academicJournals

Vol. 12(15), pp. 1820-1827, 10 April, 2013
DOI: 10.5897/AJB12.1572
ISSN 1684–5315© 2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic transformation of *cry1EC* gene into cotton (*Gossypium hirsutum* L.) for resistance against *Spodoptera litura*

R. Pushpa*, T. S. Raveenderan, S. Rajeswari, P. Amalabalu and D. Punitha

Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore-641 003, India.

Accepted 16 October, 2012

Cotton is the chief fibre crop of global importance. It plays a significant role in the national economy. Cotton crop is vulnerable to a number of insect species, especially to the larvae of lepidopteron pests. 60% insecticides sprayed on cotton are meant to control the damage caused by bollworm complex. Transgenic technology has become a popular option for the development of bollworm resistant cotton. *Spodoptera litura* is one of the notorious emerging pests in cotton. Introduction of *cry1EC* gene into commercial variety have very significant importance. This research work was carried out to transform chimeric δ-endotoxin *Cry1EC* into cotton. The tobacco cutworm (*Spodoptera litura*) is a polyphagous foliage insect which is susceptible to the chimeric δ-endotoxin *Cry1EC*. Six month-old highly friable embryogenic calli derived from cotyledonary explants of Coker 310 were used for transformation using *Agrobacterium* tumefaciens strain LBA4404 harboring plasmid pBI101.1 carrying the marker gene neomycin phosphotransferase II (*npt II*) and a synthetic *cry 1EC* gene under a constitutive 35 S promoter. *Agrobacterium* treated calli were selected on MS medium containing, 50 mg/L kanamycin, 500 mg/L cefotaxime, 30 g/L maltose and 0.4% phytagel. Embryos developed on kanamycin resistant calli were maintained on the same medium till somatic embryos matured. The cotyledonary stage embryos (3 to 5 mm size) were germinated on MS basal slat with 0.1 mg/L GA3 + 1.0 mg/L IAA, 30 g/L sucrose and solidified with 0.4% phytagel. The regenerated putative transgenic plants were hardened and transferred to the transgenic green house. Transgenic plants were confirmed by polymerase chain reaction (PCR) amplification of 800 bp npt II fragment, and 578 bp amplification of *cry1Ec* gene. Transgenic plant with single copy insertion of *cry1EC* was selected in T_0 by southern blot hybridization. Insect bioassay using *Spodoptera litura* larvae of first instar stages on T_0 plants showed 70% mortality. Not much data has been published on the toxicity of the endotoxins to *S. litura*, which is a common pest in warm and humid climates. Efforts are aimed at recovering more efficient transgenic plants through efficient transformation system and developing high resistant transgenic cotton against *S. litura* and will paved a way for promising future in cotton production.

Key words: *Agrobacterium* transformation, *cry 1EC*, molecular analysis, insect bioassay.

INTRODUCTION

Cotton (*Gossypium* spp.) is an important source of natural fibre, and plays a significant role in the Indian economy. *Spodoptera litura* is a polyphagous insect pest affecting various economically important crops and in

*Corresponding author. E-mail: Pushpa.saravanan@gmail.com.

case of severe infestation has caused significant loss to cotton crop in india. It primarily feed on foliage but also feed on fruiting parts of cotton plant (Eveleens et al., 1973). Larval feeding on cotton leaves can cause devastating losses in yield. The insect pest management in cotton is mainly dominated by the use of broad-spectrum insecticides, which disrupt the beneficial insect fauna too thus leading to pest resurgence and outbreaks of secondary pests, as well as risk to human health and environment (Yousefi, 2000). It is estimated that 60% of the insecticides sprayed on cotton are meant to control the damage caused by bollworm complex (Chaudhary and Loroia, 2001). These pests have developed high level of resistance due to repeated application of insecticides leading to heavy expenditure, crop failures and vicious cycle of debt for farmers. Development of new genetic material possessing resistance to insects, especially for bollworms has not made the required impact in achieving complete resistance due to lack of genetic variation for resistance and ploidy differences limit the utilization of wild species too.

One option to reduce the insecticide use on cotton is the exploitation of transgenic *Bt* cotton as a component of integrated pest management (Gore et al., 2001). Bt cotton, the first transgenic non-food crop, has provided a specific, safe and effective tool for the control of lepidopterous pests (Wu and Guo, 2005). Transgenic cotton cultivars expressing *Cry1Ac* have been released for commercial cultivation; worldwide this endotoxin is moderately toxic to *Helicoverpa zea* but poorly effective *against Spodoptera exigua* and *S. frugiperda* (Perlak et al., 2001). The genus *Spodoptera* contains five primary species (*litura, littoralis, exigua, frugiperda* and *exempta*), that occur worldwide. These are the pests of cotton, tobacco, castor, groundnut, a number of grain legumes and vegetable crop. The primary natural δ-endotoxin reported to show significant toxicity to *S. exigua* (Sanchis et al*.,* 1988; Visser et al*.,* 1990) and *S. littoralis* (Hone´e et al., 1991) is *Cry1C*.

Plants expressing *Cry1Ca* at a high level were reported to cause mortality and effectively give protection against *Spodoptera* sp. (Strizhov et al*.,* 1996; Mazier et al., 1997). However, transgenic Bt cotton with *Cry1Ac* proved not to be effective against *Spodoptera* spp. (Yu et al., 2004). A number of studies have shown that *Spodoptera* sp is not susceptible to Bt cotton containing Cry1Ac toxin so, there is a chance that this pest may become the major and alarming pest in Bt cotton field. As transgenic crops may perform differently under different ecological conditions so, there is a need to determine the efficacy of transgene against *Spodoptera*

Currently, cotton varieties containing Bt do not offer acceptable control of *Spodoptera.* A synthetic *cry1EC* gene has been designed by NBRI specifically against *S. litura* by replacing amino acids 530 to 587 in a poorly active Cry1Ea protein with a homologous 70 amino acid stretch of Cry1Ca in domain III (Singh et al., 2004).

Expression of the synthetic *cry1EC* gene in transgenic tobacco and cotton gave significant levels of resistance against *S. litura* (Singh et al., 2004). This elite Bt Cotton carrying *cry1EC* will give broader insect resistance coverage, particularly against *Spodoptera* (tobacco caterpillar) and expected to provide season long uniform protection of the target pests throughout the entire field. The present study reports introduction of *cry1EC* gene into cotton plants through *agrobacterium* mediated transformation and efficient regenerating of plants *via* somatic embryogenesis

MATERIALS AND METHODS

Bacterial strain and vector

The *Agrobacterium* strain, LBA4404 harbouring plasmid pBI101.1 and carrying synthetic *cry1EC* (Source: NBRI; Figure 1) was used in this study. The plasmid contains (i) the *cry1EC* gene, which encodes an insecticidal protein *Cry1EC*, (ii) 35S promoter from cauliflower mosaic virus to drive the expression of *cry1EC* gene in all parts of the plants (iii) *nptII* gene, the plant selectable marker, which encodes the enzyme, neomycin phosphotranferase II (NPTII).

Plant material

Acid-delinted cotton seeds of cotton varieties, Coker 310 were obtained from the germplasm collections of the Department of Cotton, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore. Seeds were surface sterilized with 70% ethanol for 2 min and then washed three times with sterile distilled water. They were again surface-sterilized with 0.1% mercuric chloride for 10 min followed by three washes with sterile distilled water. The seeds were germinated on half strength MS (Murashige and Skoog, 1962) medium supplemented with 1% (w/v) sucrose and 10 g/L agar. The pH of the medium was adjusted to 5.7 to 5.8 (by using 0.1 N KOH or 0.1 N HCl) prior to autoclaving the media for 15 min at 121°C and 15 psi pressure.

Callus induction

Explants from five to seven day-old seedlings were used for callus induction. Both hypocotyl (4 to 6 mm) and cotyledonary leaf (16 mm^2) sections were plated onto callus induction medium MS basal salt, supplement with 0.1 mg/L of 2,4-D and 0.5 mg/L of kinetin, 30 g/L maltose and solidified with 10 g/L agar. Vigorously growing loose and light yellow callus was transferred to a fresh somatic embryo induction medium containing MS salts with 1.9 g/L KNO_3, 30 g/L sucrose and solidified with 0.4 % phytagel (Sigma) (pH 5 to 8). Embryogenic calli with high proliferation activity were obtained after about three or four rounds of subculture with an interval of 2 weeks. Six month-old highly friable embryogenic calli derived from cotyledonary explants of Coker 310 were used for transformation.

Transformation and regeneration

Preculture

Six month-old highly friable embryogenic calli were precultured on somatic embryogenic medium containing (MS salts, 1.9 g/L KNO_3,

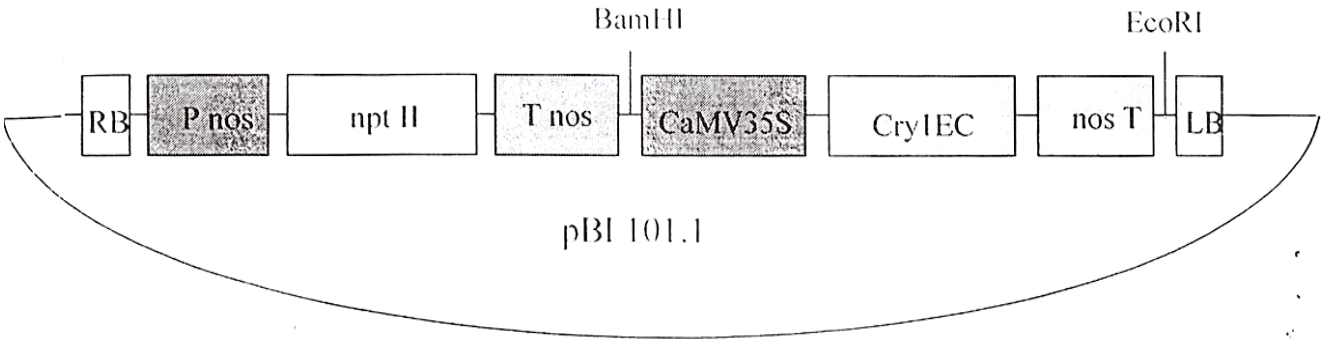

Figure 1. Linear map of T-DNA of pBI 101.1 carrying *cry1Ec* gene.

30 g/L maltose and 0.4 % phytagel) for seven days prior to cocultivation.

Cocultivation

A loopful of *Agrobacterium* was inoculated in 3 ml of AB medium (Chilton et al., 1974) containing kanamycin (100 mg/L) and grown at 28°C in a rotary shaker at 175 rpm. From the overnight culture, 0.5 ml was transferred to 30 ml AB liquid medium with kanamycin (100 mg/L) and was allowed to grow for 12 h to 0.5 to 0.6 OD (optical density) at 600 nm. The culture was centrifuged at 4000 rpm for 10 min and the pellet was resuspended in 20 ml of AAM medium (Hiei et al., 1994) containing 100 µM acetosyringone. After preculture, the embryogenic calli were treated with the bacterial suspension by shaking vigorously for 30 min. The bacterial suspension was then removed using pipette and the calli were blot dried on sterile tissue paper towels. The nearly dry calli were placed on Whatman No.1 filter paper which was overlaid on somatic embryogenic medium containing 100 µM acetosyringone (cocultivation medium). Cocultivation of calli was carried out under the dark for three days.

Selection and regeneration

After cocultivation, the calli were blot dried on sterile tissue paper towels and transferred to Somatic embryogenic medium containing, 50 mg/L kanamycin, 500 mg/L cefotaxime, 30 g/L maltose and 0.4% phytagel. After two months, embryos developed on kanamycin resistant calli were maintained on the same medium till somatic embryos matured. The cotyledonary stage embryos (3 to 5 mm size) were germinated on plant regeneration medium (MS basal slat) with 0.1 mg/L GA_3 + 1.0 mg/L IAA, 30 g/L sucrose and solidified with 0.4% phytagel. The regenerated putative transgenic plantlets attained adequate growth by producing 4 to 6 leaves with sufficient root system; they were removed from the bottles and washed in running tap water carefully to remove the media adhering to roots. They were subsequently transplanted in pots containing sterilized soil. The plants were covered with polyethylene bags and kept in the culture room. After 15 days, the polyethylene bags were removed and well established plants were transferred to transgenic greenhouse.

Polymerase chain reaction (PCR) and Southern blot analysis

Total DNA was extracted from leaves of putative transgenic plants and non transgenic wild type plants following the method described by Permingeat et al. (1998). In the extraction buffer, 0.5 M glucose was added to avoid browning by polyphenolic compounds and improve the quality of DNA samples for further molecular analysis.

PCR was performed as described by Surekha et al. (2005) with a few modifications. Reactions were performed in a final volume of 25 µl and the mixture contained 50 to 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 200 nmM of each of dNTPs, 70 ng of forward and reverse primers and two units of Taq DNA polymerase. Amplification was performed in a MYCycler, (Biorad, USA). The primer used for amplification for *cry1EC* gene was Forward primer 5' CCA GAG AAC GAG ATC TTG GAC 3', and Reverse primer 5' AGT ATT GTA CCA TCT AAC AGC GTA 3' and for amplifying *nptII* gene, the sequence was 5' ATG ATT GAA CAA GAT GGA TTG CACG 3', 5' TCA GAA GAA CTC GTC AAG AAG GC 3'. PCR amplification condition consist of pre-incubation at 94°C for 5 min leading to 30 cycles of melting at 94°C for 1 min, annealing at 55°C for 1 min, and synthesis at 72°C for 1 min followed by an extension at 72°C for 15 min. After amplification, 10 µl of the product was used for electrophoretic analysis on 0.8 % agarose gel. For southern analysis, 10 µg of genomic DNA extracted from putative transformants, untransformed control plant and 10 ng of plasmid DNA were digested with the *Eco*RI and *Hind*III enzyme, electrophoresed on 1% agarose gel and transferred onto a nylon membrane and probed with *cry1EC* gene. All standard procedures were followed as described by Sambrook et al. (1989).

Insect bioassay

Detached leaf feeding tests were done on T_0 plants and non-transformed (control) plants for insect resistance using 1st instar larvae of the pest *Spodeptera litura*. Leaves collected from the transgenic plants, were washed thoroughly using double distilled water to remove any fungicide, pesticide or dirt, blot dried, and were placed in sterile Petri dishes on a wet filter paper. Two replications were carried out for each transgenic plant individually. Five larvae were released into each Petri dish and observations were made every 24 h regarding the mortality, and growth of the larvae. After 3 days, insect mortality % age was calculated as given below:

$$\text{Mortality (\%)} = \frac{\text{Number of larvae dead}}{\text{Total Number of larvae released}} \times 100$$

Analysis of T_1 plants

To test the functional expression of the *nptII* gene in the T_0 progeny a germination test performed. Seeds collected from T_0 plants and

untransformed plants were germinated on the medium containing 75 mg/L kanamycine. PCR analysis was carried out in the germinated seedling to check the presence of transgene.

RESULTS AND DISCUSSION

Transformation, selection and regeneration

Genetic transformation in cotton has so far resulted in the development of commercially successful insect and herbicide resistant cultivars (Perlak et al., 1990; Rajasekaran et al., 1996 and Keller et al., 1997). *Agrobacterium*-mediated transformation followed by regeneration *via* somatic embryogenesis remains the method of choice (Wilkins et al., 2004). This method is, by far, the most efficient means for generating transgenic upland cotton in most of the laboratories (Leelavathi et al., 2004; Light et al., 2005; Jin et al., 2006). It is a multi-step process involving labour intensive work over a 10 to 12 month period starting from cocultivation of *Agrobacterium* with cotyledonary or hypocotyl explants, followed by production and maintenance of several hundreds of calli derived from independent transformation events on a selection medium, induction of embryogenesis in each callus line and development and germination of somatic embryos into normal plants. The above mentioned hurdles have been overcome partially by the use of embryogenic calli with a defined embryogenic potential as the explants source for transformation. The embryogenic calli offers a large population of cell competent for the *Agrobacterium* transformation, resulting in a high frequency of transformation; such a high frequency of transformation is accompanied by the shortening of the culture period to 3 to 5 months as compared to the conventional transformation method (Leelavathi et al., 2004; Wu et al., 2005; Jin et al., 2006). Based on this background, *Agrobacterium* transformation method was followed to transform the Coker genotypes in the present study. Six month-old highly friable embryogenic calli derived from cotyledonary explants were used for *Agrobacterium* mediated transformation.

The aminoglycoside, kanamycin has been extensively used as a selective antibiotic in transformation experiments, mainly because several plant transformation vectors include neomycin phosphotransferase II (*nptII*) gene as a selectable marker. Only transformed cells can grow in the presence of kanamycin. Barring a few reports, most of the workers on cotton have used kanamycin as a selection agent (Wu et al., 2005; Jin et al., 2005; Jin et al., 2006). In the present study, the minimum lethal concentration of kanamycin on cotyledon, hypocotyls and embryogenic calli explants was 50 mg/L. Higher level of kanamycin (75 and 100 mg/L) killed most of the explants. Therefore, a concentration of 50 mg/L kanamycin was used to select transformed cells or tissues in this investigation. Zhao et al. (2006) described the development of method for the recovery of genetically transformed cotton *via Agrobacterium* mediated transformation without using any antibiotics, while most workers used kanamycin as antibiotic (Tohidfar et al., 2005). During the first selection on SEM3K50 medium, the cocultivated calli become dark brown or black. Fresh growth was observed from the kanamycin resistant calli in the third selection while non transformed calli did not produce new calli and dried off. The newly formed calli were selected and subcultured. The globular somatic embryos were formed on the kanamycin resistant calli after one month of culture. The embryos developed on kanamycin resistant calli matured and germinated normally, while the non-transformed embryos became brown and subsequently died. In the present study, out of 30 embryogenic calli explants cultured in the first experiment, 10 plants were regenerated at a regeneration frequency of 33.3 % while in the second experiment, out of the 42 embryogenic calli cocultivated, five plants were regenerated at a regeneration frequency of 11.9% and in subsequent experiment, the regeneration frequency was 14.1 and 5.0 and the transformation efficiency of 6.6, 7.1 and 2.5% was recorded in the first, second and third experiment respectively. The number of calli co-cultivated and number of putative transgenic plants regenerated are summarized in the Table 1 and Figure 2.

Molecular confirmation and insect bioassay

Transformation with different *cry* genes developed for resistance against a variety of insect pests has been reported earlier in other crops. Chickpea expressing *cry1A(c)* show resistance to pod borer (*Helicoverpa armigera*) (Sanyal et al. 2005). Pigeonpea expressing *cry1EC* show resistance to tobacco caterpillar (*Spodoptera litura*) (Surekha et al. 2005). Transformation with different *cry1EC*genes has been reported earlier in cotton (Kumar et al., 2009). Putative transgenic plants were subjected to various molecular analysis techniques to determine the presence of gene of interest in the putative transgenic plants and its expression. 50 plants were shifted to sterile soil in which only 10 plants survived till maturity. Their DNA samples were used in PCR to identify their transgenic nature. The results indicate that, eight plants were PCR positive and showed amplification of the expected 800 bp of the *nptII* gene with *nptII* specific primers (Figure 3) and 578 bp of *cry1EC* gene (Figure 4). No amplified product was detected from non transformed plant and the transformation efficiency measured as % of confirmed transgenic plant out of total number of plants raised and it was found to be 80% in T_0 generation.

Transgenic plant showing highest larval mortality rate has been selected for southern blotting. In Southern blot hybridization experiment incubation of the membrane in

Table 1. *Agrobacterium* mediated transformation in Coker 310.

Experiment number	Number of calli cocultivated	Frequency of explant survival (%)	Frequency of somatic embryogenesis (%)	Number of plants regenerated	Regeneration frequency (%)	Number of plants positive for PCR (*cry1Ec*)	Transformation frequency (%)
1	30	50.0	26.6	10	33.3	2	6.6
2	42	59.5	33.3	5	11.9	3	7.1
3	120	41.6	28.3	17	14.1	3	2.5
4	118	46.6	38.1	6	5.0	-	-
5	32	40.6	28.1	12	37.5	-	-
6	46	54.3	32.6	-	-	-	-
7	89	22.4	31.4	-	-	-	-
Mean	-	45.0	31.2	-	-	-	-

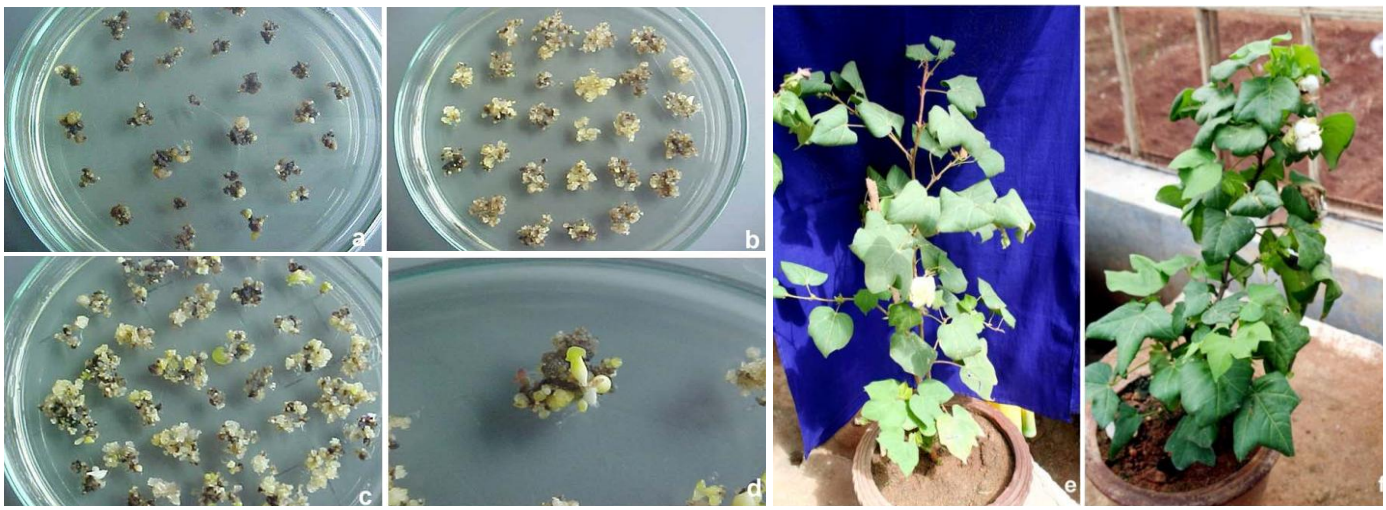

Figure 2. *Agrobacterium* mediated transformation at Coker 310 using embryogenic calli as explants. **a,** Embryogenic calli after cocultivation. **b,** Fresh growth of embryogenic calli on selection medium. **c,** Globular stage embryos development on SEMK25. **d,** Maturation and germination of somatic embryos on SEMK25. **e,** regenerated plant. **f,** Fertile transgenic plants with flowering and boll setting.

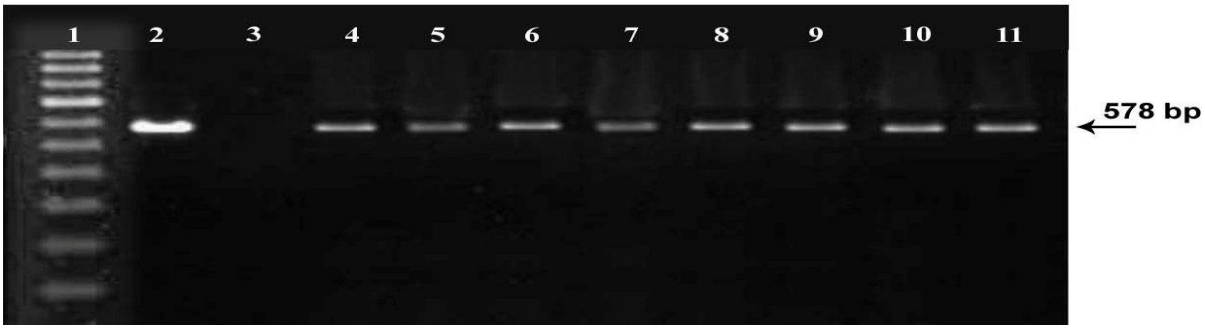

Figure 3. PCR amplification for c*ry1EC* gene in transgenic cotton plants. Lane 1, 100 bp ladder; lane 2, plasmid DNA (positive control); lane 3, non transgenic control plant- Coker 310; lane 4 -11, putative transgenic plants.

solution containing radiolabelled *cry1EC* specific probe resulted in hybridization signal in transgenic plants while such signal could not detected in non-transformed control plants. The hybridization signal could be detected in plasmid

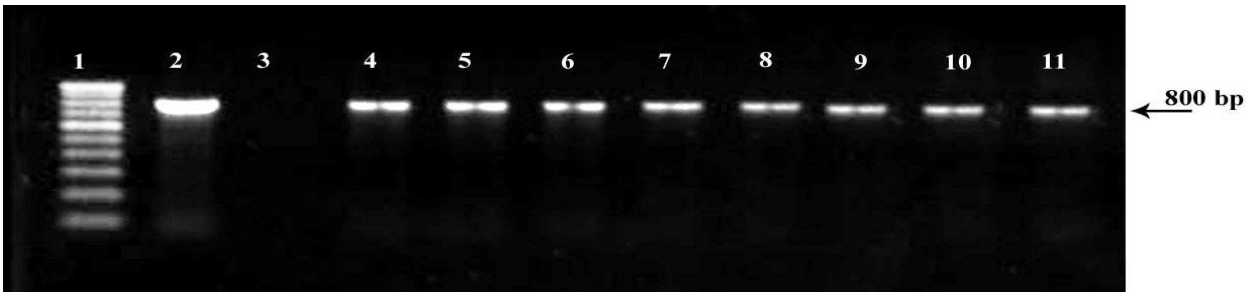

Figure 4. PCR amplification for *nptII* gene in transgenic cotton plants. Lane 1,100 bp ladder; lane 2, plasmid DNA (positive control); lane 3, non transgenic control plant- Coker 310; lane 4 -11, putative transgenic plants.

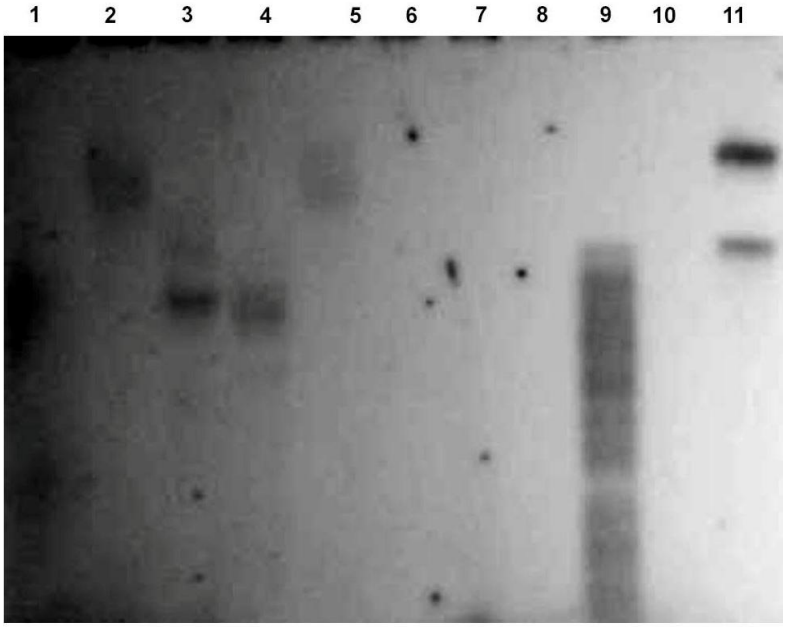

Figure 5. Southern blotting. Lane 1, MW (Lamda Hind III); lane 2, uncut genomic DNA of transgenic plant; lane 3, genomic DNA of transgenic plant digested with EcoR1; lane 4, genomic DNA of transgenic plant digested with Hind III; lane 5, uncut genomic DNA of transgenic plant, lane 6, blank, lane 7, genomic DNA of non transgenic control plant digested with Hind III; lane 8, blank; lane 9, plasmid control digested with Hind III; lane 10, blank; lane 11, uncut plasmid control.

control as well. In the present study, PCR and southern blot hybridization analysis confirmed the stable integration of plasmid DNA into plant genome (Figure 5).

The transforment possessed a single copy insertion. The toxicity of T_0 plants expressing *cry 1EC* protein was tested using feeding bioassays with first instar larvae of *S. litura*. PCR confirmed positive plants with healthy plant morphology were subjected to feeding. Mortality and loss in weight/retardation in growth were recorded for assessing the effect of the protein on the larvae. All the experiments were conducted along with a non-transformed cotton plant for comparison. Bioassay on detached leaves showed significant variation from the control wild-type plants (Table 2). The highest mortality of the larvae found in the transgenic plants was 70 %. Singh et al. (2004), Surekha et al. (2005) and Tiwari et al. (2008) recorded 100% mortality. All the larvae survived on the control plant.

The larvae survived on transgenic plant showed a greater reduction in size and loss of weight. Sujatha et al. (2009) observed varying degrees of larval mortality and slow growth in larvae fed on transgenic leaf tissue of castor. The growth of surviving larvae was also found inhibited. This could be because of lower level of expression of the synthetic gene. It is possible that effective plants could be recovered with the develop-

Table 2. Insecticidal activity of transgenic plants containing *cry1EC* gene to *Spodeptera litura.*

Transgenic plant	% mortality (mean ± SD)
1	70 ± 0.2[a]
2	50 ± 0.2[abc]
3	70 ± 0.2[a]
4	60 ± 0.2[ab]
5	50 ± 0.2[abc]
6	30 ± 0.2[c]
7	40 ± 0.2b[c]
8	30 ± 0.2[c]
Non transgenic –control plant	0 ± 0.2[d]

Insect bioassays were performed with *S. litura* first instar larvae. The readings were taken after 3 days of feeding on the leaves of transgenic T_0 plants.

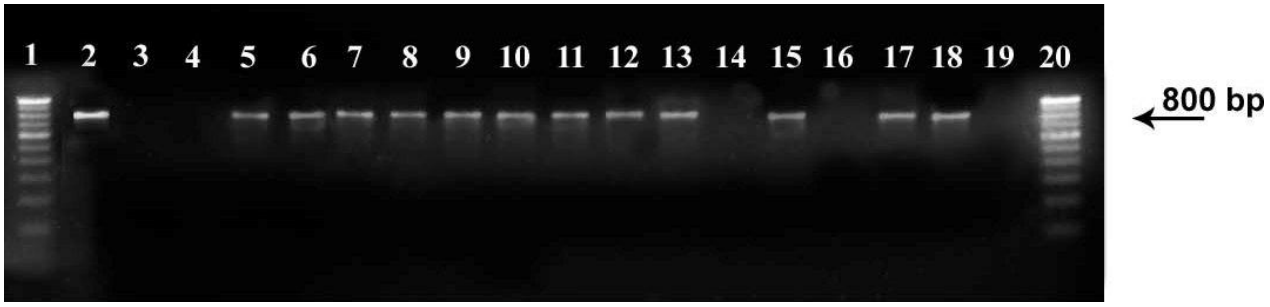

Figure 6. PCR amplification for *cry1EC* gene in T_1 transgenic cotton plants. Lane 1, 100 bp ladder; lane 2, plasmid DNA (positive control), lane 3, non transgenic control plant- Coker 310; lane 4-20, putative T_1 transgenic plants.

ment of more number of independent transgenic plants.

Transgenic inheritance analysis

The selected T_0 plant was fertile and set seeds, but the boll size was much reduced and number of boll was low when compared to non-transformed control plant. T_1 seeds were obtained from the glasshouse-grown T_0 transgenic plants. The inheritance of the introduced genes in the T_1 generation was studied using germination test. A total of 16 T_0 seeds were used for germination, in which 12 seeds were germinated. None of the seeds from non-transformed plants germinated on medium containing 75 mg/L kanamycin. For transformed plants tested, the ratio of resistance to sensitive was 3:1; the expected ratio for single dominant gene. The seedlings were scored for analyzing the segregation of *nptII* reporter gene in the progeny. PCR result also confirmed 3:1 ratio, out of 16 T_1 plants, and 12 plants were found to be positive (Figure 6).

Thus, the development of transgenic cotton against *S. litura* and the use of a hybrid protein with *Cry1E* backbone in a transgenic crop are useful developments. Not much data has been published on the toxicity of the δ-endotoxins to *S. litura*, which is a common pest in warm and humid climates. Efforts are aimed at recovering more efficient transgenic plants through efficient transformation system and developing high resistant transgenic cotton against *S.litura* will paved a way for promising future in cotton production.

REFERENCES

Chaudhary B, Loroia G (2001). Technological developments and cotton production in India and China. Curr. Sci. 80(8):8-25.

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974). *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumours. Proc. Natl. Acad. Sci. USA. 71:3672-3676.

De Maagd RA, Weemen-Hendriks M, Stiekema W, Bosch D (2000) *Bacillus thuringiensis* delta-endotoxin Cry1C domain III can function as a specificity determinant for *Spodoptera exigua* in different, but not all, Cry1-Cry1C hybrids. Appl. Environ. Microbiol. 66:1559-1563.

Eveleens KG, Van Den Bosch R, Dehler LE(1973). Secondary outbreak induction of beet armyworm by experimental insecticide applications

in cotton in California. Environ. Ent. 2:497-503.
Gore J, Leonard BR, Damczyk JJ (2001). Bollworm (Lepidoptera: Noctuidae) survival on 'Bollgard' and 'Bollgard II' cotton flower buds and flower components. J. Econ. Entomol. 94:1445-1451.
Hiei Y, Ohta, S, Komari T, Kumashiro T (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271-282.
Hone´e G, Convents D, Rie JV, Jansens S, Peferoen M ,Visser B (1991) The C-terminal domain of the toxic fragment of Bacillus thuringiensis crystal protein determines receptor binding. Mol Microbiol. 5:2799-2806.
Jin S, Zhang X, Liang S, Nie, Yichun, Guo X, Huang C (2005). Factors affecting transformation efficiency of embryogenic callus of Upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens*. Plant Cell Tiss. Org. Cult. 81:229-237.
Jin S, Zhang X, Nie Y, Guo X, Liang S , Zhu, H (2006). Identification of novel elite genotypes for *in vitro* culture and genetic transformation of cotton. Biol. plant. 50 (4):519-524.
Kumar M, Shukla AK, Singh H, Tuli R(2009). Development of insect resistant transgenic cotton lines expressing *cry1EC* gene from an insect bite and wound inducible promoter. J. Biotechnol. 140(3-4):143-148.
Lambert B, Buysse L, Decock C, Jansens S, Piens C, Saey B (1996). A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family noctuidae. Appl. Environ. Microbiol. 62:80-86.
Leelavathi S, Sunnichan VG, Kumria R, Vijaykanth, GP, Bhatnagar, RK, Reddy VS (2004). A simple and rapid *Agrobacterium*-mediated transformation protocol for cotton (*Gossypium hirsutum*): Embryogenic calli as a source to generate large numbers of transgenic plants. Plant Cell Rep. 22:465-470.
Light GG, Mahan JR, Roxas VP , Allen RD (2005). Transgenic cotton (*Gossypium hirsutum* L.) seedlings expressing a tobacco glutathione S-transferase fail to provide improved stress tolerance. Planta. 222:346-354.
Mazier M, Chaufaux J, Sanchis V, Lereclus D, Giband M and Tourneur J (1997) .The cryIC gene from Bacillus thuringiensis provides protection against *Spodoptera littoralis* in young transgenic plants. Plant Sci. 127:179-190.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:474-497.
Perlak FJ, Oppenhuizen M, Gustafson K, Voth R, Sivasupramaniam S, Heering D (2001) Development and commercial use of Bollgard cotton in the USA – early promises versus today's reality. Plant J. 27:489-501.
Permingeat HR, Romagnoli VM, Vallejos RH (1998). A simple method for isolating high yield and quality DNA from cotton (*Gossypium hirsutum* L.) leaves. Plant Mol. Biol. Rep.11:122-127.
Rajasekaran K, Grula JW, Hudspeth RL, Pofelis S, Anderson DM (1996). Herbicide-resistant Acala and Coker cottons transformed with a native gene encoding mutant forms of acetohydroxy acid synthase. Mol. Breed. 2:307-319.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning. A laboratory manual. II edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
Sanchis V, Lereclus D, Menou G, Chaufaux J, Lecadet MM (1988). Multiplicity of δ- endotoxin genes with different insecticidal specificities in *Bacillus thuringiensis* aizawai. Mol. Microbiol. pp. 393-404.
Sanyal I, Singh AK, Kaushik M, Amla DV (2005). *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis* cry1Ac gene for resistance against pod borer insect *Helicoverpa armigera*. Plant Sci. 168:1135-1146.
Singh PK, Kumar M, Chaturvedi CP, Yadav D, Tuli R (2004). Development of a hybrid δ-endotoxin and its expression in tobacco and cotton for control of a olyphagous pest *Spodoptera litura*. Transgenic Res. 13:397-410.
Strizhov N, Keller M, Mathur J, Koncz-Kalman Z, Bosch D, Prudovsky E (1996). A synthetic cryIC gene, encoding a *Bacillus thuringiensis* δ-endotoxin, confers Spodoptera resistance in alfalfa and tobacco. Proc. Natl. Acad. Sci. USA 93:15012-15017.
Sujatha M, Lakshminarayana M, Tarakeswari M, Singh PK, Tuli R (2009). Expression of the cry1EC gene in castor (*Ricinus communis* L.) confers field resistance to tobacco caterpillar (*Spodoptera litura* Fabr) and castor semilooper (*Achoea janata* L.). Plant Cell Rep. 28:935-946.
Surekha CH, Beena MR, Arundhati A, singh PK, Tuli R, Dutta gupta A, Kirti, PB (2005). *Agrobacterium* mediated genetic transformation of pigeon pea (*Cajanus cajan (L*.) Millsp.) using embryonal segments and development of transgenic plants for resistance against *Spodoptera*. Plant Sci. 169:1074-1080.
Tiwari S, Mishra D K, Singh A, Singh PK, Tuli R (2008). Expression of a synthetic *cry1EC* gene for resistance against *Spodoptera litura* in transgenic peanut *(Arachis hypogaea L.)*. Plant Cell Reports 27(6):1017-1025.
Tohidfar M, Mohammadi M, Ghareyazie B (2005). *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a heterogous bean chitinae gene. Plant Cell Tiss. Organ Cult. 83:83-96.
Visser B, Munsterman E, Stoker A, Dirkse WG (1990). A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua* – specific crystal protein. J. Bacteriol. 172:6783-6788.
Wilkins TA, Mishra R, Trolinder NL (2004). *Agrobacterium*-mediated transformation and regeneration of cotton. Food Agric. Environ. 2:179-187.
Wu J, Zhang X, Nie Y, Luo X (2005). High-efficiency transformation of *Gossypium hirsutum* embryogenic calli mediated by *Agrobacterium tumefaciens* and regeneration of insect-resistant plants. Plant Breed. 124:142-146.
Yousefi (2000). Agrochemical in South Africa. Available: http://ww.occuphealth.fi/e/info/anl/199/agro03.htm.
Yu Y, Kang X, Lu Y, Liang J, Wang H, Wu J, Yang Y (2004). Effects of the transgenic Bt cotton on the increase in population of *Spodoptera litura* Fabricius. Jiangsu J. Agric. Sci. 20:169-172.
Zhao FY, Li YF, Xu P (2006). *Agrobacterium* – mediated transformation of cotton (*Gossypium hirsutum* L. cv. Zhongmian 35) using glyphosate as a selectable marker. Biotechnol. Lett. 28:1199-1207.