Construction of plant expression vector of synthetic \textit{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.

**ABSTRACT**

To construct the plant expression vector containing synthetic \textit{B. thuringiensis-cry1Ac} gene for crop plants transformations to resist the insect \textit{Helicoverpa armigera}. A newly constructed binary vector containing the T - DNA left border, Kanamycin (kan) as marker gene, glucuronidase (uidA) reporter gene and \textit{bt-cry Ac 1} gene which transformed to \textit{A. tumefaciens} by helper plasmid pRK2013, which provides tra and \textit{mob} genes required to transfer the DNA. The plasmid constructed from basic vector pUC118 containing synthetic \textit{Bt-cry 1 Ac} gene and pGPTV was restricted digested by EcoRI and XbaI. 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 \textit{A. tumefaciens} to \textit{E.coli}. The confirmation of \textit{Bt-cry 1 Ac} gene in the construct was done by Polymerase Chain Reactions (PCR). Construction of a \textit{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.

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**Introduction**

Insecticidal Cry proteins, produced as protoxins (65–140 kDa) in parasporal crystals of \textit{Bacillus thuringiensis} \textit{(Bt)}. The crystalline \textit{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 \textit{cry} coding sequences is essential to express these \textit{Bt} toxin genes in plants (Sutton et al., 1992). The insecticidal spectrum of \textit{Bt} toxins thus far expressed in transgenic plants is limited. Therefore, the engineering of \textit{Bt} toxins with novel specificity is essential for the biological control of recalcitrant plague insects, such as \textit{Helicoverpa armigera}. Members of the \textit{Helicoverpa} genus (Lepidoptera) feed on over 40 different plant families worldwide, 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 \textit{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 \textit{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 \textit{Bt-cryIC} gene was designed based on the sequence of the corresponding wild-type gene (\textit{cry 1 Ac}; GenBank accession no. AF177675 ). Modifications of the synthetic \textit{cry 1 Ac} gene sequence did not alter the amino acid sequence of the minimal toxic fragment of the \textit{Bt} protoxins. The designed DNA sequence of the \textit{cry 1 Ac} gene (see Fig. 1; GenBank accession no. AF177675) was divided into three blocks separated by \textit{Hin}cII and \textit{Bgl}II cleavage sites. This synthesized gene was ligated in pUC118 and maintained in \textit{Escherichia coli} (\textit{E. coli}) K12 DH5α at 4°C.

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

According to GenBank, accessible \textit{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 \textit{Bt-cry 1 Ac} gene. The primer was synthesized by Eurofins Company.

Primer: forward 5’CTGAATGAACTGCAGGAGG3’ 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 (\textit{uidA}) reporter gene. The marker and \textit{uidA} are divergently transcribed, and can be easily removed or replaced and it was maintained in \textit{E. coli} strain HB101. \textit{E. coli} DH5α strain contains the helper plasmid pRK2013, which provides \textit{tra} and \textit{mob} genes required to transfer the DNA. These vectors were supplied by Eurofins private Ltd. All plasmids were stored at 4°C.
**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 by centrifugation 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 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 sample was diluted 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 DNA was represented in fig. 1

**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, XbaI and 0.75 µl of BSA were added and mixed by tapping the tubes. The reaction was stopped by adding 0.5 mM EDTA (Ethylene diamine tetra acetic acid) (pH 8.0) to a final concentration of 10 mM. The DNA samples were analyzed directly on 0.8% agarose gel containing ethidium bromide. After the run was complete, the gel was observed under UV transilluminator. The λ DNA is digested with Hind III for molecular ladder comparison (Hartl Daniel and Jones, 2001).

**Ligation**

Double distilled water was added to each tube to make 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

**Sequence Details**

ATGGCTATCGAGACGCGTTTACACTCAAATTCGACATCTCT
CTTGTTCCCTTACACAGTTCAGCGTGTGCTC
CAGTGTGCTGATCATCTCCACAGG
GATACTTACTAACCAGCTTGAGATTTTGCGC
GAGTTCGCGTCTGCGTCCACACAGG
GTCGAGTTACGACCGTCGGTCTGCG
ATCTACTACTGTCATTGAGCAATAGGT
GATGATTCGCGTCTTCTGCCATGCACTG
CTGGTACAGGACCGTAGCTTCTGAGGAACATAGATAGCT
GATGACGAGCCCATAGCTTCTGGTACAGG
GCTAATCTTCATCTGACAGGAGAGG
GCAAATCCCATGACTTCTGCAGGAGAGAGG
GCTTCTTCTGAGGAGAGAGG
GACGAGGACGACGAGG
GCTTTCTTCACAGGACGAGGAGAGG
GCTTCTTCTGAGGAGAGAGG
GACGAGGACGACGAGG
GCTTTCTTCACAGGACGAGG
GCTTCTTCTGAGGAGAGAGG
GACGAGGACGACGAGG
GCTTTCTTCACAGGACGAGG
GCTTCTTCTGAGGAGAGAGG
GACGAGGACGACGAGG
TGGAGTAAACCTGCAGAATAGGATAGTATTTGAAGTT
CCAAATCTCATTTCTCTCCATACCTCCAGATATAGAT
TGCGTTGGAGTATGCTCTGTAACTCTTTTCTCTCA
CGTTAAATTGGGATTCATCTATCTCTCAGCAACACAG
TCCTACTACAGCTAACCTTCTTGGATATAAACCTCA
ACCGAATTTGGACTTTTGAGAGGCGACACGCTTTCAC
ATCTCTACCTCGGACATATGGTTGTTAGAAACTTTA
GTGGAACGTGAGGTGATCATAGCATATGGTGTC
ATTTCCAGCTACAGCTACCTCCTTGGATAACCTCCAATC
G

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, 1 pg of DNA having 1000 bp = 1.52 picomoles pGPTV (DNA) has 13,000 bp => Therefore 1 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 (\( \sim 0.6 \) 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 100 mM CaCl₂, 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 remained in 1.5 ml of 100 mM CaCl₂. 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:1:1:2 molar ratio), tube 5 contains 150 µl of competent cells (DH5α) + 20 µl of digested samples (V:1:1:2 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 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 (100 mM Tris - HCl, pH-8.3, 500 mM KCl, 15 mM MgCl₂, 0.1 % Gelatin), 0.225mM dNTP mixture (mixture of dATP, dCTP, dGTP, dTTP), 0.3 µM of forward primer 5'CTGAAATGAACTGCGAGAGCGAGG3' and reverse primer 5'GCC AACGTTATGCTCTGTATACG3'. 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 XbaI and EcoRI, Lane 3 – pUC18/Bt-cry1Ac gene undigested, Lane 4 – pUC18/Bt-cry1Ac gene digested with XbaI and EcoRI, Lane 5 pUC18 digested. Lane 6, 8 – empty, Lane 7 – λ DNA with HindIII digest. pGPTV vector from Lane – 2 13 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 10⁻³. 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, Rifampicin 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 Bi–cry 1 Ac in A. tumefaciens. Fig 8 proved the transformed plasmid pGPTV of A. tumefaciens have Bi–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′CTGAATGAACCTGCAGGACGAGG3′ and reverse primer 5′GCC AACGCTATGTCCTGATGC 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 Bi–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 Bi–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 Bi–cry1Ac gene was corrected, and the Bi–cry 1 Ac eukaryotic expression vector has been constructed successfully.

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References