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# An Efficient *Agrobacterium* Binary Vector System to Transfer Bt 6e *cry* Gene

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***ABSTRACT.*** *Agrobacterium binary vector system remains by far the most effective gene transfer method. It's the most worked up on transfer system in the history of transgenic production. Incorporation of Bt cry gene provides a highly specific and very effective pest control method, which could be easily integrated with other IPM tools. Plasmid DNA was isolated from local Bt strain 6e and digested with DraI. The cry gene (3.5–4.0 kb) was isolated and purified. This DNA fragment was ligated with Agrobacterium vector pABK01 via XbaI adapter. Recombinant plasmid was transferred to Agrobacterium strain LBA4404 by electroporation. DNA extracted from recombinant strain was hybridized with Dig labelled cry probe. Friable Ixora (Ixora odorata) calli were co-cultivated with recombinant Agrobacterium. Transformants were selected on hygromycin medium and screened for GUS assay using Salmon-beta-D-glc-A. DNA from transformed calli were Dot blot analysed with the Dig labeled DNA probe. Bt cry gene was successfully incorporated into the Agrobacterium vector pABK01. GUS gene expression was absent in the transformed calli. Constructed recombinant vector successfully transferred Bt cry gene to Ixora calli.*

## INTRODUCTION

Plant transformation with *cry* genes from the entomopathogenic bacterium *Bacillus thuringiensis* provides an exiting approach to insect control, in which transgenic plants produce their own protective proteins (Perlak and Fischoff, 1993). To date more than 100 crystal protein gene sequences have been published. Dozens of plants have been successfully transformed with Bt *cry* genes, a number of them being even commercialized. Although majority of *cry* proteins are active against Lepidopteran larvae, more recent reports suggest effective activity against many other orders like Diptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga (Schneff *et al.*, 1998). Therefore, Bt *cry* gene transfer serves as a very versatile tool in IPM programs.

*Agrobacterium* vectors are now available for routine introduction of genes into dicotyledonous plants. Among all the transformation methods *Agrobacterium* mediated transformation stands out for its efficacy, making it the most widely worked gene transfer system. Woody ornamental *Ixora odorata* is hitherto been untouched in molecular improvements. Its popularity is very much on the rise but suffers from a number of

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Lepidopteran pest attacks. Therefore, the primary objective of this study was to develop an efficient *Agrobacterium* transfer system of Bt 6e *cry* gene to *Ixora odorata*.

## MATERIALS AND METHODS

### Bacterial strains

Local *Bacillus thuringiensis* strain 6e was used to isolate the *cry* gene. *E. colli* strain TGI was used as a host strain in the intermediate steps of the vector construction. Bt strain 6e was routinely maintained on semi-defined Luthy's growth medium at 35°C (Starr *et al.*, 1981). *E. coli* strain TGI was maintained on LB medium at 37°C. *Agrobacterium* strain LBA4404 (pABK01) was maintained on YEB medium (Luis and Simpson, 1988) with 50 mg $l^{-1}$ hygromycin and 50 mg $l^{-1}$ kanamycin at 28°C. All strains were obtained from the Microbiology Laboratory of the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya.

### Vector

*Agrobacterium* binary vector pABK01 was used as the initial vector. This vector has the *hpt* and intron GUS genes driven by CaMV 35 S promoter and a *nptII* gene driven by nos promoter.

### Isolation and purification of Bt 6e *cry* gene

Extraction and purification of plasmids from Bt var. 6e was done according to the large plasmid extraction method (Sambrook *et al.*, 1989). Bt cultures were grown overnight at 35°C on Luthy's growth medium. Bacterial cells were lysed and plasmid DNA extracted, treated with RNase (Sigma, USA) and purified once with an equal volume of Phenol:Chloroform:Iso-amyl alcohol (25:24:01). DNA was quantified using the spectrophotometric method. One micro gram of DNA was digested with *Dra*I (Sigma, USA) keeping the total volume at 20 µl. After 3 h at 37°C, 0.5 M EDTA was added to a final concentration of 10 mM. Restricted DNA fragments were resolved by electrophoresis on a 1.2% low melting point agarose gel (Sigma, USA) containing 0.5 µg $ml^{-1}$ ethidium bromide. One kilo base synthetic DNA ladder (MBI, Fermantas, Lituania) was used as the DNA marker (Fig. 1).

The intact *cry* gene of Bt var. aizawai HD–133 is found in the 3.8 kb *Dra*I fragment (Chak *et al.*, 1987) and this fragment, when southern blotted with 6e *Dra*I digestion, hybridized only with a fragment of similar size (Damayanthi, 1999). Hence, *Dra*I fragment in the region of 3.5–4.0 was located under UV using the DNA marker. This band was separated and purified as described by Sambrook *et al.*, 1989.

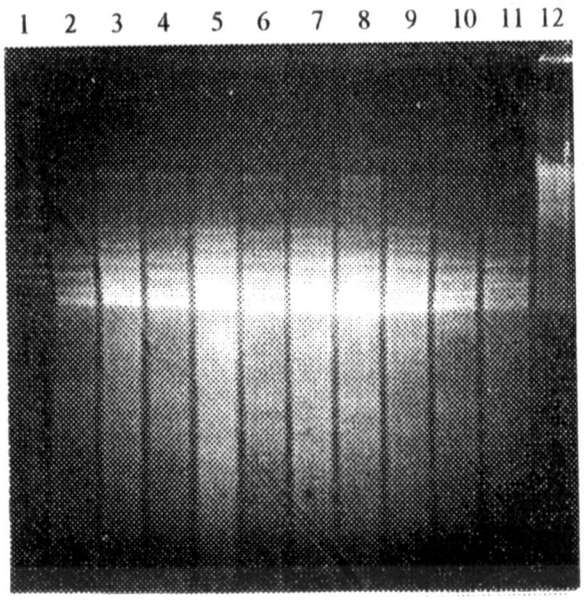


**Fig. 1. *Dra*I digested plasmid DNA of Bt 6e and HD133 on a 1.2% agarose gel.**
[Note: 1 - 1 kb ladder; 2, 4, 6, 8 and 10 - Bt 6e; 3, 5, 7, 9 and 11 - HD133, 12 - undigested Bt 6e].

## Cloning of Bt 6e *cry* gene into binary vector pABK01 *via XbaI* adapter

Vector plasmid pABK01 (Fig.2) was digested with *Xba*I (Sigma, USA) according to the manufacturer. One micro gram of vector plasmid was digested with *Xba*I for 1 h at 37°C. After complete digestion the sample was purified once with phenol:chloroform followed by ethanol precipitation at 0°C. Finally DNA was redissoloved in 90 µl of 10 mM


**Fig. 2. T-DNA region of pABK01.**
[Note: BR - right border, BL - left border, NOS - nopaline synthase, NPT - neomycin phosphotransferase, TNOS - NOS terminator, 35S - 35S promoter, GUS - β glucuronidase, HPT - hygromycin phosphotransferase, H - *Hind*III, XB - *Xba*I, S - *Sal*I, Sc - *Sac*I, E - *Eco*RI, B - *Bam*HI].

Tris. HCl (pH 8.0). Recircularization was prevented with CIP (Boehringer Manheim Biochemica Germany) according to Sambrook *et al.*, 1989. CIP was inactivated at the end by heating at 75°C for 10 min with 5 mM EDTA (pH 8.0). Purification was done once with phenol:chloroform.

Flush ended *cry* gene termini are incompatible to those of the digested pABK01. Therefore, *Xba*I adaptor (Sigma, USA) was used in the construction procedure. Phosphorylated *Xba*I adaptor was ligated to linearized dephosphorylated vector pABK01 as given by Sambrook *et al.*, 1989. Ligation reaction mixture was kept below 10 µl and incubated for 4 h at 16°C. DNA was purified with phenol:chloroform. Recombinant plasmid was electroporated to *E. coli* strain TGI to amplify, and repair any nicks that may remain.

Recombinant vector (pABK01+*Xba*I) was extracted and digested with *Sma*I (Sigma, USA) at 37°C for 3 h followed by dephosphorylation with CIP. Linearized vector was ligated with flush-ended Bt *cry* gene using $T_4$ DNA ligase (Sigma, USA), 0.5 mM ATP, 5 mM $MgCl_2$ and PEG 8000. After ligation DNA was purified with phenol:chloroform.

Recombinant plasmid was transferred to *Agrobacterium* strain LBA4404 by electroporation using Invitrogen electroporator at 1500V, 50 µF capacitance and 200 Ω resistance. LBA4404 was made electrocompetant according to ICGEB Plant Transformation Handbook 1996. Transformants were selected on YEB containing 50 mg $l^{-1}$ hygromycin and 50 mg $l^{-1}$ kanamycin.

**Dot blot analysis of the transformants**

Plasmid DNA isolated from the transformants were Dot blot analysed using DIG labeled 6e *cry* probe. Plasmid DNA were blotted on to positively charged nylon membranes and DIG labeled *cry* gene was *in situ* hybridized with target DNA according to the manufacturer (Boehringer Manhiem Biochemica Germany).

**Co-cultivation of *Ixora* calli with *cry* inserted binary vector pABK01**

*Ixora* calli (3rd subculture) obtained from leaf disks, cultured on ½ MS with 2.5 mg $l^{-1}$ 2, 4-D, were co-cultivated with recombinant *Agrobacterium* LBA4404 according to the protocol used at ICGEB (ICGEB, 1996). Co-cultivated *Ixora* calli were transferred to ½ MS with 500 mg $l^{-1}$ cefotaxime to kill the bacteria. These were then transferred to ½ MS with 100 mg $l^{-1}$ hygromycin to select the transformants.

**GUS assay**

Hygromycin selected calli were assayed with 5-bromo-6-chloro-3-indolyl-beta-D-glc-A (Biosynth International, USA) calli were incubated under dark conditions for 2 weeks.

**Dot blot analysis of transformed *Ixora* calli**

Genomic DNA was isolated from both the transformed and untransformed calli using the method described by Walbot, 1988. Extracted DNA were precipitated with isopropanol followed by a 70% ethanol wash. Finally DNA were resuspended in 100 µl of TE and probed with DIG labeled Bt 6e *cry* probe.

## RESULTS AND DISCUSSION

**Identification of Bt 6e *cry* gene**

Intact *cry* gene of Bt var. aizawai HD–133 is found within the 3.8 kb *Dra*I fragment (Chak *et al.*, 1987). Southern hybridization of Bt 6e *Dra*I restricted fragments with HD–133 *cry* probe produced a single band within the region of 3.5–4.0 kb (Damayanthi, 1999). Therefore, it is possible that the Bt 6e *cry* gene is located within the 3.5–4.0 kb *Dra*I fragment. There are a high percentage of conserved DNA sequences between the 2 fragments. On this basis Bt 6e 3.5–4.0 *Dra*I fragment was selected as the *cry* gene. This would be confirmed through a western blot analysis after cloning the fragment in an expression vector.

**Cloning of Bt 6e *cry* gene into *Agrobacterium* vector pABK01**

*Xba*I adaptor was included in the procedure due to the termini incompatibility between flush ended *cry* fragment and *Xba*I digested pABK01. *Xba*I adaptor ligated pABK01 was electroporated to *E. coli* strain TG1 to repair the nick resulted due to the 2–OH groups. A control series without this step produced negative results when Dot blot analysed (Fig. 3).

When hygromycin and kanamycin resistant transformants were Dot blot analysed with the Dig labeled 6e *cry* probe, some of the transformants gave negative results. These colonies could either be contaminants (Satellite colonies) or colonies without the *cry* insert. Out of the colonies analysed, 60% gave positive hybridization (Fig. 4).

From the results it is obvious that the vector 6 has a higher molecular weight than vectors 2, 3, 4 and 5. This proves that the *cry* gene was inserted stably to vector 6 but not to others on lanes 3, 4 and 5. Infact their sizes are almost similar to that of the basic vector pABK01. The results of the Dot blot also confirm this.

**Co-cultivation of *Ixora* calli with *cry* inserted pABK01**

Overall objective of the project is to produce a Lepidopteran pest resistant *Ixora* plant with the transfer of Bt 6e *cry* gene. Hence, *Ixora* calli were co-cultivated with recombinant vector to observe its efficacy. About 55% of the co-cultivated calli showed hygromycin resistance at 100 mg $l^{-1}$ after 2 subcultures. Hygromycin was used as the selective marker because *Ixora* showed natural kanamycin resistance even at 700 mg $l^{-1}$.

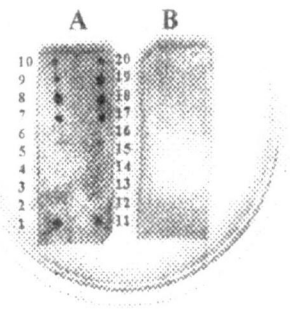


**Fig. 3. Dot blot analysis with Bt 6e *cry* probe.**
[Note: A : 1 and 11 - Bt 6e DNA, 2 and 12 - 6e *cry* fragment, 3 and 13 - pABK01 (without *cry*), 4–10 and 14–20 - recombinant pABK01; B : control series].


**Fig. 4. DNA extracted from vector construction steps run on a 0.8% agarose gel.**
[Note: 1 and 2 - pABK01, 3 - pABK01+*Xba*I, 4–6 - fully constructed vectors].

## GUS assay

None of the hygromycin selected transformants developed salmon pink color during the incubation period (2 weeks). Phenolic compounds are known to interfere with GUS activity (Jefferson *et al.*, 1989). Since *Ixora* produces a relatively high amount of phenolic compounds this could have led to the negative results.

## Dot blot analysis

Sixty per cent of the hygromycin positive transformants showed positive results when dot blot analyzed with the Bt 6e *cry* probe. This shows that the length of the T-DNA fragment transferred could be variable. This assumption could be made because of the natural hygromycin susceptibility of *Ixora* at 100 mg $l^{-1}$.

Dot blot positive transformants could be true or transient. Authenticity of the transformation could be proven with a southern blot analysis, on which we are currently working.

## Future aspects

We hope to classify the 6e *cry* gene through immunoassay. This knowledge would definitely widen the prospects of this gene as an effective tool to control pests. Furthermore, 6e *cry* fragment is currently being cloned into an expression vector to deduce its sequence, upon which it could be used to construct a synthetic *cry* gene with a higher GC content. This then would be better suited for a eukaryotic expression system, reducing the production of truncated inefficient proteins.

# CONCLUSIONS

Bt 6e 3.5–4.0 kb *Dra*I fragment could be cloned into vector pABK01 successfully *via Xba*I adaptor and Friable *Ixora* calli could be transformed by *cry* inserted pABK01.

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