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Standarizing in *planta Agrobacterium tumefacience* mediated genetic transformation protocol to develop new events by transforming *G. hirsutum* cotton based on *Cry1Ac-Cry1Ec* genes

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Abstract: Cotton breeding for insect resistance has been limited by a lack of sufficient genetic variation in the existing germplasms. Therefore, genetic engineering provides the possibility of creating varieties carrying new properties coming even from heterologous source. Exogenous pesticidal transgenes can be introduced into plants. *Agrobacterium* mediated plant transformation offers advantages like reducing copy number of the transgene and little co-suppression. Inter specific hybrids are known to be more susceptible to biotic stress. It is hence important to develop *Bt* version for inter specific hybrid. Presently, the *Bt* gene commercialized are owned by private sector. It is necessary to develop public sector's *Bt* event and commercialize them. UAS Dharwad is involved in developing public sector *Bt* cotton genotypes. One variety, RCR4 (*Gossypium hirsutum, L.*) was used in the present investigation. *Cry1Ac-Cry1Ec* genes are to control *Helicoverpa armigera* and *Spodoptera litura*. The seedlings in pots were co-cultivated with solid *Agrobacterium* culture after cutting the meristematic tip with sharp knife. The number of seedlings co-cultivated, number of seedlings established and the number of seedlings showing transformed status are presented in this study. PCR was performed to confirm the presence of the transgene in the plants that were selected to be advanced further. The results showed that non of plants had trangenes *Cry1Ac- Cry1Ec* as detected through PCR amplification. *In planta* genetic transformation was carried out and the plants were tested in T_0 generation by means of PCR amplification for the genes *Cry1Ac- Cry1Ec*. The results obtained were not amplified the *Cry1Ac- Cry1Ec.* Hence the transformation of the genes was not up to mark and the plants of T_1 generation are also not confirmed.

Keywords: Genetic Transformation, Agrobacterium Tumefacience, G. Hirsutum, Cry1Ac-Cry1Ec Genes

1. Introduction

Recent advances in plant molecular biology techniques have given birth to unprecedented opportunities for the introduction of novel traits into crops. The potential impact of these powerful methodologies on the genetic improvement of crop plants of economic importance has generated considerable interest, enthusiasm and optimism in the scientific community and is in part responsible for the rapid expansion of biotechnology industry. The anticipated role of plant biotechnology in agriculture is attributable to the production of genetically superior plants as well as elegant demonstrations in model experimental systems that new hybrids, mutants and genetically engineered plants can be obtained by these methods. The world wide pre-harvest losses due to insect pests despite the use of insecticides are 15% of total production representing over US$ 100 billion (Krattiger and Anatole, 1997).

Genetic transformation requires penetration of the transgene through the plant cell wall, facilitated by biological or physical methods (Electroporation, Biolistics, Vacuum infiltration, Ultrasound-mediated transformation, Shock wave-mediated transformation, Silicon carbide whisker-mediated transformation, Microinjection, Macroinjection, Lasermicrobeams and Electrophoresis). There are various methods available to breed genetic

resistance against insects and pests, these include conventional breeding and transgenic technology. Exogenous pesticidal transgenes can be introduced into plants by *Agrobacterium* mediated transformation. *Agrobacterium* mediated transformation offers advantages like reducing copy number of the transgene, little co suppression (Konez *et al*., 1994; Hansen *et al*., 1997, Enriquez Obregon *et al*., 1998). However, *Agrobacterium tumefaciens* infects naturally only dicotyledonous plants and also many economically important plants including the cereals are also made to infect with difficulty.

Bacillus thuringiensis is a gram positive, rod shaped spore forming soil bacterium. It produces the crystal insecticidal proteins during sporulation called delta endotoxins. These crystal proteins are toxic to larvae of different insects. e.g, Lepidopterans (Krieg *et al*., 1983; Herrnstadt *et al*., 1986) and dipteran insects, causing eventual death of the larvae (Hoftey and Whiteley 1989; Aslam *et al*., 2000). Many different crystal protein genes called *Cry* genes have been isolated and classified on the basis of amino acid sequence homologies. At least 90 genes encoding protoxin from a wide range of *Bt* isolates have been isolated and sequenced (Maizer *et al*., 1997).

Technology for gene delivery plays an important role in the process of plant genetic engineering. Foreign genes are introduced into genome of recipient species either by physical, chemical or biological means. Since the first transgenic plant appeared in 1983, studies on plant transformation techniques have achieved a great progress.

Plant transformation mediated by the soil plant pathogen *Agrobacterium tumefaciens* is simple method for plant transformation. There are two tumorgenic species *i.e. Agrobacterium tumefaciens* and *Agrobacterium rhizogene. Agrobacterium tumefaciens* is a gram negative soil bacterium, causes crown gall tumors (neoplastic disease) on many dicotyledonous and some monocotyledonous plants (Broer *et al*., 1995). During plant infection *A. tumefaciens,* transformed plants by transferring a part of its DNA called transferred DNA (T-DNA) from its tumour inducing (Ti) plasmid to the plant genome. The virulence (Vir) region of the Ti plasmid codes for the function required for processing and transfer of T-DNA (Lyer *et al*., 1982; Stachel and Nester, 1986). The discoveries that T-DNA codes for oncogene which is only transferred to plant cell genome (Bevan *et al*., 1983a) and non virulent or disarmed strains *i.e.* containing T-DNA from which oncogenes have been removed and replaced by any other gene of interest, behave in the same way as virulent strain do, that opened a new avenue in transformation of interested gene to higher plants (Fig. 1).

Katageri *et al.* (2007) reported development of transgenic cotton using shoot apical meristems, which was isolated from seedlings as explant. Synthetic gene encoding *Cry*1*Ac* endotoxin of *Bacillus thuringiensis* was used for transformation. Regeneration of shoots was carried out in selection medium containing kanamycin (100 mg/l) after co-cultivation of the explants with *Agrobacterium tumefaciens*. Progeny obtained by selfing T_0 plants was grown in the greenhouse and screened for the presence of neomycin phosphotransferase (*npt*II) and *Cry1Ac* genes by polymerase chain reaction (PCR) and Southern hybridization. Expression of *Cry1Ac* in the leaves of the transgenic plants was detected by strips and quantified by Quan-T ELISA kits. Insect bioassays were performed with the larvae of cotton bollworm (*Helicoverpa armigera*). Results of the field tests showed considerable potential of the transgenic cotton for resistance against cotton bollworm.

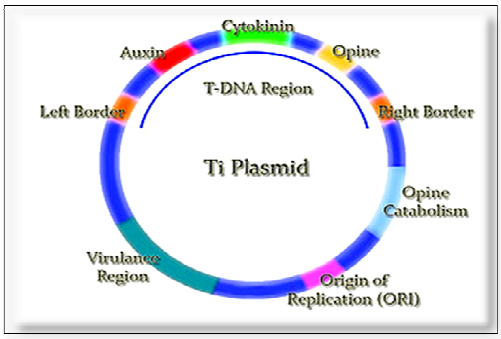

Fig. 1. *Ti Plasmid Construction in Agrobacterium tumefaciens*

The main objective of this study is: Standarizing `in *planta Agrobacterium tumefacience* mediated genetic transformation protocol to develop new events by transforming *G. hirsutum* cotton based on *Cry1Ac-Cry1E*c genes.

2. Materials and Methods

In this study, genotype independent *in planta* transformation of cotton (*G. hirsutum*) variety RCR4, using injection method and cut method has been reported. *Agrobacterium tumefaciens* strain carrying the *npt*II and *Cry1Ac-Cry1Ec* genes was used for plant transformation. The integration of the genes was confirmed by using PCR (T_0 generation).

The present investigations were carried out at Agricultural Research Station, Dharwad farm, University of Agricultural Sciences, Dharwad during 2012.

2.1. Materials

2.1.1. Genotype

One variety, RCR4 (*Gossypium hirsutum, L.*) was used in the present investigation.

- This line is productive line with high Relative Growth Rate (RGR).
- Developed by Dr. S.S.Patil, Senior cotton breeder, Agricultural Research Station, Dharwad farm, University of Agricultural Sciences.
- It is known to be productive in rainfed situation.
- The parentage of this line RAH 100 X RAH 101.

2.1.2. Cry Genes

The genes used for transformation was obtained from

NBRI Luknow and it was spared by Dr. P.K.Singh under NMITLI project funded by Counsil of Scientific and Industrial Research New Delhi, India.

2.2. Methodology

2.2.1. Preparation of Explants

- Delint the genotype seeds by using sulfuric acid (H_2SO_4) were dipped for 10 minutes with constant stirring followed by the repeated washes with water. Keep the seeds under the sunshine to dry completely.
- Delinted and surface sterilized seeds soaked in sterile water over night were used to establish the plants for transformation.

2.2.2. Sowing and Establishment of Plants

The seeds were sown on 1-1-2012 in 200 plastic packets containing sterilized black soil mixed with Bavistin (5 g/1L), 2 seeds were dibbled per packet and thinning was attended to retain one healthy plant per plastic packet at 25 days after sowing. All the recommended package of practices were followed to rise healthy crop.

2.2.3. Agrobacterium Strain and Binary Vectors

The disarmed *Agrobacterium* strain LBA 4402 harbouring binary vector pCAMBIA, carrying *Cry1Ac-Cry1Ec* genes linked to the CaMV35S promoter, the *nos* transcription terminator (amplified from Pb 101.1 with *MfeI* and *EcoRI* restriction sites at the ends) and *npt*-II gene under the control of nopaline synthase (nos) promoter and terminator was used in transformation studies. Hygromycin resistance as selection marker and *Cry1Ac-Cry1Ec* genes are used to control *Helicoverpa armigera* and *Spodoptera litura*.

2.2.4. Maintenance of Agrobacterium

The *Agrobacterium* LBA 4402 containing above mentioned genes was maintained on solid Yeast Extract Mannitol Agar (YEM) medium containing kanamycin at 50 mg/ml and 25 mg/ml rifampicin. It was subcultured once in every 30-40 days on fresh medium and incubated at 28 ^{0}C temperature for 48 hours followed by 4-6 ^{0}C for rest of the period.

2.2.5. Preparation of Agrobacterium Culture for Co-Cultivation

2.2.5.1. Liquid Culture

A colony of bacteria grown for 48 hours was taken from petridish and was inoculated in 150 ml of liquid YEM medium containing 50 mg/l of Kanamycin and 25 mg/l rifampicin and incubated for 45-48 hours at 22 ^{0}C under orbital shaker with 150 rpm. When bacterium growth reached to OD (600 nm) of 0.6 g pellet of bacterium obtained after centrifuge at 8000 rpm for 5 minute. It was resuspended in 150 ml of MS medium and 150 µM of acetosyringone was added to the *Agrobacterium* culture before 30 minute of its use.

2.2.5.2. Solid Culture

A load of bacteria from a stock plate was taken from petridish to streak on a fresh petridish having solid YEMA medium with 50mg/l of Kanamycin and 25 mg/l refampicin. The dish was incubated at 22 ^{0}C for 48 hours in dark. The bacteria were carefully collected after 48 hours into a 2 ml new autoclaved eppendorf tube and 100 µM Acetosyringone was mixed before 30 minute of its use by vortexing for few seconds.

2.2.6. Transfer the Genes to the Plant tissue and Establishment in the Greenhouse

1. The seedling shoot is embedded in the stem between the cotyledons, break off one cotyledons to expose shoot apex and cut the tissue in the shoot apex to make wound (Fig. 2).
2. The disarmed *Agrobacterium* strain LBA 4402 harbouring binary vector pCAMBIA, carrying *Cry1Ac-Cry1Ec* genes linked to the CaMV35S promoter, *nos* terminator and *npt* II gene transfer to the injuried tissue on 01/2/2012 by using micropeppite (Plate 1).
3. Seedlings covered with plastic bags to maintain high humidity (Plate 1).
4. They were watered twice a week. Seedlings were then transferred to earthen pots on 5/3/2012 containing soil and vermicompost in equal proposition and pots were placed in transgenic green house for flowering.

2.2.7. Screening Through PCR

The putative transformants generated through *in vivo* genetic transformation were subjected to PCR analysis.

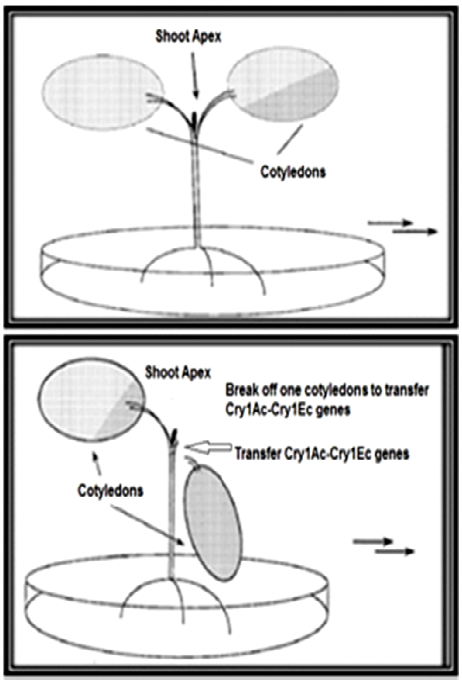

Fig. 2. *Transfer the genes (Cry1Ac- Cry1Ec) to the plant tissue and establishment in the greenhouse*

A

B

C

D

Plate 1. *Transfer the genes (Cry1Ac- Cry1Ec) to the plant tissue and establishment in the greenhouse*

2.2.7.1. DNA Extraction

DNA extraction was done with CTAB method with few modifications.

1. One gram of fresh 2nd or 3rd top most leaf was taken for DNA extraction, leaf was harvested and kept in the 1.50 ml eppendorf tube.
2. By using extraction buffer leaf samples were crushed and kept in water bath for 45 minute at 65 ^{0}C.
3. After 45 min, samples were taken out from water bath. The solution was centrifuged at 8,000 rpm for 10 minutes and supernatant was transferred to fresh eppendorf tube.
4. Supernatant was mixed with 500 µl of phenol: chloroform solution and centrifuged at 8,000 rpm for 10 min.
5. By using micropipette, supernatant was collected and transferred to new eppendorf tube. To that eppendorf tube, 500 µl chloroform solution was added and centrifuged at 8,000 rpm for 10 min.
6. Once again supernatant was collected to new eppendorf tube and 800 µl isopropanol was added and kept at 20 ^{0}C for precipitation for 1-2 hours.
7. After 2 hours, tubes were removed from the deep freezer and kept out side to attain room temperature. After that, centrifuged at 5,000 rpm for 5 min.
8. Supernatant was decanted without disturbing the pellet. To that, 70% alcohol was added and centrifuged for 5,000 rpm for 5 min. After centrifuge alcohol was decanted without disturbing the pellet.
9. Pellet was dried and suspended in 100 µl of 1 X $T_{10}E_1$ buffer.
10. 100 µl RNase was added (1 mg/ml) to the DNA and incubated at 37 ^{0}C in water bath for half an hour.
11. DNA was precipitated using 1/10th volume of 3 M sodium acetate and ethanol and incubated over night at 4 ^{0}C.
12. The solution was centrifuged at 13,000 rpm for 2 min and pellet was dried again.
13. Pellet was suspended in 50 µl 1X $T_{10}E_1$ buffer.

2.2.7.2. Estimation of Quality and Quantity of DNA

The concentration and quality of DNA was assessed spectrophotometrically. It was also assessed with 0.8 % agarose gel.

In spectrophotometric analysis, 5 µl of DNA sample diluted with TE buffer and volume made upto 3000 µl was subjected to spectrophotometer reading at absorbance of 230 nm, 260 nm and 280 nm. A good DNA preparation generally exhibits the following spectral property: A260/A280 > 1.80 DNA concentration was calculated using following formula. Concentration of DNA (µg/ml) = O.D. at 260 x 50. To test the quality and quantity of DNA, samples were run on 0.80 per cent agarose in 1x TAE (Tris Acetic acid EDTA) buffer and stained with ethidium bromide and checked for contamination by RNA and the DNA was evaluated by comparing it with a standard

undigested DNA sample.

2.2.7.3. Plasmid Isolation Procedure

1. Inoculate 10 ml media with suitable antibiotic and inculate with shaking at 3 ^{0}C overnight.
2. Take 1.5 ml of culture in fresh tube and pellet at 13,000 rpm for 1 minute.
3. Discard the supernatant and take again 1.5ml of culture in fresh tube and pellet at 13,000 rpm for 1 minute.
4. Resuspend the pellet in 150 µl of ice cold solution I by vortexing.
5. Add 250 µl of freshly prepared solution II and mix by inventing for 5 times and keep in ice for 5 minutes (don't vortex).
6. Add 200 µl of solution III, mix by vortexing and keep in ice for 4-5 minutes.
7. Spin at max speed (13,000 rpm) for 10 minutes at 4 ^{0}C.
8. Transfer supernatant to fresh tube and equal volume of chloroform: Isomyl Alcohol (24:1) and mix by vortexing.
9. Centrifuge at max speed at 4 ^{0}C for 10 minutes.
10. Transfer aqueous phase to fresh tube.
11. Add 2 volume of pre chilled absolute isopropanol and mix by inverting and incubate at room temperature for 5 minutes.
12. Pellet the DNA at 13,000 rpm for 10 minutes.
13. Wash the pellet with 70% ethanol (90 µl) at 13,000 rpm for 3 minutes.
14. Dry the pellet and dissolve in 20-25 µl of $T_{10}E_1$ or sterile water.
15. Add Rnase at 20 mg/ µl.

2.2.7.4. PCR Amplification

DNA extracted from leaves of transformants was used as template DNA, Taq DNA polymerase (Bangalore Genei), Taq Buffer (Bangalore Genei), dNTPs (Bangalore Genei), $Mgcl_2$ (Bangalore Genei) and Primers were used for cyclic amplification of DNA. Following *Cry1Ac-Cry1Ec* specific primers were used for confirming transgenic.

Sequence of *Cry1Ac-Cry1Ec* primers:

Forward 5^1 CCAGAGAACGAGATC TTGGAC 3^1

Reverse 3^{1}AGTATTGTACCATCTAACAGCGTA 5^1

Sequence of *npt*-II primers:

Forward 5^1 GAG GCD ATT CGG CTA TGA CTG 3^1

Reverse 3^1 ATC GGG AGG GGC GAT ACC GAT 5^1

The PCR mix was made fresh in bulk depending on the number of samples each time. Each 20 µl mix contained:

Taq polymerase	0.33 µl
Taq Buffer	2.5 µl
dNTPs	1 µl
$MgCl_2$	0.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Template	1 µl
SDDW	12.67 µl
Total	20 µl

The PCR amplification steps were as follows

Stage	Step	Temperature (C^0)	Duration (min)	No. of cycle
I	1. Initial Denaturation	94	5	1
II	1. Denaturation	94	1	39
	2. Annealing	70	1	
	3. Extension	72	1	
III	1. Final Extension	72	10	1
	2. Hold	4	-	-

After the completion of required cycles of amplification, the samples were stored at 4 ^{0}C in a refrigerator until further use. Here, for amplification of plasmid we standardized the protocol by using 6 temperatures (60.6 ^{0}C, 62.3 ^{0}C, 57.2 ^{0}C, 56.5 ^{0}C, 55.0 ^{0}C and 54.1 ^{0}C) to find out which temperature is suitable for amplification.

2.2.7.5. Agarose Gel Electrophoresis of DNA

1. Sufficient 1x electrophoresis buffer was prepared from 50 x stock.
2. Agarose powder was added (1%) to TAE buffer (1x) and was dissolved by melting at 100 ^{0}C. The solution was cooled to 50 ^{0}C and ethidium bromide was added (0.5 µg/ml) and the comb was positioned at 0.5-1.0 mm above the plate. Then agarose solution was poured into the gel frame and was allowed to polymerize. The gel tank was filled with TAE buffer (1x) just enough to cover the surface of the gel to a depth of 1 mm.
3. The DNA sample was mixed with gel loading buffer and it was slowly loaded into the wells of the submerged gel using a disposable microtips. DNA /Eco RI + Hind II double digest were used as molecular weight marker.
4. The system was connected to the power supply and electrophoresis was carried out at 100 volts for 30-45 min.
5. It was examined by gel documentation system.

3. Results and Discussion

Cotton breeding for insect resistance has been limited by a lack of sufficient genetic variation in the existing germplasms. Therefore, genetic engineering provides the possibility of creating varieties carrying new properties coming even from heterologous source (Lycett and Grierson 1990; Dhaliwal *et al.,* 1998). Exogenous pesticidal transgenes can be introduced into plants. *Agrobacterium* mediated plant transformation offers advantages like reducing copy number of the transgene and little co-suppression (Konez *et al.,* 1994; Hansen *et al.,* 1997).

3.1. Agrobacterium Strain and Binary vectors

The disarmed *Agrobacterium* strain LBA 4402 harbouring binary vector pCAMBIA, carrying *Cry1Ac-Cry1Ec* genes linked to the CaMV35S promoter, the *nos*

transcription terminator (amplified from Pb 101.1 with *MfeI* and *EcoRI* restriction sites at the ends) and *npt*-II gene under the control of nopaline synthase (nos) promoter and terminator was used in transformation studies. Hygromycin resistance as selection marker. *Cry1Ac*-*Cry1Ec* genes is to control *Helicoverpa armigera* and *Spodoptera litura*.

The *Agrobacterium* LBA 4402 containing above mentioned genes was maintained on solid Yeast Extract Mannitol Agar (YEM) medium containing kanamycin at 50 mg/ml and 25 mg/ml rifampicin. It was subcultured once in every 30-40 days on fresh medium and incubated at 28 ^{0}C temperature for 48 hours followed by 4-6 ^{0}C for rest of the period.

A colony of bacteria grown for 48 hours was taken from petridish and was inoculated in 150 ml of liquid YEM medium containing 50 mg/l of Kanamycin and 25 mg/l rifampicin and incubated for 45-48 hours at 22 ^{0}C under orbital shaker with 150 rpm. When bacterium growth reached to OD (600 nm) of 0.6 g pellet of bacterium obtained after centrifuge at 8000 rpm for 5 minute. It was resuspended in 150 ml of MS medium and 150 µM of acetosyringone was added to the *Agrobacterium* culture before 30 minute of its use.

3.2. Designing and Chemical Synthesis of the Chimeric Cry1 Ac and Cry1EC Genes

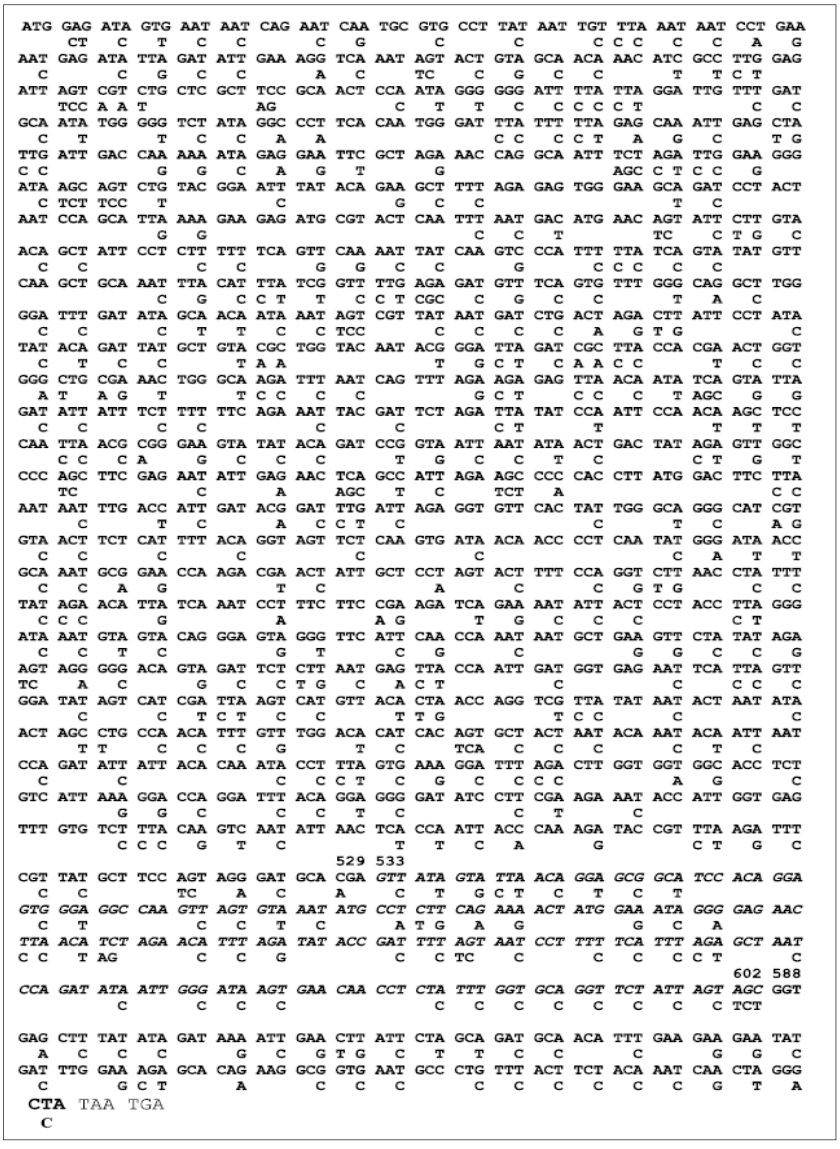

Fig. 3. *Designing of the gene coding for chimeric δ-endotoxin Cry1Ec*

A 1.9 kbp double stranded DNA was theoretically designed (Fig. 3) by modifying the sequence of natural *Cry1Ea* gene, to encode the chimeric d-endotoxin *Cry1EC*. Plant-preferred translation initiation context (Sawant *et al*., 2001) and codons were used in designing the gene. The putative transcription termination signals (AAUAAA and its variants), mRNA instability elements (ATTTA) and potential splice sites were eliminated and long hairpin loops were avoided. The whole sequence was synthesised as 58 overlapping oligonucleotides.

The oligonucleotides were synthesised on Gene Assembler Special DNA synthesiser, purified on urea –

polyacrylamide gel and assembled in four parts (Fig. 4) by assembly polymerase chain reaction (Singh *et al*., 1996). Eight oligonucleotides were assembled into a BamHI – XhoI fragment (340 bp long), 12 into a XhoI – AccI fragment (511 bp long), 24 into a AccI – ApaI fragment (680 bp long) and 14 into a ApaI – EcoRI fragment (519 bp long). These were cloned in pBluescript SK+ cloning vector.

At least four clones were sequenced in each case to locate the errors in synthesis. Sequencing was done on model 373 automatic DNA sequencer using fluorescent – dye termination cycle sequencing kit. The errors were corrected by exchanging the regions containing mutations with those from correct clones. Finally, the error-free DNA fragments were stepwise ligated to give about 1.9 kbp full length gene.

Cry1Ac protein (endotoxin) produced in *Bt* transgenic cotton is effective in controlling *Helicoverpa armigera*. The *Cry1Ac* (1915bp) gene cloned earlier from native *B. thuringiensis* at UAS, Dharwad was considered for modification (Fig. 5) (Kumaraswamy, 2005). The purpose of redesigning *Cry1Ac* was to create a synthetic gene that would be expressed at high level in plant cells.

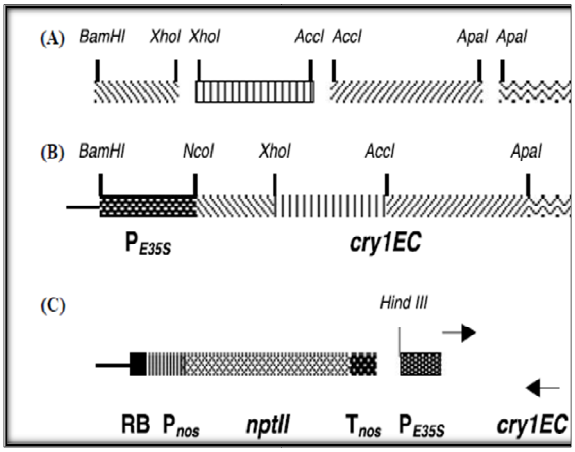

Fig. 4. *Construction of Cry1Ec for plant transformation*

GGCGAATTGGGTACCGGATCCATGGATAACAACCCAAACATTAACGAGTGCATTCCATACAACTGCTTGAGCAACCC
AGAGGTTGAGGTTCTTGGAGGAGAGCGCATTGAGACCGGATACACTCCCATCGACATCTCCTTGTCCTTGACTCAG
TTCCTCCTCAGCGAGTTCGTGCCAGGAGCTGGGTTCGTTCTCGGACTTGTTGACATCATCTGGGGAATCTTCGGAC
CATCTCAATGGGACGCCTTCCTCGTGCAAATTGAGCAGTTGATCAACCAGAGGATCGAGGAGTTCGCCAGGAACCA
GGCCATCTCTAGGTTGGAGGGATTGAGCAACCTCTACCAAATCTACGCTGAGAGCTTCAGAGAGTGGGAGGCCGA
TCCAACTAACCCAGCTCTCCGCGAGGAAATGCGTATTCAATTCAACGACATGAACAGCGCCTTGACCACTGCTATCC
CATTGTTCGCCGTGCAGAACTACCAAGTTCCACTCTTGTCCGTGTACGTTCAAGCTGCTAACCTTCACCTCAGCGTG
CTTCGTGACGTTAGCGTGTTCGGCCAAAGGTGGGGATTCGATGCTGCAACCATCAACAGCCGTTACAACGACCTTA
CTAGGCTCATTGGAAACTACACCGACCACGCTGTTCGTTGGTACAACACTGGCTTGGAGCGTGTCTGGGGACCCGA
TTCTAGAGATTGGATCAGGTACAACCAGTTCAGGAGAGAGTTGACCCTCACTGTTTTGGACATTGTGTCTCTCTTCC
CCAACTACGACTCCAGAACCTACCCTATCCGTACTGTGTCCCAACTTACCAGAGAGATCTACACTAACCCAGTTCTTG
AGAACTTCGACGGTAGCTTCCGCGGATCTGCTCAGGGCATCGAGGGCTCCATCAGGAGCCCACACTTGATGGACAT
CTTGAACAGCATAACTATCTACACCGATGCTCACAGAGGAGAGTACTACTGGTCTGGACACCAGATCATGGCCTCTC
CAGTTGGATTCAGCGGGCCCGAGTTCACCTTCCCACTCTACGGAACTATGGGAAACGCCGCTCCACAACAACGTAT
CGTTGCTCAACTTGGACAGGGAGTCTACAGGACCTTGTCTTCCACCTTGTACAGAAGGCCCTTCAACATCGGAATC
AACAACCAGCAACTTTCCGTTCTTGACGGAACTGAGTTCGCCTACGGAACCTCTTCCAACTTGCCATCCGCTGTTTA
CAGAAAGAGCGGAACCGTTGATTCCTTGGACGAGATCCCACCACAGAACAACAATGTGCCACCCAGGCAAGGATT
CTCCCACAGGTTGAGCCACGTGTCCATGTTCCGTTCCGGATTCAGCAACAGTTCCGTGAGCATCATCAGAGCCCCTA
TGTTCTCTTGGATTCACCGTTCTGCCGAGTTCAACAACATCATCGCTTCTGATAGCATTACTCAGATCCCAGCCGTGA
AGGGAAACTTCCTTTTCAACGGAAGCGTTATCAGCGGACCAGGATTCACTGGCGGAGACCTTGTGAGACTTAATA
GCTCTGGCAACAACATTCAGAATAGAGGCTACATCGAGGTTCCTATCCACTTCCCATCCACATCTACTAGATATAGAG
TTAGGGTTAGATACGCCTCTGTGACCCCAATCCACCTTAACGTGAACTGGGGCAACTCATCTATCTTCTCCAACACCG
TTCCAGCTACTGCTACCTCTCTTGATAACCTTCAATCCAGCGATTTCGGATACTTCGAGAGCGCCAACGCTTTCACTT
CTTCCTTGGGCAACATCGTGGGAGTTAGGAACTTCAGCGGTACTGCAGGAGTGATCATTGACAGATTCGAGTTCAT
TCCAGTTACTGCCACTCTTGAGGCTGAGTACAACCTTTAAGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATT

Fig. 5. *Gene Sequence of M Cry1Ac (1915bp)*

3.3. PCR Amplification

DNA extracted from leaves of transformants was used as template DNA. Taq DNA polymerase, Taq Buffer, dNTPs, Mgcl2 and *Primers* were used for cyclic amplification of DNA. Following *Cry1Ac-Cry1Ec* specific primers were used for confirming transgenic.

Sequence of *Cry1Ac-Cry1Ec* primers:

Forward 5^1 CCAGAGAACGAGATC TTGGAC 3^1

Reverse 3^1 AGTATTGTACCATCTAACAGCGTA 5^1

Sequence of *npt*-II primers:

Forward 5^1 GAG GCD ATT CGG CTA TGA CTG 3^1

Reverse 3^1 ATC GGG AGG GGC GAT ACC GAT 5^1

After the completion of required cycles of amplification, the samples were stored at 4 ^{0}C in a refrigerator until further use. Here, for amplification of plasmid we standardized the protocol by using 6 temperatures (60.6 ^{0}C, 62.3 ^{0}C, 57.2 ^{0}C, 56.5 ^{0}C, 55.0 ^{0}C and 54.1 ^{0}C). The results showed that the plasmid has amplified at four temperatures (57.2 ^{0}C, 56.5 ^{0}C, 55.0 ^{0}C and 54.1 ^{0}C) (Plate 2).

3.4. In Planta Genetic Transformation

Several methods of injuring the plant target tissue for transformation and regeneration were examined in an effort to improve the efficiency of production of transgenic cotton. *Agrobacterium tumefaciens* strain carrying *Cry1 Ac-Cry1Ec* was used for transformation of RCR4 genotype. The effect of wounding on established seedling, effect of vertical cut on well established seedling and regeneration was studied.

The seedlings in pots were co-cultivated with solid *Agrobacterium* culture after cutting the meristematic tip with sharp knife. The number of seedlings co-cultivated, number of seedlings established and the number of seedlings showing transformed status are presented in Table 1. PCR was performed to confirm the presence of the transgene in the plants that were selected to be advanced further. The results showed that non of plants had trangenes *Cry1Ac- Cry1Ec* as detected through PCR amplification.

In planta genetic transformation was carried out and the plants were tested in T_0 generation by means of PCR amplification for the genes *Cry1Ac- Cry1Ec*. The results obtained were not amplified the *Cry1Ac- Cry1Ec.* Hence the transformation of the genes was not up to mark and the plants of T_1 generation are also not confirmed.

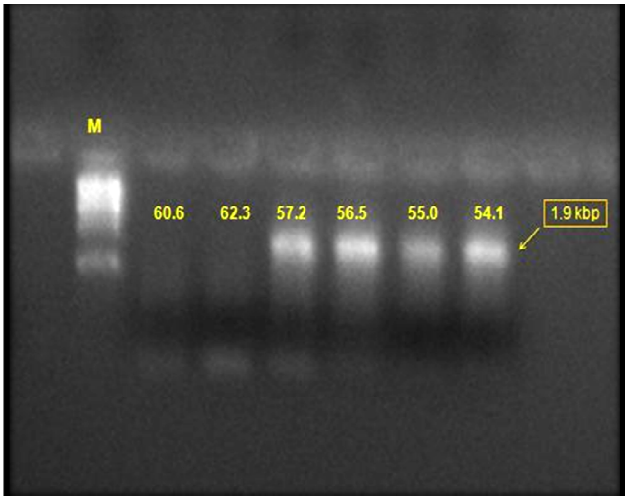

Plate 2. *Plasmid amplification at four temperature*

Table 1. *Number of cotton seedlings co-cultivated, established and transformed in planta genetic transformation study*

Genotype	Group	Total number of seedlings	Number of seedlings co-culivated	Number of plants established	Number of plants transformed
RCR4	Group 1	100	92	81	Nil
	Group 2	111	97	88	Nil
	Group 3	117	87	75	Nil
	Group 4	90	77	62	Nil
Total		418	353	306	Nil

4. Discussion

Genetic engineering offers a directed method of plant breeding that selectively targets one or a few traits for introduction into the crop plant. The first transgenic cotton plants were obtained in 1987 (Umbeck *et al.*, 1987). The deployment of endotoxins for the protection of crops against insect pests requires the availability of proteins that cause high toxicity to target pests at low level of expression. It is also important to develop novel toxins that bind to different receptors and can therefore be valuable in pyramiding for durable pest resistant. Since crops are damaged by a variety of herbivores, it is desirable to identify endotoxins that can cause mortality or growth inhibition of different target pest species. In this respect, the development of *Cry1Ec* in this work is important since both Spodoptera and Helicoverpa are common agricultural pests and serious bollworms of cotton. Several proteins, especially *Cry1Ac* cause very high toxicity to Helicoverpa sp. Other proteins with satisfactory activity against Helicoverpa are *Cry1Ab, Cry1Ia$_5$, Cry2Ab2* etc. (Chambers *et al.,* 1991; von Tersch *et al*., 1991; Lambert *et al.,* 1996; Selvapandiyan *et al.*, 1998; Perlak *et al*., 2001). The *Cry1C* has been reported to show significant toxicity to Spodoptera sp. (Hone´e *et al.,* 1988; Hofte and Whiteley, 1989; Gill *et al*., 1992; Kalman *et al*., 1993) at reasonably low level in larval diet. Within the subgroup of *Cry1 d*-endotoxins, a few other proteins that resemble *Cry1C* (Visser *et al*., 1990; Kalman *et al*., 1993) and *Cry1F* (Chambers *et al.,* 1991) have been reported to show toxicity to Spodoptera sp., though at relatively higher LC_{50} values. The *Cry1E* resembles the *Cry1C* subgroup to the extent of about 70%. It does not cause significant toxicity to *S. exigua* (Visser *et al*., 1990; Masson *et al*., 1992; Bosch *et al.,* 1994) but appears to bind to receptors different from *Cry1C.* Therefore, modification of *Cry1E* and search for proteins with high toxicity to Spodoptera sp. have been objectives in several earlier studies.

Recent advances in transgenic technology now make it possible to transfer and express various genes in agriculturally important species like cotton. The rapid development of cotton transformation technology not only provides a valuable method for introducing useful genes into cotton to improve important agronomic traits, but also helps in the study of gene function and regulation. Although transformation rates have been significantly improved since the first report of success in the transformation of cotton (Firoozabady *et al*., 1987; Umbeck *et al*., 1987), increase in transformation efficiency is still needed.

Transformation techniques that evade tissue culture (Graves and Goldman, 1986) therefore become important in recalcitrant crops such as cotton. In the present study, *in planta* transformation protocol was used to develop transformants (Rohini and Sankara Rao, 2000a; Rohini and Sankara Rao, 2000b; Rohini and Sankara Rao, 2001). In this method, the seedling shoot is embedded in the stem between the cotyledons, break off one cotyledons to expose shoot apex and cut the tissue in the shoot apex to make wound and *Agrobacterium* is targeted to the wound. Therefore, *Agrobacterium tumefaciens* transfers the gene into the genome of diverse cells which are already destined to develop into specific organs and the meristematic cells

still to be differentiated. This results in the primary transformants (T_0) being chimeric in nature. This is the reason for the analysis of the transgenic plants to be carried out in the T_1 generation.

In *in planta* method of transformation, invariably the transformation of a few cell/cells happen and such cell/cells of targetted tissue (like shoot apical) will become part of the regenerated shoot where in the transformed cells prevail as chimeric. These plants/ shoots are refered as primary transformed and such transformed chimeric becomes flowers and seeds are harvested, they will have whole germ transformed status. In the present study the tissue choosen for transformation status was basically chimeric / mosaic in related for transformation status and it would be likely that a non- transformed tissue resulted in no PCR amplified with gene specific primers.

Future Line of Work

In planta genetic transformation needs to be tested by refining the protocol meant for cotton.

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