Transgenics in groundnut (*Arachis hypogaea* L.) expressing *cry1AcF* gene for resistance to *Spodoptera litura* (F.)

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Abstract Large number of primary transgenic events were generated in groundnut by an *Agrobacterium* mediated, *in planta* transformation method to assess the efficacy of *cry1AcF* against the *Spodoptera litura*. The amplification of required size fragment of 750 bp with *npt* II primers and 901 bp with *cry1AcF* gene primers confirmed the integration of the gene. The expression of the *cry* gene was ascertained by ELISA in T₂ generation, and the maximum concentration of cry protein in transgenic plants reached approximately 0.82 µg/g FW. Further, Southern blot analysis of ten T₂ transgenic plants proved that transgene had been integrated in the genome of all the plants and Northern analysis of the same plants demonstrated the active expression of *cry1AcF* gene. The highest mean % larval mortalities 80.0 and 85.0 with an average mean % larval mortalities 16.25 ($n=369$) and 26.0 ($n=80$) were recorded in T₁ and T₂ generations, respectively. Segregation analysis of the selected lines in the T₃ generation demonstrated homozygous nature. This clearly proved that though there is considerable improvement in average mean % larval mortality in T₂ generation, the *cry1AcF* gene was effective against *S. litura* only to some extent.

Keywords Groundnut · *In planta* transformation · *cry1AcF* · *Spodoptera litura*

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Introduction

Groundnut (*Arachis hypogaea* L.) is a premier oil seed crop of India and shares about 30 % of the area and 35 % production of all the oil seed crops of the country. India is the world's second largest producer of groundnut. The lepidopteran insect pests on groundnut remain a great challenge to manage despite an array of strong management practices in place. *Cry* genes from *Bacillus thuringiensis* are now widely being used for development of insect, particularly Lepidoptera, resistant transgenics (Tiwari et al. 2011). Groundnut crop improvement programmes are being followed actively worldwide using the transgenic approach (Singsit et al. 1997; Beena et al. 2008; Tiwari et al. 2008, 2011).

Though the *cry* genes play an important role in pest management, the specificity of *Bt* cry toxins and the deployment of transgenic crops expressing a single specific *Bt* toxin may lead to resistance development by the target pest in the field. Hence, the concept of gene pyramiding has been hailed as one of the *Bt* resistance management strategy. Further, an alternative to gene pyramiding would be to use synthetic genes of multiple efficacies. Cry proteins share a common three domain structure (Li et al. 1991). In simple words, hybrid (Synthetic) toxins produced through inclusion of a domain from another toxin results in increased potency of the fused protein by the shift in receptor binding (Bosch et al. 1994). In this direction, a chimeric *Bt* gene *cry1AcF* with *cry1Ac* (domain I & II) and *cryIF* (domain III) was developed to improve the insecticidal property of the toxin (Chakrabarti et al. 1998).

To understand the efficacy of the chimeric *Bt cry1AcF* gene in groundnut, a tissue culture-independent *in planta* transformation strategy (Rohini and Rao 2000) was used. Transgenics were validated for the efficacy against *S. litura*.

Materials and methods

Plant material and bacterial strains Seeds of groundnut variety (cv. K-134) were used for transformation. Mature seeds were soaked overnight in distilled water and were surface sterilized first with 1 % Bavastin for 10 min and later with 0.1 % HgCl₂ for few seconds; washed thoroughly with distilled water after treatment with each sterilant. The seeds were later placed for germination in petriplates at 30 °C. Two-day-old seedlings were taken as explants for *Agrobacterium* infection.

The disarmed *Agrobacterium tumefaciens* strain EHA105, harboring the binary vector, pBinBt8, that carries a chimeric *cryIACF* gene (1.863 kb) (containing domains from *cryIAC* and *cryIF*) cloned at *EcoRI* and *HindIII* sites under the control of CaMV35S promoter and OCS terminator (Fig. 1) was used for transformation. The selectable marker, *nptII* gene, is regulated by *nos* promoter and terminator. EHA105/pBinBt8 was grown in LB medium (pH 7.0) containing 50 µgml⁻¹ kanamycin. The bacterial culture (3 ml) was later re-suspended in 100 ml of Winans' AB medium (pH 5.2) (Winans et al. 1988) and grown for 18 h. For *vir* gene induction treatments, wounded tobacco leaf extract (2 g in 2 ml sterile water) was added to the *Agrobacterium* suspension in Winans' AB medium, 5 h before infection (Cheng et al. 1996).

Transformation and recovery of transformants *In planta* transformation protocol standardized by our group (Rohini and Rao 2000; Keshamma et al. 2008; Kumar et al. 2009) was followed to obtain groundnut transformants. The seedlings with just emerging plumule were infected by pricking

at the shoot apical meristem with a sterile needle and subsequent immersion in the culture of *Agrobacterium* and incubated at 28 °C, 40 rpm for 60 min. Following infection, the seedlings were blotted on sterile paper towels and transferred to autoclaved soilrite (vermiculite equivalent) moistened with sterile nutrient solution for germination under aseptic conditions in a growth room in wide mouth capped glass jars of 300 ml capacity, 4 seedlings per jar. After 6 to 7 days, the seedlings were transferred to soilrite in pots and were allowed to grow under growth room conditions for at least 7 days before transferred to the greenhouse. The growth chamber was maintained at 28±1 °C under a 14 h photoperiod with florescent light of intensity 35 µmol m⁻² s⁻¹.

Molecular analysis

Grid PCR analyses of putative transgenic plants in T₁ generation

Seeds from each individual plant were maintained as separate lines. T₁ groundnut plants were grown in green house following recommended package of practices (Anonymous 2000). Plants were labeled with aluminium tags. They were divided into different grids containing 100 plants, each such that 10 plants each along the rows or columns could be counted. Samples from each such 10 plants either along the row or the column formed a composite sample. As a result, from each grid of 100 plants numbered from 1 to 100, 20 composite samples originated.

DNA extraction and PCR analysis Total genomic DNA was isolated from young leaves of untransformed (wild type), putative transformants (T₁) and T₂ progeny derived from PCR positive T₁ plants using the CTAB method (Dellaporta et al. 1983). PCR analysis was carried out with primers for *cryIACF*, *virD* and *nptII* genes. The PCR reaction mixture (20 µl) contained 0.3 U Taq DNA polymerase, 1X assay buffer (10 mM pH 9.0 TRIS–HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin), 150 µM of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 0.25 µM and 100 ng template DNA. The DNA extracted from wild type plants was used as a negative control, the pBinBt8 vector as a positive control while the reaction mix without DNA as water blank. The PCR reaction profile comprised of 30 cycles, with strand separation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The program was extended for 10 min at 72 °C. The products were electrophoresed on a 1 % agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Southern analysis Purified genomic DNA (15 µg) of transgenics and wild type plant was digested overnight with the

Fig. 1 Vector map of the binary vector pBinBt8 (13 kb) carrying *cryIACF* and *nptII* genes

appropriate restriction endonuclease for copy number Southern analysis. The digested DNA samples were electrophoresed on a 0.8 % agarose gel in TAE buffer and blotted on to a positively charged nylon membrane (Pall Pharmed Filtration Pvt. Ltd., Mumbai, India). The 450 bp amplified product of *cryIAcF* was labeled by random priming using α - 32 P-dCTP (Fermentas Inc, USA). Hybridization and washing was carried according to Sambrook et al. (1989). Membrane was exposed on FUJI Image Plate (IP) overnight and IP was read using phosphor imager (FUJI FILM FLA-5100, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Expression analysis

ELISA Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) was carried out for detection of *CryIAcF* protein in the leaf tissue samples of PCR positive putative transformants. The Envirologix *CryIAb/Ac* kit designed for the qualitative laboratory assay which detects chimeric *cryIAcF* as well was used. Samples of 100 mg fresh leaf tissue from 2 month old T_1 transformants and wild type plants were washed, blotted and homogenized in 0.5 ml of extraction buffer in a microcentrifuge tube and processed as per the instructions (Envirologix, USA). The plate was read within 30 min of the addition of stop solution. Observations were recorded on microplate reader (Sunrise™ -Tecan Group Ltd., Switzerland) set to read the plate at 450 nm.

Northern analysis Total RNA (20 μ g) from T_2 generation transgenic plants and wild type plants was electrophoresed on 1 % formaldehyde agarose gel and probed with the *cryIAcF* 450 bp PCR product amplified by *cryIAcF* gene specific primers.

Analysis of the transgenic plants for resistance to *S. litura*

Insect feeding assays *S. litura* feeding assays were carried out for transformants by placing neonate (freshly hatched larvae) on detached groundnut leaves. The larvae were provided by Pest Control (India) Pvt. Ltd. (PCI), Bangalore, India. Two tetra foliate third well expanded leaves from 40 to 50 days old plants were used. The leaf stalk was plugged with wet cotton and transferred individually to 200 ml plastic containers. Each tetra foliate leaf represented one replication and at least 2 replications were maintained from each plant in all bioassays. Ten neonate larvae were then released into each container and the observations recorded at an interval of 24 h for at least 4 days. % mortality of the released larvae and the extent of leaf damage were the two parameters recorded during the course of bioassays. The most tolerant plants identified were then selected for further advancement.

Analysis of the T_3 generation plants for the transgene segregation

T_3 generation plants were raised from the selected T_2 generation plants and subjected to PCR for the amplification of the 901 bp *cryIAcF* gene fragment. Segregation analysis of the transformants was carried out based on the chi-square analysis of the PCR data.

Statistical analyses

Data were analysed using MS excel. Means and standard deviations were worked out for all values depending on the need. Correlation and regression analyses were done following Snedecor and Cochran (1967). Scatter plots and frequency distribution graphs were generated where necessary for representing the data. T-tests were carried out to analyse the bioefficacy against *S. litura* in the respective generations.

Results

Analysis of T_1 generation plants

In T_1 generation, 1,034 seeds of putative transformants from 40 T_0 plants were raised in greenhouse.

Molecular analysis by grid PCR

All the 1,034 plants were divided into groups of 100 as grids to form 206 composite samples for PCR analysis. PCR analysis with *npt II* specific primers (Fig. 2a; a representative gel) revealed the possibility of presence of the gene in 369 plants.

Laboratory bioassay of *cryIAcF* transgenic plants for efficacy against *S. litura*

All the 369 putative transformants, were subjected to bioassay by releasing 10 neonate (larvae) larvae of *S. litura* in each replication separately. Observations were made on % larval mortality and % leaf damage. The range of mean % larval mortality of *S. litura* observed on leaves of the putative transformants was from 4.17 to 80.0 among the different plants studied. The average mean % larval mortality recorded in transgenic plants was 16.25 ± 13.18 ($n=369$) compared to 2.80 ± 4.58 in non-transgenic plants ($n=25$) and the difference was highly significant ($t=9.84$; $p<0.01$; Table 1). Correspondingly, the mean % leaf damage caused by *S. litura* varied from 5.0 to 35.0. The average mean % leaf damage recorded in transgenic plants was 14.82 ± 3.62 ($n=369$) compared to 20.80 ± 6.23 in non-transgenic plants

Fig. 2 Analysis of T₁ generation plants. **a** Representative gel showing PCR analysis of grid samples with *nptII* 750 bp primers in T₁ generation K-134 groundnut variety generated by *in planta* transformation method. Lane M: 1Kb ladder; Lane 1–20: Transgenic plants; Lane Wt:

Wild type; Lane P: Positive control; **b** Performance of the putative transformants against *S. litura*. (i–iii): transgenic plants with *cryIAcF* gene and iv: wild type plant

($n=25$) and the difference was significant ($t=3.88$; $p<0.01$; Table 2; Fig. 2b). The results thus indicated a high degree of variability among the putative transgenics for the degree of insecticidal property and the extent of leaf damage (Fig. 3a & b).

It is anticipated that the extent of damage suffered by the test leaves should broadly indicate the extent of mortality caused by the feeding of these leaves by the test insect. A strong linear negative association between mean % larval mortality of *S. litura* (x) and mean % leaf damage (y) ($r=-0.454$, $n=369$; $p<0.01$) was observed in the putative transgenic plants (Fig. 3c). It was evident that the groundnut plants with high mean % mortality suffered less damage by the larva.

Accordingly, 27 plants with two important parameters such as highest mean % larval mortality ranging between 25.00 and 80.00% and minimum leaf damage ranging from 5.00 to 12.50 were considered for further analysis (Fig. 3d).

Analysis of T₂ plants

In the T₂ generation, 412 plants could be raised from 27 selected T₁ generation plants in the greenhouse. The plants were analysed for the stability of integration, expression and efficacy against *S. litura*.

Expression analysis of *cryIAcF*

Expression levels of the chimeric Cry1AcF protein in transgenic groundnut plants was verified by ELISA. A detectable level of Cry protein was seen in 80 plants. The Cry1AcF

protein detected by ELISA ranged from 0.01 to 0.82 $\mu\text{g/g}$ tissue among the transgenic T₂ generation plants (Fig. 4a). The efficacy of the plants with high *cryIAcF* gene expression was further confirmed against *S. litura*.

Bioefficacy of *cryIAcF* transgenic T₂ plants against *S. litura*

The T₂ generation plants with detectable Cry1AcF protein expression were subjected to insect bioassay under laboratory conditions. The range of larval mortality of *S. litura* on leaves of transgenic T₂ generation plants varied from 10.0 to 85.0 among the different plants studied (Fig. 4b). The average of mean % larval mortality recorded in transgenic plants was 26.00 ± 17.05 ($n=80$) compared to 1.43 ± 3.78 seen in non-transgenic plants ($n=7$) and the difference was highly significant ($t=9.33$; $p<0.01$; Table 2; Fig. 4c). The mean % leaf damage caused by *S. litura* of T₂ generation transgenic plants with *cryIAcF* gene varied from 5.0 to 20.0 among the different plants. The average mean % leaf damage recorded in transgenic plants was 16.25 ± 4.71 ($n=80$) compared to 27.14 ± 3.93 in non-transgenic plants ($n=7$) and the difference between the damage in the transformants and wild type was highly significant ($t=7.29$; $p<0.01$; Table 3; Fig. 4d (i) & (ii)). Further, a strong linear negative association between mean % larval mortality of *S. litura* in a plant (x) and mean % leaf damage (y) ($r=-0.754$; $n=80$; $p<0.01$) was observed (Fig. 5a). Based on the bioassay, 14 plants with high mean % larval mortality 40.00 to 85.00 and less mean % leaf damage 5.00 to 12.50 (Fig. 5b) were selected as promising lines to confirm the stability of the gene integration.

Table 1 Mean % larval mortality and mean % leaf damage observed in *Spodoptera litura* when tested against T₁ generation transgenic plants carrying *cryIAcF* gene and non-transgenic plants of K-134 groundnut variety

	n	<i>Spodoptera litura</i>			
		% larval mortality		% leaf damage	
		Mean ± SD	Range	Mean ± SD	Range
Transgenic plants	369	16.25±13.18 (23.8)	4.17–80 (11.8–63.8)	14.82±3.62 (22.6)	5–35 (12.9–36.3)
Non-transgenic plants	25	2.8±4.58 (9.6)	0–10 (0–18.4)	20.8±6.23 (27.1)	15–35 (22.8–36.3)
t- test ^a		9.84		3.88	
P values		<0.01		<0.01	

Values in parentheses are arc sine transformed values

^a ‘t’ test : two samples assuming unequal variances

Molecular analysis for the stable integration and inheritance of the transgenes

The PCR amplification was seen in all the 14 individual plant DNA samples for *cryIAcF* gene and 13 individual plant DNA samples for *nptII* gene confirming the stable integration of *cryIAcF* gene in the T₂ plants (Fig. 5c and d).

In order to ascertain the authenticity of the transgene amplification of the integrated DNA, *virD* amplification was carried out in randomly selected events. The absence of the 250 bp fragment in the genomic DNA samples ascertained the absence of *Agrobacterium* contamination in the tissues (Fig. 5e).

To assess the integration pattern of the transgene, 10 plants representing the 10 T₁ events were selected. Southern analysis of genomic DNA (10 µg) digested with restriction enzyme *HindIII* was carried out to examine the copy number of the gene in the transformants and probed with random prime labeled 450 bp *cryIAcF* gene fragment. The Southern hybridization analysis clearly demonstrated integration of

the transgene into groundnut genome (Fig. 5f). The DNA from wild type plant did not show any signal.

Northern analysis of the *cryIAcF* gene in transgenic K-134 T₂ generation plants

Northern analysis of total RNA from Southern positive plants was carried out to check the transcript accumulation of *cryIAcF* gene. Total RNA (20 µg) from transgenic plants was electrophoresed on 1 % formaldehyde agarose gel and probed with the random prime labeled 450 bp *cryIAcF* PCR product. Transcript accumulation was confirmed by the strong hybridization signals at ~2 kb position. The transgenic plants showed higher level of transgene expression at the transcript level when compared to no signal in the wild type (Fig. 5g). Following the analysis of T₂ generation plants for bio-efficacy and gene integration, 14 promising lines from 10 T₁ events were selected for further advancement.

PCR analysis of the T₃ generation plants from the selected T₂ generation plants demonstrated that the *cryIAcF* gene was

Table 2 % larval mortality and % leaf damage observed in *Spodoptera litura* when tested against T₂ generation transgenic plants with *cryIAcF* gene and non-transgenic plants of K-134 groundnut variety

	n	<i>Spodoptera litura</i>			
		% larval mortality		% leaf damage	
		Mean ± SD	Range	Mean ± SD	Range
Transgenic plants	80	26.00±17.05 (30.7)	10–85 (18.4–67.2)	16.25±4.71 (23.8)	5–20 (12.9–26.6)
Non-transgenic plants	7	1.43±3.78 (6.9)	0–10 (0–18.4)	27.14±3.93 (31.4)	20–30 (26.6–33.2)
t- test ^a		9.33		7.29	
P values		<0.01		<0.01	

Values in parentheses are arc sine transformed values

^a ‘t’ test : two samples assuming unequal variances

Fig. 3 Frequency distribution of % transgenic plants (*cry1AcF* gene) for (a) Mean % larval mortality; b Mean % leaf damage in the bioassay against *Spodoptera* in T₁ generation; c Relation between mean % larval mortality and mean % leaf damage for putative transgenic plants of K-

134 groundnut variety; d Relation between mean % larval mortality and mean % leaf damage in the T₁ generation plants selected for advancement

Fig. 4 Analysis of T₂ generation plants. Frequency distribution of (a) protein expression by ELISA; b Mean % larval mortality; c Mean % leaf damage in the bioassay against *Spodoptera* in T₂ generation. d

Performance of the T₂ generation plants in the bioassay against *S. litura*. (i): transgenic plants; (ii): wild type plant

Table 3 Transgene inheritance in the progeny of the selected T₃ generation plants based on PCR analysis

Plant number (T ₃ events)	Total analysed	No. of +ves ^a	No. of -ves	χ^2	P
2.01.03	7	5	2	0.04	0.85
2.29.17	15	15	0	1.25	0.3
2.32.13	9	7	2	0.037	0.95
3.20.05	6	4	2	0.06	0.85
4.04.01	6	4	2	0.22	0.7
4.04.02	8	6	2	0	1
4.04.04	7	5	2	0.04	0.85
4.04.05	5	5	0	1	0.3
9.35.02	6	5	1	0.21	0.69
14.12.09	5	3	2	0.6	0.4
14.13.11	5	4	1	0.06	0.8
14.21.08	5	4	1	0.06	0.8
27.12.06	6	3	3	2	0.15
27.12.09	7	4	3	1.19	0.3

^aThe segregation ratio was calculated based on the PCR analysis for the amplification of the 901 bp *cryIACF* gene fragment

Fig. 5 **a** Relation between mean % larval mortality and mean % leaf damage for transgenic plants of K-134 groundnut variety; **b** Relation between mean % larval mortality and mean % leaf damage in plants selected for advancement. PCR analysis of T₂ generation plants with gene specific primers (**c**), *npt II* primers (**d**) and *virD* gene specific primers (**e**); Lane M: 1Kb ladder; Lane 1–15: Transgenic plants; Lane wt: wild type plant; Lane P: Positive control. **f** Southern blot of ten T₂

transgenic plants carrying the *cryIACF* gene presented resistance to *Spodoptera*. Lane 1–10: Transgenic plants; Lane wt: wild type plant. Each plant genomic DNA is Hind III digested and probed with *cryIACF*; **g** Northern analysis of *cryIACF* transgenics. Twenty μ g of total RNA from young leaves was electrophoresed on 1 % formaldehyde agarose gel and probed with *cryIACF* sequence. Lane 1–10: Transgenic plants; Lane wt: wild type plant

stably inherited in the self-pollinated plants and segregated according to 3:1 Mendelian inheritance pattern (Table 3).

Discussion

The identification and deployment of *cry* genes in transgenic crops for pest management has turned out to be a major accomplishment. However, transgenic crops expressing a single specific *Bt* toxin can lead to resistance development by the target pest in the field. Introduction of additional *Bt cry* genes or novel *Bt* genes into the crop can afford protection against a wider range of pests. However, mixtures of these toxins are expected to act synergistically. A major limitation has been the specificity of the *Bt* toxins to certain groups of Lepidoptera and if several such receptor specific toxins are combined, the activity levels will be higher and more effective than the natural sequences that provide a one to one relationship of toxin to insect specific receptors. For example, a mixture of *Cry1Ac* and *Cry1F* toxins will show synergistic effect and the EC_{50} of *Cry1Ac* will be lowered by 13 times due to the presence of *Cry1F*. As a result, there are suggestions that *cry1Ac* and *cry1F* genes be expressed together in transgenic crop plants for effective control of *H. armigera* (Chakrabarti et al. 1998). Therefore, it is envisaged that gene pyramiding in transgenic plants could be a potentially more viable strategy for simultaneous expression of two or more *cry* genes (Cao et al. 2002; Greenplate et al. 2000; Datta et al. 2002). However, from current theoretical evidence and practical experiences, it is clear that gene pyramiding is useful in broadening the range of insect species controlled in one transgenic variety but it is not a panacea to Cry protein resistance.

Among many strategies available in delaying insect resistance to single Cry protein, an alternative to gene pyramiding would be to use synthetic genes of multiple efficacies (Naimov et al. 2003; Chakrabarti et al. 1998). In this direction, to improve the insecticidal property of the toxin, *cry1AcF*, a chimeric *Bt* gene with *cry1Ac* and *cry1F* domains was developed. The synthetic *cry1AcF* gene has domain I and domain II from *cry1Ac* and domain III from *cry1F*.

To assess the efficacy of the gene of interest, *cry1AcF* in groundnut cv. K-134, transgenics were developed by a tissue culture independent *Agrobacterium*-mediated *in planta* transformation strategy. The *in planta* transformation protocols have been developed for transformation in many crop species, *Arabidopsis thaliana* (Feldmann and Marks 1987), soybean (Trick and Finer 1997), sunflower (Rao and Rohini 1999), safflower (Rohini and Rao 2000), rice (Supartana et al. 2005), maize (Chumakov et al. 2006) and cotton (Keshamma et al. 2008). Earlier an *in planta* transformation protocol was developed in groundnut by Rohini and Rao (2000) and proved its reproducibility by Keshamma et al. (2008). In the present

study, selection of stable transformants by employing the *in planta* transformation strategy in groundnut was ascertained by PCR analysis of the plants for selectable marker gene in T_1 generation, and for both marker gene and *cry1AcF* gene in T_2 generation. The amplification of required size fragment of 750 bp with *npt II* primers and 901 bp with *cry1AcF* gene primers was confirmed the integration of the gene.

The presence of the transgene was further ascertained by expression analysis of the T_2 generation plants by ELISA, which is expected to be crucial in understanding the *Bt* toxin expression pattern. Since the *cry1AcF* gene has *cry1Ac* domain, an attempt was made to check on the possibility of using available *cry1Ac* antibodies for the ELISA. The maximum Cry protein concentration of *cry1AcF* transgenic plants reached approximately 0.82 $\mu\text{g/g}$ leaf fresh weight in T_2 generation. It has been earlier seen that concentrations of expressed protein show large differences among different independent transgenic plants (Maqbool et al. 2001; Ramesh et al. 2004). The integration of the transgene was confirmed at the molecular level by PCR, genomic Southern and Northern analysis. Identical hybridization pattern in some of the transformants has also been observed in other legumes like chickpea (Kar et al. 1997; Indurker et al. 2007).

Segregation analysis of the T_3 generation plants raised from the selected T_2 events indicated the stable integration and homozygous nature of all the selected transformants.

It was observed that the performance of the transgenic plants based on the bioefficacy against *S. litura* improved with generations. Detached leaf bio-assays of plants with detectable *cry1AcF* expression under laboratory conditions showed significant larval mortality of *S. litura* compared to wild type plants. Bioassays revealed that the highest mean % larval mortality was 80.0 with lowest mean % leaf damage 5.0 in the T_1 generation plants. There was an improvement in the average mean % larval mortality from 16.25 ($n=369$) in T_1 generation to 26.0 ($n=80$) in T_2 generation. Similar trend was also observed in the extent of damage to the leaves by *S. litura*. The reduced damage to the leaves as the generations advanced indicated the stability and efficacy of the gene. The increased average mean larval mortality of *S. litura* in T_2 generation is important. However, the larval mortality was quite low when compared to the earlier studies where the level of expression of *Cry1Ac* protein in some of promising transgenic chickpea plants showed significantly high level of resistance (>90 %) against the target pod borer insect *H. armigera*. (Sanyal et al. 2005) and the highest mortality of the *S. litura* larvae found in the transgenic pigeon pea plants was 80% (Surekha et al. 2005).

Further, substantial reduction in the larval size was evident among the larvae tested as observed in the bioassays of present study. This could be because of low levels of feeding of the leaves, which in turn resulted in the reduced accumulation of toxins in insects that did not cause the mortality but brought

about the reduction in size. Hence these bioassays are only indicative and not the absolute representations of the toxin levels and consequently the tolerance levels in the transgenics developed. The present study emphasizes mainly on development of stable transformants in groundnut cv. K-134 using *in planta* transformation protocol and the utility of the chimeric *cryIAcF* gene. The transformation efficiency using the *in planta* transformation strategy in the present study is 35 %, the superior transformants were 10, which is 1 % of the T₁ events developed. This indicates the necessity for the development of a large number of primary and subsequently T₁ generation plants in order to obtain more number of superior events. By and large, these experimental efforts demonstrate that *cryIAcF* gene is partially effective against *S. litura* in groundnut.

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