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A Synthetic *cry1AcF* Gene Imparts Resistance to *Spodoptera litura* (F.) and *Amsacta albistriga* Walker in Peanut (*Arachis hypogaea* L.) cv. VRI-2

G. Keshavareddy[1*], A.R.V. Kumar[1], Vemanna S. Ramu[2], Rohini Sreevathsa[3] and M. Udayakumar[2]

[1]Department of Agricultural Entomology, [2]Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru-560065, Karnataka, India
[3]National Research Centre on Plant Biotechnology, New Delhi-110012, India

**Corresponding author*

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A B S T R A C T

Transgenic VRI-2 peanut plants were generated by following an *Agrobacterium* mediated, *in planta* transformation protocol with *cry1AcF* gene for efficacy against *Spodoptera litura* and *Amsacta albistriga*. Putative transgenics were selected by analyzing composite samples by GRID PCR method. The transgenic nature of the putative transgenics was further ascertained by molecular analysis for the presence of the gene of interest and selectable marker followed by efficacy against *S. litura* and/or *Amsacta albistriga*. The plants that showed 57.9-100 % mortality against *S. litura* were selected for further analysis. The stability of the selected T_1 plants in the subsequent generation was confirmed by not only integration and efficacy but also expression by ELISA. Further, Southern analysis of T_2 generation plants revealed the integration pattern of the transgene in the peanut genome and improved larval mortality in the range of 25-78.94%. The study demonstrates that overexpression of *cry1AcF* in peanut provide resistance to *S. litura* and *A. albistriga*.

Introduction

Peanut (*Arachis hypogaea* L.) is cultivated in more than 100 countries spread across 40° N to 40° S latitudes in about 26.4 million hectares with a total production of about 37.1 m tonnes, and an average productivity of 1400 kg /ha. India is the world's largest producer of peanut and is the premier oil seed crop (GOI, 2008). Economically, peanut is relatively a high input crop due to high seed cost and is mainly cultivated in marginal lands as a rainfed crop. Groundnut cultivar VRI-2 is of the Spanish bunch type and was bred and released at the Tamil Nadu Agricultural University, Coimbatore. This variety was used for transformation studies, mainly due to its suitability for cultivation both under rainfed and irrigated conditions. This variety have a duration of 100-110 days and high yield potential (1400- 1500 kg/ha). Insect pests, particularly lepidopterans cause severe

damage to the crop and remain a great challenge to manage despite an array of strong management practices. As the crop is mostly cultivated by small and marginal farmers under rainfed conditions, high seed cost deters further investment on crop protection. Vagaries of monsoon further accentuate the investment problem. Consequently, economical seed based management strategies would greatly benefit the crop productivity, particularly under rainfed conditions. Alternative to chemical control of insect pests, a novel delivery system of insecticidal toxins in crop plants, with suitable insecticidal gene/s, has emerged important option for management of crop pests. The *Bt* cotton plant in India and other countries is the evidence of success for this method. The *cry* genes from *Bacillus thuringiensis* are very important among the potential genes available for insect pests management. The *cry1* series *Bt* genes are effective against Lepidoptera, but are likely to be species specific (Chakrabarti *et al.,* 1998). For example, *cry*1Ac is effective against *Helicoverpa armigera* (Hubner), *cry1B* and *cry1C* are likely to be good for diamondback moth, *Plutella xylostella* (L.) and *cry1F* is effective against *Spodoptera litura* (Fab.). However, hybrid *Bt* genes have shown to provide increased protection to plants against a single insect pest or against a few closely related insect species (de Maagd *et al.,* 1996; Frutos *et al.,* 1999; Baisakh *et al.,* 2006). The hybrid gene from *cry1Ba* and *cry1Ia* in transgenic potato showed resistance to both a coleopteran and a lepidopteran pest (Naimov *et al.,* 2003).

The synthetic constructs suitable elements of *Bt* genes provided protection against a number of lepidopteran pests besides being more effective against known susceptible pests. The transgenic Pigeon pea plants expresiing synthetic *cry1AcF* showed resistance against *Helicoverpa armigera* with high larval mortality also showed resistance to *Spodoptera litura* in peanut and cotton (Ramu *et al.,* 2012; Keshavareddy *et al.,* 2013). Peanut transgenics with such novel hybrid construct therefore, would be ideal to tackle a range of caterpillar pests that attack them in the semi-arid tracts of South India. The *cry1AcF* gene is a synthetic construct with the elements of *cry1Ac* and *cry1F*, may be effective against a range of lepidopteran caterpillars including *S. litura* and *A. albistriga.* Peanut is a recalcitrant crop and do not respond to tissue culture. Hence, a tissue culture-independent *Agrobacterium* mediated *in planta* transformation procedure was developed for peanut (Rohini and Rao, 2000). Following the *in planta* transformation protocol, *cry1AcF* gene was transformed to peanut genotype VRI-2, which is popular in southern India. The emphasis of the study has been to prove the stability of the transgene and it's efficacy against *S. litura* and *A. albistriga*.

Materials and Methods

Plant material and bacterial strains

Seeds of peanut variety (cv. VRI-2) were used for transformation. Mature seeds were soaked overnight in distilled water and were surface sterilized first with 1% Carbendazim for 10 minutes and later with 0.1% $HgCl_2$ for few seconds; washed thoroughly with distilled water after treatment with each sterilant. The seeds were later placed for germination in petriplates at 30ºC. Two-day-old seedlings were taken as explants for *Agrobacterium* infection.

The disarmed *Agrobacterium tumefaciens* strain EHA105, harbouring the binary vector, pBinBt8, contains a chimeric *cry1AcF* gene (1.863 kb) (containing domains from *cry1Ac* and *cry1F*) cloned at *Eco*RI and *Hind*III sites; under the control of CaMV35S promoter and OCS terminator. The selectable marker, *npt*II 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 Rohini and Rao (2001) and tested by Keshamma *et al.,* 2008 was followed to obtain peanut 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 28ºC, 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 transferring to the greenhouse. The growth chamber was maintained at 28±1ºC under a 14 h photoperiod with florescent light of intensity 35 $\mu mol\ m^{-2}\ s^{-1}$.

Molecular analysis

Grid PCR analyses of putative transgenic plants in T_1 generation

Seeds from each individual plant were maintained as separate lines. T_1 peanut 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 (T_1) and T_2 progeny derived from PCR positive T_1 plants using the CTAB method (Dellaporta *et al.,* 1983). PCR analysis was carried out with various primers as outlined in Table 1. 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 $MgCl_2$, 0.01% gelatin), 150 µM of each dNTP, 1 µl each of forward and reverse primers 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 sec, annealing at 60°C for 30 sec 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. The primer sequences for *npt*II and *cry1AcF* are mentioned in Table 1.

Southern analysis

Fifteen micrograms of purified genomic DNA of transgenics and wild type plant were digested overnight with appropriate restriction

endonuclease for determining copy numbers by 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 Pharmalab Filtration Pvt. Ltd., Mumbai, India). The 450 bp amplified product of *cry1AcF* was labeled by random priming using ά-^{32}P-dCTP (Fermentas Inc, USA). Hybridization and washing was carried out 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 Cry1AcF protein in the leaf tissue samples of PCR positive putative transformants. The Envirologix Cry1Ab/Ac kit designed for the qualitative laboratory assay which detects chimeric Cry1AcF as well was used. Samples of 100 mg fresh leaf tissue from two 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. 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 µg) from T_2 generation transgenic plants and wild type plants were electrophoresed on 1% formaldehyde agarose gel and probed with the *cry1AcF* 450 bp PCR product amplified from *cry1AcF* with gene specific primers.

Analysis of the transgenic plants for resistance to *Spodoptera litura* and *Amsacta albistriga*

Insect feeding assays

Spodoptera litura and *Amsacta albistriga* feeding assays were carried out for transformants by placing neonate larvae (freshly hatched) on detached peanut leaves. The larvae were provided by Bio-control Research Laboratories, Pest Control (India) Pvt. Ltd. (PCI), Bangalore, India.

Two tetra foliate medium aged leaves were used. The leaf stalk was then 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 in to each container and the observations recorded at an interval of 24 h for at least four days. Per cent 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. In T_2 generation due to non-availability of neonates of *Amsacta*, bio-assay was not done.

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.

Results and Discussion

Analysis of T_1 generation plants

In T_1 generation, 530 seeds of putative transformants of VRI-2 peanut genotype from 39 T_0 plants were raised in greenhouse with suitable labeling. Plants were arranged in a grid fashion for the purpose of PCR analysis. Growth of all the plants was taken care to be uniform.

Molecular analysis by grid PCR

PCR analysis was done for 106 composite DNA samples (grid method) of T_1 generation with *npt* II specific primers revealed the presence of the gene (around 750 bp DNA fragment as seen in Fig. 1) in 40 samples. Thus the study indirectly demonstrated the presence of *cry1AcF* transgene in a sizable number of T_1 plants obtained from *in planta* transformed VRI-2 peanut plants using *cry1AcF* gene. The successful 40 grid positive composite samples provided 138 potentially positive plants (Fig. 2).

The insect bioassays were done using *Spodoptera litura* neonates for all the 138 putative transformants. Further, 31 of these plants that gave an assay result of more than 40 percent larval mortality of *S. litura* were identified and assayed against *Amsacta albistriga*.

Laboratory bioassay of *cry1AcF* transgenic plants against *S. litura*

The extent of larval mortality of *S. litura* observed on leaves of every putative plant with *cry1AcF* gene varied from 0.0 to 100 per cent among the different plants studied. The average mean per cent larval mortality recorded in transgenic plants was 32.76 ± 24.86 (n =138) compared to that of 2.49 ± 3.94 in Wild type plants (n = 7). The 't' test indicated significant differences between the transgenic and Wild type plants ($t = 8.43$; $p < 0.01$; Table 2).

The range of mean per cent leaf damage varied from 5.0 to 22.5 among different plants studied. The average mean per cent leaf damage recorded in transgenic plants was 12.73 ± 4.62 (n = 138) compared to that of 24.29 ± 3.45 in Wild type plants (n = 7). The 't' test indicated significant difference between transgenic and Wild type plants ($t = 5.69$; $p < 0.01$; Table 2). It is anticipated that more the mean per cent larval mortality observed in a plant, less would be the mean per cent leaf damage. This was checked by associating the two parameters for the putative *cry1AcF* transgenics. A strong linear negative association between mean per cent larval mortality of *S. litura* in a plant (x) and mean per cent leaf damage (y) ($r = -0.798$; $n = 138$; $p < 0.01$) was observed in the putative transgenic plants (Fig. 3a).

Therefore, plants with higher mean per cent larval mortality are ideal for further advancement due to their higher resistance against *S. litura*. The observed high correlation coefficient clearly suggests a straight forward relation between the toxicity and the corresponding leaf damage.

Laboratory bioassay of *cry1AcF* transgenic plants against *A. albistriga*

To assess the broad-spectrum resistance mediated by expression of *cry1AcF*, peanut plants that showed more than 40 per cent larval mortality against *S. litura* were assayed against *A. albistriga*. The mean per cent larval mortality of *A. albistriga* varied from 0 to 82.3 among the different plants studied. The average mean per cent larval mortality recorded in transgenic plants was 15.78 ± 25.25 (n = 31) compared to that of 0.07 ± 1.89 in Wild type plants (n = 7). The 't' test

indicated significant difference between transgenic and Wild type plants ($t = 3.66$; $p < 0.01$; Table 2).

The extent of leaf damage caused by *A. albistriga* larvae varied from 10.0 to 72.5 per cent among the different plants. The average mean per cent leaf damage observed in transgenic plants was 50.40 ± 19.74 (n = 31) compared to 78.57 ± 3.78 in that of Wild type plants (n = 7) 96 h after bioassay initiation. The 't' test indicated significant difference between the transgenic and Wild type plants ($t = 7.67$; $p < 0.01$; Table 2).

A strong linear negative association was observed between mean per cent larval mortality of *A. albistriga* (x) and the mean per cent leaf damage (y) ($r = -0.894$; $n = 31$; $p < 0.01$; Fig. 3b) in a plant. The results further substantiate the toxicity to be the primary effect of the products of the transgene in VRI-2 peanut plants.

Basis for plants selection for advancement

On the basis of the above bio-assay results, six plants of T_1 generation were selected for further advancement (Table 3). Among the plants selected, the highest mean per cent *S. litura* larval mortality of 100 per cent was observed in the plant lines, 5-19 and 29-6 (where 5 & 29 represents T_0 plants number; 19 & 6 represents T_1 generation plants from respective T_0 plants). The lowest mean per cent larval mortality was observed in the plant 28-16 with 57.9. The average mean per cent larval mortality recorded in selected transgenic plants for further advancement was 81.25 ± 17.71 (n = 6) compared to that of 2.49 ± 3.94 in wild type plants (n = 7) 96 h after bioassay initiation. The 't' test indicated significant difference among the T_1 generation transgenic plants selected for further advancement and the wild type plants ($t = 8.17$; $p < 0.01$; Table 3).

The extent of mean per cent leaf damage caused by *S. litura* varied from 5.0 to 10.0 among different plants selected for further advancement. The lowest mean per cent leaf damage of 5.0 was observed in the plant lines 5-19 and 29-05. The highest mean per cent leaf damage was observed in the plants, 28.16 and 29.06. The average mean per cent leaf damage recorded in selected transgenic plants for further advancement was 7.5 ± 2.24 (n = 6) compared to 24.29 ± 3.45 in wild type plants (n = 7) and the difference was highly significant ($t = 10.30$; $p < 0.01$; Table 3). Thus the plants selected were the superior most available among the putative transgenics both from the point of view of high mortality of *S. litura* larvae and in keeping the leaf damage to the minimum.

The six plants of T_1 generation that were selected for further advancement on the basis of *A. albistriga* larval mortality and leaf damage had the following traits. The mean per cent larval mortality varied from 55.0 to 82.3 among the plants selected. The highest mean per cent larval mortality of 82.3 was observed in the plant line 5-19. The lowest mean per cent larval mortality was observed in the plant line 28-02. The average mean per cent larval mortality recorded in selected transgenic plants for further advancement was 63.90 ± 9.56 (n = 6) compared to 0.07 ± 1.89 in wild type plants (n = 7) 96 h after bioassay initiation and the difference was significant ($t = 16.60$; $p < 0.01$; Table 3). Similarly, among the plants selected, the average mean per cent leaf damage caused by *A. albistriga* varied from 10.0 to 32.5. The lowest mean per cent leaf damage was observed in the plant 28.16. The highest average per cent leaf damage was observed in the plant 29-06. The average mean per cent leaf damage recorded was 17.92 ± 7.97 (n = 6) compared to 78.57 ± 3.78 in wild type plants (n = 7) and the difference was highly significant ($t = 14.85$; $p < 0.01$; Table 3).

Analysis of T_2 plants

In the T_2 generation, 99 plants could be raised from six selected T_1 generation plants in the green house. All the plants were analysed for the stability of integration, expression and efficacy against *S. litura* only, since the *A. albistriga* larvae were unavailable for the test.

Expression analysis of cry1AcF

Expression levels of the chimeric Cry1AcF protein in all 99 transgenic peanut plants was verified by ELISA. A detectable level of Cry protein was seen in 87 plants. The Cry1AcF protein detected by ELISA ranged from 0.13 to 1.31 µg equivalent of cry1Ac/g tissue among the transgenic T_2 generation plants (Fig. 4a)

Laboratory bioassay of *cry1AcF* transgenic T_2 plants against *S. litura*

The T_2 plants with detectable Cry1AcF protein expression were subjected to insect bioassay under laboratory conditions. The range of per cent larval mortality of *S. litura* on leaves of transgenic T_2 generation plants varied from 25.0 to 78.94 among different plants studied. The average mean per cent larval mortality recorded in transgenic plants was 42.12 ± 14.65 (n = 87) compared to 2.86 ± 4.88 in wild type plants (n = 7) and the difference was highly significant (t = 9.95; p < 0.01; Table 4). Mean per cent leaf damage caused by *S. litura* varied from 5.0 to 20.0 among the different plants. The average mean per cent leaf damage recorded in transgenic plants was 11.78 ± 3.76 (n = 87) compared to 24.29 ± 3.45 in wild type plants (n = 7) and the 't' test indicated significant difference between the two sets of plants (t = 10.18; p < 0.01; Table 4). A strong linear negative association between mean per cent larval mortality of *S. litura* (x) and mean per cent leaf damage (y) (r = -0.675; n = 87, p < 0.01) was observed in the plants (Fig. 5a).

Molecular analysis for transgene integration and inheritance

PCR analysis with *cry1AcF* gene and *npt*II specific primers of 87 individual plant DNA samples was carried out for the presence of *npt*II gene that amplifies at 750 bp DNA fragment (Fig. 5b). The PCR amplification was seen in DNA samples of 84 individual plants for *cry1AcF* gene and 78 samples for *npt*II gene confirming the stable integration of *cry1AcF* gene in the T_2 plants.

Further, stable integration of *cry1AcF* gene was confirmed by southern hybridization analysis with gene specific probe. Four plants representing the four T_1 events were selected. Genomic DNA (10µg) was digested with restriction enzyme *Bam*HI to examine the copy number of the gene in the transgenic plants and probed with random primer labeled with 450 bp *cry1AcF* gene specific fragment and hybridized on nylon membrane. The signal exhibited by radioactive p-32 CTP incorporation and hybridization was detected in autoradiogram showed the stable integration in transgenic peanut plants (Fig. 5c). The DNA from wild type plant did not show any signal.

Table.1 Primer/s sequence, annealing temperature and fragment size of *npt* II and *cry1AcF* gene

Gene	Primer/s sequence	Annealing temp.	Fragment size
***npt* II**	'Forward 5' GAG GCT ATT CGG CTA TGA CTG 3' Reverse 5' ATC GCG AGG GGC GAT ACC GTA 3'	58^o C	**750 bp**
cry1AcF	**'Forward 5'-AACCCAAACATCAACGAGTGC 3' 'Reverse 5'-TTATGCTGTTCAAGATGTC 3'**	**58^o C**	**901bp**

Table.2 Mean per cent larval mortality and mean per cent leaf damage observed in *S. litura* and *A. albistriga* when tested against T_1 generation transgenic plants carrying *cry1AcF* gene and wild type plants of VRI-2 peanut variety

		Spodoptera litura					*Amsacta albistriga*			
	n	% larval mortality		% leaf damage		n	% larval mortality		% leaf damage	
		Mean ± SD	Range	Mean ± SD	Range		Mean ± SD	Range	Mean ± SD	Range
Transgenic plants	138	32.76 ± 24.86 (34.92)	0-100 (0-90)	12.73 ± 4.62 (20.90)	5-22.5 (12.9 -28.32)	31	15.78 ± 25.25 (23.41)	0-82.3 (0-65.12)	50.40 ± 19.74 (45.23)	**10-72.5 (18.4-58.4)**
Wild type plants	7	2.49 ± 3.94 (9.08)	0-9.1 (0-17.56)	24.29 ± 3.45 (29.53)	20-30 (26.6-33.2)	7	0.07 ± 1.89 (1.52)	0-5 (0-12.92)	78.57 ± 3.78 (62.42)	**75-85 (60-67.2)**
t- test *		8.43		5.69			3.66		7.67	
P values		**< 0.01**		**<0.01**			**< 0.01**		**< 0.01**	

* 't' test : two samples assuming unequal variances
Values in parentheses are arc sine transformed values

Table.3 Mean per cent larval mortality and mean per cent leaf damage observed in *S. litura* and *A. albistriga* when tested against T_1 generation transgenic plants carrying *cry1AcF* gene selected for advancement and non-transgenic plants of VRI-2 peanut variety

		Spodoptera litura					*Amsacta albistriga*			
	n	% larval mortality		% leaf damage		n	% larval mortality		% leaf damage	
		Mean ± SD	Range	Mean ± SD	Range		Mean ± SD	Range	Mean ± SD	Range
Transgenic plants	6	81.25 ± 17.71 (64.34)	57.9- 100 (49.6- 90)	7.5 ± 2.24 (15.89)	5-10 (12.9-18.4)	6	63.90 ± 9.56 (53.07)	55-82.3 (47.87-65.12)	17.92 ± 7.97 (25.04)	**10-32.5 (18.43-34.76)**
Non-transgenic plants	7	2.49 ± 3.94 (9.08)	0-9.1 (0-17.56)	24.29 ± 3.45 (29.53)	20-30 (26.6-33.2)	7	0.07 ± 1.89 (1.52)	0-5 (0-12.92)	78.57 ± 3.78 (62.42)	**75-85 (60-67.2)**
t- test *		8.17		10.30			16.60		14.85	
P values		**<0.01**		**<0.01**			**< 0.01**		**< 0.01**	

* 't' test : two samples assuming unequal variances
Values in parentheses are arc sine transformed values

Table.4 Per cent larval mortality and per cent leaf damage observed in *S. litura* when tested against T_2 generation transgenic plants carrying *cry1AcF* gene and wild type plants of VRI-2 peanut variety

		Spodoptera litura			
	N	% larval mortality		% leaf damage	
		Mean ± SD	Range	Mean ± SD	Range
Transgenic plants	87	42.12 ± 14.65 (40.47)	25-78.94 (30.0- 62.7)	11.78 ± 3.76 (20.1)	5-20.0 (12.9-26.6)
Wild type plants	7	2.86 ± 4.88 (9.74)	0-10 (0-18.4)	24.29 ± 3.45 (29.53)	20-30 (26.6-33.2)
t- test *		9.95		10.18	
P values		<0.01		<0.01	

* 't' test : two samples assuming unequal variances
Values in parentheses are arc sine transformed values

Fig.1 Analysis of T_1 generation plants. a Representative gel showing PCR analysis of grid samples with *npt*II 750 bp primers in T1 generation VRI-2 peanut variety generated by *in planta* transformation method. Lane Wt: Wild type; Lane 1-40: Transgenic plants; Lane P: Positive Control; Lane M: 1 Kb ladder; b Performance of the putative transformants against *S. litura* and *A. albistriga*. *i & iii*: transgenic plants with *cry1AcF* gene and ii & iv: Wild type. (Note: i & ii: assay against *S. litura* and iii & iv against *A. albistriga*)

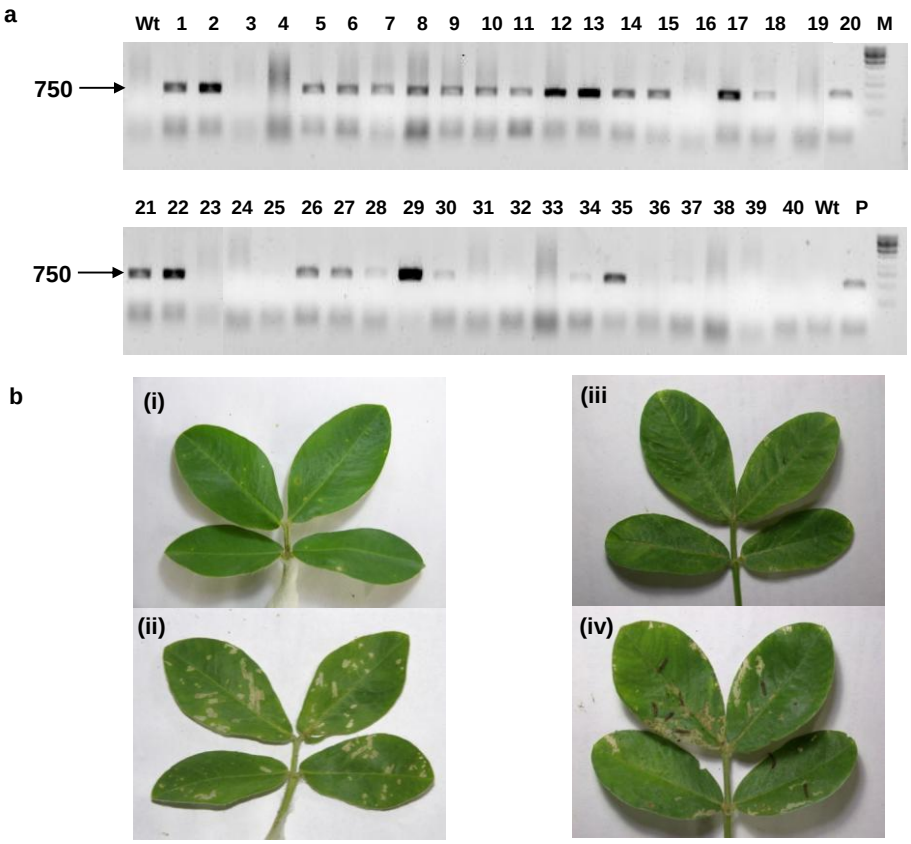


Fig.2 Frequency distribution of % transgenic plants (*cry1AcF* gene) for (a) Mean % larval mortality; (b) Mean % leaf damage in the bioassay against *Spodoptera litura*; (c) Mean % larval mortality; (d) Mean % leaf damage in the bioassay against *A. albistriga* in T_1 generation of VRI-2 peanut variety

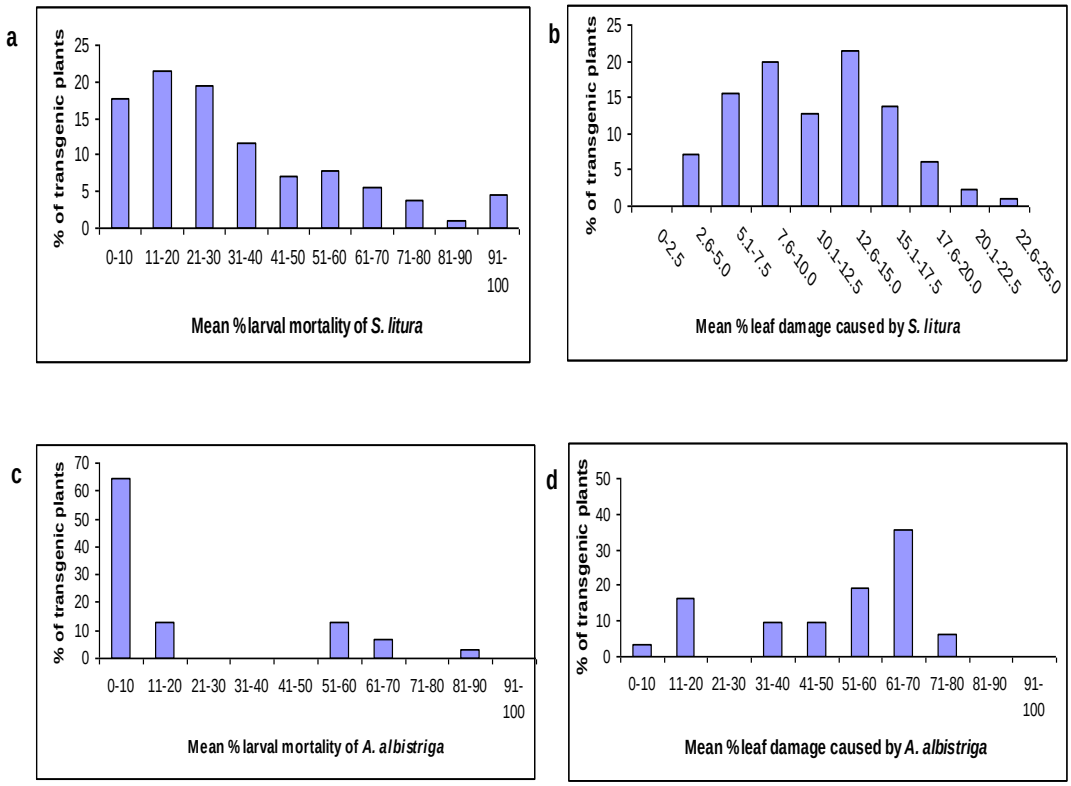


Fig.3 Relation between (a) mean % larval mortality of *S. litura* and mean % leaf damage for putative transformants; (b) Mean % larval mortality of *A. albistriga* and mean % leaf damage for putative transformants of VRI-2 peanut variety

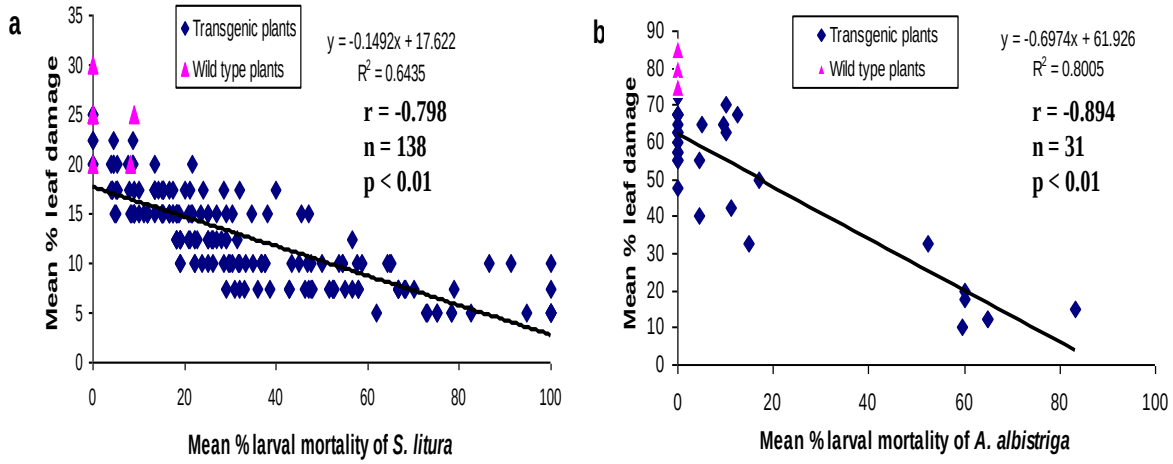


Fig.4 Analysis of T_2 generation plants. Frequency distribution of (a) protein expression by ELISA; (b) Mean % larval mortality; (c) Mean % leaf damage in the bioassay against *S. litura* in T_2 generation. (d) Performance of the T_2 generation plants in the bioassay against *S. litura.* (i): transgenic plants; (ii): wild type

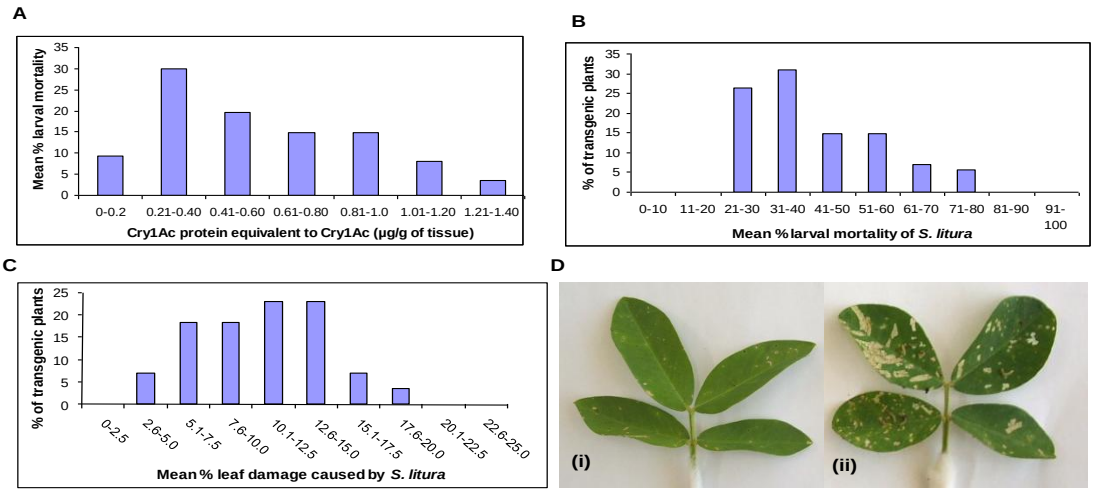
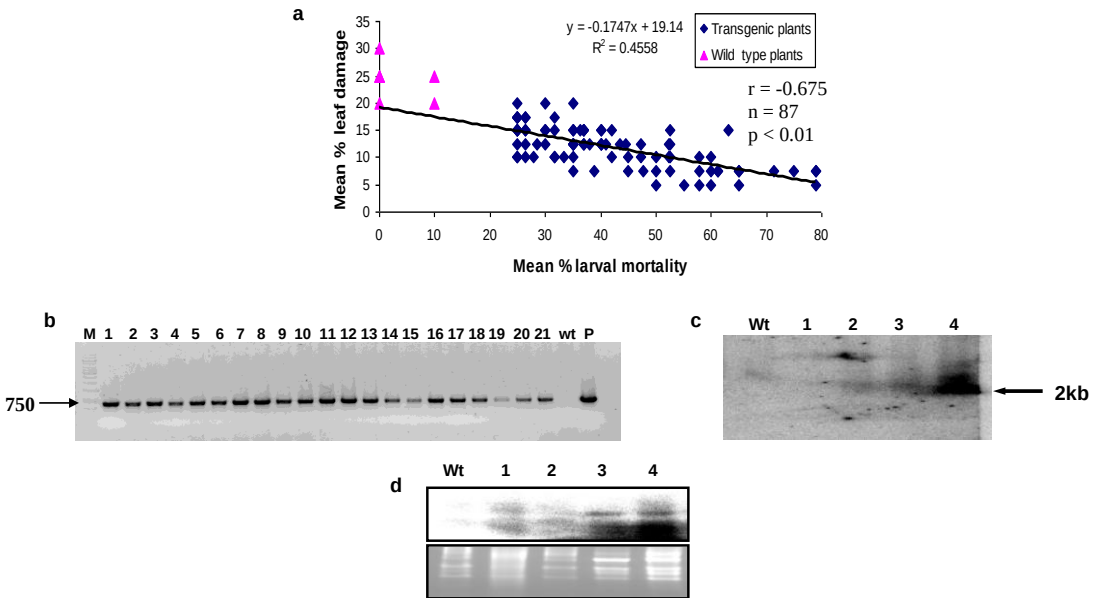


Fig.5 (a) Relation between mean % larval mortality and mean % leaf damage for transgenic plants of VRI-2 peanut variety; (b) PCR analysis of T_2 generation plants with *npt*II primers; (c) Southern blot of four T_2 transgenic plants carrying the *cry1AcF* gene presented resistance to *Spodoptera* and *Amsacta*. Lane wt: wild type plant. Lane 1-4: Transgenic plants; Each plant genomic DNA was digested with *BamH*I and probed with ^{32}P labelled 660 bp Nested PCR product of the *cry1AcF* gene; Lane wt: wild type; (d) Northern analysis of *cry1AcF* transgenics. Twenty µg of total RNA from young leaves was electrophoresed on 1% Formaldehyde agarose gel and probed with *cry1AcF* sequence. Lane 1-4: Transgenic plants; Lane wt: wild type plant



Northern analysis with total RNA from the same 4 plants was carried out to check the transcript accumulation of the *cry1AcF* gene. Twenty µg of total RNA from transgenic and wild type plants was electrophoresed on 1% formaldehyde agarose gel and probed with the random primer radio-labeled with p-32 CTP for 450bp *cry1AcF* PCR fragment. Transcript accumulation was confirmed by the strong hybridization signals at ~2 kb position. The transgenic plants showed higher levels of transgene expression at the transcriptional level and no signal in the wild type (Fig. 5d).

The specificity of *Bt* Cry toxins towards target pest species is a major advantage in agriculture because effects on non-target insects and other organisms in the ecosystem are minimized. However, deployment of transgenic crops expressing a single specific *Bt* toxin can lead to development of resistance in target insect pest. Introduction of additional *Bt cry* genes or novel *Bt* genes into the crop can afford protection against a wider range of pests. Commercial cultivation of transgenic cotton containing two *Bt* genes began in 1999, in the USA. Cotton plants expressing both Cry1Ac and Cry2Ab proteins were more toxic to bollworm (*Helicoverpa zea*; target pest) and two species of armyworms (*Spodoptera frugiperda* and *Spodoptera exigua*; secondary pests) than cotton expressing Cry1Ac alone (Chitkowski *et al.,* 2003).

Similarly many *cry* genes particularly active against Lepidoptera with varying specificity and activity levels have been identified. A major limitation has been the specificity of the *Bt* toxins to certain groups of lepidoptera. If several such receptor specific toxins are combined, the activity levels are likely to be higher and more effective than the natural sequences that provide a one to one relationship of toxin to insect specific receptors. Transfer of the carbohydrate binding domain III generated a Cry1Ab-Cry1Ca hybrid that was highly toxic to armyworm, *Spodoptera exigua*, an insect resistant to Cry1A series of toxins; the presence of the Cry1Ca domain III was sufficient to confer toxicity towards *Spodoptera* (de Maagd *et al.,* 2000) More remarkably, a hybrid Cry protein containing domains I and III from Cry1Ba and domain II of Cry1Ia conferred resistance to the lepidopteran potato tuber moth, *Phthorimaea operculella*, and the coleopteran, Colorado potato beetle, *Leptinotarsa decemlineata*, when expressed in transgenic potato (Naimov *et al.,* 2003). The parental Cry proteins in this hybrid are lepidopteran specific with no known toxicity towards coleopterans such as the potato beetle, demonstrating the creation of a novel specificity. Further, hybrid toxins produced through inclusion of a domain from another toxin results in increased potency of a fused protein by the shift in receptor binding (Bosch *et al.,* 1994). Considering this possibility, in order to improve the insecticidal property of the toxins, *cry1AcF* has been developed. The novel synthetic gene has elements of *cry1Ac* and *cry1F* toxins built into it. As a result, the potency and breadth of activity are expected to be much higher than any of the conventional natural toxins of *Bt*, against lepidoptera. Hence this *cry1AcF* gene was tried out in the present study to explore the possibility of developing transgenic peanut variety resistant to *Spodoptera litura* and also against the *Amsacta albistriga*, which are major leaf eating caterpillars. The focus of this study has been to analyse the reproducibility of the *in planta* transformation protocol and the efficacy of the *cry1AcF* gene in the peanut variety, VRI-2 against *S. litura* and *A. albistriga.*

The primary transformants (T_0) could be successfully established under greenhouse conditions. Since the *in planta* transformation protocol gives rise to a large number of primary transformants, the selection of

putative transformants requires stringent screening. In this direction, grid PCR, a technique where composite samples are analysed by PCR was used for primary identification of putative transformants. Grid PCR-based screening has been used successfully in the identification of putative transformants in our earlier studies (Keshamma *et al.,* 2008; Sundaresha *et al.,* 2010). In the present investigation, based on grid PCR, carried out in T_1 generation in VRI-2 peanut variety, the potential success rate was 26.03 per cent. However the percent of actual transformants can be arrived at only by observing the PCR results of individual plants of the positive grids and finally based on their efficacy. Fairly good transformation efficiencies have been reported for *in planta* transformation protocols. Efficiencies up to 40% with *Agrobacterium* inoculation of germinating seeds of rice have also been reported (Supartana *et al.,* 2005). Further, the development of stable transformants by employing the *in planta* transformation strategy was ascertained by PCR analysis of the plants in the T_2 generation for both the gene of interest and the selectable marker. Development of stable transformants using the *in planta* transformation protocol has earlier been established in other crop species (Sundaresha *et al.,* 2010).

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. Although, alternative methods do exist, ELISA has been standardized for the Cry proteins and is expected to be the most ideal technique for quantification of the Cry protein levels in *Bt* transgenic plants. 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 1.31 µg/g leaf fresh weight in T_2 generation. It has been seen that concentrations of foreign protein usually show great differences among different independent transgenic plants despite identical construct (Mabqool *et al.,* 2001; Ramesh *et al.,* 2004). *Bt* protein level expressed in commercial *Bt* transgenic cultivars (cotton, maize and potato) is generally 1 to 11 µg/g leaf fresh weight, which is adequate to meet the requirement of the high-dose/refuge strategy (Cohen *et al.,* 2000).

Detached leaf bioassays of plants with detectable Cry1AcF expression under laboratory conditions showed significant larval mortality of *S. litura* compared to wild type plants. The average mean per cent larval mortality was 32.76 with average per cent leaf damage of 12.73 in T_1 generation. In T_2 generation, the average per cent larval mortality rates showed increasing trend, 42.12 with 11.78 mean per cent leaf damage. The increased mean larval mortality with significant low mean leaf damage is important. But the larval mortality was 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 per cent (four out of five larvae released) (Surekha *et al.,* 2005).

Secondly, in the present study, although not documented quantitatively, substantial reduction in the larval size was evident among the larvae tested. 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 ELISA positive plants with high mean larval mortality of *S. litura* and low leaf damage were taken as a criterion to select the plants in T_2 generation for further advancement. The associational studies between mean per cent larval mortality and mean per cent leaf damage were observed to be very strong for *S. litura* in both T_1 & T_2 generations of peanut transgenic plants (observed in the selected lines). These results substantiate that plants expressing high *Bt*-toxin cause higher larval mortality, in turn reducing the leaf damage. Bio efficacy of transgenics done against *S. litura* was good as there was sequential increase in the grand mean per cent mortality observed through two generations.

Cry1AcF protein levels expressed in the transgenic peanut plants as estimated in the present studies were low compared to the previous reports. Murray *et al.,* (1991), for example, observed the tomato plants with truncated *cry1Ab* gene to tolerate tobacco horn worm [*Manduca sexta* (L.)], tomato fruit borer [*Helicoverpa zea* (Boddie)] and tomato pinworm [*Keiferia lycopersicella* (Walshingham)].

But in these plants, the *cry* protein was immunologically undetectable. Consequent to the above, expression analysis by ELISA using plates coated with cry1Ac antiserum for the Cry1AcF protein can only provide some indications of the protein levels but may not provide the true picture of the quantitative content of the Cry1AcF protein. Therefore there is a need to develop specific antisera for Cry1AcF protein and further standardization of ELISA protocols.

At the molecular level, Southern blot analysis with *BamH*I proved that one copy of the transgene had been integrated in the genome of representative transgenic events that were analysed. Earlier studies have also shown similar integration proof which showed that majority of the transgenic plants have identical signal in Southern hybridization (Kar *et al.,* 1997). In addition, single-copy insertion in transgenic plants is extremely important for transgenic breeding, because multiple gene copies can lead to instability of expression and inheritance of the transgene or even gene silencing (Stam *et al.,* 1997). Further, Northern analysis for PCR and Southern positive plants demonstrated that the *cry1AcF* transgene was actively expressed.

It is now firmly established that transgenics are bound to become the mainstay of the pest management technology in the years to come because of many advantages associated with their use. However, most of these efforts are limited to the crops that can potentially provide better market rewards for the investors. Dry land crops are generally low input crops in the first place and next, due to vagaries of monsoon, risk associated with further investment can be very high and thus insect pests remain a potential perennial problem. As a result, seed borne solutions at meaningful cost prices would facilitate better performance of the crops against insect pests. The study demonstrates that transgenic peanut plants showed reasonably good resistance against defoliators especially, *S. litura* and *A. albistriga.*

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