Available online at www.elixirpublishers.com (Elixir International Journal)

Bio Technology

Elixir Bio Tech. 53 (2012) 11736-11740

ISSN: 2229-712X

Construction of plant expression vector of synthetic *bt-cry1ac* gene for genetic transformation

Elumalai Sanniyasi*, Ramganesh Selvarajan, Prakasam Velu and Prabhakaran Mylsamy
Department of Plant Biology and Plant Biotechnology, Presidency College, Chennai- 600 005.

ARTICLE INFO

Article history:
Received: 9 April 2012;
Received in revised form:
19 November 2012;
Accepted: 28 November 2012;

Keywords
Agrobacterium tumefaciencs,
Bt-cry 1 Ac gene,
PCR, pGPTV,
pUC118.

ABSTRACT
To construct the plant expression vector containing synthetic *B. thuringiensis-cry1Ac* gene for crop plant transformations to resist the insect *Helicoverpa armigera*. A newly constructed binary vector containing the T - DNA left border, Kanamycin (*kan)* as marker gene, glucuronidase (*uidA*) reporter gene and *bt-cry Ac 1* gene which transformed to *A. tumefaciens* by helper plasmid pRK2013, which provides *tra* and *mob* genes required to transfer the DNA. The plasmid constructed from basic vector pUC118 containing synthetic *Bt-cry 1 Ac* gene and pGPTV was restricted digested by *EcoRI* and *Xbal*. The digested plasmids were purified, quantified and ligated before triparental mating method of competent cell transformation. The triparental mating efficiency can be observed through back transformation of gene from *A. tumefaciens* to *E.coli*. The confirmation of *Bt-cry 1 Ac gene* in the construct was done by Polymerase Chain Reactions (PCR). Construction of a *Bt-cry Ac1* expression vector was successful and this study will be a feasible approach for the genetic improvement of an economically important Crop plants.

Introduction

Insecticidal Cry proteins, produced as protoxins (65–140 kDa) in parasporal crystals of *Bacillus thuringiensis* (*Bt*). The crystalline *Bt* protoxins are solubilized and activated in the midgut of insects by proteolysis. The activated toxins (60–70 kDa) bind to the membrane of midgut columnar cells and form ion channels, inducing osmotic lysis of the epithelium (Schnepf, E. H. 1995., Knowles, B. H. 1994., Grochulski, P *et al* 1995). Engineering of insect resistance in maize, rice, cotton, tomato, potato, and tobacco shows that a significant modification of the bacterial *cry* coding sequences is essential to express these *Bt* toxin genes in plants (Sutton *et al.,* 1992). The insecticidal spectrum of *Bt* toxins thus far expressed in transgenic plants is limited. Therefore, the engineering of *Bt* toxins with novel specificity is essential for the biological control of recalcitrant plague insects, such as *Helicoverpa armigera*. Members of the *Helicoverpa* genus (Lepidoptera) feed on over 40 different plant families world-wide, including at least 87 species of economic importance (Hill, D. S. 1983).

The expression of the full-length (3.5 kb) wild-type gene could not be achieved mainly due to the presence of sequences that could lead to premature transcription termination and consequent transcript instability (Perlak *et al.* 1990). In several studies the expression of the cry1Ac gene is not sustained uniformly during the life cycle of the plant. For example, Kranthi et al. (2005) observed that in eight Bt-cotton Bollgard hybrids, the Cry1Ac protein expression was found to be highly variable in different plants, and more importantly, it declined progressively over the life cycle of the plant.

Here we constructed the high expression synthetic *Bt-cry 1 Ac* gene in binary vector pGPTV, so as to establish a solid foundation for further plasmid transformation study.

Materials and Methods:

Synthetic Bt-cry 1 Ac gene

Oligonucleotides were synthesized and obtained from Invitrogen, India. The synthetic *Bt-cryIC* gene was designed based on the sequence of the corresponding wild-type gene (*cry 1 Ac*; GenBank accession no. AF177675). Modifications of the synthetic *cry 1 Ac* gene sequence did not alter the amino acid sequence of the minimal toxic fragment of the *Bt* protoxins. The designed DNA sequence of the *cry 1 Ac* gene (see Fig. 1; GenBank accession no. AF177675) was divided into three blocks separated by *Hin*cII and *Bgl*II cleavage sites. This synthesized gene was ligated in pUC118 and maintained in *Escherichia coli* (*E. coli*) *K12 DH5α* at 4˚C.

Design and synthesis of *Bt-cry 1 Ac* gene primer

According to GenBank, accessible *Bt-cry 1 Ac* gene mRNA sequence data (serial accession: AY AF177675), using the primer design software Premier 5.0 to design primers containing Bgl II / EcoR I restriction site fragment for amplification of the ORF length of *Bt-cry 1 Ac* gene. The primer was synthesized by **Eurofins Company**.

Primer: forward 5'CTGAATGAACTGCAGGACGAGG3' and
reverse 5'GCCAACGCTATGTCCTGATAGC3'

Plasmids

pGPTV (plasmid Glucuronidase Plant Transformation Vector) molecular weight of 13,400 bp is a binary vector with plant selectable marker near the T - DNA left border and a glucuronidase (*uidA*) reporter gene. The marker and *uidA* are divergently transcribed, and can be easily removed or replaced and it was maintained in *E. coli* strain HB101. *E.coli* DH5α strain contains the helper plasmid pRK2013, which provides *tra* and *mob* genes required to transfer the DNA. These vectors were supplied by Eurofins private Ltd. All plasmids were stored at 4˚C

Tele:
E-mail addresses: ananandal@gmail.com

Isolation of plasmids

100 µl of bacterial glycerol stock, containing the required plasmids were added into 100 ml of LB broth media containing the respective antibiotics, *Kanamycin* for pGPTV plasmid and *Ampicillin* for pUC18 / *Bt-cry1Ac* gene, and incubated overnight at 37 °C with shaking. The cells were harvested at 6,000 rpm (rotation per minute) for 5 minutes at 4 °C. To the pellet, 3 ml of solution I was added (Solution I contains 50 mM glucose, 25 mM *Tris* (pH - 8.0) and 10 mM EDTA). It was placed in ice box for 10 minutes and brought to room temperature. Six ml of freshly prepared solution II (0.2N NaOH and 1 % SDS) was added, mixed by inverting the tube 5 to 6 times and incubated at room temperature for 10 minutes. Five ml of solution III, which has 3M potassium acetate, was added and mixed by inverting gently. Later the sample is incubated on ice both for 10 minutes, here plasmid DNA denaturation induced by solution II gets renatured by adding solution III. After the incubation of the sample in solution III is centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to fresh tubes without disturbing the cell debris. The content was shifted into 2 ml eppendorf tubes. 10 µl of RNase 'A' (10 mg/ml) was added into each tube and incubated at 37 °C for 30 minutes. Extracted supernatant is added with equal volumes of phenol: chloroform: isoamylalcohol in the ratio (25: 24: 1 v / v / v) and centrifuged at 12,000 rpm for 12 minutes at room temperature. To the supernatant added 0.1 volume of 3M sodium acetate and mixed gently, two volumes of absolute alcohol was added and kept at – 20 °C for overnight. The vial was centrifuged at 12,000 rpm for 12 minutes at 4 °C. The pellet DNA was washed with ice cold 70 % alcohol. Finally pellet was dried in speed vacuum desiccators for 3 - 5 minutes and then it was dissolved in 50 µl of 1X TE (Sambrook, *et al.*, 1989).

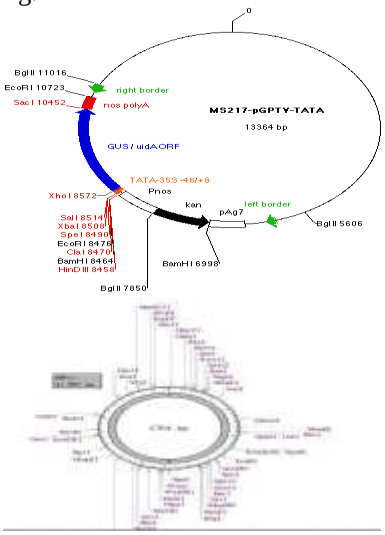
Bt – cry 1 Ac gene Cloning

DNA (approximately 1.5 µg) samples 14 µl of pGPTV and 8 µl of pUC 18 containing *Bt-cry1Ac* were taken in a sterile microfuge tubes and mixed with sufficient double distilled water to give the required final volume. 2.5 µl of appropriate 10 X restriction digestion OPAB (One Phor All Buffer) was added and mixed by tapping the tubes. 0.75 µl each of restriction enzymes *EcoRI*, *Xbal* and 0.75 µl of BSA were added and mixed by tapping the tubes. The tubes were spun for few seconds. Both the tubes were incubated at 37 °C overnight in a water bath. The reaction was stopped by adding 0.5 M EDTA (Ethylene diamine tetra acetic acid) (pH 8.0) to a final concentration of l0 mM. The DNA samples were analyzed directly on 0.8% agarose gel by adding gel loading dye to each tube containing the reaction mixture. The tubes were spun briefly and the mixture was loaded along with undigested samples for comparison into the slot cast on 0.8% agarose gel containing ethidium bromide. After the run was complete, the gel was observed under Ultra Violet (UV) transilluminator. The λ DNA is digested with *Hind III* for molecular ladder comparison (Hartl Daniel and Jones, 2001).

After that we have followed the recovery of DNA by ammonium acetate method (Sambrook, *et al.*, 1989). The eluted DNA concentration was calculated against Standard solutions of known DNA concentration (0 to 60 ng/µl in TE buffer) by Eppendorf Biophotometer.

Ligation

0.16 µl (15 ng) of insert DNA (*Bt-cry1Ac*) was taken and to it 1 µl (50 ng) of vector DNA (pGPTV) and 2 µl of 10 X ligase buffer and 0.5 µl of ligase enzyme was added in separate vials. Double distilled water was added to each tube to make up the reaction volume to 20 µl and incubated at 12 °C to 14 °C overnight. The ligation mixture was used to transform competent *E. coli* cell to check for successful ligation reaction (Saxon *et al.*, 2000). The ligated plasmid with *Bt-cry 1 Ac* gene represented in fig. 1



ATGGCTATCGAGACCGGTTACACTCCAATCGACATCTC
CTTGTCCTTGACACAGTTTCTGCTCAGCGAGTTCGTGC
CAGGTGCTGGGTTCGTGCTCGGACTAGTTGACATCATC
TGGGGTATCTTTGGTCCATCCCAATGGGACGCATTCCT
GGTTCAAATTGAACAGCTCATCAACCAGAGGATCGAA
GAATTCGCCAGGAACCAAGCCATTTCTAGGTTGGAAG
GACTCAGCAATCTCTACCAAATCTATGCAGAGTCTTTC
AGAGAATGGGAGGCCGATCCTACTAATCCAGCTCTCA
GGGAGGAGATGCGTATTCAATTCAACGATATGAACAG
CGCCTTGACCACTGCTATCCCATTGTTCGCAGTCCAGA
ACTACCAGGTGCCTCTCTTGTCCGTGTACGTTCAAGCC
GCTAATCTTCATCTCAGCGTGCTTCGAGACGTTTCAGT
GTTTGGACAGAGGTGGGGATTCGATGCTGCAACCATC
AATAGCAGATACAACGACCTTACTAGGCTCATTGGAA
ACTACACCGACTATGCTGTTCGTTGGTACAACACTGGT
TTGGAGCGTGTCTGGGGTCCTGATAGCAGAGATTGGGT
GAGATACAACCAGTTCAGGAGAGAATTGACCCTTACA
GTTTTGGATATCGTGGCTCTCTTCCCGAACTATGACAG
CAGAAGGTACCCAATCCGTACTGTTTCCCAACTTACCA
GAGAGATCTATACTAACCCAGTTCTTGAGAATTTCGAC
GGTAGCTTCCGTGGTTCTGCCCAGGGTATAGAAAGAA
GCATCAGGAGCCCTCATCTCATGGACATCTTGAACAGC
ATAACTATCTACACCGATGCTCATAGAGGATACTACTA
TTGGTCTGGACACCAGATCATGGCCTCTCCAGTTGGAT
TCAGCGGGCCCGAATTCACCTTCCCTCTCTATGGAACT
ATGGGTAACGCCGCTCCACAACAAAGGATCGTTGCTC
AACTAGGTCAGGGTGTCTACAGAACCTTGTCTTCCACT
TTGTACAGAAGGCCATTCAATATCGGTATCAACAACCA
GCAACTTTCCGTTCTCGATGGAACAGAGTTCGCCTATG
GAACCTCTTCTAACTTGCCATCCGCTGTTTACAGAAAG
TCCGGAACCGTTGATAGCTTGGACGAAATTCCACCACA
GAACAACAATGTGCCACCCAGGCAAGGATTCAGCCAC
AGGTTGAGCCATGTGTCCATGTTCCGTTCCGGTTCTAG
CAACAGTAGCGTGAGCATCATCAGAGCTCCTATGTTCT
CTTGGATACATCGTAGTGCTGAGTTCAACAATATCATT
GCATCCGATAGCATCACTCAAATTCCTGCAGTTAAGGG
AAACTTTCTCTTCAATGGTTCAGTCATTTCAGGACCAG
GATTCACAGGAGGAGACCTCGTTAGACTCAACAGCAG

TGGAAATAACATCCAGAATAGAGGGTATATTGAAGTT
CCAATTCATTTCCCTTCCACATCTACCAGATATAGAGT
TCGTGTGAGGTATGCTTCTGTAACTCCTATTCATCTCAA
CGTTAATTGGGGTAATTCATCTATCTTCAGCAACACAG
TTCCAGCTACAGCTACCTCCTTGGATAACCTCCAATCC
AGCGACTTCGGATACTTTGAGAGCGCCAATGCTTTCAC
ATCTTCACTCGGCAACATAGTGGGTGTTAGAAACTTTA
GTGGAACTGCAGGTGTGATCATAGACAGATTTGAGTTC
ATTCCAGTTACTGCAACACTCGAATAA

Fig. 1 Structure of *Bt – cry 1 Ac* cloned pGPTV (plasmid Glucuronidas Plant Transformation Vector) &synthetic gene sequence of *Bt – cry 1 Ac* and its restriction sites

Ligation Calculations

The standard statement goes that, 1μg of DNA having l000 bp = 1.52 picomoles pGPTV (DNA) has 13,000 bp => Therefore l μg of pGPTV = 0.116 picomoles *Bt-cry1Ac* (insert DNA) has 1363 bp =>Therefore 1 μg of *Bt-cry1Ac* = 0.95 picomoles.

As the Molecular weight or number of base pairs per U-G of DNA decreases, the molarity increases Concentration of pGPTV vector DNA = 50 ng/μl, Concentration of *Bt-cry1Ac* (insert DNA) = 15 ng/μl.

Competence Cell preparation and transformation

The DH5α cells (~0.6 OD) were recovered by centrifugation at 6,000 rpm for 6 minutes at 4 °C. The pellet was resuspended in 2 ml of ice cold l00 mM $CaCl_2$ stored on ice for 10 - 15 minutes and gently mixed well. The cells were recovered by centrifugation at 6,000 rpm for 6 minutes at 4 °C. The pellet responded in 1.5 ml of l00 mM $CaCl_2$. Five sterile eppendorf tubes were taken, Tube 1 and Tube 2 contains 150 μl of competent cells (DH5α) alone, tube 3 contains 150 μl of competent cells (DH5α) + 4 μl of diluted pUC - 18 DNA (5 ng/μl), tube 4 contains 150 μl of competent cells (DH5α) + 20 μl of eluted samples (V : l 1 : 2 molar ratio), tube 5 contains 150 μl of competent cells (DH5α) + 20 μl of ligated samples (V : l 1 : 2 molar ratio). The tubes were stored on ice for 20 minutes. The tubes were transferred to a rack placed in a circulating 42 °C water bath for 100 seconds and rapidly transferred to an ice bath for 1 – 2 minutes. 900 μl of LB broth was added to each tube, incubated on a shaker at 37 °C for 1 hour. The tubes were centrifuged at 6,000 rpm for 3 minutes, the pellet is dissolved in 150 μl of fresh LB broth and spread plating of the cells. Two tubes containing only competent cells were plated on LB (*Ampicillin*) and LB (*Kanamycin*) plates respectively to check for contamination. Ligated mixtures containing the cells were plated on LB broth (*Kanamycin*) plates. Tube containing cells with pUC-118 DNA and eluted DNA was plated on LB broth (*Ampicillin*) plate. The plates were incubated overnight at 37 °C.

Triparental mating (TPM)

A. tumefaciens LBA4404 was streaked to get isolated colonies on AB minimal medium with 10 μl *Rifampcin and Streptomycin* (final concentration 100 μg/ml) incubate at 28 °C. *E. coli* having pRK2013 and pGPTV with gene of interest were separately streaked to get isolated colonies on LB agar medium containing 50 μg/ml *Kanamycin* and incubated at 37 °C overnight. One loopful each of pRK2013, pGPTV - *Bt-cry1Ac* and *A. tumefaciens* strain LBA4404 were mixed on, Yeast Extract Peptone plate (YEP) without any antibiotics and incubated at 28 °C for 12 - 18 hours (Charles H. Shaw., 1995).

Evaluation of *Bt-cry 1 Ac* recombinant plasmid

Amplification reactions (Barlett and Stirling, 2003) were performed in a total volume of 25 μl containing 10 X buffer (l00 mM *Tris* - HCl, pH-8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1 % Gelatin), 0.225mM dNTP mixture (mixture of dATP, dCTP, dGTP, dTTP), 0.3 μM of forward primer 5'CTGAATGAACTGCAGGACGAGG3' and reverse primer 5'GCC AACGCTATGTCCTGATAGC 3', Taq DNA polymerase, 10 ng of genomic DNA and double distilled water so as to make up the volume to 25 μl. DNA amplifications were performed in a thermal cycler of an initial denaturation of 94 °C for 5 minutes followed by annealing of 55 °C for 30 - 60 seconds followed by extension of 72 °C for 1 minute. This temperature is determined by optimum temperature for polymerize activity. The instrument was programmed, such that the steps would be repeated for 35 cycles to get the amplified DNA and a final extension for 5 mins at 72 °C. This temperature is determined by optimum temperature for polymerize activity. Samples were loaded onto 0.8 % agarose gel for observation.

Results and Discussions

Identification of Digested Gene fragments

In Fig 2. The first lane undigested pGPTV is loaded which is again circular and lobed, in the second lane pGPTV is digested with *Xbal and EcoRI*. The molecular weight of pGPTV is 13 kb and it shows a sharp band around 13200 bp. In Lane 3 pUC18 / *Bt-cry1Ac* gene undigested sample is loaded , as it is not restricted and digested it is in circular form, in Lane 4 pUC18 / *Bt-cry1Ac* gene digested is loaded it shows a sharp band around 2636 bp in the molecular ladder. And *Bt-cry1Ac* gene released shows a band around 1363 bp in the molecular ladder. Lane 5 is pUC18 digested sample it also shows 2636 bp molecular weight in the molecular ladder.

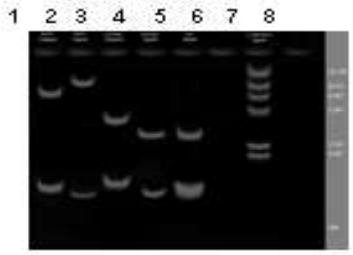


Fig 2. Comparison of digested and undigested plasmids. Lane 1 – pGPTV undigested, Lane 2 – pGPTV digested with *Xbal and EcoRI*, Lane 3 – pUC18/*Bt-cry1Ac* gene undigested, Lane 4 – pUC18/*Bt-cry1Ac* gene digested with *Xbal and EcoRI*, Lane 5 – pUC18 digested. Lane 6, 8 – empty, Lane – 7 λ DNA with *HindIII* digest.

Evaluation of cloned DNA

The DNA of the vector and gene are retrieved using ammonium acetate method. The Concentration of the vector and insert is determined. The eluted samples (Fig 3.) showed undigested and digested pGPTV in lane 1 and 2, undigested pUC18/*Bt-cry1Ac* and digested pUC18/*Bt-cry1Ac* with *Xbal and EcoRI* in lane 3 and 4 shows molecular weight 2.6kb and 1.3 kb. The lane 5 contains digested pUC118. After ligation the competent DH5α a basic strain of *E.coli* is used as vector, it was sensitive to antibiotics, so the culture of DH5α can be tested using kanamycin and ampicillin.

During transformation the desired gene (*Bt-cry1Ac*) from the basic vector was sub cloned into the binary vector (pGPTV). To check the transformation efficiency we can further proceed with *LAXI* (*LB, AMPICILLIN, X-GAL, IPTG)* plate preparation which will result in blue colonies, as the binary vector pGPTV lacks the *LacZ* gene the experiment is not possible with this binary vector. In Fig 4. Competence Cells (CC) which has

undergone transformation process is spreaded on LB plain plate (Plate - 1) and LB with *Kanamycin* plate (Plate - 2) and are used as control to check the contamination rate of Competence Cell. In Fig 5. competence cell with Diluted Plasmid DNA (pGPTV) which has been isolated from binary vector is spreaded on LB with *Kanamycin* plate (Plate - 3), competence Cell with Eluted sample (pGPTV) is spreaded on LB with *Kanamycin* plate (Plate - 4), and competence cell with ligated sample (pGPTV with *Bt-cry1Ac* gene) is spreaded on LB with *Kanamycin* plate (Plate - 5).

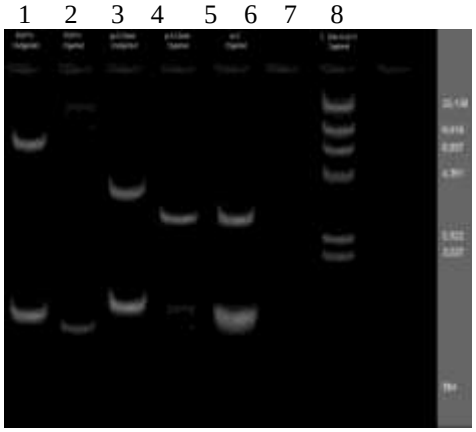


Fig 3. Gel elution. Lane 1 – pGPTV undigested, Lane 2 – pGPTV digested with *Xbal and EcoRI*, Lane 3 – pUC18/*Bt-cry1Ac* gene undigested, Lane 4 – pUC18/*Bt-cry1Ac* gene digested with *Xbal and EcoRI*, Lane – 5 pUC18 digested. Lane 6, 8 – empty, Lane 7 – λ DNA with *HindIII* digest. pGPTV vector from Lane – 213 kb and *Bt-cry1Ac* gene released from pUC18 showing 1363 bp are eluted from the gel for further process.

In LB Plain Plate (Plate - 1) with Competence Cell alone as there is no antibiotics, lawn or mat type growth is seen, which ensures the growth of competence cell. In LB with *Kanamycin* plate (Plate - 2), Competence Cell alone shows no growth because the basic strain DH5α is sensitive to antibiotics, no growth in this Plate - 2 ensures that the competence cells is not contaminated. In LB with *Kanamycin* plate (Plate - 3), Competence Cells with diluted plasmid (pGPTV) shows individual colonies. In LB with *Kanamycin* plate (Plate 4), competence cells with eluted plasmid (pGPTV) shows no growth as the plasmid is linearised. In LB with *Kanamycin* plate (Plate - 5), Competence Cells with ligated sample (pGPTV with *Bt-cry1Ac* gene) shows individual colonies but the number of colonies are less than that of Plate - 3. The formation of colonies depends on percentage of ligation that has taken place. The ligated colonies are used for triparental mating, for the gene to be transferred from binary vector to *A. tumefaciens*.

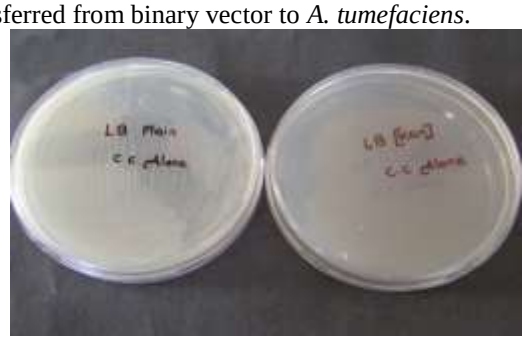


Fig 4. Competence Cells (CC) after transformation spreaded on plain LB plain plate (Plate – 1) and on LB with *Kanamycin* plate (Plate – 2)

Fig 5. Transformation plates Competence Cell with diluted plasmid (Plate – 3) on *Kanamycin* plate, eluted sample on *Kanamycin* plate (pGPTV, Plate – 4), ligated sample (pGPTV + *Bt-cry1Ac* gene, Plate - 5)

Triparental Mating

Transformed ligated sample was used in triparental mating for transferring the *Bt-cry1Ac* gene to disarmed *A. tumefaciens*. Two conjugations are expected to take place. One conjugation takes place between pGPTV + *Bt-cry1Ac* gene and pRK2013 helper strain and the second conjugation takes place between pRK2013 and *A. tumefaciens* LBA 4404.

After 14 hours of triparental mating lawn or mat type growth is observed (Fig 6) on the triparental mating plate 2, the inoculum from Fig 6 plate 2 has to be diluted serially from 10^{-1} to 10^{-5}. The serially diluted samples are spreaded on AB minimal media plate an efficient media for the growth of *A. tumefaciens*. Fig 6 (plate -1) was used as a control to check the growth of all the three strains in room temperature. After serial dilution the serially diluted samples are spreaded on AB minimal media with antibiotics *Kanamycin*, *Rifampcin* and *Streptomycin* plate (AB (KRS)) at $10^{-3}$and 10^{-5} dilutions, large single colonies are observed. They are again streaked on fresh AB (KRS) plate (Fig 7) The plates are stored on 4 °C for further transformation studies.

Fig 6. Triparental mating plates, from left to right (plate – 1) master plate , (plate – 2) triparental mating (TPM) plate, mixture of *A. tumefaciens* (LBA 4404) strain, helper strain (pRK2013) and binary vector with desired gene (pGPTV+*Bt-cry1Ac*)

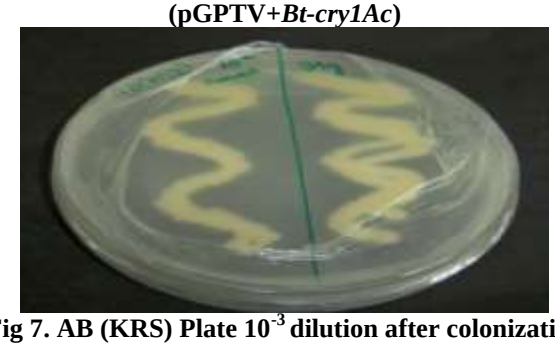


Fig 7. AB (KRS) Plate 10^{-3} dilution after colonization streaked on a new AB (KRS) plate and can be used for co – cultivation

Evaluation of PCR products

The transformants are evaluated using Polymerase chain reaction to confirm the presence of cloned gene *Bt-cry 1 Ac* in *A. tumefaciens.* Fig 8 proved the transformed plasmid pGPTV of *A. tumefaciens* have *Bt – cry 1 Ac* gene of 2.6kb in lane 1, whereas the non transformants in lane2 showed no bands, it was compared with λ DNA. Based on PCR assay method the transformed pGPTV plasmids were confirmed.

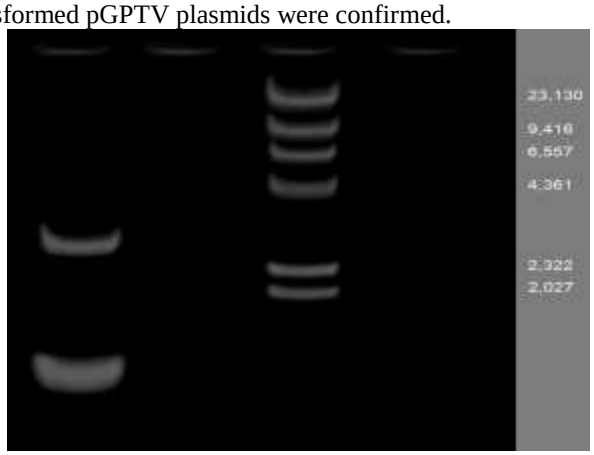


Fig 8. Detection of Transgenes. Plasmid DNA was isolated and analysed by PCR using the forward primer 5'CTGAATGAACTGCAGGACGAGG3' and reverse primer 5'GCC AACGCTATGTCCTGATAGC 3'. Amplified products were fractionated by agarose gel electrophoresis. Lane 1,Transformed *A. tumefaciens* formed band of 2.6 kb size, Lane 2, Non transformants does not produce bands, Lane 3, λ DNA.

Back Transformation

The triparental mating efficiency can be observed through Back transformation (Fig 9.). Again transferring the gene from *A. tumefaciens* to *E.coli* to check the presence of the gene. Back transformation is the method to confirm the transformation of desired transgene in *A .tumefaciens* during triparental mating process. This is the preliminary confirmation method of gene transfer before it is transferred into the plant genomic system. This method ensures us to precede *Agrobacterium* mediated transformation within the plant system (Yukiko Sawasaki *et al.,* 1996).

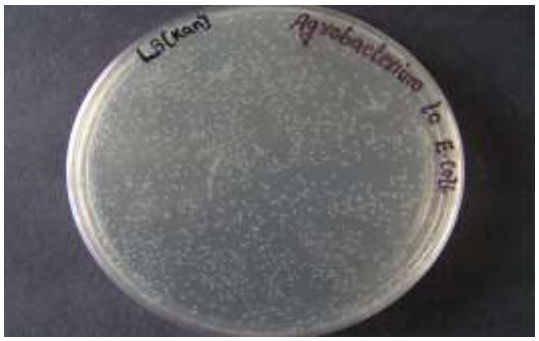


Fig 8. Back transformation transferring the gene from *A. tumefaciens* to *E.coli*, to check the presence of the gene

Conclusion

In the present study we generated a binary vector pGPTV that contains synthetic *Bt – cry 1 Ac* gene for insect resistant and *uid A* as reporter and *kan* as marker gene. This will be suitable vector for transformation of economically important crop plants. It is important to develop transgenics with high, uniform levels of Cry1Ac toxin during the entire period of the plant growth and development. Modifications of *Bt – cry 1 Ac gene* to improve stable expression in plants, achieving higher level expression. The result shows that the ligated fragments were about 13 kb, which reveals that the *Bt – cry1Ac* gene was corrected, and the *Bt – cry 1 Ac* eukaryotic expression vector has been constructed successfully.

Acknowledgement

We thank the Principal and Head, Plant Biotechnology Department, Presidency College (Autonomous), Chennai 05 and the authors also expressed sincere thanks to Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India for their financial support.

References

1. Schnepf, E. H. 1995 *Curr. Opin. Biotechnol.* 6, 305–312.
2. Knowles, B. H. 1994 *Adv. Insect Physiol.* 24, 275–308.
3. Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.-L., Brousseau, R. & Cygler, M. 1995 *J. Mol. Biol.* 254, 447–464.
4. Sutton, D. W., Havstad, P. K. & Kemp, J. D. 1992 *Transgenic Res.* 1, 228–236.
5. Hill, D. S. 1983) *Agricultural Insect Pests of the Tropics and Their Control* Cambridge Univ. Press, Cambridge, U.K..
6. Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Greenplate JT and Fischhoff DA 1990 Insect Resistant cotton plants. Bio/Technology 8 939–943.
7. Kranthi KR, Naidu S, Dhawad CS, Tatwawadi A, Mate K, Patil E, Bharose AA, Behere GT, Wadaskar RM and Kranthi S 2005 Temporal and intra-plant variability of Cry1Ac expression in Bt-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera). Curr. Sci. 89 291–298.
8. Sambrook, J., Fritsch, E.F and Maniatis, T. 1989. 'Molecular cloning: a laboratory manual'. *Cold Spring Harbor Laboratory Press, ColdSpring Harbor, N.Y.*
9. Hartl Daniel, L. and Jones Elizabeth, W. 2001, '*Genetics: Analysis of Genes and Genomes*', Fifth Edition. ISBN 0-7637-0913-1.
10. Saxon, E. and Bertozzi, C.R. 2000. 'Cell Surface Engineering by a Modified Staudinger Reaction'. *Science* ; 287: 546.
11. Bartlett and Stirling. 2003, 'A Short History of the Polymerase Chain Reaction'. *Methods Mol Biol*; 226: 3-6.
12. Charles H. Shaw., 1995. '*Agrobacterium tumefaciens* Chemotaxis Protocols' *Methods Mol Biol;* 44: 29-36.
13. Yukiko Sawasaki, Koji Inomata and Kazuo Yoshida 1996. Trans-Kingdom Conjugation between *Agrobacterium tumefaciens* and *Saccharomyces cerevisiae,* a Bacterium and a Yeast, *Plant Cell Physiol.* 37(1): 103-106.
14. Charles H. Shaw 1995. Introduction of Cloning Plasmids into *Agrobacterium tumefaciens.* Plant Gene Transfer and Expression Protocols Methods in Molecular Biology, Volume: 49, Pub. Date: Sep-27-1995, Page Range: 33-37, DOI: 10.1385/0-89603-321-X: 33.