Research Article

Received: 18 August 2009 Revised: 28 April 2010 Accepted: 19 July 2010 Published online in Wiley Online Library: 27 October 2010

(wileyonlinelibrary.com) DOI 10.1002/ps.2041

Expression of δ-endotoxin Cry1EC from an inducible promoter confers insect protection in peanut (*Arachis hypogaea* L.) plants

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Abstract

BACKGROUND: *Spodoptera litura* (F.) is a polyphagous foliage insect and a major pest on peanut (*Arachis hypogaea* L.). Constitutive expression of δ-endotoxin Cry1EC gives protection against *S. litura*, as reported earlier. In this study, insect bites and salicylic acid induced high-level expression of Cry1EC was achieved in peanut. In order to achieve this, the expression of pathogenesis responsive promoter *PR-1a* was enhanced by placing it downstream of the *CaMV35S* promoter in the pCAMBIA 1300 backbone. The resultant promoter *CaMV35S(r)PR-1a* expressed a high level of insecticidal δ-endotoxin Cry1EC. The Gus expression under the control of *CaMV35S(r)PR-1a* served as a convenient marker for evaluation of promoter response to different treatments.

RESULTS: Transgenic events that showed a very low level of uninduced expression and no expression in seeds were selected. The Cry1EC expression in leaves increased nearly eightfold in the selected event, following induction by salicylic acid. Both the salicylic-acid-treated and the *S. litura*-bitten leaves showed the highest expression after 2 days. Leaves from salicylic-acid-induced transgenic plants caused 100% mortality of *S. litura* at all stages of larval development.

CONCLUSION: The results suggest that high expression of inducible promoters provides a good strategy for the development of safer transgenic food and feed crops.

Supporting information may be found in the online version of this article.

Keywords: δ-endotoxin Cry1EC; inducible promoter; peanut transformation; *Spodoptera litura*

1 INTRODUCTION

Peanut or groundnut (*Arachis hypogaea* L.) is an important source of edible proteins and oil. Peanut crop worth about $US 2 billion is lost annually owing to fungal pathogens, viruses, bacteria and insect pests.[1] Conventional breeding has contributed to the improvement of peanut.[2–4] Many wild species of *Arachis* harbour valuable traits,[5–7] but strong interspecific reproductive barriers, low recovery of hybrids and linkage with undesirable traits limit their use in developing varieties resistant to pests and diseases.[8,9] Insecticidal crystal proteins (δ-endotoxins) of *Bacillus thuringiensis* specific to target pests have been deployed in the development of transgenic crop plants for integrated insect pest management.[10–13] The δ-endotoxin-expressing crops such as cotton and maize have resulted in economic benefits to growers globally and have reduced the use of conventional insecticides.[14]

Spodoptera litura (F.) is a polyphagous pest and causes up to 71% yield losses to peanut in southern parts of India.[15] Its polyphagous nature, high reproductive capacity and ability to migrate over large distances have led it to become a serious pest of many agricultural crops the world over. It was one of the first agricultural pests reported in India to have developed resistance to insecticides.[16] A novel chimeric δ-endotoxin Cry1EC was designed earlier in the present authors' laboratory specifically against *S. litura*, and expressed constitutively in transgenic cotton and tobacco,[17] pigeon pea[18] and peanut.[19] A strategic alternative is the use of promoters tightly regulated to give temporal and/or spatial expression of the gene of interest.[20–22] The promoter of the tobacco pathogenesis responsive gene *PR-1a* remains completely repressed until it receives a signal elicited by pathogen infection or is experimentally induced by salicylic acid (SA).[23–25] However,

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its low level of expression even after induction limits its utility for the control of insect pests. The present study establishes the feasibility of deploying a modified *PR-1a* promoter for the selection of transgenic lines that express the target gene at a high level only at the site of an insect bite. The strategy was used to produce safer, insect-protected transgenic peanut plants.

2 MATERIALS AND METHODS

2.1 Plasmid constructions

Two plasmid constructs were prepared by inserting 1.8 kb *gusA* (*β-glucuronidase*) or 1.9 kb *cry1EC* genes downstream of a *PR-1a* promoter in the binary vector pCAMBIA 1300. The 1.5 kb *PR-1a* promoter was amplified from the genomic DNA of tobacco[25] and inserted in pCAMBIA 1300 at *Bam*HI-*Xba*I/*Hind*III sites (Figs 1A and B). The promoter construct comprising *PR-1a* at *Bam*HI-*Xba*I/*Hind*III sites was named as *CaMV35S(r)PR-1a* to acknowledge the possible effect of *CaMV35S* enhancer leading to enhanced expression of the chimeric *PR-1a* promoter in pCAMBIA background. The vectors carrying *gusA* and *cry1EC* were named P_{1332} and P_{1334} respectively (Figs 1A and B). The marker in the modified vectors was *hygromycin phosphotransferase* (*hptII*) for the selection of transgenic events. *Agrobacterium tumefaciens* (Smith & Townsend) Conn. strain EHA101 containing the virulence helper plasmid pEHA101[26] was transformed with P_{1332} and P_{1334} by electroporation using a Gene-Pulser device (Bio-Rad, Hercules, CA).

2.2 Plant transformation, selection and regeneration

Plant transformation, selection and regeneration methods were as described previously.[19] The seeds of high-yielding peanut cultivar JL-24 were sterilised.[27,28] Batches of 120 and 80 de-embryonated cotyledon explants were prepared and transformed with *A. tumefaciens* harbouring binary vectors P_{1332} (*CaMV35S(r)PR-1a-gusA*) and P_{1334} (*CaMV35S(r)PR-1a-cry1EC*) respectively.

After 5 days cocultivation, explants were placed with the distal ends in contact with shoot induction medium (SIM). The SIM contained Murashige & Skoog (MS) medium, 30 g L^{-1} sucrose supplemented with 88.77 μM 6-benzylaminopurine (BAP) (for the initial 2 weeks) and 66.58 μM BAP (for the third and fourth weeks), pH 5.8, and solidified with 8 g L^{-1} agar. Augmentin (GlaxoSmithKline, India) 200 mg L^{-1} and cefotaxime (Lupin, India) 200 mg L^{-1} were added to the medium to eliminate overgrowth of *A. tumefaciens*. Multiple shoots were elongated on the elongation medium containing 13.32 μM BAP, 250 mg L^{-1} cefotaxime and 40 mg L^{-1} hygromycin for four cycles of 3 weeks each. Elongated shoots were rooted on rooting medium supplemented with 4.95 μM α-naphthylacetic acid and 250 mg mg L^{-1} cefotaxime. The acclimatised plants were planted in sandy loam soil and kept in a glasshouse till maturity.

All biochemicals and media constituents, unless stated otherwise, were molecular biology/cell culture grade from Sigma Chemical Company (St Louis, MO).

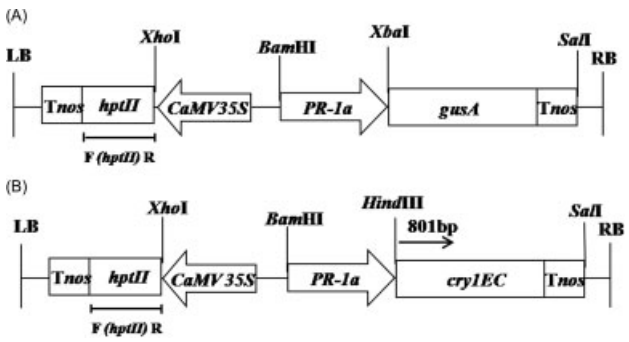

Figure 1. Gene constructs used for genetic transformation of peanut. Partial maps of the binary vectors P_{1332} carrying *gusA* (**A**) and P_{1334} carrying *cry1EC* (**B**) are shown. Arrow indicates position of the probe used in Southern blot hybridisation. F and R indicate the positions of the primers used to amplify the sequence of *hptII*. T*nos*, nopaline synthetase termination sequence; *hptII*, hygromycin phosphotransferase gene; *CaMV35S*, promoter of Cauliflower mosaic virus; *PR-1a*, promoter of tobacco pathogenesis related protein gene; *gusA*, *β*-glucuronidase coding sequence; *cry1EC*, synthetic *δ*-endotoxin gene; LB, left border; RB, right border.

2.3 Polymerase chain reaction (PCR) and Southern blot analysis

Putative (T_0) transgenic peanut plants, which showed resistance to hygromycin, were screened for the presence of the introduced genes by PCR and Southern blot analysis. PCR amplification of *hptII* and *vir*G genes was carried out as in Tiwari *et al.*[19] The *vir*G primers were used to account for the false positives, resulting from persistent and contaminating *Agrobacterium* cells in T_0 plants.[19] The position of *hptII* primers is indicated in Figs 1A and B. PCR positive *CaMV35S(r)PR-1a-cry1EC* plants were subjected to Southern blot hybridisation analysis. About 15–20 μg of genomic DNA of selected plants was digested with *Hind*III, separated by electrophoresis and blotted onto positively charged nylon membrane (Hybond N^+; Amersham Life Sciences, USA) by vacuum transfer. A fragment of 801 bp was amplified from the 5′ site of *cry1EC* gene (Fig. 1B) and radiolabelled with αP^{32}-dCTP. As there was no *Hind*III cleavage site within the selected *cry1EC* probe, the number of hybridising fragments indicated the number of insertion events. Hybridisation was carried out at 65 °C for 18 h. The blot was washed for 10 min in solution containing 2× saline sodium citrate (SSC) buffer + 0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by 20 min in 1× SSC + 0.1% SDS at 65 °C and 20 min in 0.1% SSC + 0.1% SDS at 65 °C. After washing, the blot was exposed to Fuji screen for 24 h and scanned on phosphoimager (Molecular Imager FX; Bio-Rad, Hercules, CA).

2.4 Gene segregation analysis

Transgenic (T_1) seeds were sterilised and soaked overnight in sterile water. The seeds were germinated on sterile filter paper bridges in test tubes containing half-strength MS liquid medium with 10 mg L^{-1} hygromycin for 15 days at 25 ± 2 °C under a 16 : 8 h light : dark photoperiod. The seedlings germinating in the presence of hygromycin were scored to analyse the segregation of *hptII* transgene in the progeny.

2.5 Gus expression in transgenic leaves modulated by SA and insect bite

The *β*-glucuronidase (Gus) expression in transgenic (T_1) plant leaves was monitored (0–6 days) in response to water (uninduced), insect-bite (*S. litura*) and 2 mM SA treatments. Leaves from 6–10-week-old non-transformed and transgenic plants were used for each treatment. The leaves were floated on water (uninduced) or water containing 2 mM SA for 48 h (2 days). The treated leaves were then incubated in water for up to 6 days under a 16 : 8 h light : dark photoperiod in 60 μmol m^{-2} s^{-1} light intensity. To examine the response to larval feeding, manual infestation was carried out with second-instar *S. litura* larvae. Ten larvae were released on

fully expanded second- to third-node leaves and covered by a polythene bag. Polythene bags and insects were removed after 24 h, and the plants were again allowed to grow in the normal environment of the glasshouse. The treated leaves were scored histochemically for Gus expression.

2.6 Histochemical Gus expression analysis

The histochemical Gus activity in leaves of the treated plants was measured as described by Jefferson *et al*.[29] The treated leaves were incubated with X-Gluc solution at 37 °C for 24 h in the dark. Chlorophyll was removed by soaking the tissue in 70% ethanol and finally preserved in absolute ethanol and photographed.

2.7 Fluorometric Gus expression analysis

The quantitative Gus assay was performed with the leaves of a selected homozygous single-copy T_2 transgenic line (A50-A/1). The leaves were treated with water, insect bite and 2 mM SA, as described above. Non-transformed leaves were taken as negative controls for each treatment. The treated leaves were incubated for up to 4 days under a 16 : 8 h light : dark photoperiod in 60 μmol m^{-2} s^{-1} light intensity. The Gus activity was measured by fluorometric assay.[25] The Gus activity is expressed in nmol 4-MU h^{-1} mg^{-1} protein. The experiment was conducted in duplicate and repeated twice. Although fully expanded leaves from the second to fourth nodes were excised to keep the data comparable, some variation in replicates was noticed owing to differences in maturity of the leaves used for estimating Gus activity.

2.8 Induction of *CaMV35S(r)PR-1a-cry1EC* plants

Leaves from 6–10-week-old non-transformed and several transgenic lines were treated with 2 mM SA for the induction of *CaMV35S(r)PR-1a* promoter. Fully expanded leaves from second to fourth nodes of transgenic plants were floated on water (uninduced) or water-supplemented SA (induced) in a petri dish under a 16 : 8 h light : dark photoperiod at 25 ± 2 °C for 2 days.[23] Treated leaves were analysed in immunological and insect bioassay experiments.

2.9 Immunological analysis of δ-endotoxin

The accumulation and expression of the Cry1EC δ-endotoxin was monitored using direct antigen-coated enzyme-linked immunosorbent assay (DAC-ELISA) and western blot analysis, as described previously.[19]

2.10 Biological activity of δ-endotoxin against *Spodoptera litura*

Larvae of *S. litura* inbred for several generations in the laboratory were used in feeding studies at 25 ± 2 °C. Ten first- or second-instar larvae of *S. litura* were released on induced and uninduced detached leaves of 18 putative (T_0) or five T_1 transgenic plants respectively. Non-transformed plant leaves were used as negative control. The SA-treated leaves were washed thoroughly in distilled water, blotted dry and placed in a plastic beaker onto a wet filter paper. Each experiment was repeated twice with duplicate leaf samples. Observations on surviving larval weight, mortality, leaf area consumed and feeding inhibition were recorded up to 4 days. The leaf area consumed was measured by the Area Measurement System (Delta-T Devices Ltd, Burwell, Cambridge, UK). The data were statistically analysed by SPSS software.

A selected T_2 homozygous transgenic line (A119-B/1) was used to perform bioassay experiments at different developmental stages of the *S. litura* larvae. Ten larvae of first and second instar and five larvae of third and fourth instar were released per induced leaf. When necessary, leaves of equivalent size and physiological age from the same plants were re-fed. Photographs were taken after 4 days (first or second instar) and 8 days (third or fourth instar).

3 RESULTS

3.1 Development of transgenic peanut plants

Genetically transformed de-embryonated cotyledon explants were selected on hygromycin-containing medium. After four cycles of selection, 35 and 18 transformed shoots were recovered from ten (*CaMV35S(r)PR-1a-gusA*) and four (*CaMV35S(r)PR-1a-cry1EC*) de-embryonated cotyledon explants respectively. Putative (T_0) transgenic plants were PCR positive with respect to *hptII* gene, while the *vir*G gene specific primers did not show any amplification (data not shown). Morphologically healthy T_0 plants were selected for further analysis. Results of Southern blot hybridisation, establishing genomic integration of *cry1EC* in five T_0 transgenic plants, are shown in Fig. 2. No hybridisation signal was detected in the non-transformed plants. Different sizes of the inserts established that the transgene insertion was at different loci in different transgenic plants. Among the selected transgenic plants, A119-B/2, A119-B/1, A119-A/2 and A119-A/1 (lanes 2, 3, 4 and 5) revealed single-copy insertion, while A119-B/3 (lane 1) had two copies of *cry1EC* (Fig. 2).

The selected T_0 transgenic plants grew normally and produced flowers and pods within 3–4 months, albeit the number of pods developed from T_0-tissue-culture-raised plants was low compared with that from the seed-derived non-transformed plants. However, in subsequent generations, the *in vitro* derived transgenic plants performed as well as the seed-derived plants. Transgenic progenies from the selected T_0 Gus- and Cry1EC-expressing lines were subjected to hygromycin-based segregation analysis. The positive seeds were scored for healthy rooting in the seedlings. Four Cry1EC-expressing lines (A119-A/1, A119-A/2, A119-B/1 and A119-B/2) and five Gus-expressing lines exhibited a segregation ratio close to 3 : 1, suggesting a single integration event (supporting information Tables S1 and S2). The fifth Cry1EC-expressing line

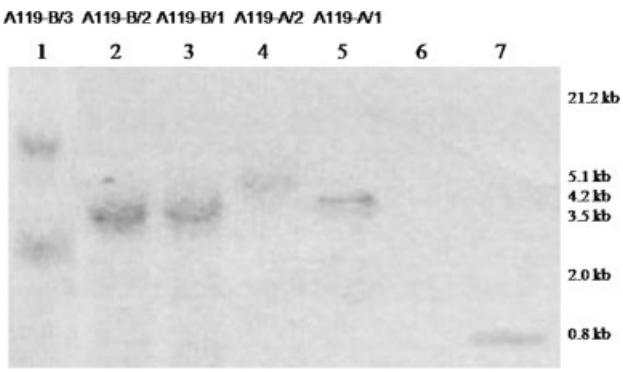

Figure 2. Southern blot hybridisation of T_0 transgenic plants carrying *CaMV35S(r)PR-1a-cry1EC* gene. Genomic DNA was digested with *Hind*III and hybridised with a ^{32}P-radiolabelled 801 bp *cry1EC* probe. Lanes 1 to 5: transgenic plants; lane 6: non-transformed plant; lane 7: positive control (PCR amplicon – 801 bp).

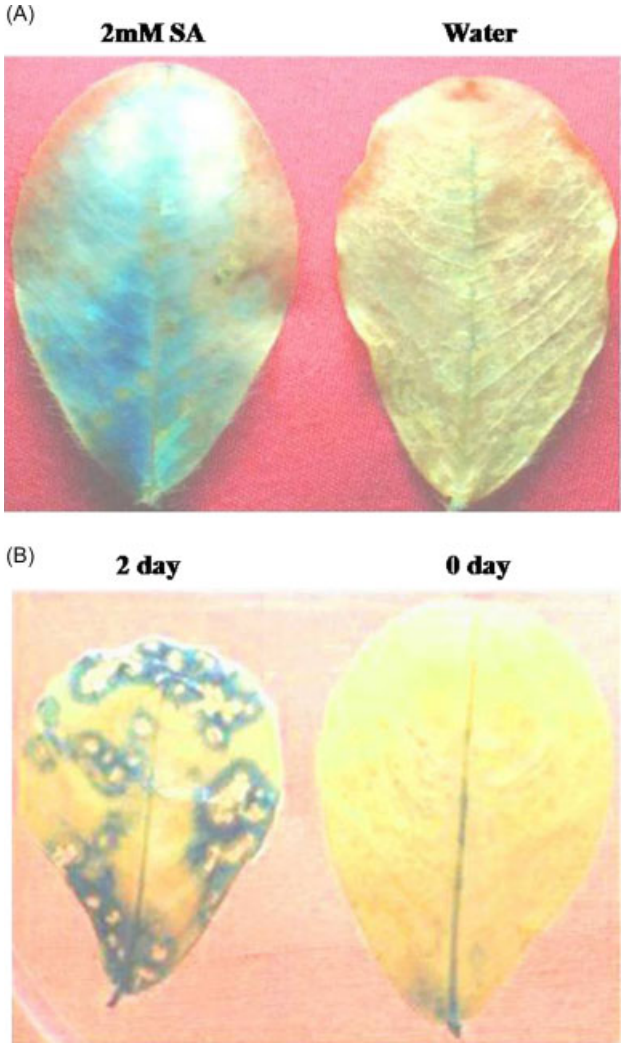

Figure 3. Histochemical Gus expression in T_1 transgenic A50-A/1 plant leaves after various treatments. (A) Gus expression in transgenic leaves after 2 days treatment with 2 mM SA (induced) and water (uninduced). (B) High-level confined Gus expression after 2 days in *S. litura*-fed leaf and negligible expression in unfed (0 day) leaf of the same plant.

(A119-B/3) showed a higher ratio, suggesting multiple integrations (supporting information Table S2).

3.2 Induction behaviour of *CaMV35S(r)PR-1a* promoter in Gus-expressing transgenic peanut lines

The results of histochemical Gus staining suggested that two (A50-A/1 and A50-D/1) out of five T_1 transgenic lines were induced by SA, while three (A50-A/2, A50-B/1 and A50-C/1) lines showed constitutive expression. The 48 h SA-induced transgenic A50-A/1 line exhibited high expression, while the corresponding water-treated plant of the same line showed very low intensity of Gus expression (Fig. 3A). The high expression persisted for up to 6 days in SA-induced leaves (data not shown). In the case of *S. litura*-fed transgenic leaves, Gus accumulation was confined only to the feeding site of the leaf tissue in A50-A/1 and A50-D/1 transgenic lines. In spite of the promoter being the same, the Gus expression was constitutive in the case of A50-A/2, A50-B/1 and A50-C/1 lines. The Gus expression in insect-fed and unfed leaves of transgenic line A50-A/1 was monitored from 0 to 6 days (supporting information

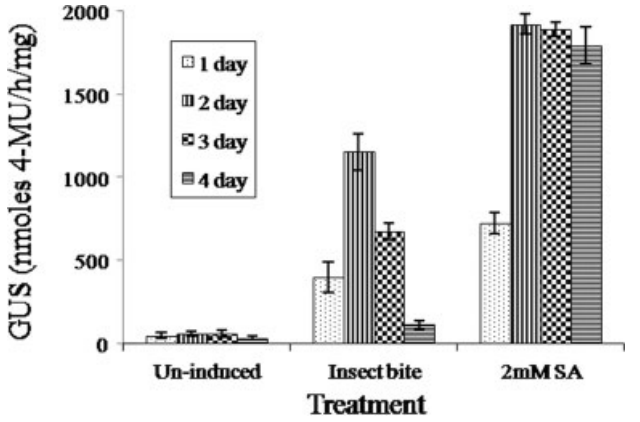

Figure 4. Fluorometric assay for quantitative Gus expression in leaves of homozygous T_2 transgenic A50-A/1 plant. Expression monitored up to 4 days following water (uninduced), insect-bite and 2 mM SA treatments.

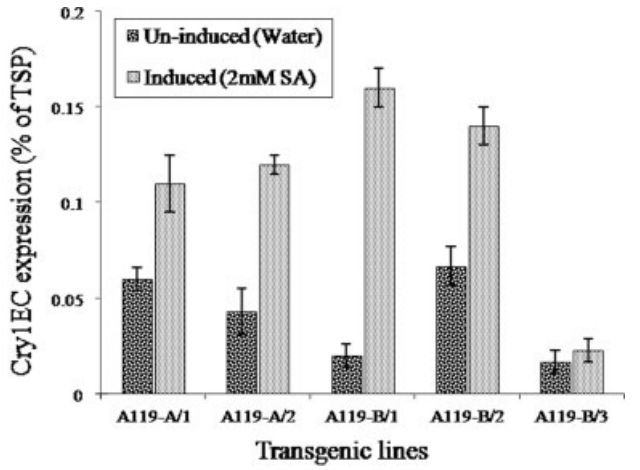

Figure 5. Expression of Cry1EC protein in five T_1 transgenic lines. The δ-endotoxin expression was monitored in induced (2 mM SA) and uninduced (water) leaves by direct antigen-coated ELISA.

Fig. S1). The expression was maximum after 2 days in the fed leaf and negligible in the unfed leaf from the same plant (Fig. 3B). The expression in the fed leaf started from day 1 and declined sharply by day 4. The Gus expression observed on days 4, 5 and 6 in fed leaves was comparable with that of unfed leaves of the same plants. The non-transformed (control) plants did not show any Gus expression.

Quantitative estimation of Gus activity in leaves of the T_2 transgenic A50-A/1 line treated with water, insect bite and 2 mM SA showed that it reached its highest level in 2 days in the SA- and insect-bite-treated leaves (Fig. 4). Very low expression was observed in the uninduced transgenic leaves.

3.3 Expression behaviour of the insecticidal gene *cry1EC* from *CaMV35S(r)PR-1a* promoter

All SA-induced T_1 transgenic lines tested by ELISA revealed the presence of Cry1EC protein in the leaves (Fig. 5). In the non-transformed plant, no expression was detected. The range of expression in single-copy-induced plants was 0.11–0.16% of total soluble protein (TSP). Without SA induction, the transgenic plants showed expression of Cry1EC, i.e. at 0.02–0.067% of TSP. The transgenic line A119-B/3 with multiple insertion events did not

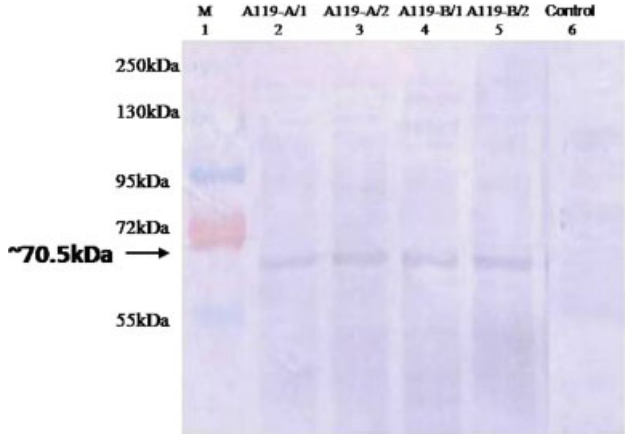

Figure 6. Western blot analysis of four T_1 transgenic lines. Four SA-induced lines A119-A/1, A119-A/2, A119-B/1 and A119-B/2 showing Cry1EC protein at 70.5 kDa (lanes 2 to 5), while it was absent in the non-transformed plant (control, lane 6).

show any significant difference in Cry1EC expression in either the induced (0.023% of TSP) or uninduced (0.017% of TSP) state (Fig. 5). The most promising line A119-B/1 gave the highest (0.16% of TSP) Cry1EC expression following 48 h of induction by SA. It showed the lowest (0.02% of TSP) uninduced expression and showed no expression in the seeds.

The high Cry1EC expression in induced single-copy (A119-A/1, A119-A/2, A119-B/1, A119-B/2) transgenic lines showed an immunoreactive protein band at 70.5 kDa in Western blot analysis (Fig. 6). Non-transformed (control) plants showed no immune-reactive band. Therefore, the expressed protein had the expected molecular mass.

3.4 Insect resistance of *CaMV35s(r)PR-1a-cry1EC* transgenic peanut lines

Leaves from all the 18 PCR positive T_0 transgenic plants caused mortality to first-instar larvae of *S. litura*. The mortality ranged from 10 to 100%. The Cry1EC expression was constitutive in uninduced leaves of T_0 plants (data not shown), in spite of no deliberate SA induction, suggesting that inductive factors resulting from the stress of tissue culture could lead to expression in T_0 plants. Uninduced Cry protein expression in tissue-culture-raised T_0 plants has been reported in other cases also.[30,31] In the T_1 generation, the expression of Cry1EC became inducible.

The insecticidal effect of the five T_1 lines expressing Cry1EC protein in induced and uninduced leaves was monitored by performing the bioassay with second-instar larvae of *S. litura* (Table 1). The induced leaves of A119-A/1, A119-A/2, A119-B/1 and A119-B/2 transgenic lines exhibited >50% and >90% mortality at day 2 and 3 respectively. The mortality reached 100% in induced leaves after 4 days of feeding. Uninduced leaves of similar lines showed 0–30%, 30–47% and 67–100% mortality after days 2, 3 and 4 respectively. The transgenic line A119-B/3, with multicopy insertions, showed 17% and 12% mortality in induced and uninduced leaves respectively, after 4 days of feeding. None of the surviving larvae showed metamorphoses, even if they were transferred onto non-transformed foliage, indicating that the surviving larvae had received a lethal dose of δ-endotoxin during the 4 days of feeding on transgenic lines. The larvae fed on non-transformed (control) plant leaves completed their larval phase in 16 days and developed into pupae.

Differences in insect feeding damage were observed in uninduced and induced leaves of transgenic plants (Fig. 7A). On day 1, the larvae showed comparable feeding on uninduced leaves of the transgenic lines and the non-transformed plants. On day 2, the uninduced leaves of the four single-copy transgenic lines showed significantly lower feeding compared with the control. The level of feeding in multicopy-inserted line A119-B/3 was similar to that in the control. The uninduced leaves of the transgenic lines showed significant feeding difference on days 3 and 4 compared with the control plants. Following SA induction, all the transgenic lines showed higher feeding differences over the non-transformed plants.

The data on feeding inhibition by uninduced and induced leaves of transgenic lines are given in Fig. 7B. Uninduced leaves fed for 1 day showed little or no feeding inhibition, while the induced leaves of the same lines inhibited feeding significantly. Lines A119-B/1 and A119-B/2 recorded 5% and 11% inhibition under uninduced condition, which increased to 80% and 70% respectively following induction with SA. All the lines caused >50% feeding inhibition on the second day after induction. However, A119-B/3 was somewhat slower, with >50% inhibition on day 4. It is apparent that single-copy transgenic plant leaves induced by SA treatment expressed a high level of Cry1EC, which resulted in significantly earlier feeding inhibition.

Table 1. Mortality of *Spodoptera litura* second-instar larvae fed on transgenic peanut leaves[a]

	Induced (2 mM SA)				Uninduced (water)			
	2 days	3 days	4 days		2 days	3 days	4 days	
Transgenic line	Mortality (%) (± SD)	Mortality (%) (± SD)	Average weight of surviving larvae (mg) (± SD)	Mortality (%) (± SD)	Mortality (%) (± SD)	Mortality (%) (± SD)	Average weight of surviving larvae (mg) (± SD)	Mortality (%) (± SD)
A119A/1	52(±9.57)	92(±5)	–	100(±00)	27(±12.58)	40(±8.16)	4.8(±0.4)	92(±9.57)
A119A/2	52(±9.57)	92(±9.57)	–	100(±00)	15(±5.77)	30(±8.16)	20(±4.73)	67(±9.57)
A119B/1	77(±5)	100(±00)	–	100(±00)	0	47(±9.57)	–	100(±00)
A119B/2	65(±5.77)	95(±5.77)	–	100(±00)	30(±10)	42(±5)	3.01(±0.07)	95(±5.77)
A119B/3	–	7.5(±9.57)	12.33(±8.55)	17(±9.57)	0	0	37(±1.95)	12(±9.57)
Control (non-transformed)	0	0	165(±0.04)	0	0	0	167(±0.14)	0

[a] The readings were taken up to 4 days of feeding on the leaves of transgenic T_1 peanut plants expressing Cry1EC protein.

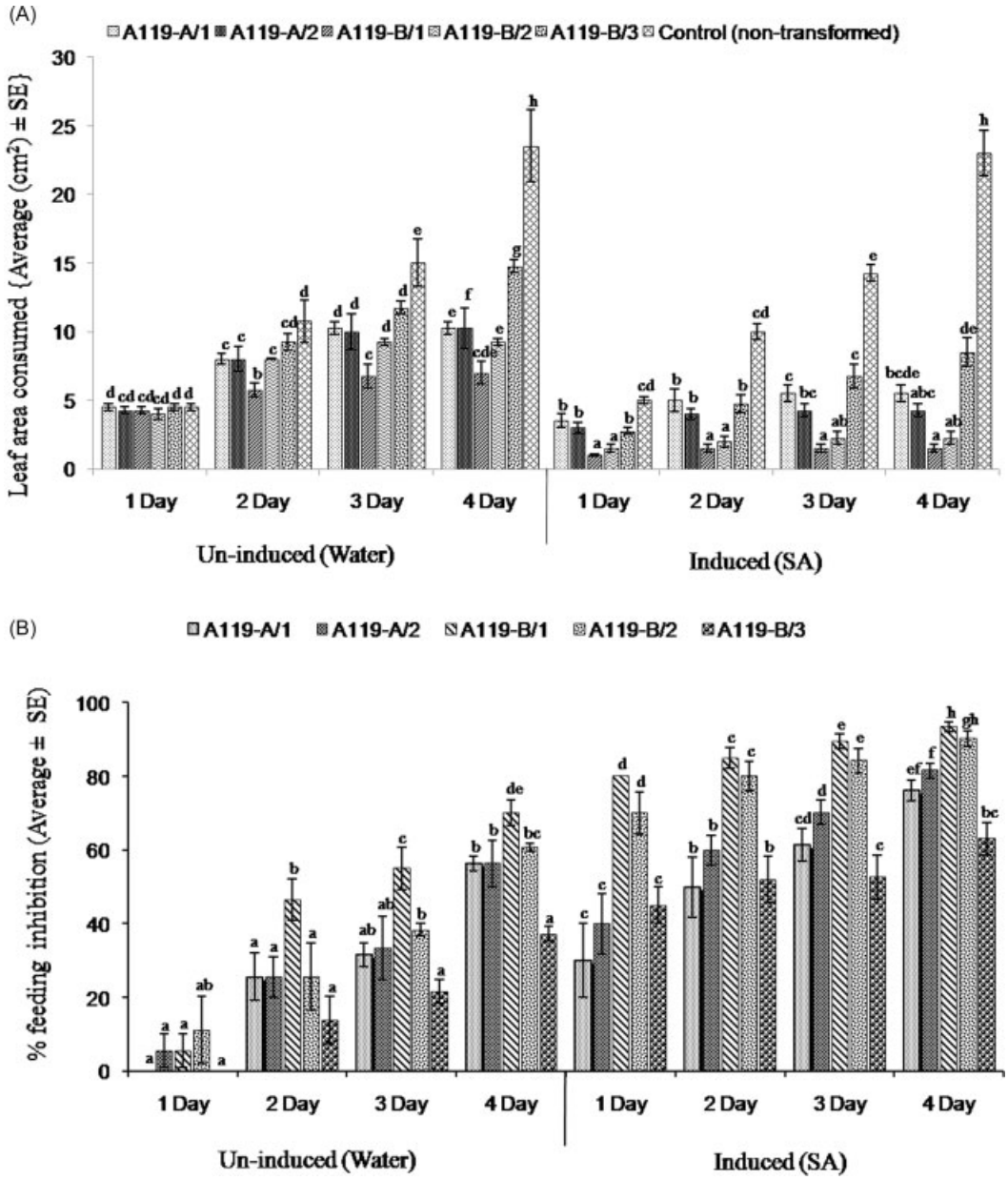

Figure 7. Insect bioassay of *Spodoptera litura* second-instar larvae fed on induced (2 mM SA) and uninduced (water) leaves of five T_1 transgenic peanut plants expressing Cry1EC protein. Feeding damage (A) and feeding inhibition (B) were monitored up to 4 days. In both graphs the bars marked with similar letters are not significantly different. The SPSS software was used to compare average by Duncan's multiple range test ($\alpha = 0.05$). Percentage feeding inhibition was calculated as 100 − (leaf area consumed in treatment/leaf area consumed in control {non-transformed} × 100).

For the recording of mortality at different developmental stages of the larvae, the most promising single-copy T_2 transgenic line A119-B/1 homozygous at *cry1EC* locus was selected. The induced leaves gave 100% mortality of first-, second-, third- and fourth-instar larvae of *S. litura* (Fig. 8). The protein expression and bioassays revealed a direct correlation between the expression of Cry1EC and induction of *CaMV35S(r)PR-1a* promoter.

4 DISCUSSION

The δ-endotoxin-expressing transgenic crops currently under cultivation express the insecticidal genes from the *CaMV35S* constitutive promoter (http://www.isaaa.org/ISAAA 2007). Field studies to date have shown negligible impact of some of the δ-endotoxins such as Cry1Ac and Cry1C on several non-target organisms in comparison with conventional insecticides.[11–13] However, negative effects of δ-endotoxins on parasitoids have been reported in laboratory[32,33] and field[34] studies. Therefore, uncontrolled expression has the risk of exposing non-target organisms to the δ-endotoxins. In addition, accumulation of a protein not required for plant growth and development unnecessarily diverts the resources of the host plant. This can put a yield penalty on the plant. An alternative resistance management strategy therefore could be to use temporal refuges where insect resistance genes should express under the control of an inducible promoter that produces δ-endotoxin only when necessary at the desired site.[35] The objective of the present study was to examine whether *CaMV35S(r)PR-1a* promoter could function as an

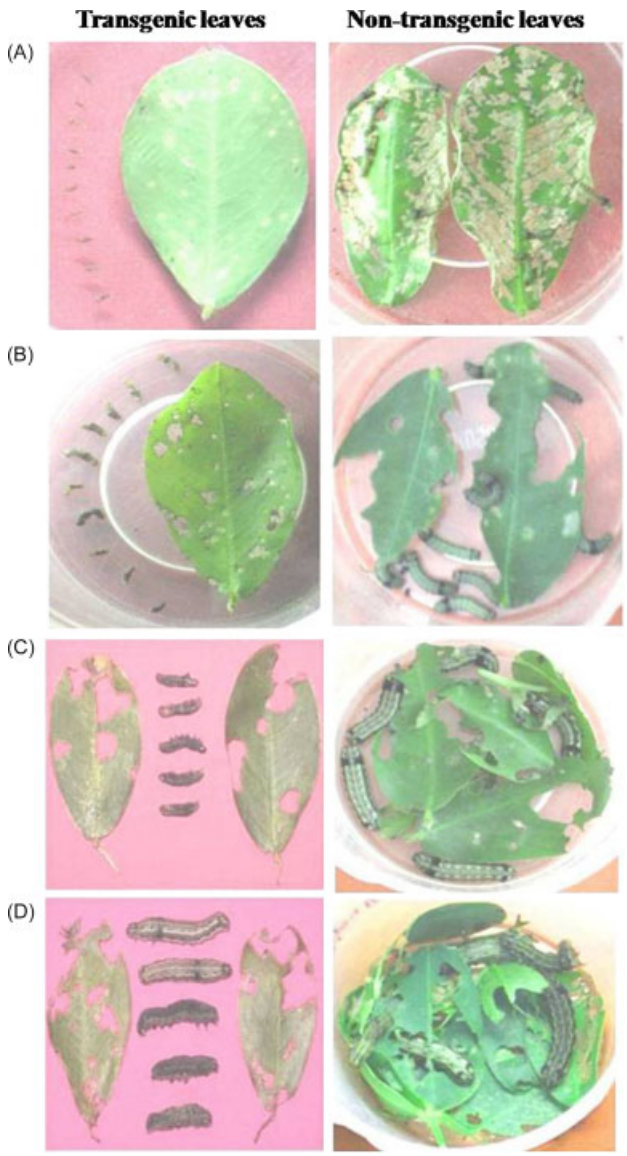

Figure 8. Resistance of T_2 homozygous transgenic peanut leaves of A119-B/1 line to feeding damage by *Spodoptera litura*. Four days after release of ten first-instar (A) and second-instar (B) larvae of *Spodoptera litura* on transgenic leaf. Eight days after release of five third-instar (C) and fourth-instar (D) larvae on transgenic leaves. Leaves of non-transformed plants are consumed vigorously by larvae in all developmental stages.

efficient system for insect-bite-induced transgene expression for the control of pests. Gus expression served as a convenient marker for evaluation of promoter response to different treatments. The induction behaviour of *CaMV35S(r)PR-1a* promoter in response to SA and *S. litura*-bitten treatments in selected Gus-expressing peanut lines showed high-level expression after 2 days of treatment. The expression was confined to the regions of insect feeding on the leaflets. The temporal pattern of Gus expression suggested that *CaMV35S(r)PR-1a* promoter followed different kinetics of expression in response to induction by SA and insect bite.

Variation in the level and pattern of Cry1EC expression was noticed in independent transgenic lines. This is known to result from differences in the position and copy number of insertion of the transgene.[36–38] In the majority of the transgenic events, Cry1EC expression was constitutive. A number of independent transgenic lines had to be screened before the line A119-B/1 was identified, which showed a very low level of expression under uninduced conditions, and an increased high level under induced conditions.

Attempts to obtain inducible insect management have been reported.[21,22,30,39] However, most of such studies have shown limited success. Jin *et al*.[30] expressed *cry1Ab3* under the control of *CaMV35S* and the inducible soybean *vspB* promoters to develop diamondback moth [*Plutella xylostella* (L.)]-resistant transgenic cabbage. The T_0 transformants showed *vspB-cry1Ab3* toxicity to the larvae, similar to that in *CaMV35S-cry1Ab3* plants. Thus, the *vspB* promoter expression was constitutive. Breitler *et al*.[21] raised striped stem borer (*Chilo suppressalis* Walker)-resistant transgenic rice lines carrying *cry1B* or *cry1Aa* gene under the control of maize-derived constitutive *ubi1* or wound-inducible *mpiC1* promoters. The wounded tissue of the selected line of *mpiC1-cry1B* showed high (0.2% of TSP) δ-endotoxin accumulation and 100% mortality to second-instar larvae of striped stem borer. Unwounded tissues showed no expression. In the case of the constitutively expressing lines (*ubi1-cry1B*), high δ-endotoxin accumulation was reported in unwounded tissues. Girijashankar *et al*.[39] developed transgenic sorghum expressing *cry1Ac* gene under the control of a wound-inducible maize *mpiC1* promoter. They reported low expression with partial tolerance to first-instar larvae of spotted stem borer (*Chilo partellus* Swinhoe). The low level of δ-endotoxin accumulation (1–8 ng g^{-1} of fresh leaf) in mechanically wounded tissue was far below the lethal dose required to confer complete protection. Cao *et al*.[22] expressed *cry1Ab* gene under the control of tobacco *PR-1a* promoter. They found low (older leaves) or no (younger leaves) Cry1Ab expression in uninduced broccoli leaves. The SA analogue ASM (*S*-methyl 1,2,3-benzothiadiazole-7-carbothioate)-treated leaves achieved high-level expression and showed complete mortality to second-instar larvae of *P. xylostella*. The δ-endotoxin accumulated at varying concentrations in different plant parts. It renders insect exposure to a low level of toxin proteins in untreated parts where *PR-1a* is induced systemically. This may result in the evolution of resistance in insects.[20] Thus, the use of *PR-1a* promoter alone for field control of insects may not be a satisfactory strategy. The inducible expression construct reported here shows substantial improvement over the previous reports. The selected transgenic lines showed high accumulation of the δ-endotoxin when induced by SA or insect bite. The insect-bite-inducible high-level expression was confined to the site of wounding. This may reduce the possibility of evolution of resistance in insects. In T_1 generation, four single-copy lines induced by SA accumulated >0.1% Cry1EC of TSP and resulted in >90% mortality to second-instar larvae within 3 days. Insect chewing may also accumulate Cry1EC, leading to >65% insect mortality in the uninduced leaves within 4 days. A relatively high level of uninduced expression of Cry1EC was observed in three of the four single-copy transgenic peanut lines. In such plants, the expression increased 2–3-fold following treatment with SA. In carefully selected transgenic lines, the expression of δ-endotoxin was limited to tissue and the period when the peanut leaves were actually damaged by pests. Earlier, the authors reported that *CaMV35S* promoter gave constitutive expression of Cry1EC, leading to its accumulation at 0.13% of TSP in transgenic peanut leaves.[19] In the present study, the A119-B/1 transgenic line expressed the δ-endotoxin at a negligible level of 0.02% of TSP until it was induced. The expression showed an eightfold increase following induction by SA and resulted in

accumulation of the δ-endotoxin at 0.16% of TSP. The selected homozygous T_2 lines showed 100% mortality to all developmental phases of the larvae.

High-level uninduced expression of *cry1EC* from *CaMV35S(r)PR-1a* promoter was reported by the authors' group in tobacco and cotton.[40] In the transgenic lines, the promoter was induced only 1.6-fold by insect bite and 2.5-fold by SA. The increase in level of expression of *PR-1a* in these promoter constructs was attributed to locating *PR-1a* immediately downstream of *CaMV35S* promoter in pCAMBIA. This could happen because the enhancer elements in *CaMV35S* promoter are known to influence the expression of promoters as far as 10 kb.[41] To examine whether the increase in *PR-1a* was actually due to *CaMV35S* located upstream, the authors cloned *CaMV35(r)PR-1a* (Fig. 1) upstream of *gusA* gene in pBI101 vector (Tiwari S *et al*., unpublished data). Thirty transgenic lines of tobacco were tested for Gus expression. None of these showed a significant basal level of expression. Thus, the higher level of expression observed in cotton and tobacco by Kumar *et al*.[40] and in peanut in the present study is not merely the effect of *CaMV35S* placed upstream. This may be due to some other unknown factor(s) present in T-DNA of pCAMBIA carrying *CaMV35S* and *PR-1a* promoters placed head to head as shown in Fig. 1. In the present study, the A119-B/1 transgenic line showed very low Cry1EC accumulation in uninduced condition. The expression substantially increased following SA and insect-bite treatments. The selected peanut lines showed inducible behaviour and caused complete mortality of the larvae even at an advanced stage of growth. A search for early, tightly regulated and highly expressing wound-inducible promoters is imperative to develop an inherently safe insect management strategy in the release of transgenic varieties.[42,43] The present report is a step forward in this direction and demonstrates its application to an important edible crop.

ACKNOWLEDGEMENTS

The authors express their gratitude to Nandita Singh for providing the leaf area measurement system, to SMH Abidi for insect bioassays, to the Council of Scientific and Industrial Research, New Delhi, for funding the study and for a fellowship to Siddharth Tiwari and Devesh K Mishra and to the Department of Science and Technology, Government of India, for a JC Bose Fellowship to Rakesh Tuli.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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