and included weight of larva, number of flowers, pods webbed/eaten and larval mortality (if any). The data were analyzed for transformed square root means and critical difference in a completely randomized design using CPCS1 software (Cheema and Singh 1993). The mean larval weight loss (%) was calculated as: [(Mean change in larval weight 4 DAI on NT flower/pod - Mean change in larval weight 4 DAI on T_1 flower/pod)/ Mean change in larval weight 4 DAI on NT flower/pod] ´ 100. The data on mean larval weight loss was plotted against mean Cry1Ab content, number of flowers and pods webbed/eaten in a linear regression model in R software (R Core Team 2021) to predict the effect of protein content, flowers and pods webbed/eaten on larval weight loss.

Segregation analysis on T_2 progeny plants

The T_1 plants displaying maximum loss in larval weight were selfed and used for the collection of T_2 seeds upon maturity. The seeds were germinated in seedling trays and the progeny plants were analyzed through PCR to know the segregation pattern of *cry1Ab* gene.

PCR analysis and qualitative ELISA on T_3 progeny plants

The T_2 progeny plants showing the presence of *cry1Ab* gene were selfed; the seeds were collected at maturity and sown. One and a half-month-old T_3 plants were analyzed for transgene inheritance through PCR and expression using qualitative ELISA kit (Immuno Farm Sciences, Hyderabad) for detection of Cry1Ab. For expression analysis, the leaf samples (20 mg) were taken in a microfuge tube, ground using pestle in 500 µL of extraction buffer provided in the kit and incubated for 30 min at room temperature. Thereafter, 50 µL of each sample was transferred to individual wells of microtiter plate. Fifty µl each of positive and negative controls (given in the kit) was also added in respective wells, followed by the addition of enzyme conjugate (50 µL) in each well. The plate was incubated for 40 min, after that the well contents were decanted in sink and the wells were washed four times with wash solution. Then 100 µL of substrate was added to each well and plate was incubated for 20 min. Finally, 100 µL of stop solution was added and the absorbance was measured at 450 nm. A leaf sample was treated as negative for *cry1Ab* expression, if absorbance was less than cut off value, i.e., 0.2 + absorbance of negative control. On the other hand, a leaf sample having absorbance more than cut off value was treated as positive for transgene expression.

RESULTS AND DISCUSSION

Among different insect pests that attack pigeonpea, major threat to its production is caused by *M. vitrata*. The limited variability within the genetic resources along with their cross incompatibility with pigeonpea cultivars make the crop improvement through conventional breeding an arduous task. Genetic transformation, on the other hand, has enabled transfer of desirable transgenes and development of transgenic plants that need to be analyzed through various methods for selecting a transgenic event(s) with stable gene inheritance and expression over generations, imperative for successful

deployment at field level (Jadhav et al. 2020). The present study was thus conducted on molecular characterization of T_1, T_2 and T_3 plants generated through selfing of *cry1Ab* carrying T_0 transformants developed earlier in our laboratory through *Agrobacterium* mediated *in planta* transformation (Singh et al. 2021). The highlighting features of the study were identification of fertile, *cry1Ab* carrying homozygous T_1 transgenic pigeonpea plants that resulted in weight loss of second instar *M. vitrata* larval post *in vitro* feeding on flowers and pods. The transgene was stably transmited and its expression was validated till T_3 generation.

Identification of *cry1Ab* carrying T_1 plants through PCR

The seeds from 15 primary pigeonpea transformants (eight of *cv.* AL 15 and seven of *cv.* AL 201) carrying *cry1Ab* were collected (Table 1). A total of 1099 seeds were placed for germination under controlled conditions in glasshouse to obtain 904 T_1 plants that were analyzed for the presence of *cry1Ab* through PCR using gene specific primers. The results revealed amplification of the transgene in 97 plants (with a transformation efficiency of 12.89% in AL 15 and 8.27% in AL 201), suggesting its inheritance in T_1 generation (Table 1; Figure S1). The previous studies reported transformation efficiencies of 13.71, 22.2 and 32.15% by PCR in T_1 pigeonpea developed through *in planta* transformation (Rao et al. 2008, Ramu et al. 2011, Parekh et al. 2014). They stressed on screening *in planta* generated pigeonpea plants in T_1 and T_2 generations due to stable integration of transgene in these generations. PCR-based method has been documented to establish genome integration of glutenin subunit *Dy10* gene, sesquiterpene synthase gene and T-DNA in genetically modified wheat, *Arabidopsis* and rapeseed events, respectively (Abdalla 2007, Ee et al. 2014, Wu et al. 2014).

RT-PCR analysis on PCR positive T_1 plants for transgene transcription

Randomly selected 12 healthy PCR positive T_1 plants (nine of AL 15 and three of AL 201) were analyzed through semi-quantitative RT-PCR for transgene transcription. The total RNA isolated from T_1 and NT plants was assessed for integrity on denaturing agarose gel. cDNA analysis using *26S rRNA* gene specific primers resulted in amplification of 534 bp fragment (Figure S2), indicating the legitimacy of synthesized product. cDNA was then analyzed with *cry1Ab* specific primers that amplified 526 bp sized fragment in all 12 T_1 plants designated as 15-210, 15-537, 15-539, 15-541, 15-542, 15-545, 15-549, 15-550, 15-552, 201-344, 201-347 and 201-353, thus proving transgene transcript accumulation in these plants (Figure 1a).

Table 1. Identification of T_1 pigeonpea plants carrying *cry1Ab* gene through PCR

S. No.	T_0 plant designation (variety)	No. of seeds sown	No. of T_1 plants analyzed by PCR	No. of PCR positive T_1 plants	Numbering of T_1 plants
1	A (AL 15)	60	51	3	15-1, 15-2, 15-3
2	B (AL 201)	65	50	0	-
3	C (AL 201)	80	62	10	201-354 to 201-363
4	D (AL 201)	75	64	0	-
5	E (AL 201)	78	68	25	201-286 to 201-302, 201-332, 201-338, 201-340, 201-344, 201-345, 201-347, 201-352, 201-353
6	F (AL 15)	82	70	35	15-474 to 15-503, 15-535, 15-537, 15-539, 15-541, 15-542
7	G (AL 15)	68	60	19	15-166 to 15-182, 15-210, 15-211
8	H (AL 15)	85	60	5	15-545, 15-549, 15-550 to 15-552
9	I (AL 15)	75	60	0	-
10	J (AL 201)	70	58	0	-
11	K (AL 201)	72	61	0	-
12	L (AL 15)	64	55	0	-
13	M (AL 15)	72	60	0	-
14	N (AL 15)	78	65	0	-
15	O (AL 201)	75	60	0	-
Total		1099	904 (481 of AL 15, 423 of AL 201)	97 (62 of AL 15, 35 of AL 201)	
Transformation efficiency				AL 15 (12.89%), AL201 (8.27%)	

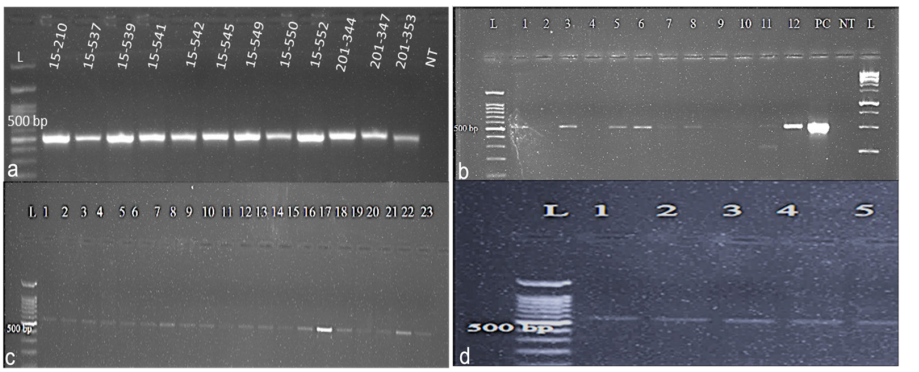

Figure 1. (**a)** Semi-quantitative RT-PCR analysis on 12 PCR positive T_1 plants using *cry1Ab* specific primers for transgene transcript accumulation. L: 100 bp DNA ladder (Cat. no. SM0243, Thermofisher Scientific, USA); the numbers 15-210 to 201-353 refer to T_1 plant samples; NT: non-transgenic plant. **(b)** PCR analysis on 12 T_2 progeny plants raised from T_1 plant 201-344. L: 100 bp DNA ladder (GeneRuler, USA); the numbers from 1 to 12 refer to T_2 samples; PC: plasmid control; NT: non-transgenic AL 201 plant. **(c)** PCR analysis on 23 T_2 progeny plants raised from T_1 plant 15-537. L: 100 bp DNA ladder (GeneRuler); the numbers from 1 to 23 refer to T_2 samples. **(d)** PCR analysis on five T_3 progeny plants (derived from T_2 plant number 15-537-5) showing 526 bp amplicon corresponding to *cry1Ab* gene. L: 100 bp DNA ladder (GeneRuler).

Quantitative ELISA on RT-PCR positive T_1 plants for Cry1Ab content

Cry1Ab protein content in flower tissues of 12 RT-PCR positive T_1 plants was estimated through quantitative ELISA. The flower tissues had protein content equal to or more than positive control at 2.5 ppb (0.49 ± 0.00) and ranged from 0.72 to 0.87 µg/g (Figure S3; Table S1), suggesting expression of the transgene in T_1 plants. The NT plant tissues did not exhibit any Cry1Ab content. ELISA has been extensively used for quantitative estimation of Cry protein in pigeonpea and other important crops, such as wheat and rice (Ramu et al. 2011, Abouseadaa et al. 2015, Xu et al. 2018). The efficacy of Cry1Ab protein produced in T_1 transgenic pigeonpea plants for conferring protection against *M. vitrata* was tested through *in vitro* insect feeding assay.

Bioassay on T_1 transgenic plants for toxicity to *Maruca vitrata*

The efficacy of *cry1Ab* in 12 T_1 transgenic plants was tested through *in vitro* feeding of second instar *M*. *vitrata* larvae on flowers and pods, where NT (AL 15, AL 201) plants' flowers and pods were used as control. The larvae fed voraciously on flowers and pods of NT plants, causing major damage to the tissues, whereas these fed slowly on T_1 transgenic plants, leading to a smaller number of webbed/eaten flowers and pods (Figure 2a, b, c, d; Table S2). Although feeding on all T_1 plants resulted in decrease in larval weight with no mortality, the larvae that fed on flowers and pods of T_1 plant 15-537 exhibited highest weight loss by up to 49.17 and 53.80%, respectively after 96 h of infestation as compared to no weight loss in larvae released on AL 15 NT plant flowers and pods (Figure S4a, b). Likewise, larval weight after feeding on T_1 plant 201-344 flowers and pods was significantly reduced by up to 44.56 and 48.32%, respectively in comparison to no larval weight loss post-feeding on AL 201 NT plant tissues (Table S2; Figure S4a, b).

Upon fitting the data on mean larval weight loss as response variable and mean number of flowers webbed/eaten, number of pods webbed/eaten and Cry1Ab content as predictor variables in regression model, the following equations were obtained: a) y = 12.38 + 3.93 × flowers webbed/eaten, b) y = 26.64 + 3.26 × pods webbed/eaten, and c) y = - 61.31 + 118.78 × Cry1Ab content, where y is mean larval weight loss (%). The regression equations indicated that for every one unit increase in the number of flowers webbed/eaten, the predicted larval weight loss increased by 3.93 units (a); for every one unit increase in the number of pods webbed/eaten, the predicted larval weight loss increased by 3.26 units (b); and for every one unit increase in Cry1Ab content, the predicted weight loss increased by 118.78 units (c), implying that the transgene had a negative effect on larval growth and development as the same is mostly predicted from index of body weight gain in insect larvae (Adesoye et al. 2008). Further, no adult emerged from pupae of larvae fed on flowers and pods of T_1 transgenic plants 15-537 and 201-344, suggesting that *cry1Ab* was effective not only in retarding larval growth, but also in reducing fitness of the larvae. On the other hand, the larvae fed on flowers and pods of remaining

Figure 2. *In vitro* feeding of second instar *M. vitrata* larvae. **(a)** Major damage on flowers of non-transgenic plant; **(b)** Minor damage on flowers of T_1 transgenic plant four days after infestation; **(c)** Major damage on pods of non-transgenic plant; **(d)** Minor damage on pods of T_1 transgenic plant four days after infestation. The larvae are marked in circles.

ten transgenic plants showed normal adult emergence. A direct relationship existed between Cry1Ab content in flower tissues and larval weight loss, i.e., the T_1 plants having high protein content, e.g., 15-537 and 201-344, induced more weight loss (Figure S3; Figure S4c).

cry1Ab gene expressed under strong CaMV 35S promoter resulted in mortality of third instar *M. vitrata* larvae within three days of infesting T_1 cowpea plants (Adesoye et al. 2008). As per Estruch et al. (1997), the transgenic plants are known to mostly hinder insect growth and development, and rarely cause 100% insect mortality. The slower growth rate coupled with reduced fitness of the insect larvae would provide a much broader window within which insecticidal intervention can be effectively and judiciously employed, leading to better management of the insect pest (Sharma et al. 2000). A partial plant resistance is also advantageous from the perspective that synergistic interactions are feasible between partially resistant plants and natural enemies of the target insect pests (Hoy et al. 1998). On the other hand, *Bt* transgenic crops causing insect mortality had adverse ecological effects on various trophic levels within and outside crop fields due to diminution of the host (lepidopteran insects) for natural enemies (parasitoids) [Schuler 2000]. Thus, the transgenic pigeonpea plants 15-537 and 201-344 plants identified to retard larval growth in the present study are a useful source of tolerance against *Maruca* pod borer.

Segregation analysis on T_2 progeny plants for identifying homozygous plants

The seeds from T_1 plants (23 from plant 5-537 and 12 from plant 201-344) demonstrating significant larval weight loss along with no adult emergence were germinated. The T_2 progenies were PCR analyzed to determine the segregation pattern of *cry1Ab* gene. Amongst twelve T_2 plants of 201-344, only eight were observed to carry the transgene (Figure 1b); however, all 23 T_2 progeny plants raised from T_1 plant number 15-537 showed the presence of amplicon (Figure 1c), suggesting stable transgene integration and homozygosity of the plant 15-537 for *cry1Ab* that was stably transmitted

to T_2 generation. The early generation transgenic plants, such as T_2 or T_3 progressing towards homozygosity, has also been reported by James et al. (2002).

PCR analysis and qualitative ELISA on T_3 progeny plants for transgene inheritance and expression

The seeds (five in number) from one of the PCR positive T_2 progeny plants 15-537-5 were germinated; the leaf tissues of T_3 progeny plants (Figure S5a) were analyzed through PCR and qualitative ELISA to determine the inheritance and expression of *cry1Ab* gene, respectively. All five T_3 plants of 15-537-5 were observed to carry the transgene (Figure 1d). The ELISA results were visualized with color development, where T_3 tissues had absorbance values of 0.270, 0.443, 0.467, 0.275 and 2.03 with a cut off value was 0.266, implying that the T_3 plants were positive for *cry1Ab* expression. The results suggested homozygosity of plant number 15-537-5 for *cry1Ab* and transmission of the transgene to T_3 generation. There was variability in *cry1Ab* expression level in different progeny plants as was evident from different absorbance values (Figure S5b). Jadhav *et al.* (2020) detected a similar type of variation through qualitative ELISA in the level of Cry2AX1 in T_3 progenies of transgenic cotton events. Reason for variation in Cry protein level among T_3 sibling progeny derived from single T_2 plant under the homozygous condition may be the effect of controlled environmental conditions under which the plants are grown (Benfey et al. 1990) or epistatic interaction between integrated transgene and endogenous plant genes (Jadhav et al. 2020).

In conclusion, T_3 progeny plant with absorbance value of 2.03 along with its progenitor T_1 transgenic plant 15-537 (homozygous for *cry1Ab* and effecting maximum larval weight loss) identified herein have broadened the genetic base of the crop. These are a valuable source for introgression of *Maruca* pod borer tolerance in pigeonpea breeding programs. Also, to our best knowledge, this is the first report on the expression of *cry1Ab* gene under the control of maize ubiquitin promoter in pigeonpea. The study will pave the way for designing genetic transformation experiments with different expression cassettes rather than using usual strong expression cassettes with unwanted high transgene expression that causes insect mortality.

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Supplementary Tables and Figures are available from the corresponding author.

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