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Agrobacterium -Mediated Transformation of Common Bean (*Phaseolus vulgaris*) var. Topcrop

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ABSTRACT. Phaseolus bean is the world's most important food legume. Insect pests are one of the most important constraints that reduce yield in beans. The production of insect resistant crops makes an important contribution to the integrated pest management. The improvement of this species by genetical transformation has been hindered by the lack of an efficient regeneration system to suit the Agrobacterium-mediated transformation. The explants used for transformation contained a cotyledon and a small portion of embryonic axis split in half. The meristematic tissue at the cotyledonary node was inoculated with the Agrobacterium culture AGL1 (PBIR$_{12}$). The recombinant binary vector harboured the cry 2 gene of the dipteran toxic Sri Lankan strain of Bacillus thuringiensis Bt 6E. Transformed explants produced shoots on shoot induction media containing MS salts, 15 µM BA and 50 µg/ml kanamycin. These shoots produced roots when cultured for 810 days on root induction medium containing MS salts, 2 µM NAA, 0.5 µM GA and 75 µg/ml kanamycin. The transformed tissues produced callus when cultured on callus induction medium containing MS salts and kanamycin 100 µg/ml. Presence of cry 2 gene in transformed plants was confirmed by probing extracted plant DNA with DIG labelled cry gene (3.8 kb fragment of HD 133). The protocols described for producing transgenic bean plants, can be modified suitably and extended to other economically important leguminous plants.

INTRODUCTION

Legumes occupy a unique position in our agricultural system and are second only to cereals as important plant food sources. Among the legumes, green bean is by far the most important with a global production of 21 Million metric tonnes annually (CGIAR, 1997). Their high protein content (18–30%) has made them an important dietary component in developing countries such as Sri Lanka. Transformation and the production of transgenic plants have benefited crop improvement (Abel *et al.*, 1986; Shah *et al.*, 1986). Modification of bean using genetic engineering techniques would facilitate the rapid development of new varieties with traits such as herbicide resistance, insect and disease resistance (Dillen *et al.*, 1999). However, regeneration technology of bean has not progressed. *Agrobacterium*-mediated transformation has been the best method available for DNA transfer to tissue explants in dicotyledonous species. The susceptibility of dry bean (*Phaseolus vulgaris*) to *Agrobacterium* infections varies depending on the cultivar or bacterial strain used (Mc Clean *et al.*, 1991). Current molecular efforts to develop insect resistant crops rely on transforming plants with single gene encoding insecticidal enzymes

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or toxins. Transgenic crop plants expressing *Bacillus thuringiensis* (Bt) *cry* genes have been developed in several laboratories (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987). We report here the development of transgenic bean plants expressing the Bt *cry* 2 gene using *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Bean seeds of variety Topcrop were obtained from the Department of Agriculture, Gannoruwa seed centre. The *Agrobacterium* strain AGL1 containing the recombinant plasmid $PBIR_{12}$ was constructed by ligating the 3.8 kb *Dra* 1 fragment of the local Bt isolate Bt 6E, to the binary vector PBI 121 at the *Sma* 1 site (Fig. 1). The 3.8 kb fragment of Bt 6E harbours the *cry* 2 gene which encode for the dipteran toxic proteins (Chak and Ellar, 1987).

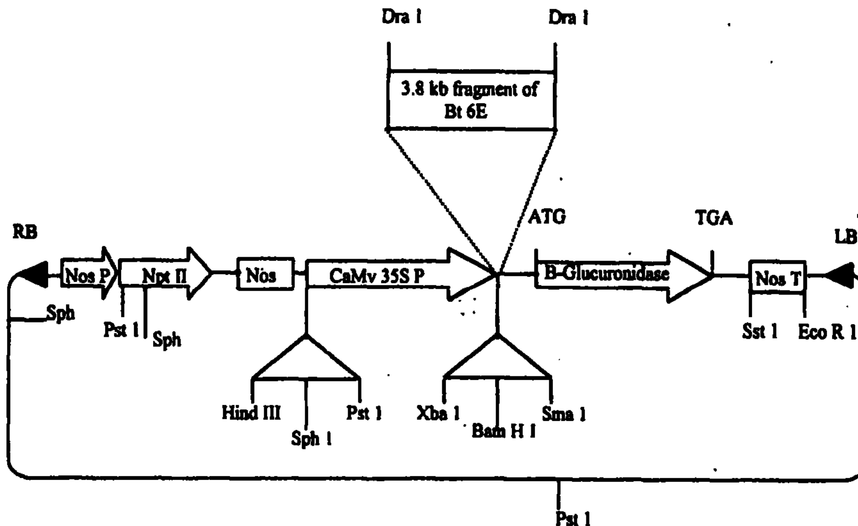

Fig. 1. Restriction map of the recombinant plasmid $PBIR_{12}$.

Preparation of explants

The regeneration protocol described by Franklin *et al.*, 1991, for green bean (cv. Dark Red Kidney) was modified suitably for the variety Topcrop. In order to minimize variation between seeds and seed borne contamination, only those seeds that were uniform in size and without cracks in the seed coat were used. Seeds were surface disinfected in 3% sodium hypochlorite solution containing few drops of Tween 20®, for 15 min, followed by rinsing in 3% NaOCl without detergent. Seeds were washed 3 times in sterile distilled water, 10 min each. Seeds were germinated in sterile culture tubes containing the

germination medium (GM) (Table1) under fluorescent light with a 16 h photoperiod at 23±2°C for 5 days. When the radicle was 15–25 mm long and 2–3 mm thick below the cotyledonary node, the seeds were taken for explant preparation. Explants were prepared only from those seeds where the hypocotyl was green. After removing the seed coat, the embryonic axis was sliced into 2 halves while still attached to the cotyledons. The epicotyl and hypocotyl were removed 1 mm from the cotyledonary node in such a way that the explant contained one cotyledon and a small portion (2–3 mm) of split embryonic axis attached to it (Plate 1). The prepared explants were kept in culture tubes containing CC1 medium (Table 1) under fluorescent light with a 16 h photoperiod at 23±2°C for 3 days.

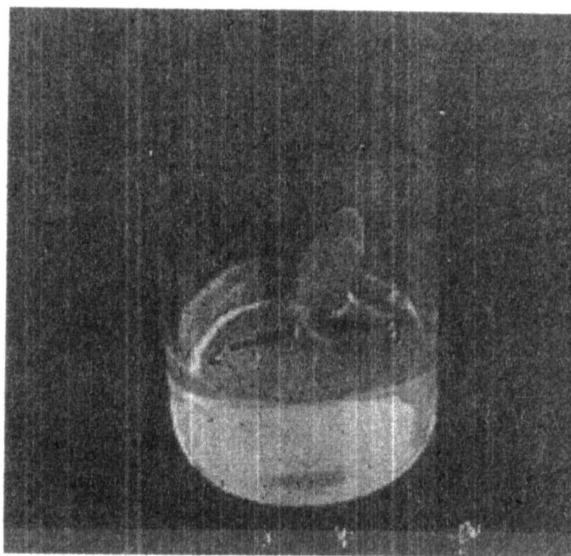

Plate 1. Explant used for transformation with AGL1 ($PBIR_{12}$).
[Note: 1 - cotyledon, 2 - split embryonic axis, 3 - axil].

Preparation of bacterial culture

The *Agrobacterium* strain AGL1 containing recombinant plasmid $PBIR_{12}$ was grown overnight in 50 ml YEB containing 50 µg/ml kanamycin. The culture was pelleted and resuspended in 10 ml of YEB without antibiotic.

Inoculation

The cotyledonary nodes were punctured with sterile 25–gauge needle. Wounded regions were inoculated with 15 µl of the concentrated culture.

Co-cultivation

The explants were transferred to culture tubes containing co-cultivation medium (CC2) (Table 1) and maintained under dark conditions at 23±2°C for 1 day.

Table 1. Different hormones used to supplement the MS medium (Murashige and Skoog, 1962).

Medium	Composition
Germination medium (GM)	5 µM BA, 3% (w/v) sucrose and 0.3% (w/v) agar
Shoot induction (SI) medium	15 µM BA, 3% (w/v) sucrose and 0.3% (w/v) agar
Root induction (RI) medium	2 µM NAA, 0.5 µM GA, 3% (w/v) sucrose and 0.3% (w/v) agar
Co-cultivation 1 (CC1) medium	10 µM BA, 2 µM GA, 3% (w/v) sucrose and 0.3% (w/v) agar
Co-cultivation 2 (CC2) medium	12 µM BA, 2 µM GA, 3% (w/v) sucrose and 0.3% (w/v) agar

Selection

The prepared explants were transferred to the shoot induction (SI) medium (Table 1). Explants were placed abaxial side down on the selection medium and grown under identical conditions as for seed germination. The transformed explants were selected by transferring 3 times at 3 days intervals to fresh shoot induction (SI) medium containing kanamycin 50 µg/ml and cefotaxime 600 µg/ml. Ten to 15 days after culture initiation, the 1st batch of shoots (where the stem portion of the shoots were 5 mm or taller) was excised and transferred to root induction (RI) medium (Table 1) containing kanamycin 75 µg/ml and cefotaxime 600 µg/ml. The cotyledons were discarded since they have a tendency to detach themselves from the shoots. The controls consisted of explants cultured under similar conditions without the bacterial inoculation.

Analysis of transformants - plant DNA isolation

The chromosomal DNA was isolated from the bean plants using the method described in the Plant Transformation Manual, ICGEB, 1996. The transformed plants, which produced roots in the selection medium, were chosen. Normal bean plants that were not subjected to infection with the recombinant vector $PBIR_{12}$ but grown under conditions as described for the transformed explants, were used as control. Sample of leaves (0.1 g each) from transformed and untransformed (control) plants was homogenized in a cold mortar, with solution I containing 250 mM NaCl, 50 mM Na_2EDTA, 50 mM Tris Cl (pH-8) and 15% (w/v) sucrose. The pellet obtained after centrifugation at 12000 rpm for

5 min was suspended in solution II containing 20 mM Tris HCl and 10 mM Na_2EDTA and then in 20% SDS. After incubating at 70°C for 15 min, the mixture was kept on ice and 7.5 mM ammonium acetate was added and incubated on ice bath for 30 min. The supernatant was collected by spinning at 12000 rpm for 30 min. DNA was precipitated by adding isopropanol and centrifuging. The pellet was washed with 70% ethanol, suspended in TE buffer and stored at –20°C.

Dot blot analysis

The 3.8 kb fragment of the *Bacillus thuringiensis* isolate HD 133 was labeled using the DIG DNA labeling and detection kit (Boehringer Mannheim, Germany) and used as a probe to detect the transformants. The plasmid DNA of Bt 6E and HD 133 were used as positive controls and the DNA extracted from the untransformed *in vitro* grown bean plants was used as the negative control. Positively charged nylon membranes (Boehringer Mannheim, Germany) were used.

Hybridization

The DNA was denatured by heating in a boiling water bath and chilling quickly on ice. One µl of each DNA sample were spotted on to the membrane. The DNA was bound to the membrane filter by UV cross linking with a transillumination device for 3 min. The filter was prehybridized in a sealed plastic box by incubating it in hybridization buffer at 68°C for more than 1 h. The hybridization buffer contained 5×SSC (NaCl 0.75 M, Na-citrate 0.075 M pH 7.0), 1% blocking stock solution, 0.1% (w/v) N-laurolyl sarcosine and 0.02% (w/v) SDS. The blocking stock solution was prepared by dissolving the blocking reagent in buffer 1 (0.1 M maleic acid; 0.15 M NaCl; pH 7.5) to a final concentration of 10% (w/v) with shaking and heating. It was stored at 4°C after autoclaving. Filters were not allowed to dry between prehybridization and hybridization. The solution was replaced with hybridization buffer containing freshly denatured probe DNA (DIG labeled 3.8 kb fragment of HD 133) and the filters were incubated for 4 h at 68°C. The solution was redistributed occasionally. The filters were washed twice with a solution containing 2×SSC (0.3 M NaCl pH 7.0, 0.03 M Na-citrate) and SDS 0.1% (w/v) and used for the detection of hybridized DNA.

Immunological detection

The filter was briefly washed in buffer 1 (0.1 M maleic acid; 0.15 M NaCl pH 7.5) and was incubated with buffer 2 (blocking stock solution diluted 1:10 in buffer 1) for 30 min. The antibody-conjugate (polyclonal sheep antidigoxigenin Fab-fragments conjugated to alkaline phosphatase) was diluted to 150 mU/ml and the filter was incubated in it for 30 min. The unbound antibody was removed by washing twice with buffer 1, 15 min each. The membrane was equilibrated with buffer 3 (100 mM Tris Cl; 100 mM NaCl; 50 mM $MgCl_2$ ph 9.5) for 2 min. The colour substrate solution was freshly prepared mixing NBT-solution and X phosphate solution in buffer 3. The filter was incubated in it without shaking, for colour development. When the desired spots were detected the reaction was

stopped by washing the membrane with buffer 4 (10 mM Tris Cl; 1 mM EDTA pH 8.0) for 5 min.

Callus induction assay

The leaves from transformed plants were used as explants for callus induction. Leaves were segmented and placed on callus induction media (Modified MS media containing MS salts, nicotinic acid 0.5 mg/l, pyridoxine 0.5 mg/l, thiamine 10 mg/l, myoinositol 100 mg/l and sucrose 30 g/l) supplemented with 0.02 µM NAA; 0.01 µM kinetin; 0.01 µM 2,4-D containing kanamycin 100 µg/ml. The positive controls were leaf segments of *in vitro* grown untransformed bean plants cultured on callus induction media without kanamycin. The negative controls were the leaf segments of *in vitro* grown untransformed bean plants cultured on callus induction media containing kanamycin 100 µg/ml. The cultures were maintained at 23±2°C.

RESULTS AND DISCUSSION

Plant transformation and regeneration

The regeneration of bean plant *via* organogenesis was found to be effective with the variety Topcrop. The buds produced by this method are of exogenous origin and therefore, should make it easier to achieve *Agrobacterium* infection (Franklin *et al.*, 1991). The explants were grown on CC1 medium so as to allow the meristematic ring to appear at the base of the axillary bud. Then the meristematic tissue at the cotyledonary node was inoculated with the recombinant AGL1 ($PBIR_{12}$) to get the transformed buds and shoots (Plate 2). When cultured on SI medium containing kanamycin 50 µg/ml the transformed explants doubled in length and width and the axillary bud at the cotyledonary node elongated to a size of 5–10 mm by 3–5 days. Four to seven days after culture initiation, a ring of compact, shiny light green coloured meristematic tissue (referred to as the meristematic ring) appeared at the base of the axillary bud. By 6–10 days after culture initiation, buds were produced from the meristematic ring, which continued to proliferate and produce shoots (Plate 3).

The presence of axillary bud together with the cotyledon was necessary for the development of the meristematic tissue (Franklin *et al.*, 1991). The addition of GA helps in the development of meristematic ring, which was visible to the naked eye by 4–5 days after culture initiation. The transformed shoots increased in length and grew normally even cultured on SI media containing kanamycin 100 µg/ml (Plate 4).

The shoots when cultured on RI medium produced roots after 8–10 days (Plate 5). Culturing in fresh media at 3 days interval helped to minimize the bacterial contamination. Cefotaxime 600 µg/ml was effective in controlling the bacterial growth and at the same time was not inhibitive on plant growth. Draper *et al.*, 1988, had showed that the ability of shoots to initiate roots in the presence of kanamycin is a commonly used and reliable indicator of transformation (cited by Maheshwaran *et al.*, 1992).

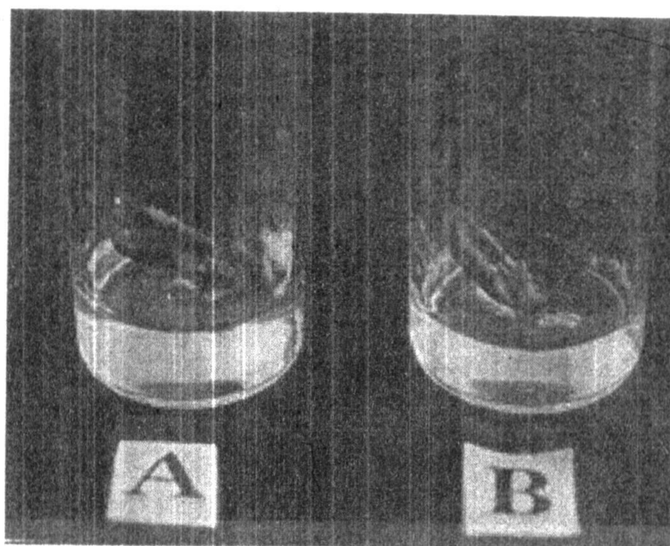

Plate 2. **Bean plant transformation.**
[Note: A - cotyledonary explant incubated with AGL1 ($PBIR_{12}$), B - control explant].

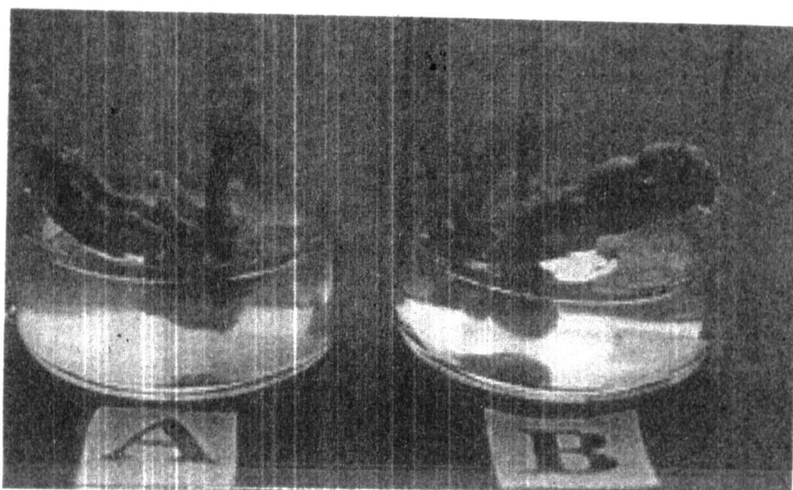

Plate 3. **Shoot induction.**
[Note: A - cotyledonary explant incubated with AGL1 ($PBIR_{12}$), B - control explant].

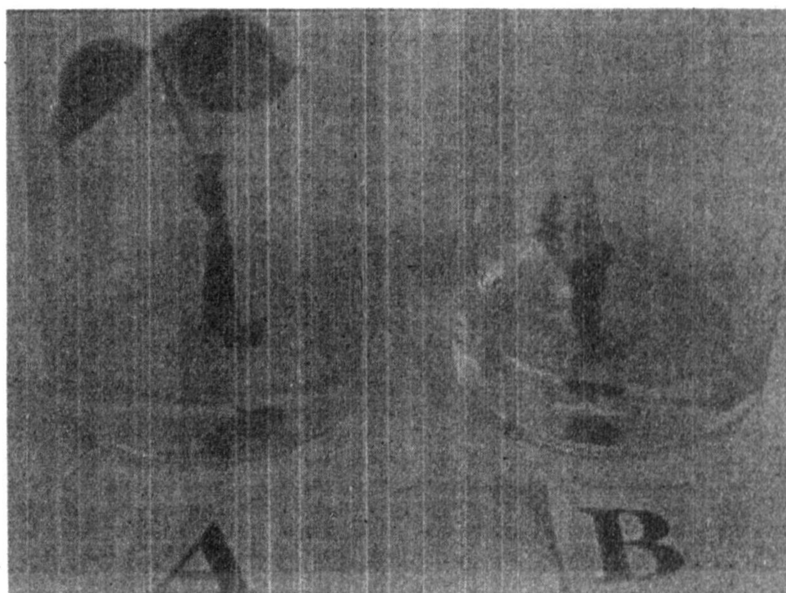

Plate 4. Shoot growth on selection medium containing kanamycin.
[Note: A - shoot produced from transformed meristematic tissue, B - control plant].

Dot blot analysis

The dot blot analysis of the plant DNA showed that the *cry* gene of Bt 6E has been successfully integrated into the bean plant genome. During the immunological detection the probe DNA hybridized with the positive controls and the transformed plant DNA resulting in the colour development. The integration of the foreign DNA is at random, which could be in single or multiple copies.

Callus induction assay

The callus induction assay further confirmed the presence of *cry* gene in bean plant genome. The transformed explants showed callus induction on selection medium after culturing for 10 days (Plate 8). The positive controls also produced callus, there by showing that the callus induction medium used is effective and the explants are viable. The negative controls showed no callus induction as expected. Christou *et al.*, 1987, have shown that in cell or friable callus cultures of legume plants, kanamycin and geneticin are useful for selection of tissues transformed with the neomycin phosphotransferase (npt II) gene.

Plate 5. Transformed plants produced roots on selection medium.

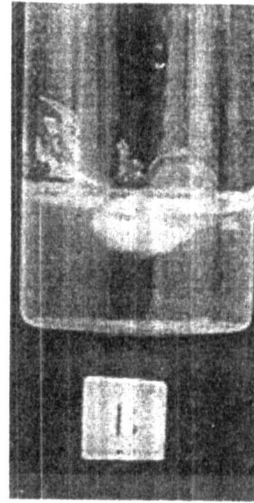

Plate 6. Control plant showing retarded growth and no root formation.

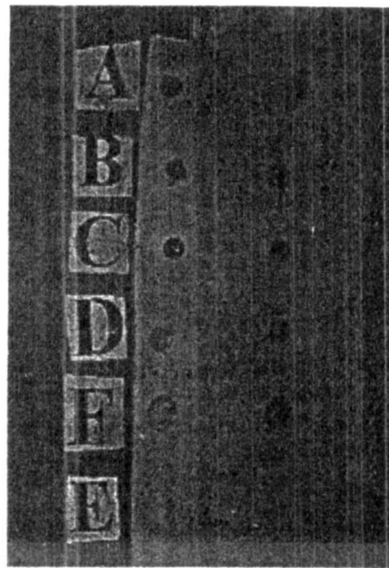

Plate 7. Dot blot analysis of bean plant DNA (DNA spots were duplicated in A-E).
[Note: A - plasmid DNA of Bt 6E, B - plasmid DNA of HD 133, C - $PBIR_{12}$, D and F - plant DNA extracted from transformed plants which produced roots on selection media, E - plant DNA extracted from control plant]

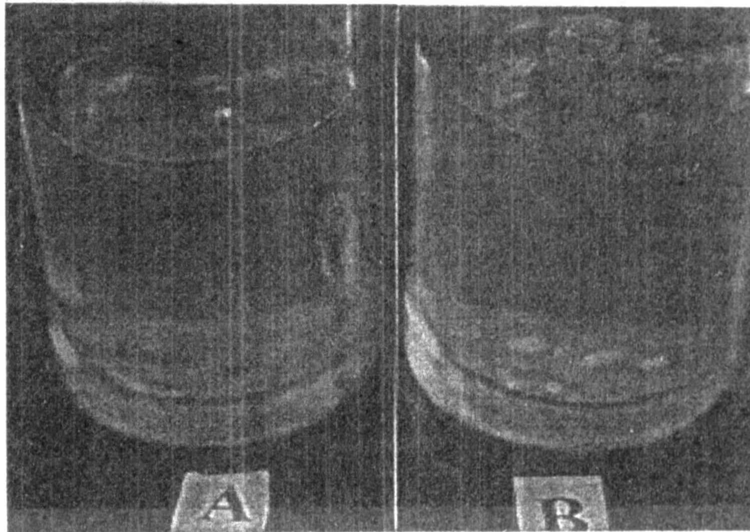

Plate 8. **Callus induction assay.**
[Note: A - control explant on kanamycin medium, B - transformed explant showing callus growth on kanamycin medium].

CONCLUSIONS

The *Agrobacterium*-mediated transformation depends on the susceptibility of the target crop to *Agrobacterium* as well as the availability of a regeneration procedure. The regeneration protocol described in our study provides a rapid regeneration system for green bean variety Topcrop. The ultimate aim is to regenerate plants identical to the parent material except for the newly inserted genes. Callus is prone to somaclonal variations and is not a reliable source for transformation and subsequent regeneration to get transgenic plants. Less variation exists if the apical or axillary meristems are used (Phillips and Hubstenberger, 1995). This regeneration system does not have a callus stage but relies on the development of new meristems during the culture process for the development of adventitious shoots.

AGL1 is an effective *Agrobacterium* strain for the transformation of bean cotyledonary nodes. Since the cotyledonary nodes are susceptible to *Agrobacterium* infections and can also be used as a source of adventitious shoot regeneration it may also be possible to obtain transformed plants from this explant. The integration and expression of *cry* gene in bean plant was shown by the hybridization of the plant DNA to the DIG labeled DNA probe.

The acclimatization of the transgenic bean plants need to be done to obtain the seeds and investigate the transmission and expression of the integrated insecticidal crystal gene in the sexual offspring.

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